CHARACTERIZING THE ROLE AND REGULATION OF GROWTH ARREST SPECIFIC FABP4 IN CHICKEN EMBRYO FIBROBLASTS

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Abstract

Conditions which promote reversible growth arrest, such as hypoxia and high cell density, lead to activation of a diverse network of proteins known as growth arrest specific (GAS) genes. Fatty acid binding protein 4 (FABP4), a lipid chaperone involved in the regulation of metabolic and inflammatory responses, has been shown to be part of the GAS program. While the induction of FABP4 in oxygen-deprived environments is well characterized, its functionality and regulation in such conditions remains unclear. In this study, we describe how mis-expression of FABP4 affects cell viability and survival within low oxygen conditions. Loss of FABP4 using shRNA was shown to be associated with a significant increase in oxidative stress and lipid peroxidation, a reduction in lipid droplet formation and a greater incidence of apoptosis. Hypoxia-mediated expression of FABP4 was also found to be positively correlated with cellular levels of C/EBP, an essential activator of p20K in quiescence. FABP4 and p20K are both lipocalins that have been shown to share similar induction patterns and ability to assist in the maintenance of lipid trafficking in cellular stress circumstances. Unexpectedly, the depletion of FABP4 or p20K results in loss of the other in limited oxygen concentrations. This occurs independently of disruption to the broad GAS gene program, suggesting the two proteins may be co-regulated in a shared hypoxic-signalling pathway. C/EBPb appears to be the transcriptional activator shared by FABP4 and p20K in quiescence, and the three may be part of an intricate system to sense and respond to reactive oxygen species and lipid radicals. However, the forced expression of either FABP4 or p20K when the other is repressed only moderately restores cell survival through alleviating oxidative stress, indicating the two are both necessary for optimal response to hypoxia. In all, these studies suggest that analogous to the p20K lipocalin, FABP4

plays a critical role in lipid homeostasis and cell survival in conditions of limited oxygen concentrations, and its stimulation is dependent on C/EBPb activity.

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This project would not have been possible with the tireless support, guidance and encouragement of my supervisor and mentor, Dr. André Bédard. André accepted me into his lab as a naïve and horribly confused undergraduate student with no research experience outside of the classroom. Over the past three and a half years, I have grown as a scientist, critical thinker, communicator and leader. André always makes time for his students, and I frequently find himself knocking on his office door to ask questions about experimental techniques, peculiar results and life. His devotion and passion for science and teaching are apparent in everything he does. I have never met another supervisor who provides such in-depth hands-on training or conducts as many experiments as André does. Thank you for always pushing me to be better and do better.

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get through an experiment, a test or a mental breakdown. I know we will be lifelong friends and I cannot wait to be by your side through all of our meltdowns and accomplishments.

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List of Abbreviations

ALV LTR	Avian leukosis virus long terminal repeat
AP-1	Activator Protein 1
BSA	Bovine Serum Albumin
bZIP	Basic Leucine Zipper
cAMP	Cyclic Adenosine Monophosphate
CDK	Cyclin Dependent Kinase
C/EBP	CCAAT-Enhancer Binding Protein
CEF	Chicken Embryo Fibroblasts
CHOP	C/EBP Homologous Protein
C.I.	Contact Inhibited
Cy	Cycling / Proliferating
DAPI	4’.6-Diamidino-2-Phenylindole
DCFDA	2’,7’- dichlorofluorescin diacetate
DMEM	Dulbeco’s Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
ER	Endoplasmic Reticulum
ERK	Extracellular Signal Regulated Kinase
FABP	Fatty Acid Binding Protein
G_1	Gap 1 Phase
G_2	Gap 2 Phase

G_0	Quiescence
GAS	Growth Arrest Specific
GFP	Green fluorescent protein
HIF	Hypoxia Inducible Factor
HRE	Hypoxia Response Element
HRP	Horseradish Peroxidase
KD	Knockdown
LAP	Liver Activating Protein
LIP	Liver Inhibiting Protein
LPA	Lysophosphatidic Acid
M	Mitosis
MAPK	Mitogen Activated Protein Kinase
MDA	Malondialdehyde
MEK	MAPK/ERK Kinase
mRNA	Messenger Ribonucleotide Acid
NF	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NFM	Neurofilament medium polypeptide
PBS	Phosphate-Buffered Saline
PUFA	Polyunsaturated Fatty Acids
QRU	Quiescence Responsive Unit
RCASBP	Replication Competent ALV LTR with a Splice Acceptor Bryan Polymerase

RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RTK	Receptor Tyrosine Kinase
S	Synthesis
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
shRNA	Short Hairpin RNA
STAT	Signal Transducer and Activator of Transcription
TBS	Tris-Buffered Saline
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
UPR	Unfolded Protein Response
UTR	Untranslated Region

Vector Acronyms

RCAS(A)	Control empty RCASBP (A) vector
RCAS(A) - GFP RNAi	Control RCASBP (A) vector containing shRNA targeting GFP
RCAS(A) - p20K RNAi	RCASBP (A) vector containing shRNA targeting p20K
RCAS(A) - T3 FABP4 RNAi	RCASBP (A) vector containing one variant shRNA targeting FABP4
RCAS(A) - T4 FABP4 RNAi	RCASBP (A) vector containing another variant shRNA targeting FABP4
RCAS(A) – NFM	RCASBP (A) vector containing cDNA for NFM
RCAS(B)	Control empty RCASBP (B) vector
RCAS(B) – p20K	RCASBP(B) vector containing cDNA for p20K
RCAS(B) – FABP4	RCASBP(B) vector containing cDNA for FABP4
RCAS(B) – 99 C/EBPβ RNAi	RCASBP(B) vector containing shRNA targeting C/EBPβ
RCAS(B) - Δ184	RCASBP(B) vector containing dominant negative mutant of C/EBPβ (over-expression of LIP)
RCAS(A) + RCAS(B)	Combined transfection of control RCASBP(A) empty vector and RCASBP(B) emprty vector
RCAS(A) T3 FABP4 RNAi + RCAS(B) p20K	Combined transfection of RCASBP(A) vector containing shRNA targeting FABP4 and RCASBP(B) vector containing p20K cDNA

RCAS(A) p20K RNAi + RCAS(B) FABP4	Combined transfection of RCASBP(A) vector containing shRNA targeting p20K and RCASBP(B) vector containing FABP4 cDNA

Literature Review

1. Cell Cycle Regulation and Growth Arrest

i. Overview

The complex mechanisms underlying the self-replication of eukaryotic cells are essential to promote and regulate successful replication while ensuring conditions are favourable for division. The eukaryotic cell cycle is comprised of four discrete events, gap 1 phase (G1), synthesis (S), gap 2 phase (G2) and mitosis (M), division and genomic replication to yield two identical daughter cells. This process is tightly synchronized by various growth factors and proteins, including interactions between cyclins and cyclin-dependent kinases (CDK) (Lodish et al., 2000). The initial phase, G1, is characterized by rapid growth and elevated biosynthetic production in preparation for cell duplication (Lodish et al., 2000; Berridge, 2014). DNA replication occurs during the longest phase of the cell cycle, S phase. A second growth phase, G2, increases protein concentration before the cell proceeds to the mitotic (M) stage, where the duplicated diploid chromosomes are condensed, aligned at the mitotic spindle and undergo equal segregation to form two identical nuclei. Cellular division subsequently occurs following the division of the genomic material during cytokinesis (Lodish et al., 2000). Upon successful proliferation, the cell may re-enter the cell cycle to continue metabolic tasks and proliferation, temporarily withdraw from division and enter G0, or terminally differentiate (Berridge, 2014).

ii. Regulators of the cell cycle

Multi-cellular organisms feature a diverse regulatory network of cell cycle checkpoints to monitor proliferative progression while ensuring cellular integrity and fidelity is maintained. These surveillance mechanisms may suspend the cycle if a phase fails to advance to completion, or if cellular errors such as DNA damage or inadequate cell size occur (Barnum and O'Connell,

2014). Negative feedback ensures succeeding stages cannot proceed unless proper conditions are fulfilled, with irreversible transitions between the events enforcing uni-directionality (Polyak et al., 1994).

