REGULATION AND ROLE OF P20K IN CEF

CHARACTERIZING THE TRANSCRIPTIONAL REGULATION AND ROLE OF THE GROWTH ARREST SPECIFIC P20K LIPOCALIN IN CHICKEN EMBRYO FIBROBLASTS

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Abstract

The p20K lipocalin was originally identified to be expressed in chicken heart mesenchymal cells entering growth arrest in response to high cell density. Preliminary studies analyzing the transcriptional activation of p20K within growth arrest revealed that it is predominantly controlled by C/EBPβ, which binds to two regions within the p20K promoter (QRU). Although the transcriptional activation of p20K has been thoroughly examined, it has yet to be defined how p20K is regulated in cycling and starved states. In this study, we observe that C/EBPβ and the MEK pathway are the dominant regulators of p20K expression. This regulation was mutually exclusive, as C/EBPβ was found to associate with the QRU and promote p20K expression during states of growth arrest, whereas the serine/threonine kinase ERK2 bound and repressed p20K expression in cycling states. Furthermore, we show that the ER stress inducible protein CHOP acts to block p20K expression in states of ER stress or starvation through the interaction with C/EBPβ.

Transcriptome analysis done in conjunction with these studies revealed that many hypoxia-responsive specific genes, including carbonicanhydrase IX, were expressed within states of contact inhibition. These studies were supported when nitroreductase activity, a marker of hypoxia, was detected in cells at confluence. These results suggest that cells at high density experience hypoxia, and as such, p20K may be induced in a hypoxia-specific manner. Studies analyzing the function of p20K in hypoxia revealed that the knockdown of p20K via shRNA negatively affected cell viability within low oxygen concentrations. This was shown to be associated with a significant increase in lipid

peroxidation and lipid accumulation within hypoxia, and ultimately resulted in a higher incidence of apoptotic cells. In all, these studies suggest that p20K plays a critical role in lipid homeostasis and cell survival within conditions of limited oxygen concentrations.

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Chapter 2: The p20K Lipocalin is Required for Lipid Homeostasis and Cell Survival in Hypoxic Chicken Embryo Fibroblasts

List of Abbreviations

4-HNE	4-Hydroxynonenal
ABL	Abelson Murine Leukemia Viral Oncogene
AGP	α1-Acid Glycoprotein
AP-1	Activator Protein 1
ATF	Activating Transcription Factor
Bcl	B-Cell Lymphoma
BCR	Breakpoint Cluster Region Protein
BHT	Butylated Hydroxytoluene
bZIP	Basic Leucine Zipper
camp	Cyclic Adenosine Monophosphate
CAK	CDK-Activating Kinase
CCRK	Cell Cycle Related Kinase
CDH1	Cadherin-1
CDK	Cyclin Dependent Kinase
C/EBP	CCAAT-Enhancer Binding Protein
CEF	Chicken Embryo Fibroblasts
ChIP	Chromatin Immunoprecipitation
CHOP	C/EBP Homologous Protein
C.I.	Contact Inhibited
CKI	CDK Inhibitor
DAPI	4',6-Diamidino-2-Phenylindole
DEAE	Diethlyaminoethyl
DFO	1,8-Diazafluoren-9-one
DMEM	Dulbeco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DR5	Death Receptor 5
DTT	Dithiothreitol

EBS	ERK2 Binding Site
EDTA	Ethlyenediaminetetraacetic Acid
eI2Fα	Eukaryotic Translation Initation Factor 2α
EMSA	Electrophoretic Mobility Shift Assay
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-Regulated Kinase
Ex-FABP	Extracellular Fatty Acid Binding Protein
G_1	Gap 1 Phase
G_2	Gap 2 Phase
G_0	Quiescence
GADD	Growth Arrest DNA Damage
GATE	INF-γ Activated Transcriptional Element
GAS	Growth Arrest Specific
GTP	Guanosine Triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
HIF	Hypoxia Inducible Factor
HO	Heme Oxygenase
HRE	Hypoxia Response Element
IGF	Insulin-Like Growth Factor
IgG	Immunoglobulin G
INF	Interferon
INK	Inhibitors of Kinase
IRF	Interferon Regulatory Factors
JAK	Janus Kinase
JNK	c-Jun-N-Terminal Kinase
LAP	Liver Activating Protein
LIP	Liver Inhibiting Protein
LPA	Lysophosphatidic Acid
M	Mitosis

MAPK	Mitogen Activated Protein Kinase
MDA	Malondialdehyde
MEK	MAPK/ERK Kinase
Mup	Major Urinary Protein
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NGAL	Neutrophil Gelatinase-Associated Lipocalin
OAS	2'-5'-Oliguadenylate Synthetase
ONPG	Ortho-Nitrophenyl-β-Galactoside
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PERK	Protein Kinase RNA-Like Endoplasmic Reticulum Kinase
PUFA	Polyunsaturated Fatty Acids
QRU	Quiescent Responsive Unit
Rb	Retinoblastoma Protein
RBP	Retinol Binding Protein
RCASBP	Replication Competent ALV LTR With a Splice Acceptor Bryan Polymerase
RET	Rearranged During Transformation
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RSV	Rous Sarcoma Virus
RTK	Receptor Tyrosine Kinase
S	Synthesis
SARP	Secreted Apoptosis Related Protein
SCF	Skp, Cullin, F-Box Containing Complex
SDS	Sodium Dodecyl Sulphate
shRNA	Short Hairpin RNA
SKP2	S-Phase Kinase-Associated Protein 2

SOD	Superoxide Dimutase
Src	Sarcoma
STAT	Signal Transducer and Activator of Transcription
TBA	Thiobarbituric Acid
TBE	Tris/Borate/EDTA
TBS	Tris Buffered Saline
TdT	Terminal Deoxynucleotidyl Transferase
TGF	Transforming Growth Factor
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
UV	Ultraviolet

Literature Review

1. The Cell Cycle

i) Overview

Multicellular organisms have developed highly complex mechanisms in order to drive and regulate proper cellular proliferation. During periods of favorable growth, such as in the presence of growth factors or mitogenic signals, somatic cells are driven through a process known as the cell cycle. The eukaryotic cell cycle functions to allow an existing cell to accurately duplicate its vast amount of genetic material and organelles, and subsequently divide into identical cells termed daughter cells (Alberts *et al.,* 2002).

The eukaryotic cell cycle is comprised of four distinct phases, which include mitosis (M), gap 1 phase (G_1), synthesis (S) and gap 2 phase (G_2). The G_1 phase functions as a time barrier for initial growth, which ultimately allows the cell to increase its mass of proteins required for cell duplication (Alberts *et al.*, 2002). This phase is critical in cell progression as it determines whether or not the cell will continue to divide, or enter quiescence (G_o phase), based on its ability to proceed past what is termed a "restriction point" (Shafer, 1998). S phase is the longest checkpoint of the cell cycle, and it is the phase in which DNA undergoes duplication (Alberts *et al.*, 2002). G_2 phase is another growth phase between S phase and mitosis, which allows cells to once again increase their protein mass in preparation for mitosis (Alberts *et al.*, 2002). During mitosis, the recently duplicated chromosomes condense, which is followed by nuclear envelope breakdown. Following nuclear envelope breakdown, sister chromatids attach and align at the mitotic spindle, which subsequently results in separation of sister chromatids to the

opposite poles where they then form intact nuclei. Once intact nuclei have been formed, the cell undergoes cytokinesis in which the cell is pinched into two, forming two identical daughter cells (Alberts *et al.*, 2002). See Figure 1 for a visual representation of the cell cycle.

ii) CDKs, Cyclins, and CKIs – Controllers of the Cell Cycle

Due to the fact that the eukaryotic cell cycle plays a critical role in the control of cell viability and proliferation, it requires a diverse network of regulatory elements in order to ensure that proper progression is guaranteed. At the foundation of cell cycle control are the cyclin dependent serine/thronine kinases (CDKs) and their regulatory subunits cyclins. During the early to mid G_1 phase, D-type cyclins (D1, D2, and D3) are synthesized in response to mitogenic stimulation, and begin to form complexes with CDK's 4 and 6 (Sherr *et al*., 1994). These active CDK/cyclin complexes proceed to phosphorylate members of the retinoblastoma (Rb) family of proteins (which include pRB and the related p107 and p130 proteins), resulting in the release of inhibition on E2F family member transcription factors (Weinberg, 1995; Bracken, 2004). With the loss of inhibition, E2F family members promote the expression of Cyclin A, Cyclin E, and other E2F family members, creating a positive feedback loop of gene expression (Cobrinik, 2005). Expression of Cyclin E allows for it to associate and activate CDK2, which further phosphorylates the Rb family of proteins and permits the cell to continue through the cell cycle in a mitogen-independent manner (essentially allowing it to pass the "restriction point") (Sherr, 1993). During late stage G_1, Cyclin E becomes maximally expressed and promotes the transition into S phase with CDK2 through the further inactivation of Rb

family proteins as well as through the phosphorylation of Cdh1, which targets a variety of S-phase promoting factors for degradation (Peters, 2002). During this time Cyclin A also begins to form complexes with CDK2 and promotes the degradation of Cyclin E, leading to inactivation of E2F family members and entrance into S-phase (Woo and Poon, 2003).

Cell cycle control is also mediated in part by the two primary families of cyclin-dependent kinase inhibitors (CKIs). During the early to mid G_1 phase, cell cycle progression is negatively regulated by the INK4 family of CKIs which include p16 (INK4a), p15 (INK4b), p18, (INK4c) and p19 (INK4d) (Cánepa *et al*, 2007). INK4 family members function to inhibit cell cycle progression through the inhibition of CyclinD-CDK4/6 complex formation by competing with Cyclin D for binding to CDK4 and CDK6 (Cánepa *et al*, 2007). They have additionally been shown to promote the redeployment of the Cip/Kip family of CKIs, ultimately allowing them to inhibit CyclinE-CDK2 activity (Morgan, 1997; Johnson and Walker, 1999; Cánepa *et al*, 2007). In the later stages of G_1, the Cip/Kip family of CKIs, which are comprised of $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, function to inhibit the activity of CDK2 complexes. $p21^{Cip1}$ is regulated by the p53 tumor suppressor and prevents CDK2 activity by inhibiting its activation through cyclin-activating kinase (CAK) (el-Deiry *et al*., 1993; Aprelikova *et al*., 1995). $p27^{Kip1}$ functions to inhibit CDK2 activity by actively binding to both CDK2 and Cyclin A, and is transcriptionally controlled through the presence or absence of proliferative signaling within the cell (Johnson and Walker, 1999; Russo *et al*., 1996). Interestingly, both $p21^{Cip1}$ and $p27^{Kip1}$ have been shown to positively regulate cell cycle

progression as well, by enhancing the ability of Cyclin D to form complexes with CDK4 and CDK6 (Cheng *et al*., 1999; Sherr and Roberts, 1999).

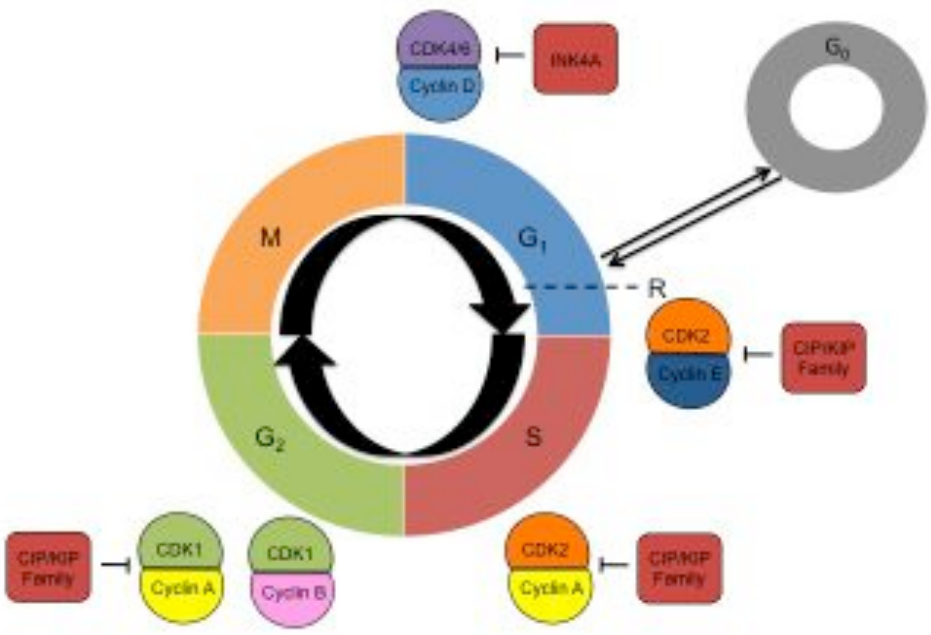

Figure 1: The Cell Cycle. The cell cycle is comprised of four distinct phases which include Gap 1 Phase (G_1), DNA synthesis (S), Gap 2 Phase (G_2), and Mitosis (M). Progression through the cell cycle is controlled primarily by the catalytic cyclin dependent kinases (CDKs) and their regulatory subunits, the cyclins. In early G_1 phase, progression is driven by CDK4/6, which associates with Cyclin D and allows for passage through what is termed the "Restriction Point" (R). Once past the restriction point, CDK2 begins to form active complexes with Cyclin E and Cyclin A which allows for progression through S phase. The G_2 and M phases are predominantly controlled by CDK1 which forms active complexes with both Cyclin A and Cyclin B. Cell cycle progression is negatively regulated by the two family of CDK inhibitors (CKIs), the CIP/KIP family (p21, p27, p57) and the INK4 family (p15, p16, p18, p19). The INK4 family predominantly inhibits the activity of CDK4/6-Cyclin D complexes while the CIP/KIP family inhibits the activity of all CDK-cyclin complexes.

iii) Mitogenic Signaling and the Cell Cycle

Cells possess a variety of signaling pathways in order to transmit extracellular signals, such as nutrients, mitogens, and cytostatic factors, into intracellular processes. One of the principal families of signaling pathways, which play a variety of key roles within the cell, are the MAP kinase (MAPK) signaling cascades. There are four primary MAPK signaling cascades and they include the c-Jun N-terminal kinase (JNK) cascade, the p38 cascade, the ERK5 cascade and the extracellular regulated kinase 1/2 (ERK1/2) cascade (Figure 2) (Yang *et al*., 2003). These cascades play roles in processes such as proliferation, development, stress response, survival, and apoptosis (Keshet and Seger, 2010). One of the key signaling pathways responsible for promoting cell proliferation and progression of the cell cycle is the ERK1/2 signaling cascade.

In the ERK1/2 signaling cascade, growth factors bind receptor-tyrosine kinases (RTKs) located on the cell surface. Once ligand binding occurs, RTKs dimerize which results in their trans-phosphorylation and activation (Lemmon and Schlessinger, 2010). Activation of RTKs results in the recruitment and activation of the small GTPase Ras, which subsequently activates Raf kinases (Meloche and Pouysseugur, 2007). Activated Raf phosphorylates and activates the MAP kinase kinases MEK1 and MEK2 which subsequently activates the MAP kinases ERK1 and ERK2 (Lenormand *et al*., 1993). ERK1/2 activation results in nuclear translocation, where they serve a variety of functions (Yoon and Seger, 2006; Lewis *et al*., 1998). ERK1/2 has been shown to promote cell cycle progression in various ways, one of these ways being through the transcriptional induction of the *Cyclin D1* gene (Albanese *et al*., 1995; Lavoie *et al*., 1996). Although

the exact mechanism of this transcriptional control has yet to be characterized, it is thought that ERK1/2 stabilizes AP-1 family members which have been shown to induce the expression of *Cyclin D1* through an AP-1 binding site in its promoter region (Albanese *et al*., 1995; Herber *et al*., 1994). ERK1/2 have also been shown to play a role in the post-transcriptional regulation of Cyclin D1 mRNA stabilization and transport as well as promote cell cycle progression through the stabilization of c-Myc (Culijkovic *et al*., 2005; Topisirovic *et al*., 2004). c-Myc is a member of the Myc family of transcription factors which can form heterodimers with Max, resulting in the expression of Cyclin D2, as well as CDK4 and p21, all of which can promote cell progression through the G_1 phase (Sears *et al*., 2000; Bouchard *et al*., 1999; Hermeking *et al*., 2000; Coller *et al*., 2000).

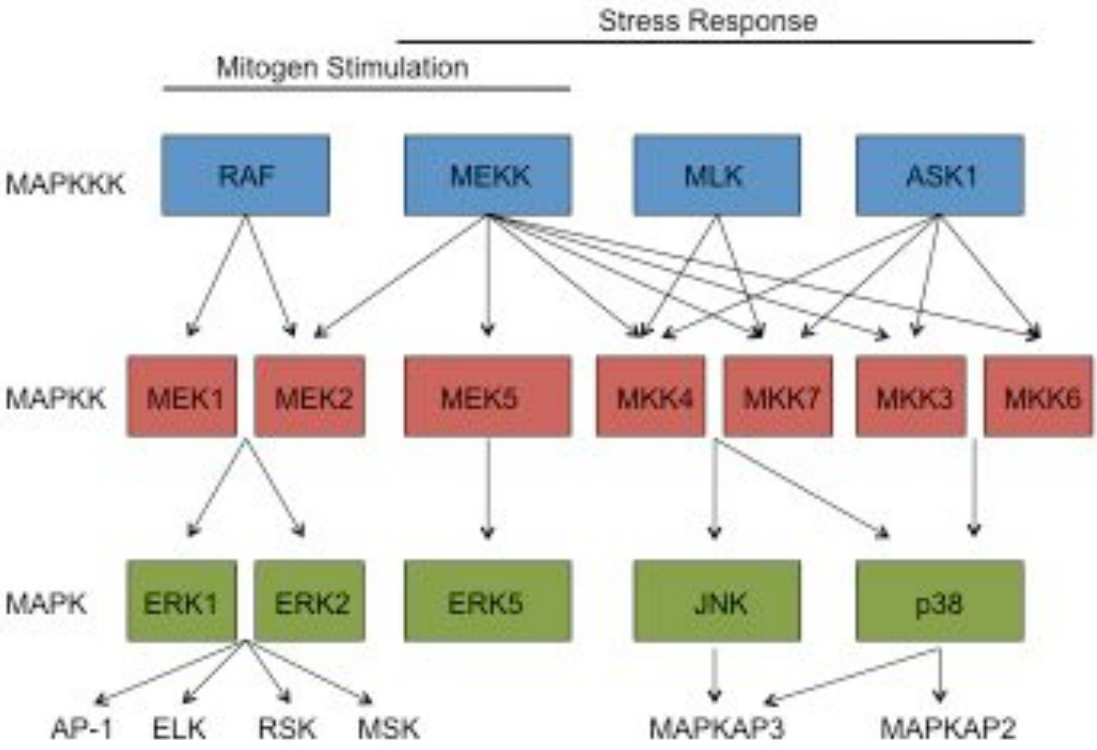

Figure 2: The MAPK Signaling Cascades. A general schematic of the four primary MAPK signaling cascades which are activated in response to mitogenic stimulation or stress response. Figure was modified from Yang *et al*., 2003.

iv) Cell Cycle Arrest (G_0)

During periods of unfavorable growth, such as cell-cell contact or lack of oxygen, eukaryotic cells require a way to ultimately prevent cell cycle progression and DNA synthesis in order to increase their chances of survival (Polyak *et al*., 1994). Cells do this by entering a reversible, nondividing state known as quiescence (G_o). Multiple cellular activities mediate this transition, one in particular being the Ras/Raf/MEK/ERK signaling cascade previously mentioned. Due to the lack of mitogenic stimulation during unfavorable growth conditions, the Ras/Raf/MEK/ERK signaling cascade fails to be activated. This results in the loss of ERK1/2 translocation to the nucleus and loss of *Cyclin D1* expression. This results in Rb family members remaining unphosphorylated and E2F transcription factors remaining repressed, and as such cells fail to pass the restriction point and transition from the G_1 to G_o phase (Ladha *et al*., 1998). The Cip/Kip family and INK4 family of CKIs have also been shown to play a role in promoting quiescence, as highly elevated levels of $p21^{Cip1}$ and $p27^{Kip1}$ have been found in quiescent states in varying cell types and INK4 induction was shown to induce growth arrest in certain cell contexts (Kwon *et al*., 2002, Reynisdottir *et al*, 1995).

It must be noted that cellular quiescence differs from states of irreversible growth arrest such as senescence. The most common forms of irreversible growth arrest initiation are usually related to telomere erosion over time in primary cells, however it is also associated with DNA double-strand breaks or strong oncogene-induced mitogenic signaling (Nakamura *et al*., 2008; Bartkova *et al*., 2006). One of the primary mediators of irreversible growth arrest is p53. During cell stress, $p19^{Arf}$ can activate p53 by binding to

and sequestering the *Mdm2* gene product, preventing its ability to associate with p53 (Weber *et al*., 1999). p53 is then free to promote senescence by inducing the expression of $p21^{Cip1}$ (Weber *et al*., 1999; Atadja *et a*l., 1995). $p16^{INK4A}$ has also been shown to be a primary moderator of irreversible growth arrest, as it is not commonly expressed at high levels in quiescent cells, but is highly expressed within senescent cells and expression levels generally increase with age in mice and humans (Alcorta *et al*., 1996; Hera *et al*., 1996).

2. Growth Arrest Specific Genes

i) Overview

The G_0 phase of the cell cycle is operationally distinct from the G_1 phase in a variety of manners. While the G_1 phase promotes cell proliferation through the expression of factors required for cell cycle progression (such as Cyclin D1, E2F factors, etc), the G_o phase requires the expression of genes that inhibit growth, enhance cell survival and suppress re-entry back into the G_1 phase. Interestingly, it has been shown that several quiescence signals arrest growth and division through varying mechanisms, implying that there are different signal dependent quiescent states (Coller *et al.*, 2006). There are however, families of genes that were found to be expressed during all states of reversible growth arrest, and these have been termed growth arrest specific (GAS) genes. GAS genes play several roles within a cell, and ultimately help prepare and maintain the cell within a growth-arrested state. The best characterized of these GAS genes have been the GAS1-6 family, which were originally discovered by Schneider *et al*, in 1998. The GAS1 protein, has been shown to play a role in growth suppression by blocking entry into S

phase in a p53-dependent manner, and as such has been considered to be a tumor suppressor. This is mediated through its interaction with two different membrane receptors, rearranged during transformation (RET) signaling and sonic hedgehog receptor patched 1 (Del Sal *et al*, 1992; Rosti *et al*, 2015; Zamerano *et al*, 2003). Another member of the GAS family of proteins, GAS6 has been shown to promote cell survival by acting as a ligand of Axl tyrosine kinase receptor (Goruppi *et al.,* 1996). Another family of GAS genes, the secreted apoptosis related proteins (SARPs), also promote cell survival by blocking apoptosis signals through the interference of the Wnt-frizzled signaling pathway (Melkonyan *et al*., 1997). GAS genes, such as superoxide dismutase-3, have also been shown to promote survival through the reduction of free radical buildup, which accumulate during these states (Suliman *et al*., 2004). Interestingly, multiple GAS gene products are also thought to have roles in priming cells for re-entry. This includes the platelet-derived growth factor α-receptor (PDGFαR), Wnt signaling proteins and the bone morphogenetic protein (Lin *et al*., 1996; Coller *et al*., 2006). In all, it is undeniably evident that once a cell enters a state of growth arrest it must initiate a critical and unique gene program in order to properly maintain this state.

ii) p20K and the QRU

One GAS gene which has been shown to be highly expressed during quiescence of chicken embryo fibroblasts (CEF) is the p20K lipocalin, also referred to as the extracellular fatty acid-binding protein (Ex-FABP) (Bedard *et al*., 1987; Mao *et al*., 1993). p20K is a member of the lipocalin protein family, and has been portrayed to have high affinity for hydrophobic molecules such as long chain unsaturated fatty acids

(Cancedda *et al*.., 1990; Cancedda *et al*., 1996). p20K has been shown to play a variety of roles in various cell types, including cell survival, inflammatory response and cell development (Cancedda *et al*., 1990; Gentili *et al*., 1998; Cancedda *et al*., 2000; Cermelli *et al*., 2000; Gentili *et al*., 2005). It also plays roles in the metabolism of lipids and transport of long chain unsaturated fatty acids (Cermelli *et al*., 2000). More recent analysis of the crystal structure of p20K revealed that it contains multiple pockets with ligand specificities for both bacterial siderophores and lysophosphatidic acid (LPA), implying that p20K plays a role in many diverse cellular activities including being a potential candidate as an antibacterial catecholate siderophore binding lipocalin (Correnti *et al*., 2011) (Figure 3) .

Characterization of p20K gene expression indicates that a 48bp region within the promoter is required for its transcriptional activation (Mao *et al*., 1993). As this region was shown to be activated by quiescence, it was ultimately termed the Quiescence Responsive Unit (QRU). Binding of the CCAAT-Enhancer Binding Protein Beta (C/EBPβ) to this region was shown to up-regulate p20K, and the forced expression of a dominant negative mutant of C/EBPβ resulted in the inhibition of p20K during quiescence states (Kim *et al*., 1999). Further characterization of this region revealed that it contained two C/EBPβ sites, termed the A and B regions, which were necessary for transcriptional activation of p20K (Kim *et al*., 1999).

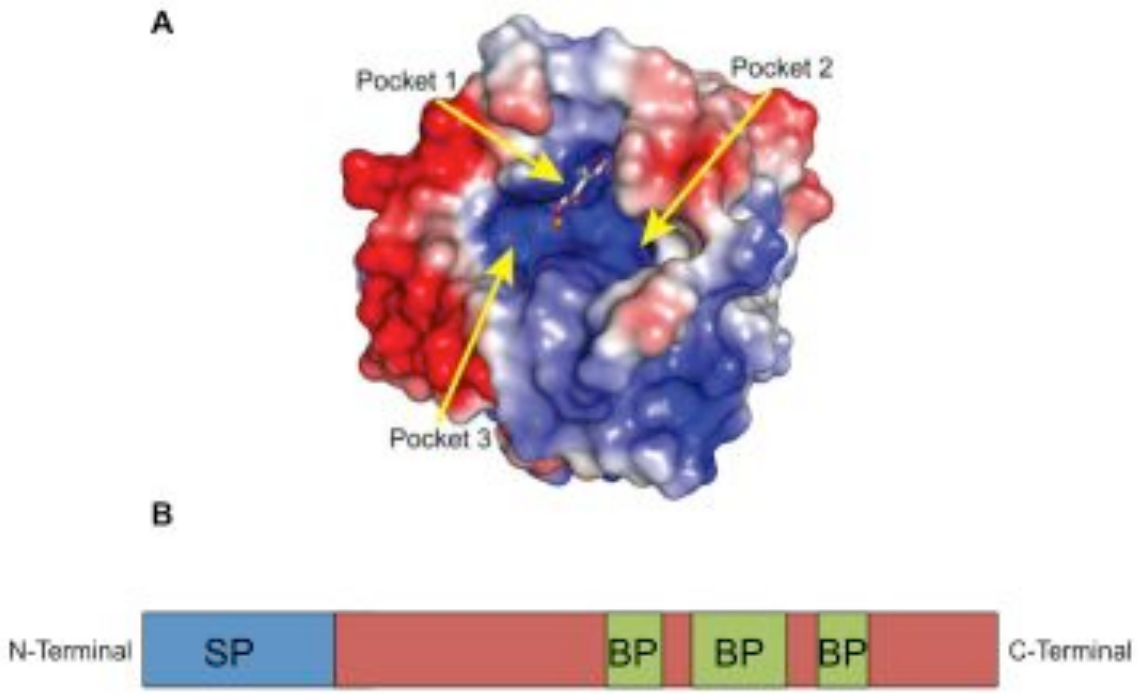

Figure 3: Protein Structure of p20K. (A) The crystal structure of p20K is shown and is colored by electrostatic potential. Individual binding pockets are labeled. This figure was adapted from Correnti *et al*., 2012. Figure (B) represents a general schematic of p20K structure. The signal peptide (SP) is located at the N-terminal of the protein. The p20K chain is highlighted in red and contains three binding pockets (BP).

iii) Hypoxia and Contact Inhibition

Multiple environmental signals can promote a cell's entrance into quiescence. Two primary conditions which have been shown to promote quiescence are low oxygen concentrations (i.e hypoxia) and cell-cell contact, also referred to as contact inhibition. As oxygen is a fundamental element for aerobic metabolism in mammalian cells, it is no surprise that cells have developed a comprehensive cellular response to adapt to and ultimately respond to environmental conditions where oxygen may be lacking. At the forefront of this response are the hypoxia inducible transcription factors (HIFs). Under cycling normoxia (21% O_2) conditions the primary HIF factor HIF-1α is degraded. During low oxygen concentrations however, this degradation is inhibited and HIF-1α can associate with HIF-1β to form an active and functional complex (Giaccia *et al*., 2004; Chi *et al*., 2006). Once active, HIF-1 serves a variety of functions such as promoting angiogenesis to scavenge for oxygen, promoting the transition to anaerobic metabolism, as well as playing roles in inflammation response. (Genbacev *et al*., 1997; Carmelret *et al*., 1998; Vaupel, 2004; Cramer *et al*., 2003). Multiple genes are highly upregulated in hypoxia which play various roles in aiding to adapt to these conditions. These include angiogenic factors, proteins responsible for glucose transport, metabolism, glycogen synthesis, cell proliferation and apoptotic factors (Chi *et al*., 2006). Many genes are also repressed during hypoxia, many of which function to promote or regulate cell proliferation (including *Cyclin D1, PCNA* and *CCRK*), ultimately indicating that cells in hypoxia conditions regulate proliferation during this state through the repression of factors required for cell cycle progression (Chi *et al*., 2006).