At the core of cell cycle regulation are cyclin dependent serine/threonine kinases (CDKs) and their regulatory subunit, cyclins. The activation of CDKs is dependent on activating and inhibitory phosphorylation events at regulatory sites as well as the binding of cyclins, which allows translocation of the CDK into the nucleus. In contrast to CDKs which are continuously transcribed but require post-translational modifications to carry out their function, cyclin induction varies throughout the cell cycle. CDKs and cyclins form complexes specific to different cell cycle stages where they drive expression of genes required for completion of the corresponding phase. Their timely activation and degradation thus functions as a mechanism of cell cycle control.

In early to mid G1, D-type cyclin (D1, D2 and D3) synthesis is induced by external mitogen stimulation, allowing cyclin D1 to bind to CDK4 and CDK6. The kinase-cyclin complexes then phosphorylate members of the retinoblastoma (Rb) protein family. The inactivation of Rb proteins releases the suppression of E2F transcription factors and ultimately permits the accumulation of factors essential for completion of a round of the cell division, including cyclin A and E. E-type cyclins interact with CDK2 to allow the cell cycle to progress in a mitogen-independent manner during late G1 (restriction point). The transition from G1 to S phase is in part mediated by the replacement of cyclin E with cyclin A in CDK2-cyclin complexes. CDK2-cyclin A units are present throughout DNA synthesis (S) and the second round of growth (G2) while triggering the degradation of cyclin E to prevent slippage back into G1. The later stages of the cell cycle (G2 and M) are predominantly controlled by CDK1, which

interacts with cyclin A and/or cyclin B. As the cell shifts from growth to mitosis, CDK2-cyclin A are lost, allowing cyclin A to interact with CDK1. CDK1-cyclin A complexes reach their peak concentrations during interphase. The destruction of cyclin A coincides with degradation of the nuclear envelope to permit the coupling of CDK1 with cyclin B to control the division of one cell into two.

iii. Cell cycle arrest

The type of cell cycle arrest eukaryotic cells undergo is dependent on a combination of extracellular and intracellular conditions. Irreversible non-proliferative states triggered by stress stimuli are termed senescence, whereas temporary growth-arrested states are known as quiescence (Blagosklonny, 2011). Senescence is often related to aging, as replication rates begin to diminish until completely halting (Mombach et al., 2014). Senescent cells are characterized by increased cellular volume (hypertrophy) through accumulation of mitochondria, lysosomes and reactive oxygen species (ROS) contributing to the altered morphology of the cells (Cho and Hwang, 2012). Mechanisms including telomere shortening, DNA damage, cytotoxic exposure and/or other degenerative properties trigger the irreversible growth arrest during the G1, G1/S or G2 checkpoints (Blagosklonny, 2011)(Terzi et al., 2016). Overall, it is conflicting signals of growth stimulation and cell cycle repression which cause loss of proliferation potential (Blagosklonny, 2011). In contrast, quiescence regulates proliferation and homeostasis through temporarily pausing cellular growth in a stable phase known as G0 (Terzi et al., 2016). Quiescence is characterized by modified expression patterns, stimulating genes which inhibit growth, enhance cell survival and suppress re-entry into G1 while simultaneously subduing macromolecular syntheses and enzymatic activity (Erb et al., 2016). If the cell recovers from the stress that paused growth, it may still re-enter the cell cycle upon mitogenic stimulation and

expression of D-type cyclins. Different pathways are activated by several quiescence signals to block division, implying diverse signal-dependent quiescence states exist (Coller et al., 2006).

2. Growth Arrest Specific (GAS) Genes and Quiescence

i. Overview

Reversible non-dividing states (G0) initiated by stress stimuli and unfavourable growth, conditions, such as a lack of oxygen, nutrients or space, are characterized by the expression of growth arrest specific (GAS) genes. These loci are responsible for stimulating, maintaining and departure from quiescence while preventing apoptosis. GAS genes are downregulated during normal growth conditions, terminal differentiation and senescence. Once the cells become non-proliferative in a rescindable manner, the transcription of these genes are detected, suggesting their expression is specific to quiescent growth arrest (Fleming et al., 1998). Six GAS genes (*gas1-6*) were first discovered to antagonize proliferation in response to environmental stress in 1988 (Schneider et al., 1988). Subsequent studies have shown GAS family members promote cell survival, block DNA synthesis, contribute to lipid metabolism and protect against oxidative stress by alleviating free radical collection (Fornace et al., 1989)(Del Sal et al., 1992)(Melkonyan et al., 1997) (Kim et al., 1999)(Suliman et al., 2004). SARP1 is exclusively expressed in quiescent cells and aid in the prevention of apoptosis through interference with the Wnt-frizzled pathway. Other loci are necessary to prime and prepare cells for cell cycle re-entry and aid in withdrawal from quiescence, like PDGFaR. (Coller et al., 2006). GAS gene expression may vary with tissue type, but in all circumstances, is critical to initiate and maintain growth blockage (Schneider et al., 1988). Notably, varying GAS gene expression profiles are detected with different types of quiescent signals, such as contact inhibition, lack of cellular adhesion, serum