Another environmental cue that promotes a cell's entrance into quiescence is contact inhibition. Like other environmental signals leading to growth arrest, contact inhibition leads to the down-regulation of multiple cell cycle progression factors including Cyclin D1 and SKP2 (which is part of the SCF complexes which mediates ubiquitination of $p27^{Cip1}$) (Dietrich *et al*., 2002; Carrrano *et al*., 1999; Nakayama and Nakayama, 2005). Interestingly, microarray analysis revealed that contact inhibited cells also exhibit unique gene expression patterns in comparison to other quiescent induced states. This includes the downregulation of the proliferating cell nuclear antigen (PCNA) DNA replication factor, biosynthetic enzymes and splicing factors (Coller *et al*., 2006). Contact inhibition was also shown to trigger increased expression of E2F4 and F4N, which may play roles in IGF-1 mediated cell survival (Coller *et al*., 2006).

3. CCAAT-Enhancer Binding Protein Family

i) Overview

The CCAAT-enhancer binding protein family is a class of transcription factors consisting of six members; C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and C/EBPζ (Akira *et al*., 1990; Roman *et al*., 1990; Cao *et al*., 1991; Williams *et al*., 1991) (See Figure 4 for schematic of family members). All six C/EBP family members display 90% sequence similarity in the C-terminal region, which has been characterized as a basic-leucine zipper domain required for protein dimerization and DNA binding (Landshulz *et al*., 1988; Landshulz *et al*., 1989; Vinson *et al*., 1989). C/EBP family members are primarily regulated at the transcriptional level, and have been shown to be expressed in response to a variety of signals including hormones, cAMP, INF-γ and TGF-β (Lekstrom-

Himes and Xanthopoulos, 1998; Alam *et al*., 1992). Regulation can also occur at the translational level through the use of alternative initiation codons, which can result in the formation of a multiple isoforms (Ossipow *et al*., 1993; Lin *et al*., 1993).

C/EBP has been found to bind a consensus sequence of RTTGCGYAAY (where R = A or G, and Y = C or T), however they tolerate some variations of this sequence (Osada *et al*., 1996). The transcriptional activation potential of C/EBP is primarily regulated through its ability to dimerize with other proteins, as C/EBP can form intrafamiliar hetero-and homodimers that can increase or decrease their activity (LeClair *et al*., 1992; Vallejo *et al*., 1993; Lekstrom-Himes and Xanthopoulos, 1998). Interestingly, many C/EBP family members have been shown to produce multiple isomers with different transcriptional activity, an excellent example being C/EBPβ. C/EBPβ has three primary isoforms, which include LAP, LAP* and LIP (Descombes and Schibler, 1991). As LIP does not contain an activation domain, it can inhibit the transcriptional activity of C/EBPβ and C/EBP family members by forming non-functional dimers (Descombes and Schibler, 1991). C/EBP can also form heterodimers with other bZIP and non-bZIP transcription factors to regulate transcription, some of which include CREB/ATF family members, NF-κB and AP-1 family members (LeClair *et al*., 1992; Vallejo *et al*., 1993; Lekstrom-Himes and Xanthopoulos, 1998).

C/EBP family members play diverse roles within a variety of cell types. C/EBPα and C/EBPβ have been shown to play roles in cell differentiation, as the ectopic expression of either promotes differentiation while their knockdown inhibits differentiation in 3T3-L1 cell lines (Freytag *et al*., 1994; Lin *et al*., 1994, Yeh *et al*.,

1995; Lin and Lane, 1992). C/EBPα has also been portrayed to play roles in many metabolic pathways through the transcriptional regulation of genes involved in glycogen synthesis and gluconeogenesis (Lee *et al*., 1997). C/EBPβ has been shown to regulate a variety of genes involved in inflammation response, including IL-1 and 6 (Akira *et al*., 1990; Poli *et al*., 1990). C/EBP family members also play both pro- and anti- proliferative roles, as C/EBPα has been shown to inhibit cell proliferation when overexpressed in cell cultures, while C/EBPβ has been shown to be highly elevated in tumors and is also activated by oncogenic Ras (Lekstrom-Himes and Xanthopoulos, 1998; Greenbaum *et al*., 1998; Zhu *et al.,* 2002). Collectively, it is obvious that C/EBP family members are a significant group of transcription factors with diverse regulatory functions within a cell.

ii) C/EBP Homologous Protein (CHOP)

The C/EBP homologous protein (CHOP) belongs to the growth arrest DNA damage (GADD) protein family and is a key mediator of ER-stress induced apoptosis (Pan *et al*., 2004). Like C/EBP family members, CHOP also contains a conserved bZIP domain however it can only regulate gene expression through the interaction with other C/EBP family members to control their activity (Oyadomari and Mori, 2004). This interaction with C/EBP is necessary for its control in promoting apoptosis, as cells lacking C/EBPβ are resistant to ER stress induced apoptosis (Zinszner *et al*., 1998). CHOP has been shown to be one of the most highly induced genes during ER stress, and its induction during these states has been shown to be predominantly controlled by the PERK/eI2Fα and ATF signaling pathways (Okada *et al*., 2002; Scheuner *et al*., 2001; Harding *et al*., 2000). Interestingly, CHOP is also regulated via post-translational

modification by the p38 MAP kinase family, which phosphorylates and stabilizes CHOP protein (Maytin *et al*., 2001).

CHOP regulates a variety of factors that ultimately play roles in promoting apoptosis. This includes the down-regulation of anti-apoptotic proteins such as Bcl2, the up-regulation of pro-apoptotic proteins such as death receptor 5 (DR5), and through the induction of oxidative stress (McCullough *et al*., 2001; Pan *et al*., 2004; Yamaguchi and Wang, 2004). Interestingly, CHOP can also interact with other bZIP proteins such as members of the AP-1 family to activate AP-1 target genes, thereby suggesting that CHOP regulates gene expression during cellular stress by interacting with more than just C/EBP family members (Ubeda *et al*., 1999).

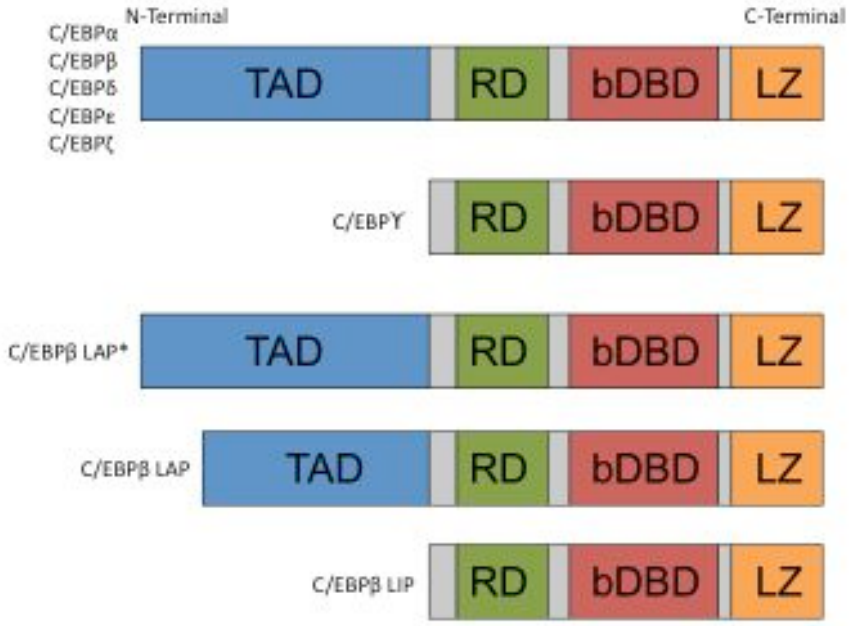

Figure 4: Structure of C/EBP family members and C/EBPβ isoforms. All members of the C/EBP protein family, with the exclusion of C/EBPγ and the C/EBPβ isoform LIP, contain a transcriptional activation domain (TAD), which is required for transcriptional activation of target genes. All members of the protein family contain a regulatory domain (RD) that is the site in which post-translational modifications occur. All members also contain a basic DNA binding domain (bDBD) and leucine zipper (LZ), which is required for DNA binding and homo-/heterodimer formation respectively.

4. ERK2 Function as a Transcriptional Repressor

Until 2009, it was thought that all activity carried out by the mitogen activated protein kinase 1 (or ERK2) was controlled through its kinase activity. A proteomic study conducted in 2009 by Hu *et al*. however revealed that ERK2 could bind to a consensus G/CAAAG/C sequence (which will be henceforth referred to as the ERK2 binding site or EBS) (Hu *et al*., 2009). Interestingly, it was shown that the DNA-binding activity of ERK2 was independent of its kinase activity, as ERK2 containing mutations within the kinase domain still exhibited DNA binding (Hu *et al*., 2009). When analyzing the crystal structure of ERK2 it was evident that it contained a DNA-binding domain spanning amino acids 259 to 277, and this DNA-binding domain was unique amongst other members of the ERK family (Hu *et al*., 2009).

Further analysis into the physiological function of ERK2 DNA-binding showed that it bound to multiple IFN-γ Activated Transcriptional Element (GATE) regulated genes that contain the consensus ERK2 binding sites (Hu *et al*., 2009). The repression by ERK2 was shown to be important for the control of these promoter regions as knockdown of ERK2 caused a de-repression of two of these IFN-γ controlled genes; IRF9 and OAS1 (Hu *et al*., 2009). Normally, IFN-γ stimulation results in the activation of C/EBPβ, which promotes the induction of these genes (Hu *et al*., 2009). This feature is interesting due to the fact that IFN-γ signaling mediates C/EBPβ activation through the MEKK1/MEK1 pathway and subsequently through ERK1/2 (Roy *et al*., 2002). Hu *et al*. proposed a model through which ERK2 mediates transcriptional repression of GATE element regulated genes in normal conditions, however when signaling (through cytokine stimulation or

other mechanisms) causes the activation of C/EBPβ through ERK2, C/EBPβ is able to rapidly out-compete ERK2 for binding to these GATE elements (Hu *et al*., 2009). C/EBPβ activity is eventually relieved once ERK2 concentrations increase in the nucleus, allowing it to once again mediate transcriptional repression (Hu *et al*., 2009). In all it is evident that the binding activity of ERK2 allows it to serve a distinct cellular function from other members of the ERK family.

5. The Lipocalin Family

i) Overview

The lipocalin family is an evolutionarily conserved family of small proteins that serve multiple functions within various cell types. Interestingly, the lipocalin family portrays low levels of sequence similarity amongst themselves, as pairwise sequence identity between members of the family falls below 20% (Flower, 1993). They all however, contain eight β-strands which forms a β-barrel structure with a unique folding pattern that is conserved amongst them (Flower, 1993). This unique β-barrel structure forms a calyx which allows the lipocalin family to undertake multiple functions (Flower, 1993). One of the primary functions of the lipocalin family is ligand binding, as they have been shown to bind a wide array of small hydrophobic ligands. Specificity of ligand binding is dictated by the amino acid composition and size of the calyx and loop scaffold (Flower, 1995). Interestingly, there is also evidence portraying that lipocalins can exert their function through receptor binding. One example of this is the retinol binding proteins (RBP), which, when bound by retinol, have been shown to bind their target cells through the use of specific cell surface receptors which allows them to be internalized via

endocytosis (Senoo *et al*., 1990; Bavik *et al*., 1992; Sivaprasadarao *et al*., 1993; Smeland *et al*., 1995). Lipocalins function through macromolecular complex formation, such as the neutrophil gelatinase-associated lipocalin (NGAL) that covalently attach to the human neutrophil gelatinase in order to regulate its activity (Treibel *et al*., 1992; Kjeldsen *et al*., 1993).

As previously stated, the lipocalin family plays a variety of roles within numerous cell types, and this activity is mediated by several ways. One of the primary roles of the lipocalin family is to function as extracellular transport proteins. The RBP lipocalins have been shown to bind retinol, where it serves to both transport it to peripheral tissues from the liver as well as protect it from oxidative damage during circulation (Flower, 1993). Another role of the lipocalin family is in pheromone activity. The major urinary protein (Mup) lipocalins have been observed to bind and transport multiple pheromones such as 2-(S-butyl)thiazoline from the liver to the urinary tract to be secreted in the urine (Cavaggioni *et al*., 1987; Bacchini *et al*., 1992; Mucignat-Caretta *et al*., 1995). The lipocalin family has also been shown to play roles in immune response regulation, such as the α1-acid glycoprotein (AGP). AGP was observed to accumulate to sites of inflammation and is thought to function as an immunoregulatory lipocalin by inhibiting platelet aggregation, neutrophil activation and phagocytosis (Costello *et al*., 1979; Bennett and Schmid, 1980; Kremer *et a*l., 1988). Multiple lipocalins have also been shown to play roles in cell cycle regulation, such as the purpurin lipocalin. Purpurin was found to be expressed in the neural cells of the retina, and is believed to function in the control of cell differentiation, adhesion and survival (Schubert *et al*., 1986; Berman et al.,

1987). Another example is the apoD lipocalin, which is thought to play a role in nerve regeneration as it was observed to increase 500-fold in expression in regenerating peripheral nerves (Boyles *et al*., 1990; Spreyer *et al*., 1990). In all, it is undoubtedly apparent that the lipocalin family is structurally and functionally diverse in comparison to other protein families which allows it to play multiple roles within various cell types.

ii) NGAL

One of the most prominently studied lipocalins is the neutrophil gelatinase-associated lipocalin. NGAL, also referred to as lipocalin 2 (Lcn2), was originally identified and isolated in neutrophil granules (Hraba-Renevey *et al*., 1989; Triebel *et al*., 1992). Interestingly, NGAL is thought to be the human homologue of p20K as they share 83% structural similarity and carry out similar functions (Holmes *et al.,* 2005; Correnti *et al*., 2011). Like p20K, NGAL has been shown to be expressed in a variety of organs, including the uterus, stomach, and colon, and was found to be highly expressed within the bone marrow (Cowland and Borregaard, 1997). Interestingly, NGAL expression is promoted by a variety of stimuli, including the presence of the oncoprotein BCR-ABL (Sheng *et al*., 2009), in neoplastic colorectal disease and in response to the treatment with reactive oxygen species (ROS) (McLean *et al*., 2013; Roudkenar *et al*., 2007).

Like p20K, NGAL has also been shown to serve a variety of functions in various cell types. NGAL is thought to have immunosuppressive properties through its regulation of neutrophil gelatinase, as cells infected with SV40 have been shown to present a 7-10 fold increase in NGAL expression (Hraba-Renevey *et al*., 1989). NGAL has also been shown to suppress bacterial growth through the sequestration of iron-containing

siderophores such as enterobactin, which results in the starvation of bacteria that require siderophores for proliferation (Goetz *et al*., 2002). Additionally, NGAL is also thought to mediate cell differentiation, as its application to rat metanephric mesenchyme was shown to promote epithelial differentiation of the mesenchymal progenitors (Yang *et al*., 2002; Schmidt-Ott *et al*., 2006). Neutrophil gelatinase-associated lipocalin has also been observed to mediate apoptosis in some cell types, however this role of NGAL is controversial and context-dependent as it has yet to be confirmed *in vivo* (Correnti *et al*., 2012; Kehrer, 2010; Berger *et al*., 2006). More recently, NGAL has also been shown to act as a protective factor against oxidative stress (Roudkenar *et al*., 2008). This role of NGAL is thought to be directed through its ability to induce the expression of heme oxygenase-1/2 (HO-(1)(2)) and superoxide dismutase-1/2 (SOD-(1)(2)), which have been shown to have protective effects against oxidative stress (Bahmani *et al*., 2010; Song *et al*., 2015; Choi and Alam, 1996; Endo *et al*., 2007).

6. Lipid Peroxidation in Hypoxia

Lipids play many fundamental roles within a cell, particularly polar lipids which act primarily as structural components of cellular membranes providing barriers for the cell and subcellular organelles (van Meer *et al*., 2008). During states of low oxygen concentrations, cells undergo oxidative stress that is a direct result of the production and accumulation of endogenous reactive oxygen species (ROS) which derive from the mitochondria, plasma membrane, endoplasmic reticulum and peroxisomes (Moldovan and Moldovan, 2004). With the buildup of ROS, free radicals begin to attack multiple targets within the cell. One of the most prominent targets of oxidation by ROS are lipids,

primarily glycolipids, phospholipids, cholesterol and polyunsaturated fatty acids (PUFAs) (Ayala *et al*., 2014). Although both hydroxyl and hydroperoxyl radicals can function to damage lipids, the hydroperoxyl radical has been observed to be the most potent oxidant with regards to lipid peroxidation (Bielski *et al*., 1983; Schneider *et al*., 2008; Browne and Armstrong, 2000). Once lipid peroxidation occurs due to an attack by a hydroperoxyl radical, the free lipid radical can rapidly elicit free radical attacks on peripheral lipids, causing a chain reaction of lipid oxidation which can only be terminated by antioxidants such as vitamin C (Yin *et al*., 2011). The process of lipid perodixation is particularly detrimental with regards to biomembranes, as lipid oxidation of biomembranes results in the loss of membrane fluidity, disrupts ion-gradients and lipid protein interactions and can additionally cause the release of bi-products which can alter gene expression and signal transduction (Watanabe and Nakamura, 1979; Halliwell and Chirico, 1993; Rice-Evans and Burdon, 1993). Interestingly, when antioxidant enzymes are unable to keep up with the load of oxidative stress, a cell may promote the formation of a biproduct of lipid oxidation 4-hydroxynonenal (4-HNE). 4-HNE has been shown to regulate many transcription factors which are involved in stress response, but has been shown to have a more prominent role in promoting apoptosis in a p53-dependent manner. (Huang *et al*., 2012; Zhang *et al*., 2010; Abarikwu *et al*., 2012; Sharma *et al*., 2008; Choudhary *et al*., 2002). In all, once a cell experiences a high accumulation of lipid peroxidation resulting in toxic conditions that cause damage and surpasses the overall repair capacity of the cell, the cell will ultimately undergo apoptosis or necrosis programmed death (Volinsky and Kinnunen, 2013).

Objectives

The p20K lipocalin gene was originally identified as a gene induced in conditions of contact inhibition (Bédard *et al*., 1987). Preliminary studies analyzing the transcriptional regulation of p20K were able to portray that its transcriptional activation was dependent on the interaction of C/EBPβ with two regions within the promoter region of p20K gene termed the 'Quiescence Responsive Unit' or QRU. Although the transcriptional activation of p20K by C/EBPβ has been well defined, we have still yet to fully characterize the transcriptional regulation of p20K in key cell contexts including cycling and starved states. Furthermore, although previous studies have been able to portray that p20K plays multiple roles within a cell, including cell survival, inflammatory response and cell development, we have still yet to elucidate its precise role in the state of reversible growth arrest.

The goal of this work is to analyze the transcriptional regulation of p20K in various cell contexts as well as analyze the role of p20K in the G_o phase. Chapter 1 focuses on analyzing the regulation of p20K expression, particularly focusing on the role of ERK2 as a transcriptional repressor of the p20K lipocalin gene during cycling states. Chapter 2 focuses on revealing the role of p20K in conditions of quiescence, predominantly in states of limiting oxygen concentrations.

Detailed Materials and Methods

1. Chicken Embryo Fibroblast Cell Culture and Culture Conditions

Chicken embryo fibroblasts (CEF) were isolated from day 10 chicken embryos. Cells were cultured in Dulbeco's modified eagle medium (DMEM) high glucose 1x (Sigma D5546) supplemented with 5% heat inactivated cosmic calf serum (Hyclone AUA33984), 5% tryptose phosphate broth (Sigma T8782), 2 mM L-Glutamine (Gibco 25030), 500 units Penicillin and 500 μg Streptomycin (Gibco 15140). Cells were split utilizing Trypsin-EDTA (Sigma 59417C). In normoxia conditions, cells were grown at 41.5°C in 5% CO_2 and 21% O_2. Hypoxia was induced by incubating cells in 1-2% O_2 for the time period described. For confluent conditions, cells were grown to 100% confluence. The day prior to cell samples being collected, the media was changed to ensure cells were grown to confluence. For MEK inhibition, cell culture medium was changed 3 hours prior to MEK inhibitor treatment. Cells were treated with 0.1 μg-2 μg of MEK inhibitor PD184352 (Sigma PZ0181) or a 0.1% DMSO diluent. For experiments requiring greater then 24 hours of treatment, the desired concentration of MEK inhibitor was added every 24 hours for the total duration of the experiment. For cell transformations with New York Rous Sarcoma Virus (NY-RSV) 72-4 cell cultures were split the day of transformation into 100 mm plates. After cells adhered (approximately 4-5 hours) the media was removed such that only 4 mL of media remained. 0.5 mL of viral stock was then added into the plate and the plate was left to incubate overnight at non-permissive temperatures (41.5°C). The next morning the media was replaced with fresh

media. Cells were split until needed and placed in both non-permissive and permissive temperatures for analysis (41.5°C and 37°C respectively).

2. SDS-PAGE and Western Blotting

2.1. Protein Sample Preparation

The desired cells to be used were first washed three times with cold 1xPBS pH 7.4 (137 mM NaCl, 2.7 mM KCL, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4). Any remaining PBS was aspirated off and cells were scraped and collected in 1 mL of 1xPBS utilizing a rubber scraper. Cell samples were centrifuged at 7000 RPM (4000 RCF) for 3 minutes and all 1xPBS was removed. The cell pellet was resuspended in 1x Sodium Dodecyl Sulfate (SDS) sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 60 mM Tris-HCL pH 6.8) containing 1x Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher 78441), vortexed for 1 minute and boiled for 3 minutes. Samples were spun down at 13000RPM (13800 RCF) for 5 minutes and the supernatant was transferred to a new microcentrifuge tube. Samples were stored at -80°C until needed.

2.2 SDS-Page and Western Blotting

Total cell protein extracts (30-60 μg) were subjected to SDS-polyacrylamide gel electrophoresis with gel concentrations ranging from 9-14% depending on the size of the proteins needing to be resolved. When resolved, gels were blotted on to a nitrocellulose membrane (Schleicher and Schuell, BA85) and subsequently blocked in a 5% solution of skim milk powder dissolved in 1xTBS (20 mM Tris pH 7.6, 140 mM NaCl) for 1 hour at room temperature. Blots were incubated with the desired primary antibody overnight at 4°C (see Table 1 for primary antibody concentrations). The following day, the membrane

was washed with a cycle consisting of one wash of 1xTBS, two washes of 1xTBS containing 0.1% Tween, and two washes of 1xTBS, each for 5 minutes. A secondary incubation was then conducted for 2 hours at room temperature with the required secondary antibody (see Table 2 for secondary antibody concentrations). The blots were once again washed utilizing the same cycle as previously described and immune complexes were visualized via Luminata Forte Western HRP Subtrate (Millipore WBLUF0100) and hyperfilm (GE Healthcare 28906839) following the manufacturer's protocols.

Table 1: Primary Antibody Dilutions

Antibody	**Application**	**Dilution**
p20K (601-Y- Bédard Lab)	Western Blotting	1:1000
p20K (601-Y - Bédard Lab)	Immunofluorescence	1:300
Chop (Tulip Bleed 2 - Bédard Lab)	Western Blotting	1:1500
C/EBPβ (8582 - Bédard Lab)	Western Blotting	1:1000
ERK2 (Millipore #05-157)	Western Blotting	1:1000
ERK2 (Millipore #05-157	Immunofluorescence	1:200
β-Tubulin (Sigma T8328)	Western Blotting	1:50,000

Table 2: Secondary Antibody Dilutions

Antibody	Application	Dilution
Anti-Rabbit IgG, HRP-Linked Antibody (Cell Signaling #7074S)	Western Blotting	1:25000
Anti-Mouse IgG, HRP-Linked Antibody (Cell Signaling #7076)	Western Blotting	1:25000
Anti-Goat IgG, HRP-Linked Antibody (Santa Cruz 2378)	Western Blotting	1:25000
Anti-Rabbit IgG FITC (ab6717, Abcam)	Immunofluorescence	1:100
Anti-Mouse IgG CF594 (20110, Biotium)	Immunofluorescence	1:100

3. Proliferation Assays

Confluent CEF were split from 100 mm plates into 24 well microtitre dishes and incubated in their respective conditions. Cells were treated with 1 mL of trypsin and diluted in 9 mL of ISOTON® II Diluent (Beckman Coulter 8546719). Cells were counted in quadruplicate samples for statistical significance utilizing a Beckman Coulter model Z2 (Coulter Corporation, Miami, FL) Coulter counter.

4. Retroviral shRNAi vector construct generation

The target sequences of the ERK2 and p20K genes were chosen using the shRNAi design tool at www.genescript.com/ssl-bin/app/rnai. Hairpins for the first miRNA cloning site (*NheI/MluI*) of the pRFPRNAiC(U6-) miRNA cassette were generated by polymerase chain reaction (PCR) utilizing 10 ng of the respective gene-specific oligonucleotide along with 100 ng of two flanking oligonucleotides A and B (see Table 3 for Oligo Design) and

Q5 High-Fidelity 2X Master Mix (NEB M0492S). Oligonucleotides C and D were used to generate short hairpins for ERK2 and oligonucleotides E and F were used to generate short hairpins for p20K. The PCR protocol for amplification was as follows: 5 minutes at 95°C followed by 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 45 seconds with a total of 25 cycles. PCR amplification was done utilizing an Applied Biosystems GeneAmp PCR System 2700. PCR products were cleaned utilizing the GenElute™ PCR Clean-Up Kit (Sigma NA1020) following the manufacturers instructions. Purified PCR products were subsequently digested at the *NheI* and *MluI* restrction sites and subcloned into the pRFPRNAiC(U6-) miRNA cassette. The miRNA expression cassettes were further subcloned into the RCASBP(A)-RNAi and RCASBP(B)-RNAi vectors utilizing *NotI*-*ClaI* digestion.

Table 3: Oligonucleotide sequences used for shRNA vector generation

A	5'-GGCGGGGCTAGCTGGAGAAGATGCCTTCCGGAGAGGTGCTGCTGAGCG-3'
B	5'-GGGTGGACGCGTAAGAGGGGAAGAAAGCTTCTAACCCCGCTATTCACCACCACTAGGCA-3'
C	5'-GAGAGGTGCTGCTGAGCGTGCTTCAGGCATGAGAATATTATAGTGAAGCCACAGATGTA-3'
D	5'-ATTCACCACCACTAGGCAGCGTTCAGGCATGAGAATATTATACATCTGTGGCTTCACT-3'
E	5'-GAGAGGTGCTGCTGAAAGATGAAGATGGTAATGGTAGTGAAGCCACAGATGTA-3'
F	5'-ATTCACCACCACTAGGCAGGCAAGATGAAGATGGTAATGGTACATCTGTGGCTTCACT-3'

5. Luciferase Assay

DNA precipitations were prepared at a total of 30 μg per 100 mm plate of CEF being transfected. 10 μg of the desired pGluc-derived reporter construct and 5 ug of a RSV-βgal control plasmid (described previously (Dehbi *et al*, 1992) were brought up to 30 μg with salmon sperm carrier DNA. The DNA solution was incubated with 0.2M NaCl and two times the total volume of 100% ethanol. The precipitate was incubated overnight at -20°C and centrifuged and washed with ice cold 70% ethanol the following morning. The DNA pellet was vacuum dried and resuspended in 30 μL of ddH_2O and 270 μL of 1xTBS and added dropwise into 120 μL of DEAE-Dextran (10 mg/mL in 1xTBS).

Culture medium was substituted with 6 mL of cosmic calf serum free media prior to the solution being added to the plate. The DEAE-Dextran DNA solution was then added to the plate and allowed to incubate for a minimum of 5 hours. After 5 hours, cells were shocked with a 10% dimethyl sulfoxide (DMSO) solution in 1xHBSP (7.5 mM Na_2HPO_4, 5 mM KCL, 140 mM NaCl, 6 mM Glucose, 25 mM HEPES, pH 7.12) and then washed twice with complete DMEM medium before being supplemented with 9 mL complete DMEM. Cells were split the following day and once adhered half the cells were placed in hypoxic conditions while the other half were placed in normoxic conditions for a period of 24 hours. CEF were lysed in 100 μL of 250 mM Tris (pH 6.8) and 1% NP-40. 10 μL of lysate was assayed for *Gaussia* luciferase activity using the *Gaussia* luciferase assay kit (NEB E3300S) as per the manufacturer's recommendations. Luminescence was quantified on a Berthold Lumat 9501 luminometer. Samples were subsequently normalized via β-gal normalization. For each β-galactosidase reaction, 20 μL of cell lysate was incubated with 3 μL of 100X magnesium buffer (100 mM $MgCl_2$, 5 M 2-mercaptoethanol), 211 μL of 0.1 M Na_2HPO_4 pH 7.3 and 66 μL of o-nitrophenyl-β-D-galactopyranoside (ONPG) at 37°C until a yellow coloration was achieved (approximately 30 minutes). A control sample of 20 μL of 250 mM Tris-HCL pH 8 was utilized in substitute for 20 μL of lysate. The reaction was terminated with 0.5 mL of 1 M Na_2CO_3 and β-galactosidase activity was analyzed colorimetrically at an OD of 410 nm.

6. Live Cell Nitroreductase Activity Assay

Nitroreductase activity was assayed for utilizing a Cyto-ID® Hypoxia Stress Detection kit provided by ENZO® (ENZ-51042, Enzo Life Sciences, Farmingdale, NY

11735, USA). Cells were seeded onto glass coverslips and either incubated in hypoxia, incubated with 0.2 mM deferoxamine (DFO) for 4 hours as a positive control, incubated in normoxia or contact inhibited and subsequently treated with a hypoxia red detection reagent at a concentration of 0.5μM for 30 minutes. The glass coverslips were then mounted onto slides in a drop of PBS and cells were imaged via a fluorescent microscope at a Ex/EM of 596/670nm.

7. ChIP Assays

7.1 Sample Preparation

Cells were fixed utilizing 1% formaldehyde in 1xPBS for 10 minutes and the remaining formaldehyde was quenched utilizing 1.25 M glycine for 5 minutes. Cells were washed and harvested in 1xPBS, centrifuged at 7000 RPM (4000 RCF) for 3 minutes, and the remaining PBS was removed. Cell pellets were resuspended in 1 mL of SDS ChIP Lysis Buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.1, 1xHalt protease phosphate inhibitor cocktail (Pierce, USA)) and stored at -80°C if needed.

7.2 Immunoprecipitation

Aliquots of 500 μL were sonicated utilizing a Sonifier Cell Disruptor 350 at 50% output for a total of 6 minutes at 20 second pulses. Samples were pre-cleared utilizing salmon sperm DNA blocked Protein A beads (Millipore #16-757) and diluted to a final volume of 1mL utilizing ChIP Dilution Buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCL pH 8.1, 167 mM NaCL, and Halt inhibitor cocktail (Pierce, USA)). A 10 μL input sample was set aside and the remaining chromatin solution was immunoprecipitated using 2 μg of Mouse IgG, ERK2, C/EBPβ, and rabbit pre-immune

serum antibodies overnight at 4°C. Following the overnight incubation, antibody complexes were precipitated utilizing blocked salmon sperm DNA blocked Protein A beads (Millipore #16-757) for 1 hour (Note: 2 μg of rabbit α-mouse IgG (Jackson Immunoresearch #315-005-003) was added to all samples to increase mouse antibody affinity for protein A beads). Following incubation, beads were centrifuged and subjected to a series of washes: one wash with Low Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCL pH 8.1, 150 mM NaCl), one wash with High Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCL pH 8.1, 500 mM NaCl), once with LiCl Immune Complex Wash Buffer (0.25M LiCl, 1% NP-40, 1%deocycholic acid, 1 mM EDTA, 10 mM Tris-HCL pH 8.1) and twice with TE Buffer (10 mM Tris-HCL, 1 mM EDTA pH 8.0). Following washes, bound complexes were eluted using 200 μL of Elution Buffer (0.1 M $NaHCO_3$, 0.005% SDS). Samples were de-crosslinked at 65°C, and subjected to RNA and protein digestion using 2 μg of RNse A and Protinase K respectively. The DNA was ethanol precipitated and resuspended in 20 μL of 1xTE buffer prior to PCR amplification.

7.3 PCR Amplification

PCR amplifications for immunoprecipitated chromatin were done on both the QRU and a control which consisted of the first intronic region of the p20K gene. The following primers were used for amplification:

p20K Intron Forward: 5'-GGTGTGCTGAGTATTTGAGGTG-3'

Reverse: 5'-AAATTACTCTGGGGGCTGA-3'

QRU Forward: 5'-CATCCCCTCTTCATTCTCCA-3'

Reverse: 5'-CACTGCTATTGTTGGCATGG-3'

PCR reactions were carried out in a total of 25 μL consisting of 12.5 μL 2X GoTaq PCR Mix (Promega M782B), 1 μL of template DNA, and 1mM forward and reverse primers. The PCR program used for amplification was as follows: 94 °C for 5 minutes followed by 55 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 35 and finished with a 10 minute incubation at 72 °C. Following amplification, PCR products were resolved on a 1.2% agarose gel and visualized in a UV transilluminator.

8. Immunoflourescence

Cell cultures were first seeded onto glass slides in 60 mm plates and incubated in their respective conditions. The slides were washed twice in 1xPBS and subsequently fixed utilizing 3.7% formaldehyde in 1xPBS for 10 minutes at room temperature. Cells were then permeabilized utilizing 0.1% Triton X-100 in 1xPBS on ice for 5 minutes. Cells were then blocked in 5% fetal bovine serum in 1xPBS for 1 hour and subsequently incubated with the desired primary antibody (see Western blotting section for antibody dilutions) overnight at 4 °C. The following morning, cells were washed 2 times with 1xPBS and incubated with the respective secondary antibody for 2 hours at room temperature in the dark. The secondary antibodies used in this study are described in Table 2. Following secondary incubation, cells were washed twice with 1xPBS and incubated with DAPI as a nuclear stain (ThermoFischer D1306) at a concentration of 300 nM for 5 minutes in the dark. Cells were finally washed twice with 1xPBS, fixed on coverslips with Aqua-Mount (Lerner Laboratories #13800) and imaged.

9. shRNAi and Overexpression Vector Transfection

A total of 30 μg of DNA precipitate was prepared for each 100 mm cell culture dish that needed to be transfected. The $CaPO_4$ method of transfection has been described previously (Graham FL & van der Eb AJ, 1973). Essentially 10 μg of the plasmid construct was brought up to a total of 30 μg with salmon sperm carrier DNA and incubated in 0.2 M NaCl. Twice the total volume of 100% ethanol was added to the sample and it was left to precipitate overnight at -20°C. The precipitated DNA was washed with 70% ethanol, vacuum dried and resuspended in 30 μL of ddH_2O. 62 μL of 2M $CaCl_2$ was added and the mixture was brought up to a total volume of 500 μL with ddH_2O. The sample was added dropwise to 500 μL of 2xHBSP (15 mM Na_2HPO_4, 10 mM KCL, 280 mM NaCl, 12 mM Glucose, 50 mM HEPES, pH 7.12) and left to incubate for 30 minutes (Note: Four hours prior to transfection, the media was replaced with 6mL of fresh complete media). After the 30-minute incubation, the DNA mixture was added dropwise to the cells in 100 mm culture dish and left to incubate for a minimum of 5 hours. Following incubation, cells were shocked for 1 minute with 4 mL of a glycerol shock solution of 15% glycerol in 1xHPSP. The shocking solution was removed and followed by two washes with complete medium. The media was replaced and cells were incubated in normoxic conditions overnight. The following morning the cells were split and cultured for four passages before they were analyzed.

10. Live Cell Imaging of Lipid Peroxidation

Cells seeded on coverslips were incubated in conditions of normoxia or hypoxia for a period of 36 hours. The fluorescent dye and indicator of lipid oxydation C-11

BODIPY$^{581/591}$ (2.5μM; ThermoFisher Scientific D3861) was added to the medium for a period of 30 min at 37°C while nuclei were stained with Hoechst 33342 for 10 minutes (ThermoFisher H1399). After washing in PBS, the cover slips were mounted on a drop of PBS and observed.

11. Malondialdehyde (MDA) Detection Assay

Lipid peroxidation was quantified though detection of malondialdehyde. Cells were incubated in conditions of normoxia or hypoxia for 36 hours and malondialdehyde levels were determined using commercially available reagents (Lipid Peroxidation (MDA) assay kit; Abcam ab118970). After the required incubation time cells were washed extensively in PBS, counted and collected by centrifugation. Cells were then resuspended in 300 μL of MDA lysis buffer containing butylated hydroxytoluene (BHT) and lyzed in a Dounce homogenizer on ice. Cell debris was eliminated by centrifugation at 13000 RPM (13800 RCF) for 10 minutes and levels of malondialdehyde were determined in 200 μL aliquots of the supernatant by adding 600 μL of thiobarbituric acid (TBA) and incubated at 95°C for 60 minutes followed by a cooling period of 10 min in an ice water bath. Samples were read in a spectrophotometer at 532nm and MDA concentrations were calculated using a standard curve which was prepared in the same manner stated above utilizing 20-100 μM MDA standard. MDA levels were compared between samples utilizing average cell counts.

12. Electrophoretic Mobility Shift Assay

12.1 Recombinant ERK2 Generation

The protocol for producing recombinant ERK2 was based off a protocol described

previously by Hu *et al*., 2009. Essentially, yeast strain B7471 was transformed with a yeast high-copy expression vector that produces GST-His_6 fusion proteins. Two vectors were transformed which contained the human ERK2 sequence (pEGH-A-ERK2) and the human MEK1 sequence to activate ERK2 (pEGH-A-MEK1) utilizing a lithium chloride transformation protocol (Ito *et al*., 1983). Transformed cultures were grown in SC-ura/glucose media and transferred to SC-ura/raffinose media after which they were induced using 2% galactose for 4 hours. Cells were lysed utilizing glass beads (Sigma G8772) suspended in lysis buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1 mM EGTA, 10% glycerol, 0.1% Triton X-100, 0.1% beta-mercaptoethanol, and Halt protease phosphatase inhibitor cocktail (Pierce, USA)). Recombinant proteins were then purified utilizing glutathione-agarose beads (Sigma G4510) and washed utilizing wash buffer I (50 mM Tris pH 7.5, 500 mM NaCl, 1 mM EGTA, 10% glycerol, 0.1% Triton X-100, 0.1% beta-mercaptoethanol and Halt protease phosphatase inhibitor cocktail (Pierce, USA)) and buffer II (50 mM HEPES pH 7.5, 100 mM NaCl, 1 mM EGTA, 10% glycerol, 0.1% beta-mercaptoethanol and Halt protease phosphatase inhibitor cocktail (Pierce, USA)) and eluted utilizing elution buffer (50 mM HEPES pH 8, 100 mM NaCl, 30% glycerol, 40 mM glutathione, 0.03% Triton X-100). Samples were aliquotted and stored at -80°C until needed.

12.2 Probe Generation

Single stranded oligonucleotides were biotin labeled using a Biotin 3' End DNA Labeling kit (89818, Pierce, USA) as per the manufacturer's protocol. Following biotinylation, equal volumes of complementary oligonucleotides were added into a single

reaction for annealing. The reaction mixture was heated to 90°C for 1 min and then allowed to cool slowly to room temperature. Once cooled, the reaction was frozen and stored at -20°C until use. The oligos utilized for the wild-type QRU and mutant QRU are as follows:

WT QRU: ACACTTTCCTCTTTCCGTAAGC

TGTGAAAGGAGAAAGGCATTCG

MT QRU: ACACTTACCTCATTCCGTAAGC

TGTGAATGGAGTAAGGCATTCG

12.3 EMSAs

Binding reactions were carried out using 100 fmol of biotinylated dsDNA probe and 500ng of purified protein in 20 μL of binding buffer (10 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 25% Glycerol, 1 mM DTT and Halt protease phosphatase inhibitor cocktail (Pierce, USA)). In order to eliminate non-specific binding, reaction mixtures were first incubated with 2 μg of poly dI-dC on ice for one hour. 100 fmol of biotinylated dsDNA probe was then added and the reaction mixture was incubated at room temperature for 30 mins. For competition assays, 25 pmol (a 250 fold excess) of unlabeled (cold) DNA was added. Reaction mixtures were loaded onto a 4.8% non-denaturing polyacrylamide gel and run in 0.5 x TBE at 100 volts at 4°C. Nucleic acids were then transferred onto a nylon membrane in 0.5x TBE. Following transfer, the membrane was crosslinked using a 312nm UV lamp for 10 min. Visualization was carried out utilizing a HRP-conjugated streptavidin Chemiluminescent Nucleic Acid Detection

Module kit (Pierce, USA) and hyperfilm (GE Healthcare #2890683) as per the manufacturers protocols.

13. TUNEL Assays

Cells were seeded onto glass coverslips in 60 mm dishes and incubated in their respective conditions for 36 hours. Cells were fixed utilizing 4% formaldehyde in 1xPBS for 1 hour at room temperature and were subsequently permeabilized utilizing 0.1% Triton X-100 in 0.1% sodium citrate. Cells were then washed 3 times with 1xPBS and incubated with terminal deoxynucleotidyl transferase (TdT) and TMR red-fluorescent labeled dUTP provided by Roche (12156792910) for 1 hour at 37°C. Samples were mounted onto glass slides with a drop of 1xPBS. Several fields of each sample were taken such that a minimum of 100 cells per sample were counted. Each condition was analyzed in quadruplicate.

14. Lipid Staining

Cells were seeded onto glass coverslips in 60mm dishes and incubated in their respective conditions for 36 hours. Cells were fixed utilizing 10% formaldehyde in 1xPBS for 10 minutes at room temperature. The lipophilic Oil Red O dye (Sigma-Aldrich 09755) was prepared by creating a 0.5% Oil Red O solution in 100% isopropanol and further diluting this stock 3:2 in ddH_2O. Cells were then treated with the diluted Oil Red O dye for 15 minutes at room temperature and washed multiple times in ddH_2O. Cells were mounted onto glass slides utilizing a drop of 1xPBS and imaged.

***Note:** All experiments were conducted in triplicate with the exception of cell counts and imaging, which were done in quadruplicate.

Results

Chapter 1: ERK2 and CHOP restrict the expression of the growth-arrest specific p20K lipocalin gene to G_o

The C/EBPβ transcription factor has been shown to play roles in many cellular processes in various cellular contexts. Our preliminary results indicate that p20K is induced in states of reversible growth arrest, and this activation is dependent on C/EBPβ binding to the p20K promoter. Interestingly, p20K is shown to be repressed within states of v-src transformation as well as during cell proliferation, two conditions in which we know C/EBPβ is active. As such, we wished to determine the specificity of gene activation of C/EBPβ and analyze the elements mediating the transcriptional repression of p20K in key cell contexts. Within this chapter we demonstrate that two repressors control the activity of C/EBPβ. In cycling states, the ERK2 kinase binds and represses p20K expression through direct DNA binding. In states of ER stress or starvation the ER stress inducible protein CHOP blocks p20K expression through the interaction with C/EBPβ. We additionally show that contact inhibited cells exhibit hypoxia specific gene signatures as well as nitroreductase activity, which is a marker of hypoxia. These results suggest that p20K is induced in a hypoxia specific manner.

Preliminary work on this chapter was conducted by Dora Camacho, Wenli Xie, Bart Maslikowski, Ben Fielding, Romita Ghosh, Flore-Anne Poujade, Mohammed Athar, and Laura Mantella. For my portion of the chapter, I characterized the expression of p20K and CHOP leading to starvation (Chapter 1 Figure 1A), analyzed nitroreductase activity in confluent cells (Chapter 1 Figure 2B-I), assessed the proliferation of cells in normoxia

and hypoxia (Chapter 1 Figure 3A), analyzed the levels of p20K expression in CHOP shRNA samples under Tunicamycin and Thapsigargin treatment (Chapter 1 Figure 6B), characterized ERK2 binding to the QRU and its function as a transcriptional repressor (Chapter 1 Figure 7, Figure 8J), analyzed the effect of ERK2 recruitment to the QRU when overexpressing C/EBPβ (Chapter 1 Figure 8A-G), and characterized the effect of MEK inhibition on the expression of p20K in both normal (Chapter 1 Figure 8H) and ts NY Rous-Sarcoma Virus transformed cells (Chapter 1 Figure 9). I additionally conducted all experiments to generate the data shown in Supplementary Figures 1-3. Lastly, I aided in the writing and editing of this manuscript for publication alongside Dr. Pierre-André Bédard.

ERK2 and CHOP restrict the expression of the growth-arrest specific p20K lipocalin gene to G_0

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ABSTRACT

The activation of the growth-arrest specific (gas) p20K gene depends on the interaction of C/EBPβ with two elements of a 48 bp promoter region termed the quiescence-responsive unit or QRU. Here we identify the ERK2 kinase as a transcriptional repressor of the p20K QRU in cycling chicken embryo fibroblasts. ERK2 binds to repeated GAAAG sequences overlapping the C/EBPβ sites of the QRU. The recruitment of ERK2 and C/EBPβ is mutually exclusive and dictates the expression of p20K. The C/EBP Homologous Protein CHOP is associated with C/EBPβ in conditions promoting ER stress and, to a lesser extent, in cycling CEF but is not detectable when C/EBPβ is immunoprecipitated from contact-inhibited cells. Forced expression of CHOP inhibited while its down-regulation promoted p20K during ER stress, indicating that CHOP is also a potent inhibitor of p20K. Transcriptome analyses revealed that hypoxia-responsive genes are strongly induced in contact inhibited but not serum-starved CEF and elevated levels of nitroreductase activity, a marker of hypoxia, were detected at confluence. Conditions of hypoxia (2% O_2) induced growth arrest in sub-confluent CEF and markedly stimulated p20K expression suggesting that the control of proliferation and gas gene expression is closely linked to limiting oxygen concentrations associated with high cell density.

INTRODUCTION

In response to unfavorable conditions of proliferation, cells exit the cell cycle and enter a state of reversible growth arrest known as G_0. The G_0 state of quiescence is characterized by the activation of a group of genes referred to as growth arrest-specific or "gas" genes (1). The products of gas genes contribute directly to growth arrest (2, 3), enhance survival in conditions of oxidative stress (4), are components of the extra-cellular matrix (5-7), are involved in lipid metabolism (8-11) and prepare the cell to reenter the cell cycle (12, 13). Our understanding of the signals and mechanisms regulating the expression of gas genes is incomplete. A subset of gas genes is regulated at the post-transcriptional level while others depend on transcriptional activation for expression in conditions of contact inhibition or serum starvation (6, 14-18).

We previously characterized the induction of the p20K lipocalin gene by cell density. In chicken embryo fibroblasts (CEF), p20K is expressed predominantly at confluence and, to a lesser extent, in response to serum/medium depletion (17, 19). Transcriptional activation of the p20K gene depends on a 48 bp region of the promoter, termed the "Quiescence-Responsive Unit" or QRU (17). C/EBPβ binds to two elements of the QRU and is sufficient to induce the expression of p20K when over-expressed in cycling cells (20).

C/EBPβ is activated in response to several stimuli and plays an important role in biological processes unrelated to growth arrest. For instance, we reported that the activity of C/EBPβ is induced in CEF transformed by the Rous sarcoma virus (RSV) i.e. in conditions where p20K is not expressed (21). Mitogenic stimulation also controls the

activity of C/EBPβ and promotes the expression of genes of the G_0/G_1 transition. AP-1, a factor controlling the expression of IL8 and cyclin D1, inhibits the induction of p20K and plays a major role in this process (22). Cells over-expressing c-Jun, JunD or Fra-2, the main components of AP-1 in cycling CEF, do not express p20K, are unable to enter G_0 and undergo apoptosis at high cell density. Normal CEF entering G_0 down-regulate the activity of AP-1 by a number of mechanisms that include the repression of c-Jun, JunD and Fra-2 expression. The expression of a dominant negative mutant of C/EBPβ blocks the induction of p20K and enhances the activity and expression of AP-1 proteins in CEF. Therefore, AP-1 and C/EBPβ play opposing roles in the expression of gas genes in CEF (22).

Despite these advances, the signals promoting the transcriptional activation of p20K and the factors interacting or antagonizing C/EBPβ in this process remain poorly understood. In this report, we identify ERK2 as a transcriptional repressor binding directly to the QRU in cycling cells and competing with C/EBPβ for recruitment to the p20K promoter. Heterodimers consisting of the "C/EBP Homologous Protein" CHOP and C/EBPβ were prominent in conditions of ER stress but were also detected, albeit at lower levels, in actively dividing cells. In contrast, CHOP-C/EBPβ dimers were absent in density arrested CEF. Forced expression of CHOP reduced p20K levels in growth-arrested cells while down-regulation of CHOP reestablished p20K expression in conditions of ER stress. Therefore, CHOP also functioned as a factor limiting p20K expression. Here we characterize the transcriptome of cycling CEF and CEF rendered quiescent by serum starvation or contact inhibition. While genes induced by starvation

were identified in these analyses, we focus the present studies on the program of contact-inhibited cells. As determined by gene profiling, hypoxia-responsive genes (CAIX, CAXII, Enolase 2) were induced markedly by contact inhibition but not starvation. Hypoxia (2% oxygen) was a potent inducer of growth arrest and p20K expression in CEF. Moreover, high cell density reduced oxygen availability as indicated by the activity of nitroreductase, a marker of hypoxia. Therefore, the expression of p20K is restricted to G_0 by ERK2 and CHOP, and is induced by conditions promoting reversible growth arrest such as high cell density and limiting oxygen concentrations.

MATERIALS AND METHODS

Cell culture. Early passages (n<10) of CEFs were cultured at 41.5ºC in "complete medium" consisting in DMEM with 5% heat-inactivated (at 57ºC for 30 minutes) "Cosmic" calf serum (CCS, Hyclone SH30067.03, Logan, UH), 5% tryptose phosphate broth, 2 mM L-glutamine, 0.2 mg/ml streptomycin and 0.2 U/ml penicillin (Life Technologies). CEFs were also starved in DMEM medium without the serum (serum-free medium) after being washed twice with the serum-free medium. Nutrient depletion was also induced by culturing cells in complete medium over several days without nutrient/medium replenishment. Hypoxia was induced by culturing CEF for 24 hrs in 1-2% O_2 as described in the text. CEF were treated with the MEK inhibitor PD184352 (0.1-2 mM; Sigma-Aldricht #CI-1040). ER stress was induced by treating CEF with 1 μg/ml tunicamycin or thapsigargin (Sigma-Aldrich # T7765 and T9033, respectively) for 12-48 hrs.

Live cell detection of nitroreductase activity. Nitroreductase activity was used as an indicator of hypoxia in CEF and examined by fluorescence microscopy using commercially available reagents (ENZ-51042, Enzo Life-Sciences, Farmingdale, NY 11735, USA). Subconfluent CEF in conditions of normoxia or subjected to 2% O_2 for 24 hrs were analyzed and compared to density-arrested CEF. CEF treated with 0.2 mM of the hypoxia-mimetic desferrioxamine (DFO) were used as a control in these experiments.

Proliferation assays. Cells were collected by treatment with trypsin. 200 μL aliquots of the cell suspension in 10 mL of diluent were counted in a Coulter counter (lower limit was set to 10 μm). The averages of triplicated samples were used to determine proliferation. The error bars represent the standard deviations of the mean.

Construction of retroviral vectors for CHOP down-regulation by shRNA. The 21 nucleotide target sequences of the CHOP gene were chosen using the design tool at www.genscript.com/ssl-bin/app/rnai. The 5' base of the sense strand was altered in all cases so that it mismatched the guide strand base to mimic the structure found in endogenous miRNA30. Results described in this report were obtained with a shRNA construct targeting nucleotides 640-660 of the CHOP mRNA (designated "640"; target sequence is underlined in Table 1) but were replicated with a separate shRNA construct targeting nucleotides 387-407 (designated '387", underlined in Table 1; data not shown). Hairpins for the first miRNA cloning site were generated by PCR using 10 ng of each gene-specific oligonucleotide A (A_{640} or A_{387}) and B (B_{640} or B_{387}) together with 100 ng of two generic flanking oligonucleotides C and D in a 50 ml reaction using ProofStart

polymerase (Qiagen; Table 1). PCR conditions were 5min at 95° followed by 25 cycles of 30s at 94°, 30s at 55° and 45s at 72° using GeneAmp PCR system 2700 (Applied Biosystems). PCR products were purified, digested with *Nhe*I and *Mlu*I and subcloned into pRFPRNAiC(U6-). The miRNA expression cassettes for CHOP were then subcloned from pRFPRNAiC(U6-)-CHOP into a modified RCASARNAi vector as a *Not*I-*Cla*I fragment (ARK-Genomics;(23) (24)).

Table 1 : Sequence of oligonucleotides used for preparation of shRNA vectors

A640	GAGAGGTGCTGCTGAGCGACAGCTGAGTGCACACAACGAGTAGTGAAGCCACAGATGTA
A387	GAGAGGTGCTGCTGAGCGTGGGGGTGCACAGCAGGAAGATAGTGAAGCCACAGATGTA
B640	ATTCACCACCACTAGGCAGCAGCTGAGTGCACACAACGAGTACATCTGTGGCTTCACT
B387	ATTCACCACCACTAGGCACGGGGGTGCACAGCAGGAAGAGTACATCTGTGGCTTCACT
C	GGCGGGGCTAGCTGGAGAAGATGCCTTCCGGAGAGGTGCTGCTGAGCG
D	GGGTGGACGCGTAAGAGGGGAAGAAAGCTTCTAACCCCGCTATTCACCACCACTAGGCA

Northern blotting and RT-qPCR analyses. Northern blotting was performed as described before (22). For RT-qPCR analyses, RNA samples were treated with DNase I, reverse transcribed and analyzed using commercially available reagents (ProtoScript cDNA synthesis kit, New England Biolabs; PerfeCTa SYBR Green FastMix low ROX, Quanta Biosciences), a real-time PCR instrument from Stratagene (MX3000P) and the following primers:

CA9 Carbonic Anhydrase IX

F : TAGGTTGGGCCAAGGGAGAACCC (n. 5-27)
R : CGGCAATGTTGAACCCGGGCA (n. 110-90)

CA12 Carbonic Anhydrase XII

F : GGCAAGGATCCTCCACCACACCT (n. 748-770)
R : TCGCCGGAGATGCCTTTCCG (n. 876-857)

ENO2 Enolase II
F : CCCCCTGCAGGTCTGAACGC (n. 1276-1295)
R : AGTTGTGTCCAGCAAAGCGTGCT (n. 1368-1346)

p20K p20K Lipocalin
F : GCCCAGCCAGGAGGAATGCA (n. 519-538)
R : AGCAGCCTCGAGCTTTGGCA (n. 618-599)

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

Western blotting analysis and immunofluorescence microscopy. Western blotting and immune-fluorescence analyses were performed as described before (22) using the following primary antibodies:

Chicken p20K	Dilution 1:2000 (Western), 1:300 (Immuno-fluorescence; previously described (19))
Chicken CHOP	Dilution 1:1500; previously described (25)
Chicken C/EBPβ	Dilution 1:1500; previously described (21)
ERK2	Dilution 1:2000 (Western), 1:100 (Immuno-fluorescence; Clone 1B3B9, Millipore #05-157).
HIF1a	Dilution 1:500; ab2185, Abcam
β-Tubulin	Dilution 1:50,000, T7816, Sigma-Aldrich (St-Louis, MO)

Following several washes, the blots were incubated with a 1:25,000 dilution of a secondary anti-rabbit, anti-mouse, or anti-goat IgG antibody conjugated with horseradish peroxidase (HRP) at room temperature in 5% milk TBS. Chemiluminescent signals were revealed by incubation with the HRP substrate Luminata Forte according to the protocol provided by the supplier (Millipore WBLUF0100).

For immunofluorescence microscopy, cells were first seeded onto glass slides in 60mm plates and subjected to various conditions of normoxia or hypoxia. Cells were fixed utilizing 3.7% formaldehyde in PBS for 10 minutes at room temperature. Cells were permeabilized with 0.1% Triton X-100 in PBS on ice for 5 minutes, incubated in 5% fetal bovine serum in PBS for 1 hour and finally incubated with the desired primary antibody overnight at 4 °C (Table 1). Anti rabbit IgG FITC or Anti mouse IgG CF594 secondary antibodies at a dilution of 1:100 were used in these experiments. Nuclei were stained with DAPI (ThermoFischer D1306) at a concentration of 300nM for 5 minutes in the dark.

EMSAs. Electrophoretic mobility shift assays were performed as described before (22). To generate probes, single stranded oligonucleotides were labelled with a Biotin 3' End DNA Labeling kit (Pierce, USA) as per the manufacturer's protocol. Binding reactions were carried out using 100 fmol of biotinylated dsDNA probe and 500 ng of purified protein in 20 uL of binding buffer (10 mM Tris pH 7.5, 100 mM NaCl, 1mM EDTA, 25% Glycerol, 1mM DTT and Halt inhibitor cocktail (Pierce, USA). Recombinant ERK2 was produced in yeast as described by Hu and co-investigators (26). The yeast strain B7471 was transformed with a high-copy expression vector encoding GST-His_6 fusion proteins. Separate vectors encoding human ERK2 (pEGH-A-ERK2) and human MEK1 (pEGH-A-MEK1) were co-transformed to generate activated ERK2.

ChIP Assays. Cells were fixed in 1% formaldehyde for 10 minutes followed by the addition of 1.25 M glycine. Cell were collected and resuspended in 1 ml of SDS Lysis Buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.1, 1X Halt protease phosphatase inhibitor cocktail (Pierce, USA). Aliquots of 400 uL were then sonicated for a total of 6 minutes in 20 sec pulses. Samples were pre-cleared using salmon sperm DNA blocked Protein A beads (Millipore #16-757) and diluted to a 1ml final volume using dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCL pH 8.1, 167 mM NaCl, and Halt inhibitor cocktail (Pierce, USA). A sample was saved and the remaining chromatin solution was immunoprecipitated using antibodies (2 ug) described in Table 1. Antibody complexes were pulled down using blocked Protein A beads for 1 hour (Note: 2 ug of rabbit α-mouse IgG (Jackson Immunoresearch #315-005-003) and subjected to a series of washes; once with low salt immune complex wash buffer (0.1%

SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCL pH 8.1, 150 mM NaCl), once with high salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20 mM Tris-HCL pH 8.1, 500 mM NaCl), once with LiCl immune complex wash buffer (0.25 M LiCl, 1% NP-40, 1% deocycholic acid, 1mM EDTA, 10 mM Tris-HCL pH 8.1) and twice with TE buffer (10 mM Tris-HCL, 1 mM EDTA pH 8.0). Following washes, bound complexes were eluted using 200 uL of elution buffer (0.1 M $NaHCO_3$, 0.005% SDS). Samples were then de-crosslinked over night at 65°C and treated with RNase A and Proteinase K. The DNA was ethanol precipitated and resuspended in TE buffer. PCR amplification of the QRU and a control region located within intron 1 of the p20K gene was performed using the following primers:

QRU Forward:	5'-CATCCCCTCTTCATTCTCCA-3'
QRU Reverse:	5'-CACTGCTATTGTTGGCATGG-3'
p20K Intron Forward:	5'-GGTGTGCTGAGTATTTGAGGTG-3'
p20K Intron Reverse:	5'-AAATTACTCTGGGGGCTGA-3'

Transient Expression Assays. Chloramphenicol acetyl transferase assays were performed as described before (22). The ^{14}C-labelled chloramphenicol species were imaged using a Typhoon scanner (Trio GE Healthcare), quantified using ImagQuant software and analyzed in a t-test. For luciferase assays, pGluc-derived reporter constructs and a RSV-βgal control plasmid were co-transfected into normal CEF utilizing the DEAE-dextran method for six hours and shocked using 10% DMSO-PBS shock for 2 minutes (27). Cells were split the following day and placed in conditions of normoxia or hypoxia (2% oxygen) for a period of 24 hours. CEF were then lysed in 100 ml of 250mM

Tris (pH 6.8) and 1% NP-40. Ten ml of lysate was assayed for *Gaussia* luciferase using the *Gaussia* luciferase assay kit as per the manufacturer's recommendations (New England Biolabs). Samples were subsequently normalized by βgal activity.

RESULTS

Control of p20K expression by hypoxia and cell confluence. Confluent chicken embryo fibroblasts (CEF) express high levels of the growth arrest-specific p20K lipocalin (Fig.1A; (19)). However, this expression is transient and inhibited when cells are kept at confluence for several days without medium/nutrient replenishment. The down-regulation of p20K is associated with the induction of the CHOP transcriptional regulator, a marker of starvation and ER stress, and a progressive decline in cell numbers (Fig.1A and data not shown (25, 28)). Interestingly, cycling CEF expressed low levels of CHOP while contact inhibited CEF are characterized by nearly undetectable expression of this protein. Similar patterns of p20K and CHOP expression were observed at the mRNA level, with high levels of p20K transcripts observed in contact inhibited CEF and high levels of CHOP mRNA detected in serum-starved but not contact inhibited CEF (Fig.1B). Basal and low levels of CHOP transcripts were also found in cycling CEF. These results suggest that CHOP and p20K are components of different biological responses regulated by different pathways.

To identify processes associated with contact inhibition, we compared the transcriptomes of cycling, serum-starved and contact inhibited, serum-starved but subconfluent, and contact inhibited CEF in complete serum-containing medium (Fig.1C). All conditions of growth arrest led to extensive changes in gene expression but

differentially expressed genes were more prominent when contact inhibited CEF were compared to cycling cells (Supplementary Table 1). Several genes associated with the response to hypoxia, including carbonic anhydrase 9, carbonic anhydrase 12 and enolase 2, were markedly up-regulated in contact inhibited but not serum-starved CEF. The induction of this class of genes by confluence was confirmed by RT-qPCR (Fig.1 D).

Genes for HIF1α and several factors with pro-angiogenic activity, including FABP4, FABP5, prokinecitin 2, angiopoietin-like 5, SFRP2 (secreted frizzle-related protein 2) and C1QTNF3 (CTRP3), were also up-regulated at confluence suggesting that oxygen deprivation is a feature of the response to high cell density (Supplem. Table 2;(29-34)). As expected, genes involved in DNA replication and progression through the cell cycle [cyclin E2, cyclin M2, PLK3, DNA primase 2, ORC4 and ORC1 (origin recognition complex subunits 4 and 1, respectively), thymidine kinase 1] were down-regulated while the cyclin-dependent kinase inhibitors p18 (CDKN2C) and $p27^{Kip1}$ (CDKN1B) were induced in conditions of growth arrest.

The induction of hypoxia-responsive genes suggests that oxygen availability regulates the expression of growth-arrest specific genes such as p20K. In agreement with this model, p20K expression was induced in sub-confluent CEF incubated in low oxygen concentrations for 24 hrs (1-2% O_2 ; Fig.2A). Increased levels of cellular nitroreductase activity, an indicator of hypoxia, were observed in confluent CEF indicating these cells were under limiting oxygen concentrations (Fig. 2B-E). These conditions of hypoxia were not associated with ER stress as the basal levels of CHOP transcripts were reduced by a 24hr-incubation in 2% oxygen (Fig.2J-K).

Incubation in 2% O_2 inhibited CEF proliferation raising the possibility that the induction of p20K was not a direct effect of hypoxia but, instead, the result of growth arrest (Fig.3A). This was further examined by looking at the kinetics of p20K and HIF1α expression in response to incubation in 2% O_2. HIF1α accumulated considerably and rapidly in response to hypoxia i.e. within two hours of incubation in reduced oxygen concentrations (Fig.3B). In contrast, p20K expression was not detected in the first 12 hrs of incubation. CEF continued to accumulate for 18 hrs but entered quiescence within 25 hrs in 2% O_2 (Fig.3C). This entry into quiescence was marked by the induction of p20K. While hypoxia had similar inhibitory effects on CEF proliferation in medium containing calf or fetal bovine serum, p20K expression was maximally induced when growth factors were more limiting (in calf serum), suggesting that its expression was still affected by the presence of mitogens in the medium.

The results described above suggest that p20K accumulation in 2% O_2 is not a direct effect of hypoxia but the result of growth arrest induced by limiting oxygen concentrations. If this model is correct, the induction of p20K by hypoxia should reflect the QRU- and C/EBPβ-dependent activation of the p20K promoter, as observed in contact inhibited CEF (20). This was examined in transient expression assays with p20K promoter constructs. Interestingly, two potential hypoxia responsive elements (HRE) consisting of the canonical HIF binding site and its ancillary sequence are located upstream of the QRU at position -415 and -458 of the p20K gene transcription start site (Fig.4A; (35)). However, constructs lacking the putative HREs were fully inducible in conditions of 2% O_2 (Fig.4B-C). In contrast, deletion of the 48 bp QRU and C/EBP

binding sites abolished the activation of the p20K promoter by hypoxia, establishing the requirement for the QRU for induction in these conditions. Consistent with the importance of the QRU, the hypoxia-dependent accumulation of p20K was inhibited by the expression of LIP, the dominant negative form of C/EBPβ (Fig.4D). These results suggest that the QRU- and C/EBPβ–dependent induction of p20K reflects the promotion of growth arrest by hypoxia and is not a direct response to low oxygen concentrations.

ER stress antagonizes the density-dependent expression of p20K. The role of CHOP in the control of gene expression by C/EBPβ is context-dependent with CHOP acting as an inhibitor for some genes but cooperating with C/EBPβ in the activation of stress-responsive promoters with variants of the C/EBP element (36). The induction of p20K at confluence coincides with a reduction in the basal expression of CHOP (Fig.1A&5A). The down-regulation of CHOP by shRNA enhanced p20K expression at confluence but had no effect in cycling CEF. CHOP down-regulation also increased saturation density raising the possibility that the effect on p20K expression was indirect (our unpublished results). To alleviate this problem, CHOP was over-expressed in sub-confluent CEF and p20K expression was examined in conditions of hypoxia. A marked inhibition of p20K expression was observed when cells infected with a retrovirus encoding CHOP were incubated in 2% oxygen for 24 hrs (Fig.5 B-C).

Conditions resulting in strong activation of CHOP, such as ER stress induced by tunicamycin or thapsigargin, abolished p20K expression at confluence (Fig.6A). A partial expression of p20K was restored when CHOP levels were reduced by a shRNA in tunicamycin- but not thapsigargin-treated cells where higher levels of CHOP expression

were observed (Fig. 6B-C). Co-immunoprecipitation experiments confirmed that CHOP is associated with C/EBPβ during proliferation, starvation and ER stress but cannot be detected at contact inhibition where CHOP levels are lowest (Fig. 6D, lane 8). Reduced levels of the two trans-activating forms of C/EBPβ (designated LAP* and LAP) were also observed in cells treated with tunicamycin and thapsigargin (Fig.6D, lanes 9&10). These results suggest that CHOP functions as a potent inhibitor of p20K by interacting with C/EBPβ in various cellular contexts.

ERK2 binds to the QRU and inhibits the expression of p20K during proliferation. The down-regulation of CHOP by shRNAs enhanced cell proliferation and the expression of p20K at confluence but had no effects in cycling CEF (Fig.5A). Therefore, other factors may interfere with the transactivation of the p20K QRU by C/EBPβ. The central region of the QRU, located between but overlapping the two C/EBPβ binding sites, is similar to the ERK2 binding element described by Hu and co-investigators (26). ChIP assays confirmed that ERK2 is recruited to the p20K promoter in conditions of proliferation but is not detectable when CEF are growth-arrested in 2% O_2 or by contact inhibition (Fig.7B and data not shown). C/EBPβ showed the opposite pattern and was only detected in response to hypoxia and in the absence of ERK2 binding.

The association of ERK2 with the p20K promoter appears to be direct as recombinant ERK2 formed a complex with the central domain of the QRU in electrophoretic mobility shift assays (Fig.7C). An excess of the central domain oligonucleotide that included substitutions of key nucleotides within the candidate ERK2

binding sites did not compete for formation of this complex (Lane 5 in Fig.7C). Binding was also impaired when recombinant ERK2 was pre-incubated with an ERK2-specific antibody (Lane 7 in Fig.7C).

The results described above suggest that ERK2 competes with C/EBPβ for QRU occupancy in conditions of proliferation. To test this model, C/EBPβ was over-expressed with a retroviral vector generating conditions leading to the ectopic expression of p20K in CEF (20). ChIP assays confirmed that over-expressed C/EBPβ displaced ERK2 on the p20K promoter (Fig.8A). ERK2 remained largely nuclear in these cells suggesting that the absence of ERK2 on the QRU was not the result of intracellular relocalization caused by C/EBPβ over-expression (Fig.8C).

We attempted to down-regulate the expression of ERK2 with RCASBP-shRNA retroviral vectors but CEF quickly entered senescence in these conditions. The shRNA-mediated down-regulation of ERK2 also led to a marked reduction in C/EBPβ expression and a lack of p20K inducibility (data not shown, Supplementary Fig.1). In contrast, chemical inhibition of MEK impaired CEF proliferation but did not cause senescence (our unpublished results). The expression of p20K was stimulated by MEK inhibition but required more than 24 hrs of treatment (Fig.8H). As determined by ChIP and immunofluorescence assays, this delayed induction of p20K reflected the slow and progressive nuclear exclusion of ERK2 (data not shown, Supplementary Fig.2). Interestingly, MEK inhibition abolished the basal expression of CHOP in actively dividing CEF (Fig.8H). As determined by transient expression assays, forced expression of ERK2 reduced the activity of the QRU in cycling and contact inhibited CEF (Fig.8I)

while substitutions of key nucleotides affecting the ERK2 binding sites enhanced the activity of the QRU in cycling cells (Fig.8J). This confirmed the inhibitory role of the central domain of the QRU. More importantly, the mutant QRU construct (designated "EBS mutant" in Fig.8J) was no longer inducible by growth arrest in low oxygen conditions.

v-Src becomes a potent inducer of p20K expression upon inhibition of MEK. The v-Src oncoprotein is a strong inducer of C/EBPβ activity but also a potent inhibitor of p20K expression (19, 21). To determine the role of the MEK pathway in the repression of p20K by v-Src, CEF infected with the temperature-sensitive mutant NY72-4 RSV were treated with 2 mM PD184352 for 24 hrs and p20K expression was analyzed by Western blotting at the permissive and non-permissive temperatures. MEK inhibition impaired morphological transformation and reduced but did not block cell proliferation at the permissive temperature (Fig.9A and supplementary Fig.3). Significantly, a marked induction of p20K was observed in response to ts $pp60^{v\text{-}Src}$ activation when CEF were treated with the MEK inhibitor for 24 hrs, consistent with the central role of the MEK pathway in the repression of p20K (Fig.9B, lane 8).

Collectively, these results indicate that the expression of p20K is under direct inhibition by the MEK pathway with ERK2 functioning as a transcriptional repressor binding to the QRU in actively dividing cells.

DISCUSSION

Control of the p20K gas gene by C/EBPβ and the MEK pathway. The pro-mitogenic and pro-survival functions of C/EBPβ have been described in several contexts but C/EBPβ also cooperates with ATF-4 to induce the expression of death-associated protein kinase 1 and the onset of apoptosis in IFNg-treated cells (37, 38). In CEF, C/EBPβ is required for the control of genes activated by the v-Src tyrosine kinase but is also critical for the expression of the growth-arrest specific p20K lipocalin gene at contact inhibition (20, 21). To exert this pleiotropic function, C/EBPβ interacts with unrelated transcription factors controlled by multiple signaling pathways and governing different programs of gene expression. In RSV-transformed CEF, C/EBPβ cooperates with AP-1 and NF-kB to mediate the constitutive induction of IL-8 (21, 24, 27, 39). In contrast, the up-regulation of c-Jun, JunD or Fra-2 and increase in AP-1 activity antagonize the action of C/EBPβ in the control of growth arrest-specific genes (22). To date, the exact mechanism underlying the opposing role of AP-1 and C/EBPβ in CEF is unclear. Likewise, the factors determining the specificity of gene activation by C/EBPβ are not completely understood. In this report, we establish ERK2 as a factor binding to the quiescence-responsive unit (QRU) and inhibiting the expression of the p20K gas gene in actively dividing cells. Using ChIP assays, we demonstrate that ERK2 and C/EBPβ interact with the QRU in a mutually exclusive manner with ERK2 binding detected in conditions of proliferation and p20K repression (Figs. 7-8). We reported previously that forced expression of C/EBPβ using the RCASBP virus is sufficient to promote strong p20K expression in cycling CEF (20). These conditions abrogated the association of

ERK2 with the p20K QRU suggesting that ERK2 and C/EBPβ compete for QRU occupancy in the control of p20K expression (Fig. 8A).

Recombinant ERK2 interacted with the repeated GAAAG element of the QRU in EMSA suggesting that ERK2 controls p20K expression directly by binding to sequences overlapping the C/EBPβ binding sites (Fig.7C). This mode of action of ERK2 was first described by Hu and co-investigators on promoters of IFNg-inducible genes in human cells (26). While our studies use different cellular models, it is intriguing that ERK2 functions as a transcriptional repressor of genes activated by C/EBPβ in both systems. The identification of additional target genes will indicate if the transcriptional repressor activity of ERK2 is limited to C/EBPβ–controlled genes or is also important for promoters regulated by other transcription factors.

To confirm these findings, we attempted to down-regulate the expression of ERK2 with shRNAs but failed to obtain cells that could be propagated in culture. CEF infected with ERK2 shRNA viruses quickly entered senescence, lost C/EBPβ expression and did not express p20K (Supplem. Fig. 1, data not shown). A similar phenotype of premature senescence has been described for mouse embryo fibroblasts lacking both *erk1* and *erk2* gene functions (40). Since a gene encoding ERK1 has not been found in the chicken genome, it is possible that the *erk1/erk2* gene functions are solely dependent on ERK2 in this species (41). Surprisingly, CEF treated with the MEK inhibitor PD184352 were viable, did not enter senescence and expressed high levels of p20K albeit after a long delay. Since phospho-ERK levels and cell proliferation were markedly reduced in PD184352-treated CEF, ERK2 may antagonize senescence at least in part in a

phosphorylation-independent manner. The slow kinetics of CHOP down-regulation (Fig.8H) and ERK2 nuclear exclusion likely account for the delay of p20K induction in response to PD184352, a model consistent with the results of ChIP assays performed with the ERK2 antibody in normal CEF (Supplem. Fig.2 and data not shown).

MEK inhibition led to aberrant and rapid activation of p20K by ts v-Src, a potent inhibitor of this gene in normal conditions (Fig.8; (19)). While C/EBPβ is activated by the ERK pathway in mammalian cells (42), the mechanism leading to the stimulation of C/EBPβ activity in v-Src transformed CEF is not fully characterized but may depend on the increase in C/EBPβ levels in these cells (21). The results shown in Fig.8 provide a striking illustration of the inhibitory function of the MEK pathway in the control of p20K expression.

Conditions generating ER stress such as prolonged starvation or treatment with tunicamycin or thapsigargin led to a marked induction of the "C/EBP HOmologous Protein" factor (CHOP) and repression of p20K (Fig.1&5). CHOP is a bi-functional protein forming heterodimers with C/EBPβ to induce the expression of stress-responsive genes such as Carbonic Anhydrase VI (36). In the CA VI promoter, the CHOP-C/EBPβ dimer binds to a variant of the C/EBP regulatory element to promote gene activation. However, the dimerization with CHOP can also interfere with the function of C/EBPβ on promoters lacking this element and normally activated by other C/EBPβ dimers, thus causing gene repression. Forced expression of CHOP inhibited p20K in response to hypoxia in agreement with a role for CHOP as an inhibitor of p20K (Fig.5C). The mechanism promoting CHOP expression in cycling cells is poorly characterized but may

reflect a direct interaction of AP-1 with a TPA response element of the CHOP promoter, as described by Holbrook and co-investigators in mammals (43). In agreement with this model, CHOP expression is abolished by the expression of a dominant negative mutant of c-Jun in cycling CEF (our unpublished results).

High cell density promotes hypoxia in CEF. Gene profiling and RT-qPCR analyses revealed that several genes associated with the response to hypoxia, including carbonic anhydrase IX, carbonic anhydrase XII and enolase 2, are induced in density-arrested CEF (Fig.1). Nitroreductase activity, an indicator of hypoxia, was also elevated at confluence indicating that high cell density results in a significant depletion of oxygen levels. Since protein synthesis and cell proliferation are dependent on oxygen availability, hypoxia may promote growth arrest in confluent CEF monolayers. A similar relationship between high cell density, hypoxia and the control of protein synthesis has been observed in mammalian cells (44). Interestingly, p20K was first identified in quiescent chicken heart mesenchymal cells where its expression was also strongly affected by cell density (19). While the function of p20K remains to be characterized, these results suggest a role for this protein in the response to hypoxia. In agreement with this model, the results of recent experiments indicated that p20K enhances cell survival in limiting oxygen concentrations (our unpublished results). Thus, the induction of p20K may be part of an adaptive response to the lack of oxygen, changing conditions of proliferation and lipid metabolism. This is currently under investigation.

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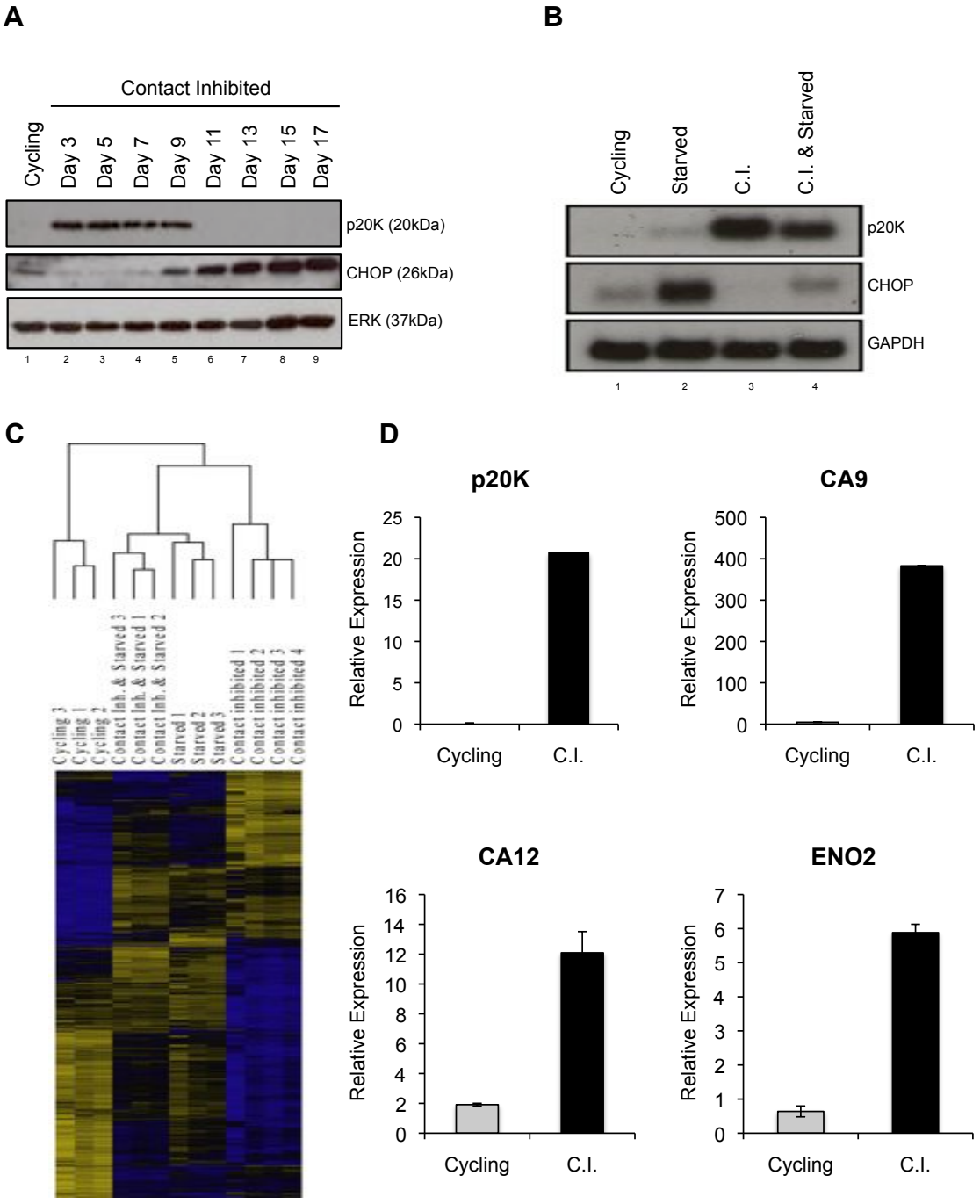

A
Contact Inhibited
Cycling
Day 3
Day 5
Day 7
Day 9
Day 11
Day 13
Day 15
Day 17
p20K (20kDa)
CHOP (26kDa)
ERK (37kDa)
1 2 3 4 5 6 7 8 9
B
Cycling
Starved
C.I.
C.I. & Starved
p20K
CHOP
GAPDH
1 2 3 4
C
Cycling 3
Cycling 1
Cycling 2
Contact Inh. & Starved 3
Contact Inh. & Starved 1
Contact Inh. & Starved 2
Starved 1
Starved 2
Starved 3
Contact inhibited 1
Contact inhibited 2
Contact inhibited 3
Contact inhibited 4
D
p20K
CA9
CA12
ENO2
Relative Expression
Cycling
C.I.

Figure 1: A) Expression of p20K during proliferation, at confluence and in response to nutrient depletion. Sub-confluent CEFs were cultured over a period of several days and protein lysates were prepared at different days after seeding. CEF reached confluence on day 3; cell numbers began to decrease on day 9 after seeding. The levels of p20K and CHOP, a marker of starvation, were examined by Western blotting. B) Transcripts for p20K and CHOP were examined in cycling CEF, sub-confluent CEF starved in serum-free medium for 48 hrs, confluent CEF maintained in complete medium (C.I.) or confluent CEF transferred to serum-free medium for 48 hrs (C.I & Starved). RNA loading was examined by probing the blot for GAPDH mRNA. C) Unsupervised hierarchal clustering of genes demonstrating a minimum of two-fold change in expression between at least two conditions is indicated. D) Validation of mRNA expression for p20K, carbonic anhydrase 9 (CA9), carbonic anhydrase 12 (CA12), enolase 2 (ENO2) by RT-qPCR. Transcripts levels are indicated in relative units for cycling and contact-inhibited (C.I) CEF.

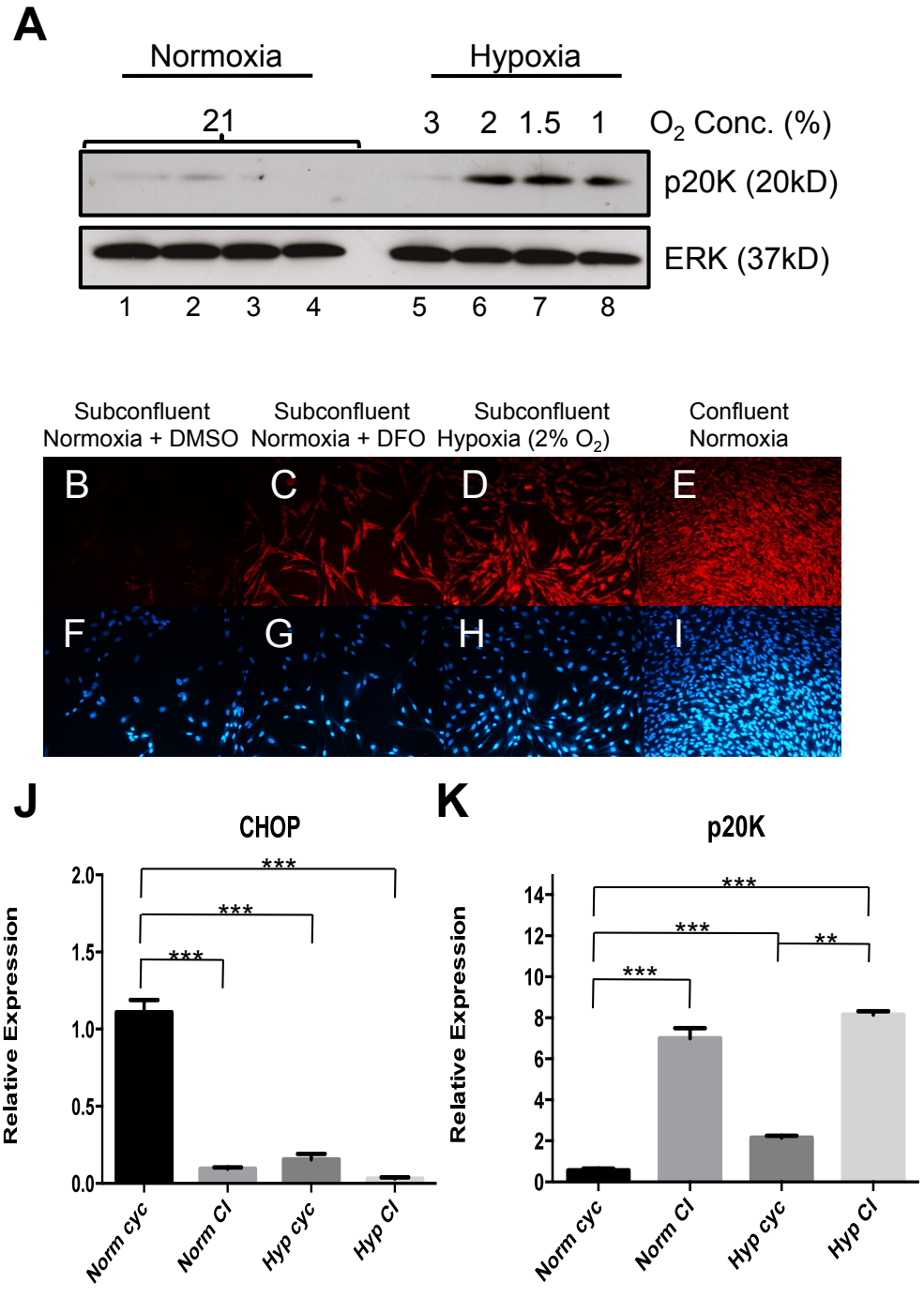

A
Normoxia
Hypoxia
21
3 2 1.5 1
O_2 Conc. (%)
p20K (20kD)
ERK (37kD)
1 2 3 4 5 6 7 8
Subconfluent Normoxia + DMSO
Subconfluent Normoxia + DFO
Subconfluent Hypoxia (2% O_2)
Confluent Normoxia
B C D E
F G H I
J
CHOP
Relative Expression
2.0
1.5
1.0
0.5
0.0
***
***
***
Norm cyc
Norm CI
Hyp cyc
Hyp CI
K
p20K
Relative Expression
14
12
10
8
6
4
2
0
***
***
**
***
Norm cyc
Norm CI
Hyp cyc
Hyp CI

Figure 2: (A) Western blotting analysis of p20K in CEF cultured in normoxia (21% O_2) and various conditions of hypoxia (1-3% O_2). ERK was utilized as a loading control. B to E) CEF treated with the fluorescent indicator of hypoxia Cyto-ID® were analyzed in CEF for different conditions of culture (panels B, C, D, E). Sub-confluent CEF treated with the hypoxia-mimetic desferrioxamine (DFO, 0.2 mM) or the diluent 0.1% DMSO were used as positive and negative control, respectively. For conditions of hypoxia, subconfluent CEF were cultured in 2% oxygen for 24 hrs; all other samples were examined at normoxia. Nuclei were stained with Hoechst stain. J-K) CHOP and p20K mRNA levels were quantified by RT-qPCR and normalized to GAPDH expression. The relative expression is depicted for sub-confluent/cycling (cyc) or contact inhibited (CI) CEF cultured in 24 hours hypoxia (labeled as "Hyp"; 2% O_2) or normoxia (labeled as "Norm", 21% O_2). Error bars represent the standard deviation of three independent experiments. *** Indicates a $p<0.001$ by one-way ANOVA with Bonferroni post hoc test versus the control (Cycling Normoxia). ** Indicates that the value is significantly different ($p<0.001$) in a separate *t-test* analysis.

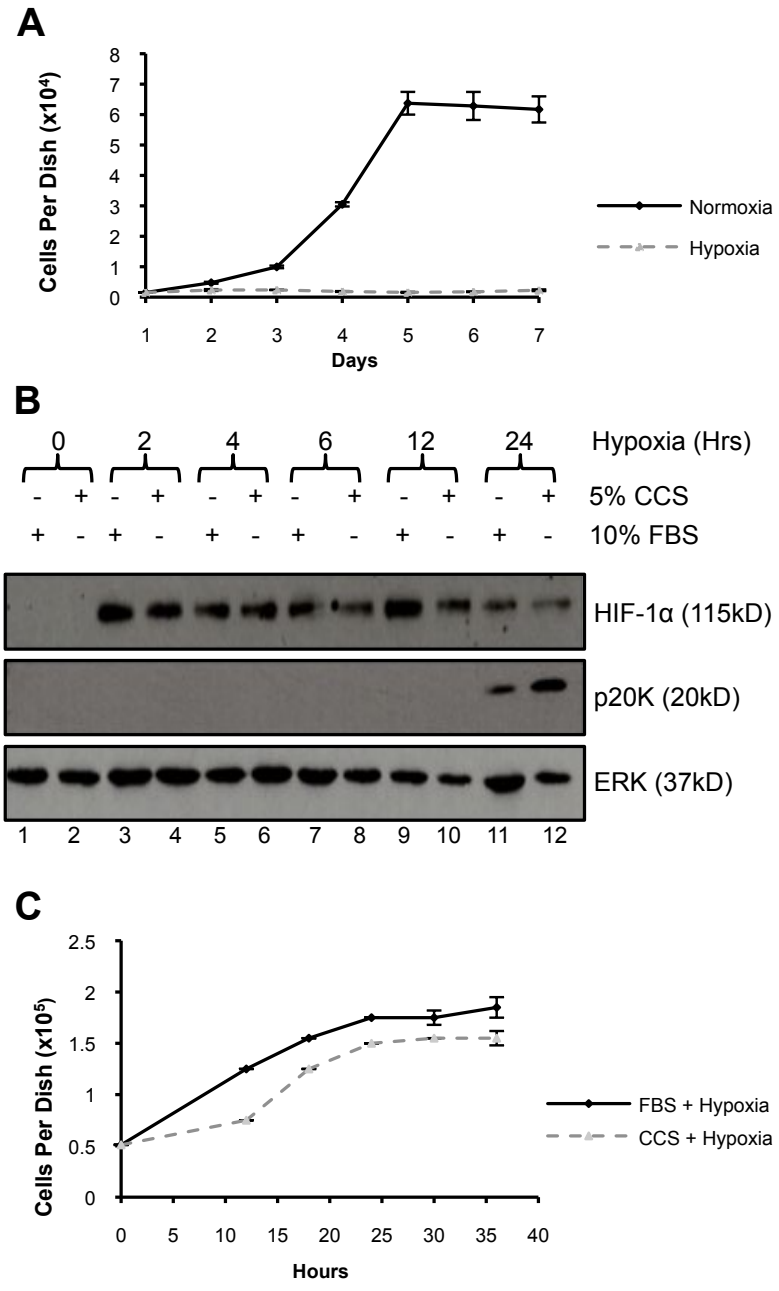

A
Cells Per Dish (x10⁴)
Days
Normoxia
Hypoxia
B
Hypoxia (Hrs)
5% CCS
10% FBS
HIF-1α (115kD)
p20K (20kD)
ERK (37kD)
C
Cells Per Dish (x10⁵)
Hours
FBS + Hypoxia
CCS + Hypoxia

Figure 3: (A) CEF proliferation in conditions of hypoxia (2% O_2) or normoxia (21% O_2) over a period of 7 days. Error bars represent the standard deviation of four independent counts. (B) Western blotting analysis of HIF-1α and p20K expression in CEF cultured in 5% calf serum (CCS for "Cosmic" calf serum; Hyclone) or 10% fetal bovine serum (FBS) and incubated in hypoxia (2% O_2) or normoxia (21%) over a period of 24 hours. ERK was used as a loading control. (C) CEF proliferation was examined over a period of 40 hours for cells in 10% FBS versus 5% CCS in conditions of hypoxia (2% O_2). The error bars represent the standard deviation of four independent samples.

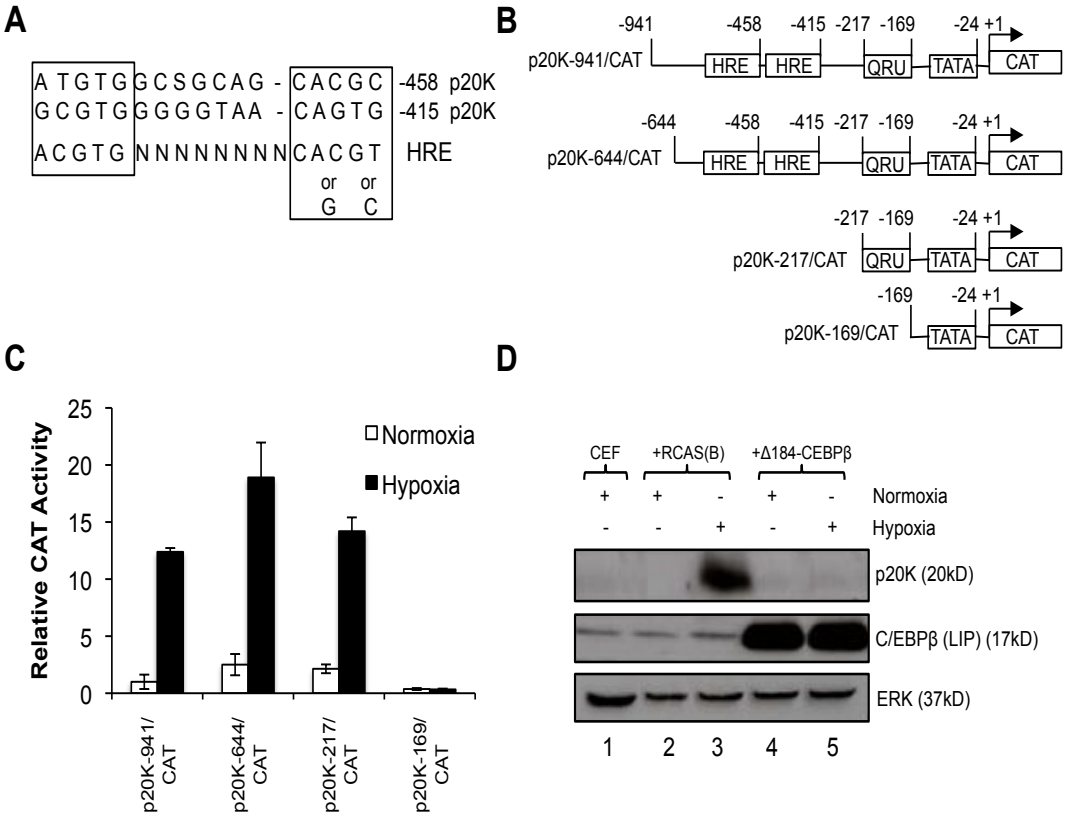

A
A T G T G G C S G C A G - C A C G C -458 p20K
G C G T G G G G G T A A - C A G T G -415 p20K
A C G T G N N N N N N N N C A C G T HRE
or or
G C
B
-941 -458 -415 -217 -169 -24 +1
p20K-941/CAT
HRE HRE QRU TATA CAT
-644 -458 -415 -217 -169 -24 +1
p20K-644/CAT
HRE HRE QRU TATA CAT
-217 -169 -24 +1
p20K-217/CAT
QRU TATA CAT
-169 -24 +1
p20K-169/CAT
TATA CAT
C
Relative CAT Activity
25
20
15
10
5
0
Normoxia
Hypoxia
p20K-941/ CAT
p20K-644/ CAT
p20K-217/ CAT
p20K-169/ CAT
D
CEF
+RCAS(B)
+Δ184-CEBPβ
+ + - + - Normoxia
- - + - + Hypoxia
p20K (20kD)
C/EBPβ (LIP) (17kD)
ERK (37kD)
1 2 3 4 5

Figure 4: A) Sequence of the putative hypoxia-responsive elements (HRE) of the p20K promoter region. B) Schematic representation of reporter constructs of the p20K promoter analyzed in transient expression assays (panel C) in conditions of normoxia (21% O_2) or hypoxia (2% O_2). (D) Western blotting analysis of p20K and dominant negative form of C/EBPβ (LIP) expression in CEF cultured in normoxia or hypoxia (2% O_2). Data are shown for uninfected CEF (labeled "CEF"), CEF infected with the control virus RCAS(B) or the virus expressing the dominant negative form of C/EBPβ (RCAS(B) Δ184-C/EBPβ). ERK was used as a loading control.

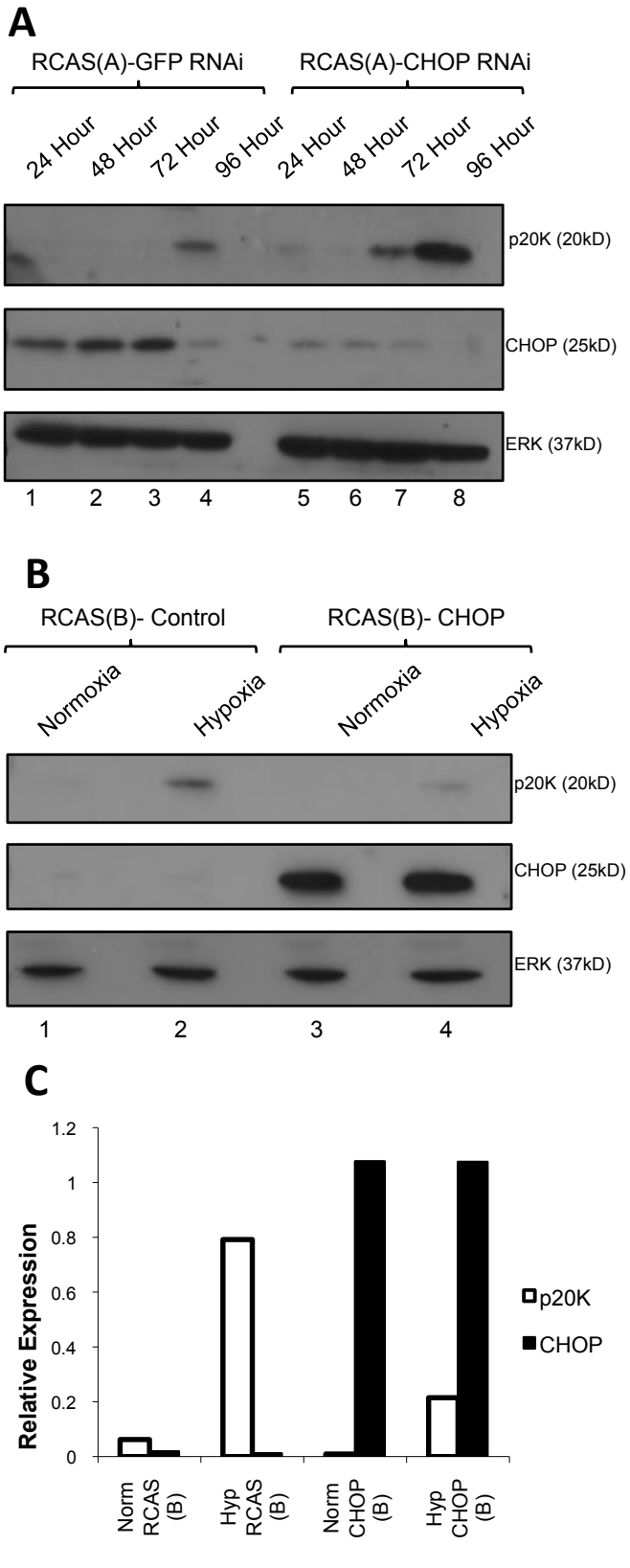

A
RCAS(A)-GFP RNAi
RCAS(A)-CHOP RNAi
24 Hour
48 Hour
72 Hour
96 Hour
24 Hour
48 Hour
72 Hour
96 Hour
p20K (20kD)
CHOP (25kD)
ERK (37kD)
1 2 3 4 5 6 7 8
B
RCAS(B)- Control
RCAS(B)- CHOP
Normoxia
Hypoxia
Normoxia
Hypoxia
p20K (20kD)
CHOP (25kD)
ERK (37kD)
1 2 3 4
C
Relative Expression
1.2
1
0.8
0.6
0.4
0.2
0
p20K
CHOP
Norm RCAS (B)
Hyp RCAS (B)
Norm CHOP (B)
Hyp CHOP (B)

Figure 5: A) The effects of downregulation of CHOP by shRNA on p20K expression were examined by Western blotting. The expression of p20K and CHOP was analyzed in both cycling and confluent CEF infected with a group A virus expressing a control shRNA for GFP (RCAS(A)-GFP RNAi) or shRNA for CHOP (RCAS(A)-CHOP-RNAi). ERK was used as a loading control. B) The proliferation of control CEF and CEF expressing the CHOP shRNA (RCAS(A)-CHOP-RNAi) is shown. C) The effects of CHOP overexpression on p20K levels was examined by Western blotting for cells in normoxia or hypoxia (2% O_2 for 24 hours). CHOP over-expression was obtained with the RCAS(B)-CHOP retrovirus (labeled as "CHOP"). ERK was used as a loading control. The relative levels of CHOP and p20K expression were determined by densitometry (shown in D).

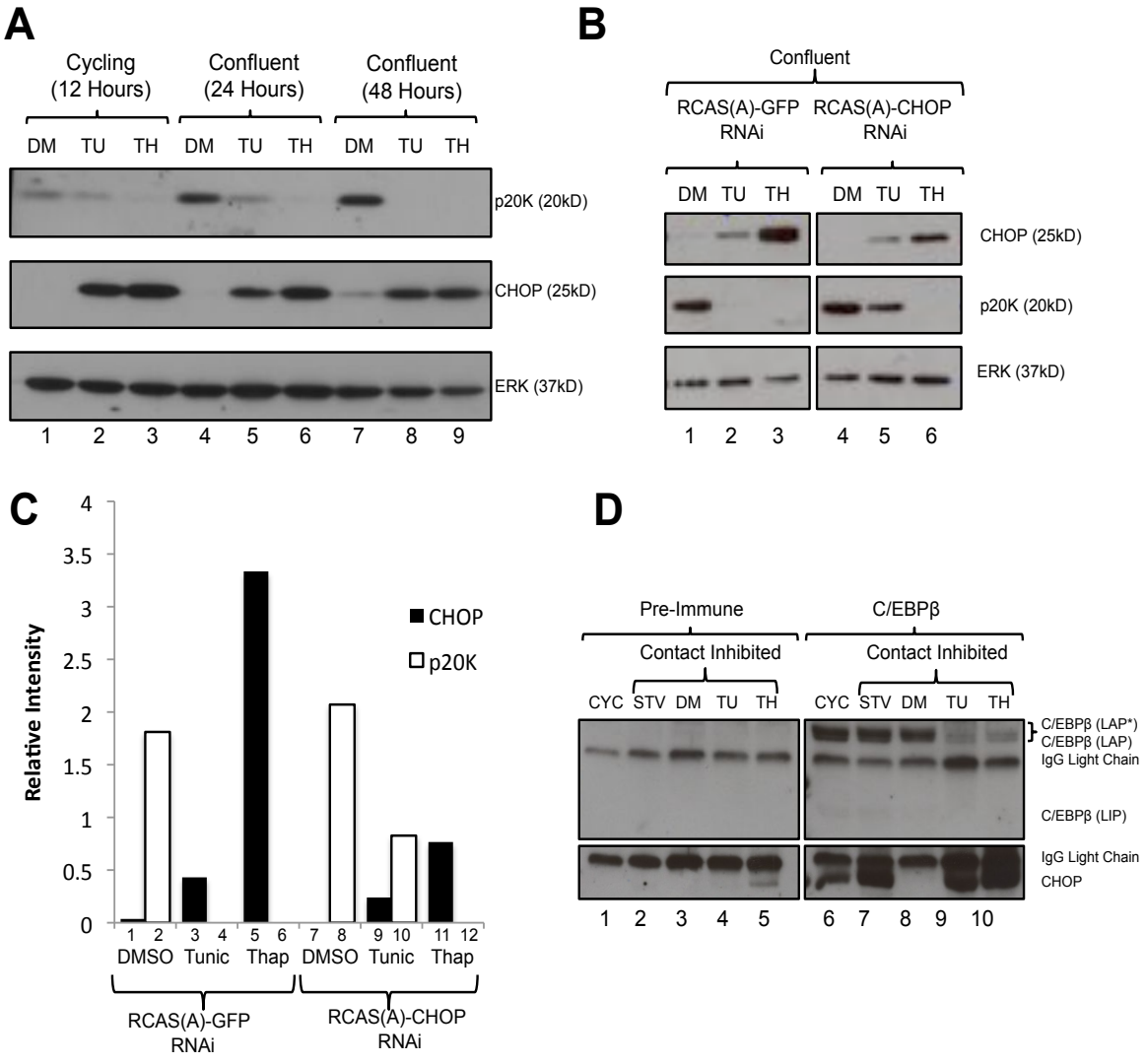

A
Cycling (12 Hours)
Confluent (24 Hours)
Confluent (48 Hours)
DM TU TH DM TU TH DM TU TH
p20K (20kD)
CHOP (25kD)
ERK (37kD)
1 2 3 4 5 6 7 8 9
B
Confluent
RCAS(A)-GFP RNAi
RCAS(A)-CHOP RNAi
DM TU TH DM TU TH
CHOP (25kD)
p20K (20kD)
ERK (37kD)
1 2 3 4 5 6
C
Relative Intensity
CHOP
p20K
1 2 3 4 5 6 7 8 9 10 11 12
DMSO Tunic Thap DMSO Tunic Thap
RCAS(A)-GFP RNAi
RCAS(A)-CHOP RNAi
D
Pre-Immune
C/EBPβ
Contact Inhibited
Contact Inhibited
CYC STV DM TU TH CYC STV DM TU TH
C/EBPβ (LAP*)
C/EBPβ (LAP)
IgG Light Chain
C/EBPβ (LIP)
IgG Light Chain
CHOP
1 2 3 4 5 6 7 8 9 10

Figure 6: A) Western blotting analysis of p20K and CHOP in cycling or confluent CEF treated with 0.1% DMSO (DM), 1μg/ml tunicamycin (TU), or 1 μg/ml thapsigargin (TH) for 12, 24 and 48 hours. CEF were confluent at time = 24 hours in this experiment. B) Western blotting analysis of p20K and CHOP in confluent cells infected with a group A virus expressing a control shRNA for GFP (RCAS(A)-GFP RNAi) or shRNA for CHOP (RCAS(A)-CHOP-RNAi) treated with the diluent 0.1% DMSO (DM), 1 μg/ml tunicamycin (TU), or 1 μg/ml thapsigargin (TH). In this experiment, the relative levels of CHOP and p20K were determined by densitometry and are represented in panel C). D) Association of CHOP with C/EBPβ as determined by co-immunoprecipitation and Western blotting analysis. Cell lysates were prepared from cycling CEF (CYC) or contact inhibited CEF treated with 1 μg/ml tunicamycin (TU), 1 μg/ml thapsigargin (TH) or the diluent (0.1% DMSO – DM). A cell lysate was also prepared from confluent CEF maintained in the same medium without replenishment and starved for 6 days (STV). C/EBPβ was immunoprecipitated and analyzed by Western blotting; the co-immunoprecipitation of CHOP was examined by probing the blot with a CHOP polyclonal antibody.

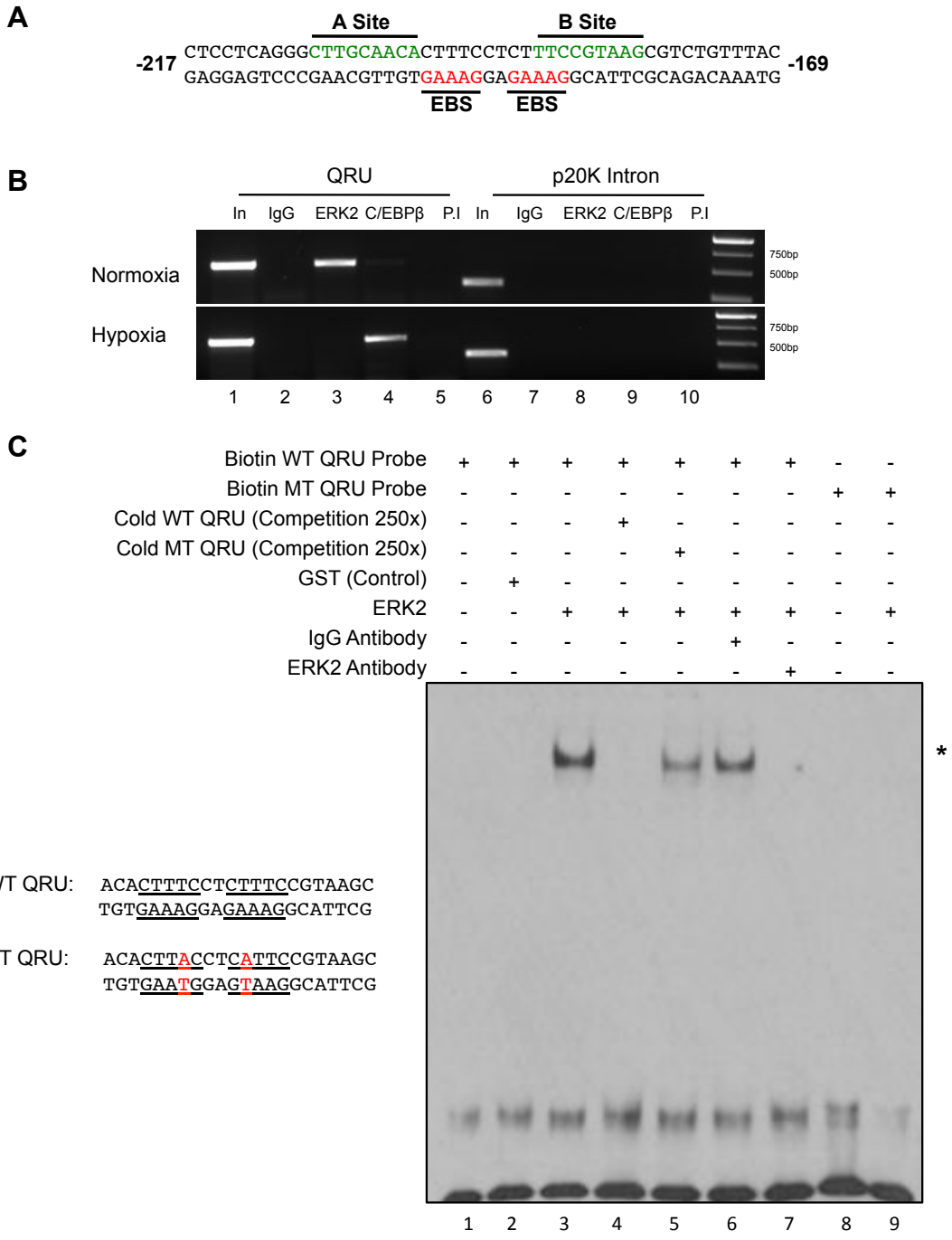

A
A Site
B Site
-217
CTCCTCAGGGCTTGCAACACTTTCCTCTTTCCGTAAGCGTCTGTTTAC
GAGGAGTCCCGAACGTTGTGAAAGGAGAAAGGCATTCGCAGACAAATG
-169
EBS
EBS
B
QRU
p20K Intron
In IgG ERK2 C/EBPβ P.I In IgG ERK2 C/EBPβ P.I
Normoxia
Hypoxia
750bp
500bp
750bp
500bp
1 2 3 4 5 6 7 8 9 10
C
Biotin WT QRU Probe + + + + + + + - -
Biotin MT QRU Probe - - - - - - - + +
Cold WT QRU (Competition 250x) - - - + - - - - -
Cold MT QRU (Competition 250x) - - - - + - - - -
GST (Control) - + - - - - - - -
ERK2 - - + + + + + - +
IgG Antibody - - - - - + - - -
ERK2 Antibody - - - - - - + - -
*
WT QRU: ACACTTTCCTCTTTCCGTAAGC
TGTGAAAGGAGAAAGGCATTCG
MT QRU: ACACTTACCTCATTCCGTAAGC
TGTGAATGGAGTAAGGCATTCG
1 2 3 4 5 6 7 8 9

Figure 7: Interaction of ERK2 and C/EBPβ with the p20K QRU. A) The sequence of the QRU with the position of the two C/EBPβ (A site and B site) and ERK2 binding sites (EBS) is indicated. B) ERK2 and C/EBPβ antibodies were used in ChIP assays to probe for the recruitment of ERK2 and C/EBPβ when CEF were cultured in conditions of normoxia or hypoxia (24 hrs in 2% O_2). Mouse IgG and the corresponding rabbit pre-immune serum (P.I) were used as negative controls for the ERK2 and C/EBPβ antibody, respectively. Primers were used to PCR-amplify the QRU while a region located in intron 1 of the p20K gene was used as a negative control. C) Recombinant ERK2 binds to the QRU in EMSA. An asterisk indicates the position of the nucleoprotein complex. The competitor was a 250-fold excess of unlabeled double-stranded oligonucleotide added to the binding reaction mixture. GST was utilized as a negative control. The consensus ERK2 binding sites are underlined while nucleotides altered in the EBS mutant oligos are highlighted in red. A control yeast lysate subjected to a mock purification was also analyzed in parallel (GST). The specificity of the ERK2 nucleoprotein complex was examined by adding ERK2-specific antibodies or a control IgG to the binding reaction.

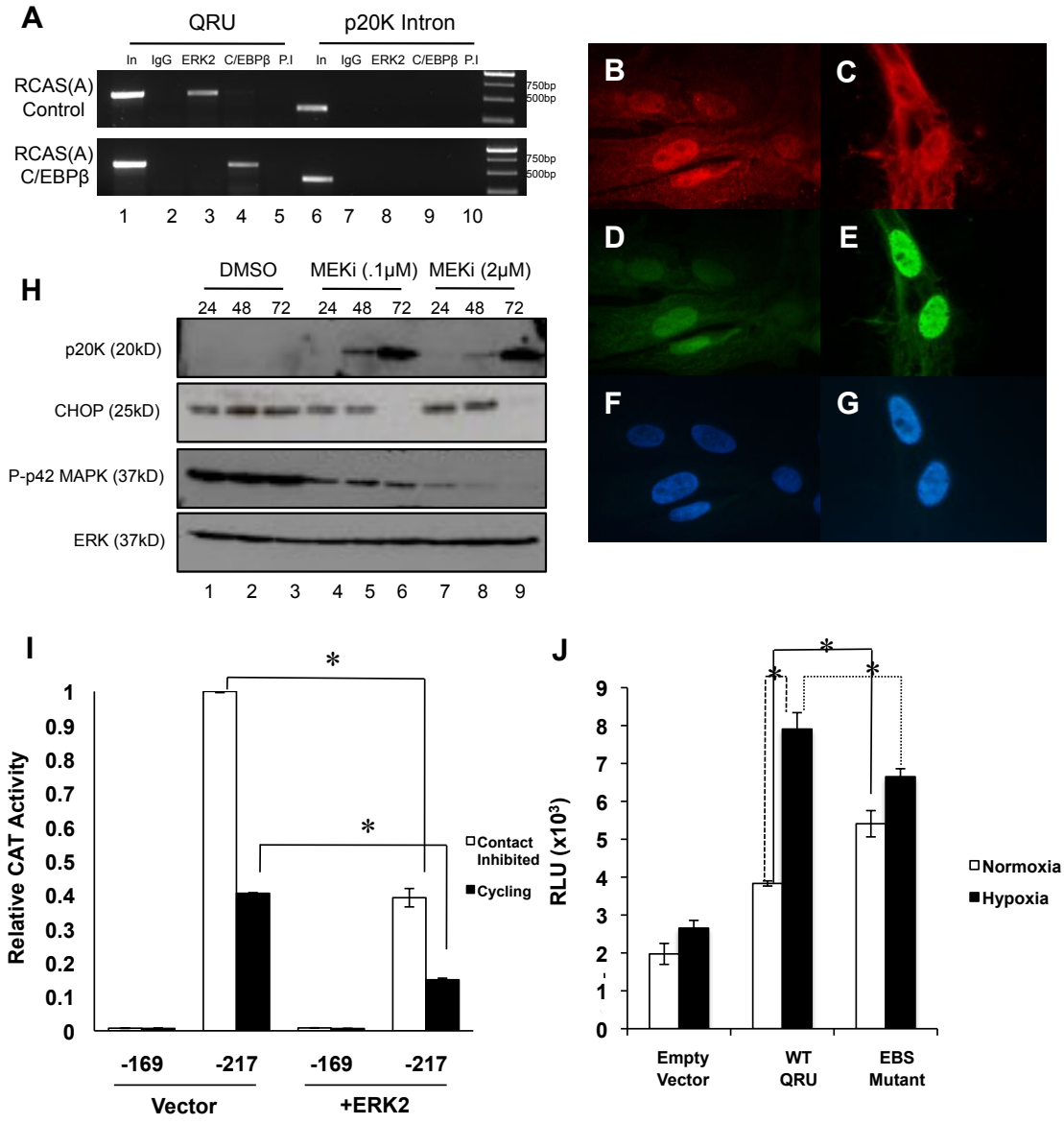

A
QRU
p20K Intron
In IgG ERK2 C/EBPβ P.I In IgG ERK2 C/EBPβ P.I
RCAS(A) Control
RCAS(A) C/EBPβ
750bp
500bp
1 2 3 4 5 6 7 8 9 10
B
C
D
E
F
G
H
DMSO
MEKi (.1μM)
MEKi (2μM)
24 48 72 24 48 72 24 48 72
p20K (20kD)
CHOP (25kD)
P-p42 MAPK (37kD)
ERK (37kD)
1 2 3 4 5 6 7 8 9
I
Relative CAT Activity
Contact Inhibited
Cycling
-169 -217 -169 -217
Vector
+ERK2
J
RLU (x10³)
Normoxia
Hypoxia
Empty Vector
WT QRU
EBS Mutant

Figure 8: Competition of ERK2 and C/EBPβ for QRU occupancy. A) The recruitment of ERK2 and C/EBPβ to the QRU was examined in ChIP assays using chromatin isolated from CEF infected with the control virus RCAS(A) or a C/EBPβ-encoding RCAS(A) virus . A murine IgG and the corresponding rabbit pre-immune (P.I) serum were used as negative controls for ERK2 and C/EBPβ immunoprecipiation, respectively. Primers were used to PCR-amplify the QRU region or a region located in intron 1 of the p20K gene as a negative control. Immuno-localization of ERK2 (B and C) and C/EBPβ (D and E) in CEF infected with a control virus (RCAS(A); panels B,D, and F) or a virus expressing C/EBPβ (RCAS(A)-C/EBPβ; panels C,E and G). DAPI was used for nuclei staining (F and G). H) Western blotting analysis of p20K and phospho-ERK (P-p42 MAPK) in CEF treated with 0.1% DMSO, 0.1 μM or 2 μM MEK inhibitor for a period of 24, 48 or 72 hours. ERK was used as a loading control. (I) The activity of reporter constructs of the p20K promoter including (-217) or lacking (-169) the QRU was determined in transient expression assay in response to ERK2 over-expression for cycling and contact-inhibited CEF. In this experiment, an expression vector for ERK2 or the parental, control vector were co-transfected with the reporter constructs. J) Promoter activity in constructs containing the wild-type QRU (WT QRU) and constructs containing mutations in the putative ERK2 Binding Site of the QRU (EBS Mutant) were analyzed by transient expression assays in conditions of normoxia or hypoxia (24 hrs in 2% O_2). The parental reporter vector, lacking a QRU region, was used as a negative control. * $P \leq 0.01$.

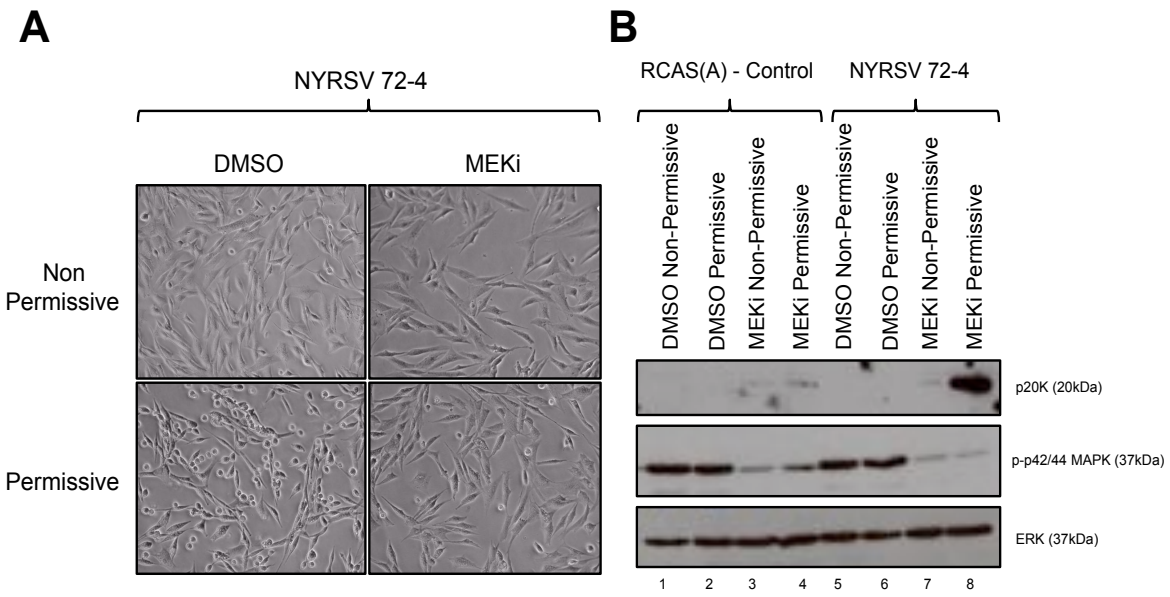
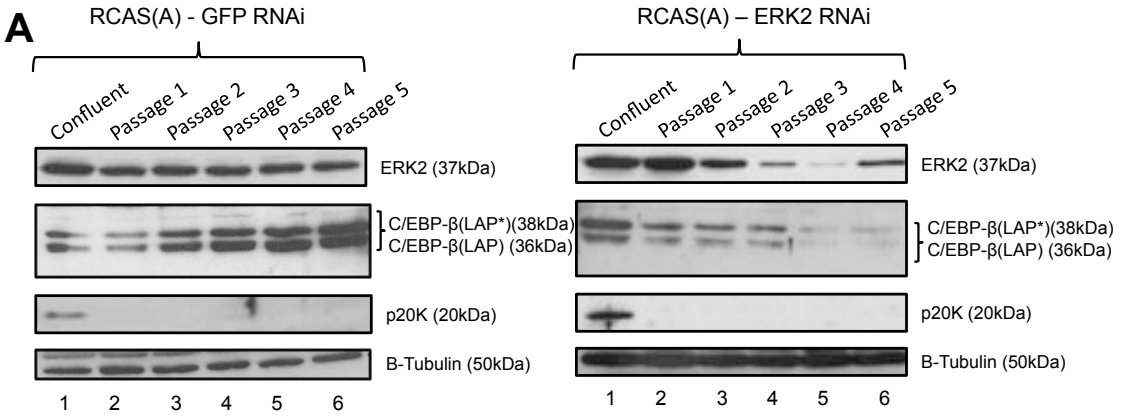
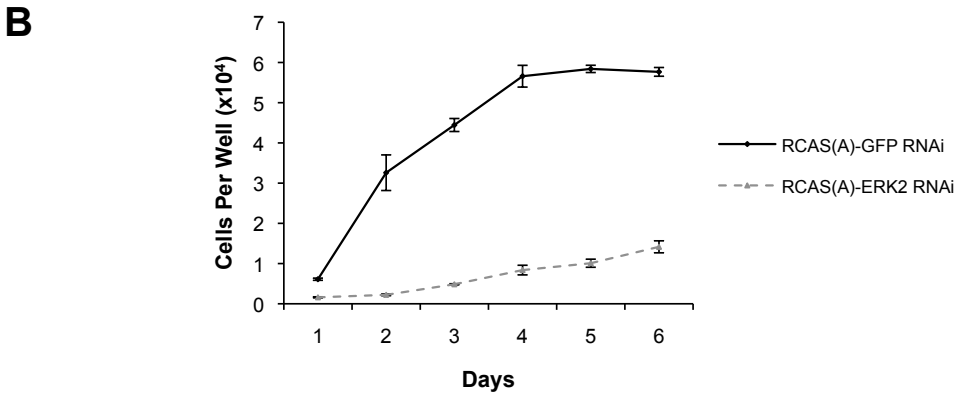
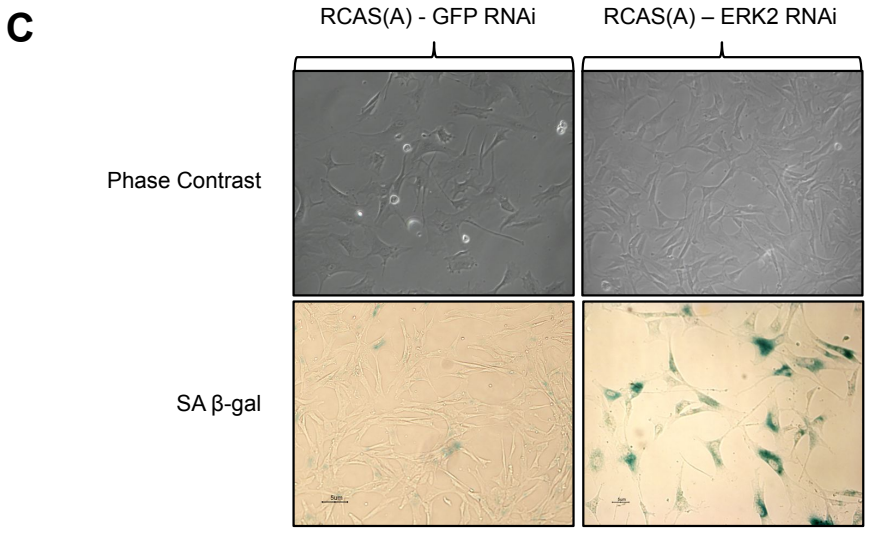

A
NYRSV 72-4
DMSO
MEKi
Non Permissive
Permissive
B
RCAS(A) - Control
NYRSV 72-4
DMSO Non-Permissive
DMSO Permissive
MEKi Non-Permissive
MEKi Permissive
DMSO Non-Permissive
DMSO Permissive
MEKi Non-Permissive
MEKi Permissive
p20K (20kDa)
p-p42/44 MAPK (37kDa)
ERK (37kDa)
1 2 3 4 5 6 7 8

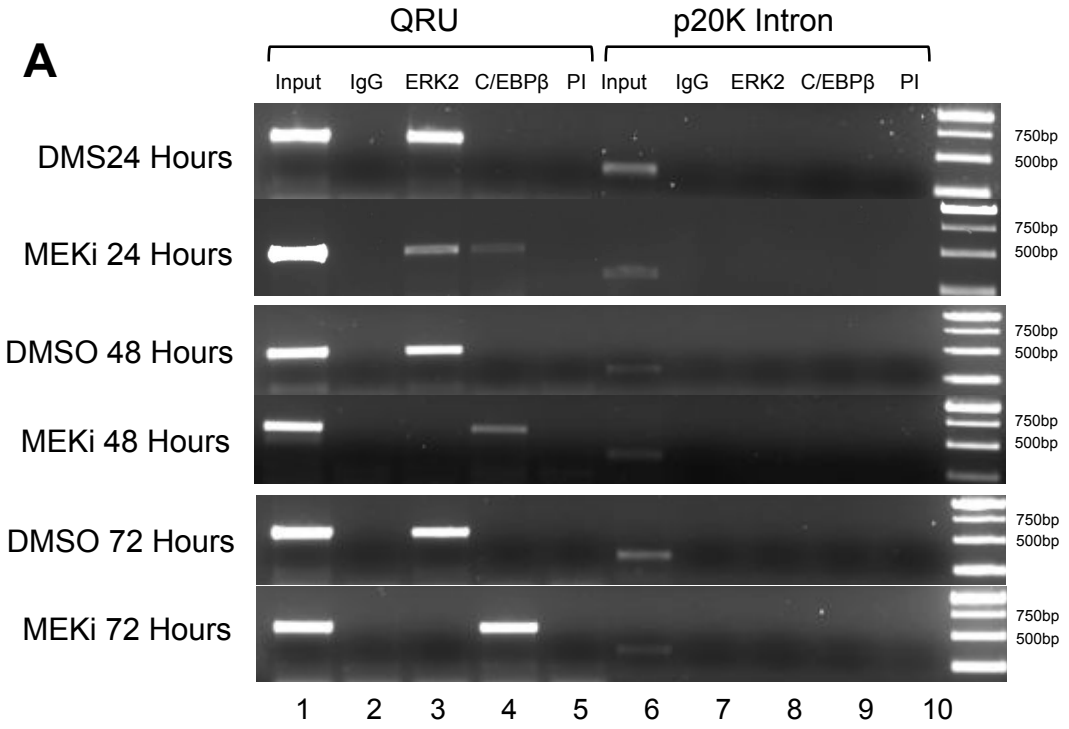
Figure 9: Induction of p20K by v-Src in conditions of MEK inhibition. A) Phase contrast micrographs of CEF infected with the temperature sensitive mutant ts NY72-4 RSV at the permissive (37.5°C) or non-permissive (41.5°C) temperatures and treated with either the DMSO diluent or 2 μM of the MEK inhibitor PD184352 for 24 hours. B) Western blotting analysis of p20K and phospho-ERK (P-p42/p44 MAPK) in CEF infected with a control virus lacking a Src gene (RCAS(A)-Control) or ts NY72-4 RSV cultured at the permissive (37.5°C) or non-permissive (41.5°C) temperatures and treated with DMSO or 2 μM of the MEK inhibitor PD184352 for 24 hours. ERK was utilized as a loading control.

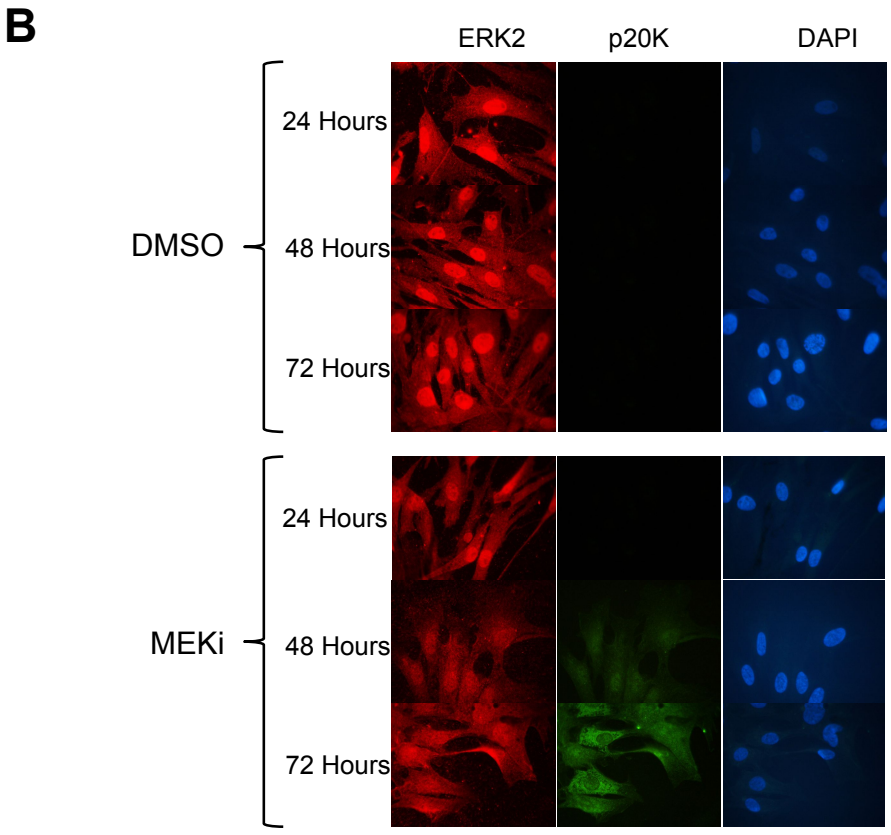

A
RCAS(A) - GFP RNAi
RCAS(A) – ERK2 RNAi
Confluent
Passage 1
Passage 2
Passage 3
Passage 4
Passage 5
ERK2 (37kDa)
C/EBP-β(LAP*)(38kDa)
C/EBP-β(LAP) (36kDa)
p20K (20kDa)
B-Tubulin (50kDa)
1 2 3 4 5 6


B
Cells Per Well (x10⁴)
Days
RCAS(A)-GFP RNAi
RCAS(A)-ERK2 RNAi


C
RCAS(A) - GFP RNAi
RCAS(A) – ERK2 RNAi
Phase Contrast
SA β-gal

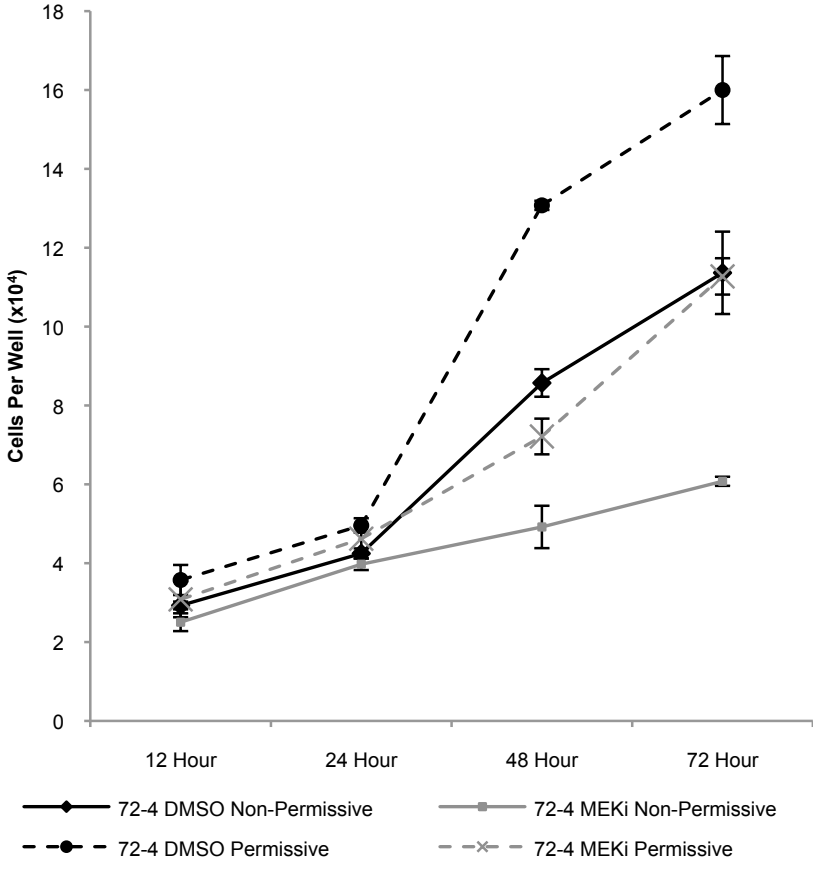
Supplementary Figure 1: (A) Western blotting analysis of p20K, ERK2, and C/EBPβ in CEF transfected with either a group A virus expressing a control shRNA for GFP (RCAS(A)-GFP RNAi) or a shRNA for ERK2 (RCAS(A)-ERK2-RNAi). Cell samples were collected at the day after transfection at confluence (confluence), and at every successive passage in cycling conditions for a total of 5 passages (Passage 1 – Passage 5). (B) Proliferation of CEF infected with either a group A virus expressing a control shRNA (RCAS(A)-GFP RNAi) or a shRNA for ERK2 (RCAS(A)-ERK2-RNAi) over a period of 6 days. Error bars represent the average+SD of four independent counts. (C) Phase contrast and bright field micrographs of CEF displaying senescence associated β-galactosidase activity for cells infected with either a group A virus expressing a control shRNA (RCAS(A)-GFP RNAi) or a shRNA for ERK2 (RCAS(A)-ERK2-RNAi) taken at 6 days post transfection of retroviral plasmid DNA.


A
QRU
p20K Intron
Input
IgG
ERK2
C/EBPβ
PI
Input
IgG
ERK2
C/EBPβ
PI
DMS24 Hours
MEKi 24 Hours
DMSO 48 Hours
MEKi 48 Hours
DMSO 72 Hours
MEKi 72 Hours
750bp
500bp
1
2
3
4
5
6
7
8
9
10


B
ERK2
p20K
DAPI
DMSO
MEKi
24 Hours
48 Hours
72 Hours

.

Supplementary Figure 2: (A) Analysis of ERK2 and C/EBPβ interaction with the p20K QRU during MEK inhibitor treatment (PD184352), as determined by ChIP assay. ERK2 and C/EBPβ antibodies were used to immunoprecipitate ERK2 or C/EBPβ protein/DNA complexes in CEF treated with either 2μm MEK inhibitor or the DMSO diluent as a control. Samples were taken every 24 hours for a total of 72 hours. Mouse IgG or the corresponding rabbit pre-immune (P.I) serum were used as negative controls, respectively. Primers were used to PCR-amplify the QRU region while a region located within intron 1 of the p20K gene was PCR-amplified as a negative control. (B) Immunolocalization of ERK2 and p20K in CEF treated with the MEK inhibitor (2 μM) or the DMSO diluent. Photos were obtained every 24 hours for a total of 72 hours. DAPI was used to stain nuclei.


18
16
14
12
10
8
6
4
2
0
Cells Per Well (x10⁴)
12 Hour
24 Hour
48 Hour
72 Hour
72-4 DMSO Non-Permissive
72-4 MEKi Non-Permissive
72-4 DMSO Permissive
72-4 MEKi Permissive

Supplementary Figure 3: Proliferation of CEF infected with the temperature sensitive mutant NY72-4 RSV at the permissive (37.5°C) and non-permissive (41.5°C) temperatures and treated with 2mM of the MEK inhibitor PD184352 or the DMSO diluent. Cells were counted for a total of 72 hours. Error bars represent standard deviations of the mean.

Supplementary Table 1

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

Pair-wise comparisons of number of differentially expressed probe-sets for cycling CEF, serum starved, contact inhibited and starved/contact inhibited CEF. Numbers are provided for up-regulated, down-regulated and the total number of differentially regulated probe-sets.

Chapter 2: The p20K lipocalin is required for lipid homeostasis and cell survival in hypoxic chicken embryo fibroblasts

The p20K lipocalin was originally identified to be induced during quiescence in response to contact inhibition. More recent evidence was able to show that cells grown in culture to high-density display hypoxic specific signatures. Combined, this data suggests that p20K is expressed in a hypoxia specific manner. Although p20K has been shown to play a variety of roles, including cell survival, inflammatory response and cell development, its role in hypoxia has still yet to be uncovered. (Cancedda *et al*., 1990; Gentili *et al*., 1998; Cancedda *et al*., 2000; Cermelli *et al*., 2000; Gentili *et al*., 2005). Within this chapter we portray that p20K acts as a survival factor in CEF cultured in limited oxygen concentrations. The knockdown of p20K via shRNA presented a higher incidence of apoptosis as well as lipid oxidation within hypoxia. Additionally, the loss of p20K also promoted the formation of large lipid-containing vesicles in limited oxygen concentrations, which suggests that lipids rapidly accumulate in the absence of p20K within these states.

All experiments conducted for this chapter were done by myself. The manuscript was prepared for publication by Dr. Pierre-André Bédard and myself.

The p20K lipocalin is required for lipid homeostasis and cell survival in hypoxic chicken embryo fibroblasts

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Running Title: Role of p20K in lipid homeostasis

Key words: Hypoxia, growth arrest specific genes, p20K lipocalin, lipid peroxidation

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ABSTRACT

The expression of the p20K lipocalin is induced in response to contact inhibition or conditions of limiting oxygen concentrations in chicken embryo fibroblasts (CEF). p20K binds avidly to poly-unsaturated fatty acids and is a member of the siderocalins binding iron catechol compounds. Despite these advances, thus far the function of p20K remains unknown. Here we report that p20K acts as a survival factor for cells in restrictive conditions of proliferation. Down-regulation of p20K mediated by shRNA resulted in a high incidence of apoptosis when CEF were incubated in limiting oxygen concentrations. Increased lipid oxidation and production of malondialdehyde (MDA), a bi-product of lipid peroxidation, were observed upon p20K down-regulation. Large lipid-containing vesicles appeared in the cytosol of CEF lacking p20K in conditions of hypoxia but not when these cells were cultured in normal concentrations of oxygen (normoxia). These results point to a role for p20K in lipid homeostasis and the control of cell survival during reversible growth arrest.

INTRODUCTION

Lipocalins define a family of diverse lipid-binding proteins sharing the "lipocalin-fold", a domain for interaction with small and predominantly lipophylic molecules (1, 2). While structurally conserved, the hydrophobic pocket or calix of lipocalins accommodates a large variety of lipophilic ligands such as retinoids, steroids, fatty acids and phospholipids but also secondary metabolites belonging to several functional and chemical classes (vitamins, cofactors, odorants, etc…;(2)). Since most lipocalins are secreted, it is perhaps not surprising that they were isolated and characterized principally from cell culture medium, blood or body fluids such as tears and genital secretions (1, 3-6). Lipocalins contribute to lipid metabolism, storage, transport and sequestration in a wide variety of tissues and extracellular environments. Thus the functions of lipocalins are also diverse but to this day remain incompletely understood. This is due in part to the wide variety of ligands associated with the lipocalins and the limited amino acid sequence similarity residing outside of the calix structure, making species comparisons difficult (1). In some cases, lipocalins are clearly cyto-protective acting as scavengers of deleterious lipid peroxidation products (3, 4, 7-9). Lipocalins can also act bi-functionally promoting survival in some contexts and apoptosis in others. In HeLa cells, the neutrophil gelatinase-associated lipocalin (NGAL or Lcn-2) induced apoptosis when added extra-cellularly but not if first loaded with iron (10). Therefore, the effect of NGAL is context-dependent and determined by iron availability and the expression of the NGAL receptor.

We first identified the p20K lipocalin as a secretory protein expressed in quiescent chicken heart mesenchymal cells or chicken embryo fibroblasts (CEF). In CEF, p20K is

expressed predominantly at contact inhibition and to a much lower extent in response to serum/nutrient depletion (5, 11, 12). Among lipocalins, p20K is rather unique since it remains associated with intracellular membrane compartments for extensive periods of time after synthesis and before secretion into the medium (5). In the cell, it is associated with the ER and Golgi apparatus but is not detectable at the nuclear envelope or plasma membrane. As a member of the lipocalin family, p20K binds avidly to poly-unsaturated fatty acids *in vitro* (13). More recently, the crystal structure of p20K was determined revealing the presence of an extended multi-chambered calix capable of binding to iron-containing siderophores and to lysophosphatidic acid simultaneously (14). Since it displayed bacterio-static activity dependent on its ability to interact with bacterial siderophores and sequester iron, 20K belongs to the lipocalin sub-class of siderocalins (2).

The results of experiments based on the micro-injection of antibodies in chicken embryos suggest a role for p20K as a trophic factor promoting survival during development (15). However, the mechanism(s) underlying the pro-survival function of p20K and its relevance to growth arrest remain unknown. Gene profiling analyses revealed that several genes associated with the response to hypoxia are expressed in density-arrested but not serum starved CEF (16). Moreover limiting oxygen concentrations (2% O_2) promote growth arrest and p20K expression, and hypoxia is a feature of density-arrested CEF, suggesting a role for p20K in these conditions (16). In this study, we describe the results of experiments based on ectopic expression or down-regulation of p20K in CEF. We establish a link between p20K expression and levels of

peroxidated lipids in the cell, on one hand, and the ability of p20K to promote cell survival on the other hand. These results indicate an important function for the p20K lipocalin in the lipid metabolism of cells entering G_0 as a result of high cell density and oxygen depletion.

MATERIALS AND METHODS

Cell culture. Early passages (n<10) of CEFs were cultured at 41.5ºC in 21% O_2 and 5% CO_2 in "complete medium" consisting of DMEM with 5% heat-inactivated (at 57ºC for 30 minutes) "Cosmic" calf serum (CCS, Hyclone SH30067.03, Logan, UH), 5% tryptose phosphate broth, 2 mM L-glutamine, 0.2 mg/ml streptomycin and 0.2 U/ml penicillin (Life Technologies). Hypoxia was induced by culturing CEF for 36 hrs in 1-2% O_2 as described in the text.

Western blotting analysis. Total protein extracts (30 μg) were subjected to SDS-polyacrylamide gel electrophoresis with a gel concentration of 12%. When resolved, gels were blotted onto a nitrocellulose membrane (Schleicher and Schuell, BA85) and subsequently blocked in a 5% solution of skim milk powder dissolved in 1xTBS (20 mM Tris pH 7.6, 140 mM NaCl) for 1 hour at room temperature. Blots were incubated with the following primary antibodies overnight at 4°C:

Chicken p20K Dilution 1:1500 (Western) – House Made (42-R)

CHOP Dilution 1:1000 (Western) – House Made (Tulip)

ERK Dilution 1:1000 (Western) - Santa Cruz sc-93

Following several washes, the blots were incubated with a 1:25,000 dilution of a secondary anti-rabbit, anti-mouse, or anti-goat IgG antibody conjugated with horseradish peroxidase (HRP) at room temperature in 5% milk TBS. Chemiluminescent signals were revealed by incubation with the HRP substrate Luminata Forte according to the protocol provided by the supplier (Millipore WBLUF0100).

Proliferation assays. Confluent CEF were split into 24 well microtitre dishes and incubated in normoxia and hypoxia for the time duration indicated. Cells were treated with 1 mL of trypsin and diluted in 9 mL of ISOTON® II Diluent (Beckman Coulter 8546719). Cells were counted in quadruplicate samples for statistical significance utilizing a Beckman Coulter model Z2 (Coulter Corporation, Miami, FL) Coulter counter.

Live Cell Imaging of Lipid Peroxidation. Intracellular lipid peroxidation was observed as per the manufacturers protocols (ThermoFisher Scientific D3861). Eseentially cells were seeded on coverslips and incubated in conditions of normoxia (21% O_2) or hypoxia (2% O_2) for a period of 36 hours. The fluorescent dye and indicator of lipid oxidation C-11 BODIPY$^{581/591}$ (5 μM; ThermoFisher Scientific D3861) was added to the medium for a period of 30 min while nuclei were stained with Hoechst 33342 (H1399, Peirece, USA) for 10 minutes. After washing in PBS, the cover slips were mounted and observed. Cells were imaged in quadruplicate in order to ensure significance.

Malondialdehyde (MDA) Detection Assay and Lipid Analysis. Lipid peroxidation

was quantified though detection of the byproduct malondialdehyde (17). Cells were incubated in conditions of normoxia (21% O_2) or hypoxia (2% O_2) for 36 hours and malondialdehyde levels were determined using commercially available reagents and protocols provided by the supplier (Lipid Peroxidation (MDA) assay kit; Abcam ab118970).

Retroviral shRNAi vector construct generation:

The target sequences of the ERK2 and p20K genes were chosen using the shRNAi design tool at www.genescript.com/ssl-bin/app/rnai. Constructs were created using a method previously described (16). The following oligonucleotides were utilized to generate short hairpins for p20K.

p20K shRNA Forward:

5'-GAGAGGTGCTGCTGAAAGATGAAGATGGTAATGGTAGTGAAGCCAC AGATGTA-3'

p20K shRNA Reverse:

5'-ATTCACCACCACTAGGCAGGCAAGATGAAGATGGTAATGGTACATCT GTGGCTTCACT-3'

TUNEL Assays: TUNEL assays were conducted as per manufacturers protocols (Roche 12156792910). Essentially, cells were seeded onto glass coverslips in 60mm dishes and

incubated in their respective conditions for 36 hours. Cells were then fixed utilizing 4% formaldehyde in 1xPBS for 1 hour at room temperature and were subsequently permeabilized utilizing 0.1% Triton X-100 in 0.1% sodium citrate. Cells were washed 3 times with 1xPBS and incubated with terminal deoxynucleotidyl transferase (TdT) and TMR red-fluorescent labeled dUTP provided by Roche (12156792910) for 1 hour at 37°C. Samples were mounted onto glass slides with a drop of 1xPBS. Several fields of each sample were taken such that a minimum of 100 cells per sample were counted. Each condition was analyzed in quadruplicate for significance.

RESULTS

Mis-expression of p20K alters saturation density in chicken embryo fibroblasts. The quiescence-specific p20K protein is a member of the lipocalin family of lipid-binding proteins. In chicken embryo fibroblasts (CEF), p20K is expressed preferentially at contact inhibition or in response to hypoxia but is poorly induced when cells are arrested by serum/medium depletion (11, 12, 16). Experiments based on the micro-injection of antibodies in embryos suggest a role for p20K in cell survival (15). However, its role during development and in growth arrested cells remains poorly understood. To characterize the function of p20K, we compared the proliferation of chicken embryo fibroblasts infected with replication-competent viruses encoding p20K or a shRNA causing its down-regulation (Fig.1A). Since hypoxia is a potent inducer of growth arrest and p20K, and a feature of contact inhibited CEF (16), we compared the effect of p20K mis-expression on CEF in conditions of normoxia or hypoxia. While incubation in 2% O_2

inhibits proliferation, these conditions of hypoxia did not cause ER stress as indicated by the absence of CHOP induction, a marker of ER stress (18, 19). In fact, CHOP levels were lower in CEF subjected to hypoxia since CHOP is also expressed albeit at a low basal level in actively dividing CEF and is repressed in conditions of reversible growth arrest such as contact inhibition (16). Forced expression of p20K enhanced CEF saturation density at normoxia (Fig.1B). In limiting oxygen concentrations (2% O_2), proliferation was inhibited within 24 hrs of incubation but continued, albeit at a lower rate, in cells over-expressing p20K. Since p20K is not expressed in sub-confluent cells, actively dividing CEF expressing the p20K shRNA accumulated normally in conditions of normoxia. In contrast, CEF accumulation was impaired when cells were transferred to 2% oxygen (Fig.1C). In conditions of normoxia, proliferation was also affected by the down-regulation of p20K in cultures approaching confluence and saturation density was reduced by the expression of the p20K shRNA (at 48-96 hrs in Fig.1C). Collectively these results indicate that p20K promotes CEF accumulation in restrictive conditions such as high cell density or hypoxia.

p20K enhances cell survival in conditions of hypoxia. Mis-expression of p20K may affect the response of confluent cells to contact inhibition and growth arrest or regulate cell survival at high cell density and/or during hypoxia. As determined in TUNEL assays, the incidence of apoptosis was low in control cells in normoxia and in response to moderate conditions of hypoxia (24 hrs in 2% O_2; Fig.2). Apoptosis was also negligible for CEF expressing the p20K shRNA in conditions of normoxia but increased markedly when cells were transferred to 2% oxygen (Fig.2). In these conditions, the

incidence of apoptosis increased by more than 10fold in response to p20K inhibition. Therefore, p20K promotes cell survival in conditions of hypoxia.

Increased lipid peroxidation is observed in conditions of p20K inhibition and hypoxia. Hypoxia is associated with the release of reactive oxygen species (ROS) and oxidation of biological macromolecules including lipids (17). To examine the role of p20K in lipid homeostasis, we performed a fluorescence assay based on the oxidative sensitivity of the lipophilic fluorophore C11-BODIPY$^{581/591}$ (20). The fluorescence of intact C11-BODIPY$^{581/591}$ dye is red but shifts to green (510 nm) when the fluorophore is oxidized, providing a read-out for the level of lipid oxidation in the cell. Control CEF treated with C11-BODIPY$^{581/591}$ showed little evidence of lipid oxidation in conditions of normoxia or hypoxia (Fig.3). Similar results were obtained with cells expressing the p20K shRNA in conditions of normoxia. However, intense green fluorescence indicative of lipid oxidation was evident when these cells were transferred to limiting oxygen conditions for 24 hrs. These results were confirmed by quantifying the levels of malondialdehyde (MDA), a bi-product of lipid peroxidation (17). MDA levels increased when CEF were transferred to 2% O_2 for 24 hrs and this increase was enhanced by the down-regulation of p20K (Fig.4, compare lanes 2 and 6). No difference was observed when control and p20K shRNA-expressing cells were cultured in conditions of normoxia. Collectively, these results indicate that p20K plays a role in lipid homeostasis by alleviating the effects of lipid oxidation caused by limiting oxygen concentrations.

Lipids accumulate in response to the down-regulation of p20K and hypoxia. Large vesicles were apparent in CEF subjected to hypoxia and expressing the p20K

shRNA (Fig.3B). These vesicles accumulated rapidly in 2% O_2 but were not detected in conditions of normoxia. They were rare in control CEF (RCASBP(A) GFP RNAi) in normoxia or hypoxia. Staining with the lipophilic dye Oil-Red-O indicated that these vesicles contain lipids (Fig.5). Small lipid-containing vesicles were observed in control cells but these were rare while large vesicles were absent. Therefore, the down-regulation of p20K causes the accumulation and peroxidation of lipids during hypoxia.

DISCUSSION

The p20K lipocalin promotes survival during reversible growth arrest. Confluent chicken embryo fibroblasts (CEF) enter G_0 as a result of contact inhibition. CEF confluence and the entry into G_0 is characterized by the abrupt induction of the p20K lipocalin gene (5). This induction is mediated by the interaction of C/EBPβ homodimers with two elements of a 48 bp region of the p20K promoter known as the Quiescence Responsive Unit or QRU (21). In actively dividing cells, the QRU is occupied by the ERK2 kinase, which binds to repeated GAAAG elements overlapping the C/EBPβ binding sites (16). Thus competition between ERK2 and C/EBPβ restricts the expression of p20K during proliferation. *In vitro*, contact inhibited CEF remain viable for extensive periods of time at confluence but begin to starve as a result of nutrient depletion. Starvation is characterized by the induction of the pro-apoptotic CHOP member of the C/EBP family, the inhibition of p20K expression, the onset of ER stress and cell death (16, 18). Thus, p20K expression is restricted to and is a marker of reversible growth arrest.

For quiescent cells to re-enter the cell cycle, they must first ensure that cell homeostasis and viability are preserved during growth arrest and that they remain responsive to mitogenic stimulation. CEF expressing the p20K shRNA proliferated normally in exponentially dividing cells (which do not express p20K) but did not accumulate at the same rate and did not reach the saturation density of control cells (Fig.1C). Cell accumulation was also reduced in conditions of limiting oxygen concentrations. As indicated by the results of TUNEL assays, CEF expressing the p20K shRNA were characterized by a high incidence of cell death in conditions of hypoxia, suggesting that p20K acts as a survival factor when oxygen becomes limiting (Fig.2). Significantly, fewer than 1% of control cells underwent apoptosis suggesting that our conditions of hypoxia (2% O_2 for 24 hrs) are well tolerated and do not result in extensive cell death in normal cells (Fig.2C). These results suggest that p20K is part of an adaptive response ensuring cell viability in conditions of limiting oxygen concentrations. Since hypoxia is a feature of contact inhibited CEF (16), p20K likely fulfills a similar role at confluence.

Several lipocalins have a cytoprotective role in various cellular contexts and in a wide range of organisms. *Arabidopsis* encodes two lipocalins, TIL (temperature-induced lipocalin) and CHL (chloroplastic lipocalin), involved in the stress response caused by heat and high light, respectively (7). Double TIL and CHL mutants are hypersensitive to temperature, drought and light stresses and exhibit increased lipid peroxidation in these conditions of stress. Murine apolipoprotein M, a lipocalin and component of High Density Lipoproteins (HDL), binds to oxidized phospholipids and accounts for some of

the antioxidant effects of HDL (9). The OBD lipocalin (odorant binding protein), expressed by nasal mucosa in mammals, binds to 4-hydroxy-2-nonetal (HNE) a cyto-toxic byproduct of lipid peroxidation, and enhance cell viability of HNE-treated nasal epithelium cells (4). Likewise, the human tear Lipocalin 1 (lnc-1) is induced in response to compounds causing lipid peroxidation such as H_2O_2 or $FeSO_4$ and exerts a cyto-protective role by scavenging lipid peroxidation byproducts (3). The NGAL lipocalin (human neutrophil gelatinase-associated lipocalin; also known as lnc-2), the closest relative of p20K in mammals, is also a siderocalin exhibiting a bi-functional activity, promoting survival when bound to iron but increasing apoptosis when lacking iron (10, 22). Therefore, lipocalins are emerging as a novel class of stress-response factors promoting lipid homeostasis and/or cell survival. Whether or not the cyto-protective activity of p20K is dependent on iron binding remains to be determined but, unlike NGAL/lnc-2, p20K has never been associated with pro-apoptotic conditions and, in fact, is strongly repressed by ER stress and the induction of the pro-apoptotic CHOP factor (16). Thus, it is likely that, like several lipocalins, p20K enhances survival by limiting the effects of lipid peroxidation. Whether or not it is able to bind and sequester oxidized lipids remain to be determined and is the subject of future investigations.

p20K is a component of a novel "*Lipid Homeostasis Response*". Significantly, the over-expression of p20K enhanced CEF survival at confluence and proliferation in conditions of limiting oxygen concentrations (Fig.1B). The fact that p20K-overexpressing cells continued to accumulate, albeit at a reduced rate, in conditions of 2% oxygen suggests that lipid peroxidation is a critical factor impeding cell proliferation in

normal CEF (Fig.1B). Membrane phospholipids may function in an analogous manner to REDD1, the oxygen-sensor in the mTOR pathway and regulator of protein synthesis (23), by restricting proliferation when oxygen becomes limiting.

Previous gene profiling analyses revealed that several factors with a role in lipid metabolism, including the Fatty Acid Binding Protein 4 and 5 (FABP4/5), the cholesterol membrane transporter ABCA1, the ectonucleotide pyrophosphatase/phosphodiesterase 1 & 2 (ENPP1, ENPP2) and sulfotransferase 1E1 (SULT1E1) are all strongly induced by contact inhibition suggesting that high cell density and perhaps oxygen depletion engages a systemic response promoting lipid homeostasis and required for cell survival (16). The results of preliminary lipid profiling analyses revealed that several lipid species including cholesterol, palmitate and stearate accumulate in response to hypoxia (our unpublished results). Since compounds such as palmitate are pro-apoptotic, the induction of factors restoring lipid homeostasis may enhance cell survival by reducing lipotoxicity (24, 25). The characterization of such "*Lipid Homeostasis Response*" may improve our understanding and treatment of conditions characterized by hypoxia such as ischemia.

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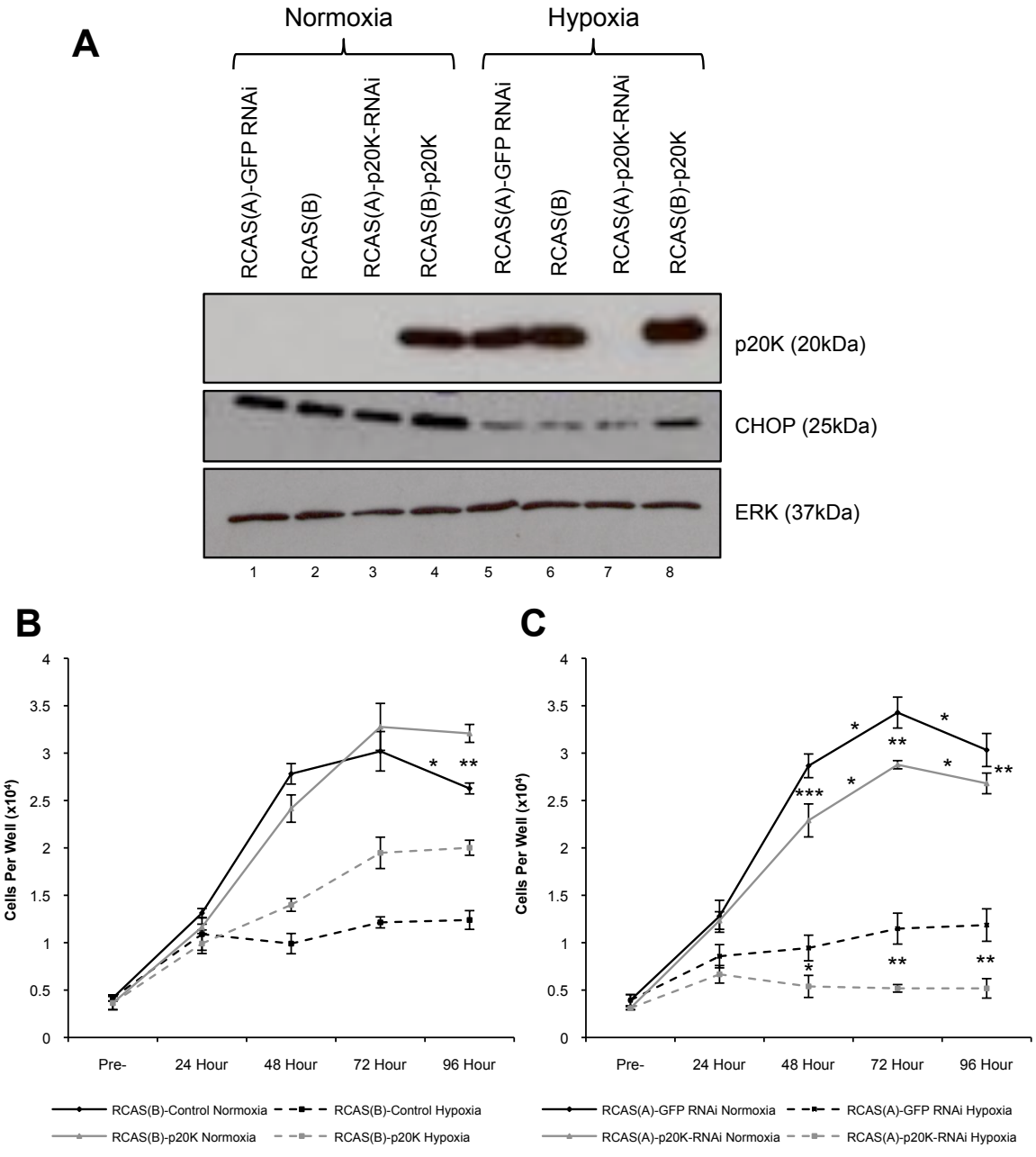

A
Normoxia
Hypoxia
RCAS(A)-GFP RNAi
RCAS(B)
RCAS(A)-p20K-RNAi
RCAS(B)-p20K
RCAS(A)-GFP RNAi
RCAS(B)
RCAS(A)-p20K-RNAi
RCAS(B)-p20K
p20K (20kDa)
CHOP (25kDa)
ERK (37kDa)
1 2 3 4 5 6 7 8
B
C
Cells Per Well (x10⁴)
Pre-
24 Hour
48 Hour
72 Hour
96 Hour
RCAS(B)-Control Normoxia
RCAS(B)-Control Hypoxia
RCAS(B)-p20K Normoxia
RCAS(B)-p20K Hypoxia
RCAS(A)-GFP RNAi Normoxia
RCAS(A)-GFP RNAi Hypoxia
RCAS(A)-p20K-RNAi Normoxia
RCAS(A)-p20K-RNAi Hypoxia

Figure 1: A) Western blotting analysis of p20K and CHOP in CEF infected with a group A virus expressing a control shRNA for GFP (RCAS(A)-GFP RNAi) or shRNA for p20K (RCAS(A)-p20K RNAi) incubated in normoxic (21% O_2) and hypoxic (2% O_2) conditions for 36 hours. ERK was utilized as a loading control. B) Proliferation of CEF infected with a group B virus control (RCAS(B)-Control) or group B virus over expressing p20K (RCAS(B)-p20K) in condtions of hypoxia (2% O_2) or normoxia (21% O_2). C) Proliferation of CEF infected with a group A virus expressing a control shRNA for GFP (RCAS(A)-GFP RNAi) or shRNA for p20K (RCAS(A)-p20K RNAi) incubated in normoxia (21% O_2) or hypoxia (2% O_2). Pre- represents cell counts taken prior to incubation in hypoxia. Error bars represent the standard deviation of the mean. Asterisks represent significance determined by pair-wise comparisons. Asterisks between error bars represent pair-wise significance between samples within the same conditions. Asterisks above lines represent significance between time points within the same sample. * Indicates $P \leq 0.05$, ** indicates $P \leq 0.01$, *** indicates $P \leq 0.001$.

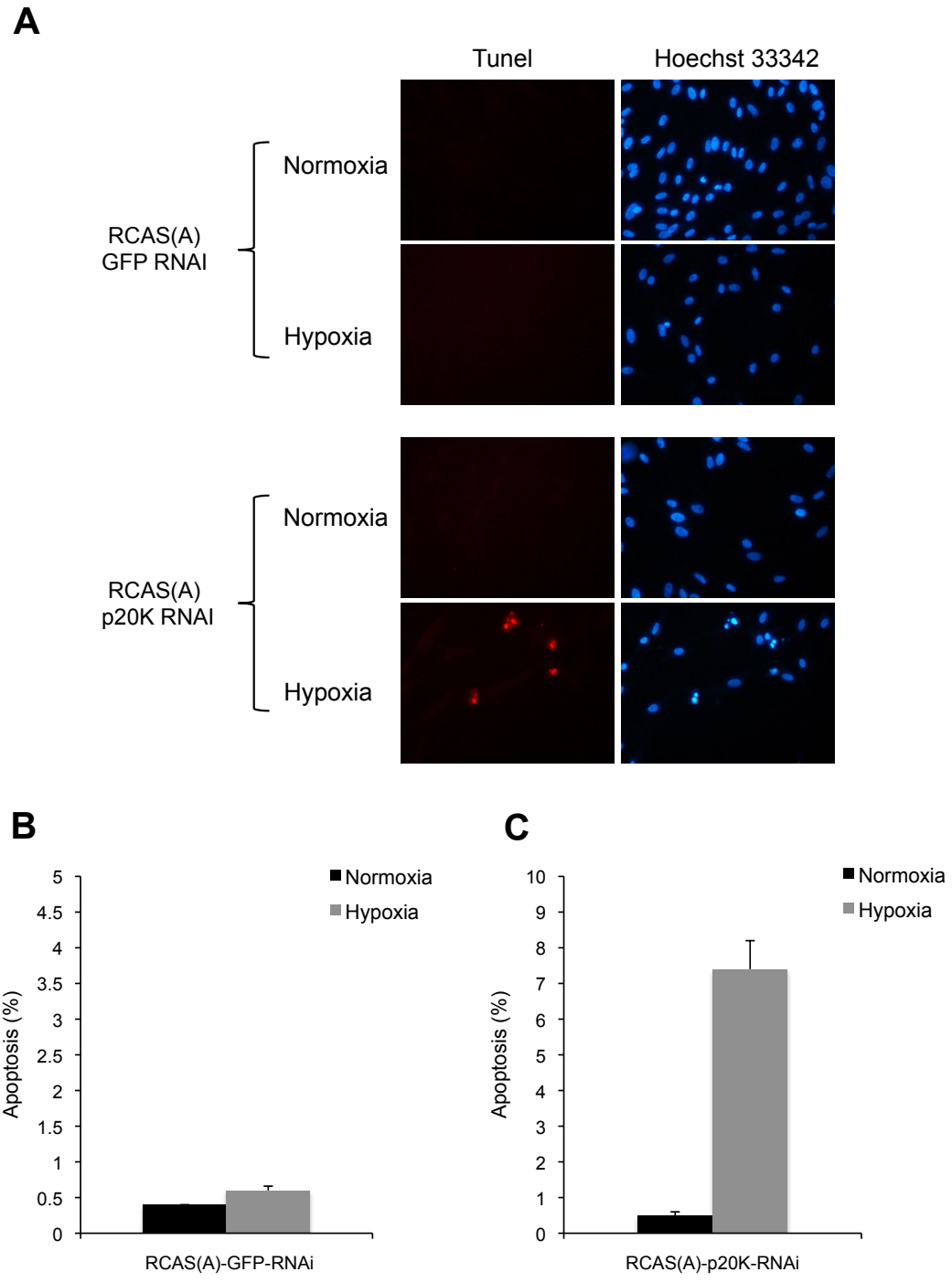

A
Tunel
Hoechst 33342
RCAS(A)
GFP RNAI
Normoxia
Hypoxia
RCAS(A)
p20K RNAI
Normoxia
Hypoxia
B
Normoxia
Hypoxia
Apoptosis (%)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
RCAS(A)-GFP-RNAi
C
Normoxia
Hypoxia
Apoptosis (%)
10
9
8
7
6
5
4
3
2
1
0
RCAS(A)-p20K-RNAi

Figure 2: A) TUNEL assays of CEF infected with a group A virus expressing a control shRNA for GFP (RCAS(A)-GFP RNAi) or shRNA for p20K (RCAS(A)-p20K RNAi) incubated in normoxic (21% O_2) and hypoxic (2% O_2) conditions for 36 hours. B) Quantitation of the levels of apoptosis as shown in (A). Error bars represent standard deviations of the mean.

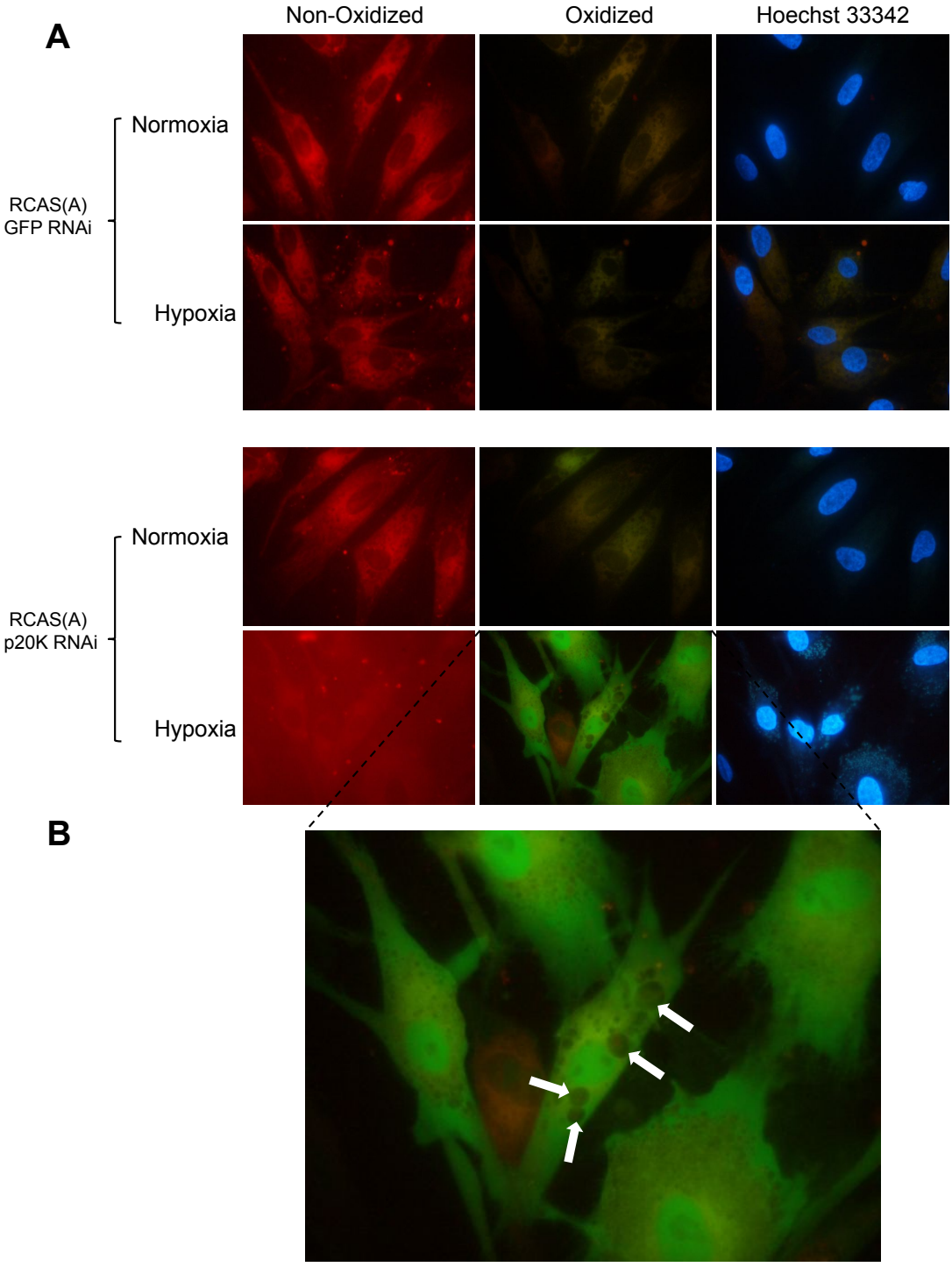

A
Non-Oxidized
Oxidized
Hoechst 33342
Normoxia
RCAS(A)
GFP RNAi
Hypoxia
Normoxia
RCAS(A)
p20K RNAi
Hypoxia
B

Figure 3: A) Fluorescent staining with C11-Bodipy$^{581/591}$ lipid peroxidation sensor. CEF were infected with a group A virus expressing a control shRNA for GFP (RCAS(A)-GFP RNAi) or shRNA for p20K (RCAS(A)-p20K RNAi) and incubated in normoxic (21% O_2) and hypoxic (2% O_2) conditions for 36 hours prior to fluorescent labeling. Non-oxidized and oxidized lipid conditions are shown. Hoechst 33342 was utilized for nuclei staining. B) Image blow-up of oxidized lipids in CEF infected with a group A virus expressing p20K incubated in hypoxia. White arrows point to vesicles accumulating in this condition.

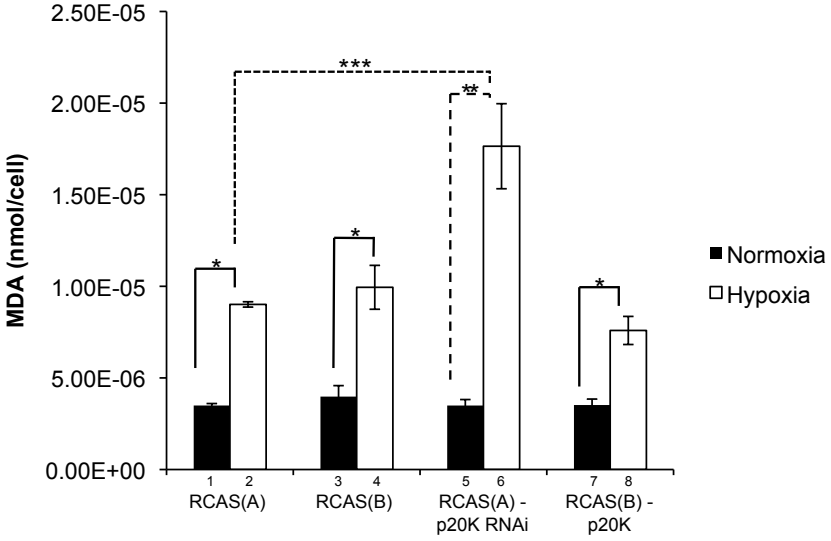
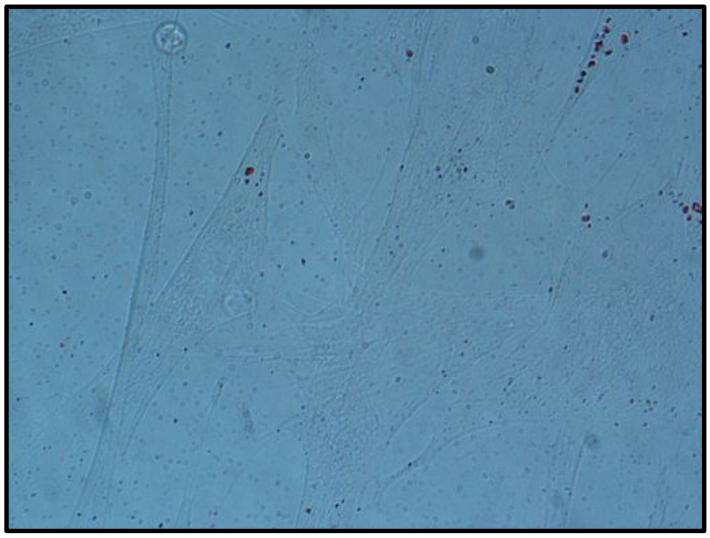

2.50E-05
2.00E-05
1.50E-05
1.00E-05
5.00E-06
0.00E+00
MDA (nmol/cell)
***
**
*
*
*
1 2
RCAS(A)
3 4
RCAS(B)
5 6
RCAS(A) - p20K RNAi
7 8
RCAS(B) - p20K
Normoxia
Hypoxia

Figure 4: Quantitation of MDA levels in CEF infected with a group A virus expressing a control shRNA for GFP (RCAS(A)) or shRNA for p20K (RCAS(A)-p20K RNAi) and CEF infected with a group B virus control (RCAS(B)-Control) or group B virus over expressing p20K (RCAS(B)-p20K). Cells were incubated in either hypoxic (2% O_2) or normoxic (21% O_2) conditions for 36 hours. Relative detection of MDA levels were analyzed using thiobarbituric acid (TBA) to create MDA-TBA adducts which were quantified colorimetrically at an OD of 532nm. * Indicates $P \leq 0.05$, ** indicates $P \leq 0.01$, *** indicates $P \leq 0.001$.

RCAS(A)
GFP RNAi



RCAS(A)
p20K RNAi

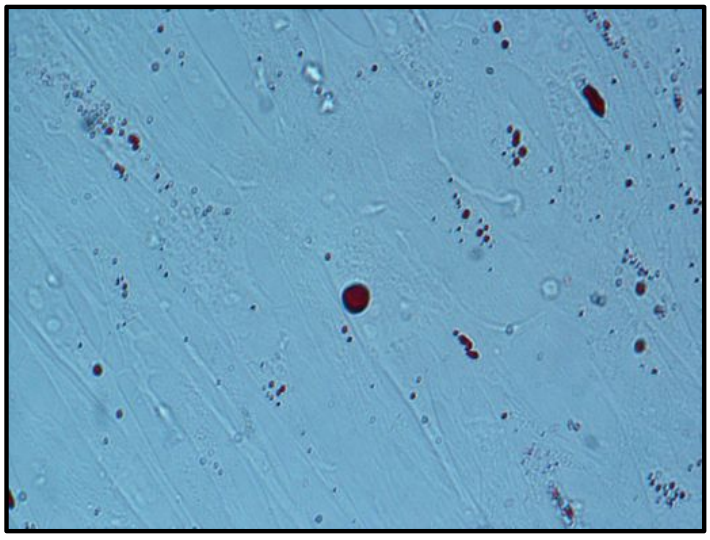


Figure 5: Staining of lipid rich vesicles with Oil Red-O in CEF infected with a group A virus expressing a control shRNA for GFP (RCAS(A)-GFP RNAi) or shRNA for p20K (RCAS(A)-p20K RNAi) incubated in hypoxic (2% O_2) conditions for 36 hours.

Discussion

Chapter 1: ERK2 and CHOP restrict the expression of the growth-arrest specific p20K lipocalin gene to G_o

1.1 p20K Expression is Growth Arrest Specific

Preliminary gene profiling studies were able to demonstrate that multiple hypoxia regulated genes, such as enolase and carbonic anhydrase IX and XII, were present in contact inhibited gene profiles. This data led us to believe that confluence may promote oxygen deprivation in culture, which in turn may lead to the expression of growth arrest specific genes such as p20K in a hypoxia dependent manner. Follow up studies on this assumption revealed that confluent cells in culture exhibited elevated levels of nitroreductase activity, an indicator of hypoxia (Chapter 1 Figure 2A). Interestingly, when analyzing the promoter region of p20K, it was apparent that there were two putative hypoxia responsive elements (HRE) located upstream of the QRU (Chapter 1 Figure 4A). Transient expression assays analyzing the activation potential of these domains revealed that these regions were not required for the transcriptional activation of p20K in low oxygen concentrations (2% O_2) (Chapter 1 Figure 4C). These results were further supported by the fact that when analyzing the protein kinetics of p20K and HIF1α, the induction of the hypoxia inducible factor HIF1α was rapid in response to hypoxia (within two hours of incubation), whereas p20K accumulation was only apparent 18 hours after incubation (Chapter 1 Figure 3B). If p20K induction were dependent on the HRE elements located within the promoter, we would expect to see p20K accumulation shortly after the induction of HIF1α. These results suggest that the induction of p20K through the

QRU by C/EBPβ is the result of cells undergoing growth arrest due to limiting oxygen concentrations, and is not a direct response to hypoxia. Collectively, these findings suggest that p20K plays a role in the response to hypoxia at growth arrest. Interestingly further analysis of the gene profiling results of contact inhibited cells revealed that many other lipid binding and lipid associated proteins were highly induced. As recent unpublished results indicated that p20K enhances cell survival in limiting oxygen concentrations (Chapter 2), these data suggest that the induction of p20K may be an adaptive response to low oxygen concentrations and lipid metabolism.

1.2 CHOP Acts as an ER Stress Response Regulator of p20K

Previous studies by Kim *et al*., 1999 were able to demonstrate that the transcriptional activation of p20K depends on the binding of C/EBPβ to two regions within the QRU. Interestingly, when subjecting the cells to starvation via prolonged nutrient depletion or ER stress it was evident that p20K expression was inhibited (Chapter 1 Figure 1A). When analyzing protein kinetics during these states it was observed that a marked induction of the ER stress-inducible protein CHOP correlated with the down regulation of p20K. As previous studies have shown that CHOP can act as a dominant-negative inhibitor of gene transcription through the dimerization with the C/EBPβ isomer LAP (Ron & Habener, 1992), we wished to observe if CHOP could function to block p20K induction through C/EBPβ during these states. Down-regulation of CHOP through the use of shRNA was shown to enhance the expression of p20K at confluence and slightly promote the expression of p20K in cycling states (Chapter 1 Figure 5A). The enhanced expression of p20K at confluence may be due in part to the fact that cells

lacking CHOP proliferate at a faster rate then controls cells. As such, CEF lacking CHOP at confluence may have been at a higher density then controls, which resulted in a greater accumulation of p20K. Furthermore, forced expression of CHOP was shown to attenuate the expression of p20K in hypoxia and conditions resulting in the strong activation of CHOP, such as treatment with tunicamycin or thapsigargin, resulted in the loss of p20K expression at confluence (Chapter 1 Figure 5B and Figure 6A). Interestingly, when knocking down CHOP during treatment with the ER stress inducing tunicamycin or thapsigargin at confluence, it was apparent that p20K expression was only reestablished during tunicaymcin treatment but not with thapsigargin treatment (Chapter 1 Figure 6B). When analyzing the levels of CHOP it was noticeable that thapsigargin treatment resulted in a greater expression of CHOP as compared to tunicamycin, which was comparable to basal cycling levels which may account for the sustained repression of p20K (Chapter 1 Figure 6C). To analyze if this regulation of p20K by CHOP was due to the direct dimerization of CHOP with C/EBPβ we conducted an immunoprecipitation assay. Results showed that CHOP was associated with C/EBPβ during proliferation, starvation, and ER stress however was not detected at confluence (Chapter 1 Figure 6D). This suggests that CHOP functions to block p20K expression through the interaction with C/EBPβ in states of starvation and ER stress.

1.3 C/EBPβ and the MEK Pathway Are Key Regulators of p20K Expression

When analyzing the kinetics p20K and CHOP in cycling states it was apparent that the knockdown of CHOP was not sufficient to promote the induction of p20K. This study led us to believe that an additional factor must be controlling the transcriptional

regulation of p20K in cycling states. When analyzing possible factors that could function to repress the activity of p20K we observed that the QRU contained two EBS which overlapped the C/EBPβ binding sites (Chapter 1 Figure 7A). ERK2 has only recently been described to have DNA binding potential (Hu *et al*., 2009), and has been shown to mediate the transcriptional repression of other C/EBPβ regulated genes. Preliminary ChIP studies analyzing this possible function of ERK2 were able to demonstrate that ERK2 associates to the QRU during cycling states however was not present during growth arrest (Chapter 1 Figure 7B). This association was shown to be mutually exclusive with C/EBPβ, as C/EBPβ and ERK2 rarely co-occupied the QRU. To show if this interaction with the QRU was direct, we employed an EMSA. Recombinant ERK2 was shown to interact with the repeated GAAAG element of the ERK2 binding site of the QRU, signifying that ERK2 binding to the QRU is direct (Chapter 1 Figure 7C). Further analysis analyzing the repressive potential of ERK2 through transient expression assays indicated that forced expression of ERK2 resulted in the repression of the QRU, even in growth arrested states (Chapter 1 Figure 8I). Likewise, mutations within the ERK2 binding sites led to constitutive activity of the QRU in cycling conditions (Chapter 1 Figure 8J). Interestingly, mutations within the ERK2 binding sites of the QRU resulted in a reduction of QRU activity at hypoxia. As the ERK2 binding sites and C/EBPβ binding sites overlap, it is possible that mutations within the ERK2 binding site also affect C/EBPβ interaction, resulting in a reduction of C/EBPβ binding potential to the QRU.

To further supplement previous findings, we wished to analyze the effect of the knockdown of ERK2 through shRNAs on p20K induction. Unfortunately CEF infected

with the shRNA quickly entered premature senescence, a cell state in which we know p20K is not expressed (Chapter 1 Supplementary Figure 1A-C). In addition, infected CEF showed a marked loss of C/EBPβ levels, which may also contribute to the lack of p20K expression observed. Interestingly, a similar senescence phenotype portrayed by these cells has been previously documented in mouse embryo fibroblasts lacking both *erk1* and *erk2* (Voisin *et al*., 2010). Due to the fact that a ERK1 gene has yet to be identified in the chicken genome, it is possible that the functions of ERK1 and ERK2 are solely dependent on ERK2 in this species, which would support previous studies analyzing the redundancy of ERK1 and ERK2 (Voisin *et al*., 2010). In addition, this would supplement the fact that ERK2 functions as a unique member of the ERK family through its ability to directly bind and regulate gene expression, a function which has to date only been shown with ERK2. To analyze if the MEK pathway was involved in controlling the transcriptional regulation of p20K, we treated cells with a MEK inhibitor (PD184352) to analyze what affect it would have on p20K expression. Interestingly, cells lacking activated ERK2 were viable, and did not enter premature senescence as seen through shRNA mediated knockdown of ERK2 (Chapter 1 Figure 8H). This may signify that ERK2 could attenuate senescence in its inactivated state or through a phosphorylation-independent manner. p20K induction was obvious after MEK inhibition, however the induction was minimal until 72 hours after treatment. This delayed induction may be due to the fact that CHOP down-regulation and nuclear exclusion of ERK2 after MEK inhibitor treatment took greater then 48 hours (Chapter 1 Supplementary Figure 2). Surprisingly, MEK inhibition in ts v-Src transformed cells showed a rapid activation of p20K (Chapter 1 Figure 9B). As

previous studies have portrayed that p20K is not expressed during ts v-Src transformation (Wang *et al*., 2011), this study highlights the inhibitory function of the MEK pathway in the control of p20K expression and the role of C/EBPβ in the induction of p20K expression.

The original model that Hu *et al*., 2009, suggested for the dynamics between C/EBPβ and ERK2 for gene regulation was that in normal cycling conditions ERK2 is able to occupy the promoter region, however when signaling (through cytokine stimulation or other mechanisms) causes the activation of C/EBPβ through ERK2, C/EBPβ is able to rapidly out-compete ERK2 for binding to these promoter elements (Hu *et al*., 2009). Based on the fact that we have previously shown that the forced expression of C/EBPβ is able to promote the induction of p20K in cycling CEF, we wished to test this model. Interestingly, we were able to portray that the forced expression of C/EBPβ abrogated the association of ERK2 with the QRU in cycling conditions (Chapter 1 Figure 8A). As the forced expression of C/EBPβ did not result in the nuclear exclusion of ERK2 (Chapter 1 Figure 8B-G), this evidence supports the original model of Hu *et al*., 2009, and suggests that ERK2 and C/EBPβ compete for QRU occupancy in the control of p20K expression.

Conclusion

From the findings described above, it is apparent that the transcriptional regulation of p20K is mediated by inhibitory factors which function through various mechanisms. As the factors determining the specificity of gene activation by C/EBPβ have yet to be fully elucidated, this report aids in the understanding of the ability of C/EBPβ to exert its

pleiotropic function in a variety of cell contexts. In this report we establish that ERK2 is a transcriptional repressor of the QRU in actively dividing cells. This transcriptional regulation is mutually exclusive with C/EBPβ, as ERK2 is detected on the QRU in conditions of cell proliferation and p20K repression and C/EBPβ is detected on the QRU in conditions of growth arrest and p20K induction. Forced expression of C/EBPβ, which resulted in the loss of ERK2 recruitment to the QRU, suggests that ERK2 and C/EBPβ compete for the QRU occupancy in the control of p20K expression. Furthermore, we were able to portray that conditions leading to ER stress, such as prolonged starvation in culture, promoted the expression of the stress-inducible CHOP protein, which directly correlated with a loss of p20K expression. This loss of p20K expression relied on the direct association of CHOP with C/EBPβ.

Gene profiling and RT-qPCR studies which were done in combination with the previously described studies revealed that several hypoxia specific gene markers, including carbonic anhydrase IX, were shown to be induced in contact inhibited CEF. Follow up studies utilizing a hypoxia specific reporter dye were able to portray that cells at high density exhibited a significant depletion of oxygen levels, suggesting that cells experience hypoxic conditions during high cell density in culture. Interestingly, other lipid binding proteins, along with p20K, were shown to also be highly induced in conditions of both hypoxia and contact inhibition. Although p20K was not shown to be induced in direct response to hypoxia, it is possible that it, along with other lipid binding proteins, play a role in response to low oxygen concentrations.

Chapter 2: The p20K Lipocalin is Required for Lipid Homeostasis and Cell Survival in Hypoxic Chicken Embryo Fibroblasts

2.1 Loss of p20K Alters CEF Viability in Hypoxia

Previous studies on the transcriptional regulation of the p20K lipocalin gene revealed that it is induced in conditions of contact inhibition or hypoxia induced growth arrest. Further analysis indicated that contact inhibited cells experience hypoxic environments in culture. This analysis led us to believe that p20K may be regulated in a hypoxia specific manner, and as such may play a role in the response to hypoxia.

To first address what function p20K plays in hypoxic states, a proliferation assay was conducted on CEF mis-expressing or overexpressing p20K. It was evident that the loss of p20K resulted in a decrease in cell viability within hypoxia as compared to controls (Chapter 2 Figure 1C). Inversely, the overexpression of p20K in these states allowed cells to continue to accumulate, albeit at a reduced rate, in hypoxia (Chapter 2 Figure 1B). Likewise, CEF lacking p20K were unable to accumulate to as high a density as control samples, whereas the overexpression of p20K allowed cells to reach a higher saturation density. To address if this loss of cell viability was due to apoptosis, a TUNEL assay was performed. From these results it was evident that there was a higher incidence of TUNEL staining in p20K knockdown cells in hypoxia as compared to both normoxia and control cells (Chapter 2 Figure 2). Normal CEF under hypoxia experienced a minimal amount of cell death (<1%), indicating that these cells are able to adapt and survive within these conditions. In all, these results suggest that p20K may play a survival role in CEF which allow cells to adapt to limited oxygen concentrations.

2.2 Loss of p20K Results in Higher Levels of Lipid Peroxidation in Conditions of Hypoxia

In states of low oxygen concentrations cells undergo oxidative stress which results in the buildup of free radicals or ROS (Moldovan and Moldovan, 2004). These ROS may elicit free radical attacks on PUFAs and membrane lipids which results in the formation of lipid radicals, which can rapidly lead to a chain reaction of lipid peroxidation and eventually apoptosis (Ayala *et al*., 2014). As p20K, like many other members of the lipocalin family, has been shown to bind fatty acids through the use of its β-barrel calyx (Flower, 1996), it was postulated that it may function to prevent or regulate the response to lipid peroxidation during hypoxia. In order to address if this pro-survival role of p20K was related to lipid peroxidation we employed the C11-BODIPY$^{581/591}$ dye developed by Molecular Probes. The C11-BODIPY$^{581/591}$ fluorescent probe is a fatty acid analogue with fluorescent properties in the red spectrum when non-oxidzed. However, when it undergoes oxidation its properties shift from a red to green fluorescence, allowing for the localization of lipid peroxidation and intensity at a subcellular level. As seen from the results, lipid peroxidation, as marked by a green fluorescence, is only seen in cells incubated in hypoxia where p20K is absent (Chapter 2 Figure 3A). Interestingly, the control construct (RCAS(A)-GFP RNAi), did not display a visible amount of lipid peroxidation in hypoxia, signifying that this fluorescent probe must require a significant increase of ROS and lipid peroxidation in order to undergo a spectral shift. When analyzing the intracellular localization of lipid peroxidation, it appears that it is mostly contained within the nucleus, with some levels of lipid peroxidation also occurring within

the ER and Golgi (Chapter 2 Figure 3B). As C11-Bodipy$^{581/591}$ has been shown to not preferentially integrate into any membrane, it can be suggested that lipid oxidation during low oxygen concentrations are primarily seen within these regions (Drummen *et al*., 2002). These results are interesting, as p20K localization is rarely seen near the nucleus. This may suggest that other shuttling proteins besides p20K may be needed in order to regulate these peroxidized lipids.

Although the C11-BODIPY$^{581/591}$ probe allowed us to measure the relative intensity and subcellular localization of lipid peroxidation, we still wished to measure the overall levels of lipid peroxidation per cell. As such, we employed a MDA colormetric assay. In essence, lipid peroxidation forms byproducts of reactive aldehydes, one of which is malondialdehyde (MDA) (Gawel *et al*., 2004). MDA levels can be analyzed using thiobarbituric acid (TBA) to create MDA-TBA adducts which can be quantified colorimetrically at an OD of 532nm (Garcia *et al*., 2005). As seen from the results (Chapter 2 Figure 4) there is an highly significant increase in MDA production when p20K is absent from CEF in hypoxic conditions as compared to normoxic conditions and the RCAS(A) empty vector control cells. Interestingly, it appears that MDA concentrations are similar within both the RCAS(B) control and p20K overexpression samples. These results may be due to the fact that similar levels of the p20K protein are seen within these two conditions, and as such a significant difference in MDA production may not be seen.

In all, these results provide a molecular mechanism accounting for the loss of viability in cells lacking p20K, as lipid peroxides have been shown to readily induce

apoptosis (Sandstrom *et al*., 1994). Another explanation for the increase in apoptosis seen within p20K knockdown CEF in hypoxia may be the possible accumulation of the secondary byproduct of lipid peroxidation 4-HNE. 4-HNE is a cytotoxic end product of lipid peroxidation, and has been shown to promote oxidative stress-induced cell death in many cell types (Choudhary *et al*., 2002). Due to the fact that we see an increase in one of the secondary byproducts of lipid peroxidation; MDA, it would not be surprising if these cells also experienced an increase in 4-HNE as well. 4-HNE has been shown to mediate apoptosis through p53 and the Bax, p21 and JNK signaling components which ultimately inhibits antiapoptotic genes such as Bcl2 (Abarikwu *et al*., 2012; Sharma *et al*., 2008; Choudhary *et al*., 2002). 4-HNE has also been shown to mediate cell death through Fas signaling on the plasma membrane which can ultimately activate downstream apoptotic signaling (Sharma *et al*., 2008).

Interestingly many other members of the lipocalin family have cytoprotective roles in various cell contexts. Some lipocalins exert their cytoprotective roles through the direct binding of lipids, an example being the RBP lipocalins which have been shown to protect retinol against oxidative damage during circulation through active binding (Flower, 1993). Other lipocalins exert their cytoprotective roles through the sequestration of lipid peroxidation byproducts, excellent examples being odorant binding protein (OBD) and tear Lipocalin 1 (Lechner *et al*., 2001; Grolli *et al*., 2006). As such, it is likely that, like other lipocalin family members, p20K enhances the survival of CEF in limiting oxygen concentrations by limiting the effect of lipid peroxidation. In all it is apparent that

the lipocalin protein family is a large and still poorly characterized family which may play direct roles in promoting lipid homeostasis.

2.3 Loss of p20K results in accumulation of lipids in low oxygen concentrations

A former study analyzing the function of p20K was able to portray that the inhibition of p20K function through the use of antibody injection resulted in the accumulation of fatty acids within chicken embryos (Gentilli *et al*., 2005). It is possible that during states of low oxygen, p20K is needed to shuttle lipids that accumulate during hypoxia/contact inhibition out of the cell. This accumulation of lipids may increase the potential targets of peroxidation or increase the presence of pro-apoptotic lipids that need to be recycled or excreted out of the cell. Pro-apoptotic lipids could increase the cytotoxic biproducts of lipid peroxidation and increase cell death. To address this, CEF were stained with an Oil Red-O lipid stain to analyze lipid vesicle accumulation. As seen from Chapter 2 Figure 5, cells lacking p20K incubated in hypoxia show an accumulation of the lipophilic dye within large vesicles, indicating that they contain a large quantity of lipids. These results suggest that cells lacking p20K have difficulties in shuttling lipids out of the cell, resulting in the accumulation of lipid rich vesicles. In all these conditions may determine the loss of viability seen in CEF lacking p20K in conditions of low oxygen concentrations. Interestingly, gene profiling analysis conducted prior to these studies were able to portray that several factors involved in lipid metabolism and homeostasis (including ectonucleotide pyrophosphatase/phosphodiesterase 1 and 2 (ENPP1/2) and the Fatty Acid Binding Protein 4 and 5 (FABP4/5)) were highly induced by contact inhibition. As aforementioned, we believe that contact inhibited cells experience hypoxic

conditions, and as such these genes may be regulated in a hypoxia specific manner. In conjunction with this, preliminary lipid profiling analysis was able to show that many lipid species such as palmitate and stearate accumulate in conditions of hypoxia (Unpublished Results). In all, the combined data suggests the existence of a novel pathway, which includes p20K, that acts in response to hypoxia and is required to restore lipid homeostasis and protect cells against lipotoxicity.

Conclusions

Previous studies conducted within our lab on the transcriptional regulation of the p20K lipocalin gene revealed that it is regulated in a hypoxia-specific manner. Although other studies indicated that p20K plays multiple roles within a cell, including cell survival, inflammatory response and cell development, the role of p20K in hypoxia has still yet to be elucidated. In this study we show that loss of p20K drastically affects the viability of CEF in conditions of hypoxia. Further analysis showed that this loss of p20K resulted in a significant increase in the levels of lipid peroxidation within these states. These results may account for the altered viability of CEF seen in this state, as increases in lipid peroxidation have been shown to promote apoptosis through various mechanisms (Volinsky and Kinnunen, 2013). This may be mediated directly by pro-apoptotic fatty acids which accumulate during this state, or it is possible that it may be mediated by secondary products of lipid peroxidation, such as 4-HNE, which has been shown to promote apoptosis through p53. In order to address if p20K helps mediate the shuttling of lipids during hypoxia, CEF were stained with a lipophilic Oil Red-O dye. It was apparent that lipid vesicles accumulated in conditions of p20K knockdown as compared to

controls. The accumulation of lipids within these states may promote the accumulation of peroxidized lipids or pro-apoptotic lipids leading to cytoxic effects.

Collectively, it is evident that p20K plays a pro-survival role in conditions of hypoxia which be in relation to its ability to protect CEF against lipid peroxidation. Although the exact mechanism of p20Ks ability to protect against lipid peroxidation is unknown, these results suggest that there is a novel pathway which has not been identified in scientific literature which deals with the response and regulation of lipid homeostasis during low oxygen concentrations. This hypothesis is currently being tested.

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