starvation or removal of mitogens. This implies the cell type and environmental condition modulate GAS genes transcription profiles (Coller et al., 2006).

The lipocalin family is of particular interest, as a number of these proteins have been identified as GAS genes, including FABP4 and p20K. Moreover, these small secreted proteins are capable of binding to and transporting hydrophobic molecules, thus aiding in cellular maintenance of lipid homeostasis.

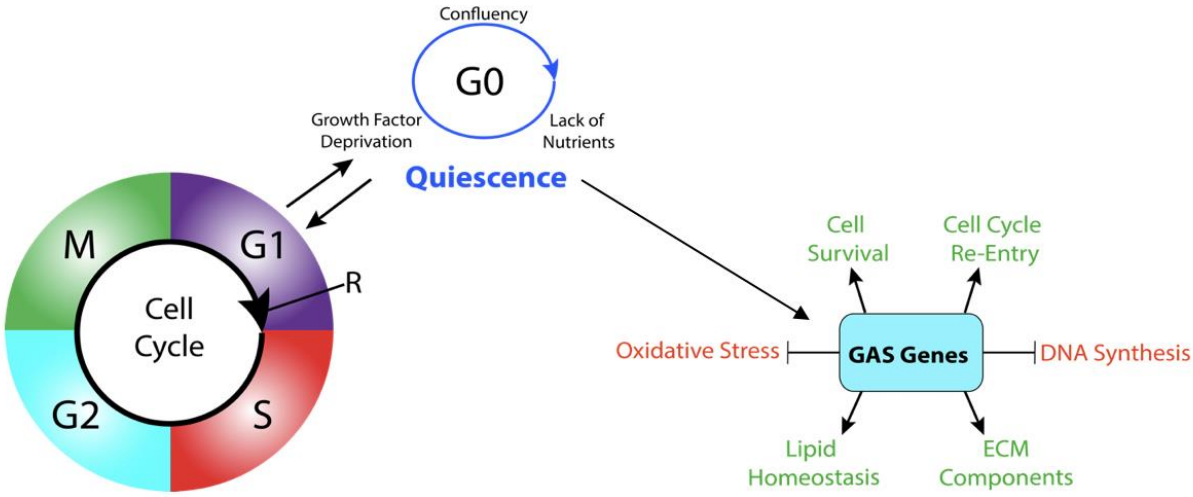


Figure 1. Quiescence and Growth Arrest Specific (GAS) Genes. The cell cycle is composed of four distinct phases: Gap 1 Phase (G1), DNA Synthesis (S), Gap 2 Phase (G2) and Mitosis (M). The Restriction Point, known as R, in late G1 is a critical checkpoint in which the cell either irreversibly commits to completing one round of the cell cycle or aborts the division. In unfavourable growth conditions, the cell may exit G1 and enter a reversible growth arrest, known as G0. Quiescence is driven by factors such as contact inhibition, nutrient limitations and growth factor deprivation to prevent proliferation. Entry into G0 induces expression of a set of genes, known as growth arrest specific (GAS) genes. These loci modulate activities such as lipid homeostasis, cell survival, maintenance of the ECM, and are necessary for re-entry into the cell

cycle. They also help to inhibit oxidative stress and DNA synthesis, allowing the cell to be maintained during hostile circumstances.

ii. Fatty Acid Binding Protein 4 (FABP4)

FABP4 (also known as adipocyte-binding protein aP2/A-FABP) is one of nine intracellular fatty-acid-binding proteins (FABPs) distinguished for their role in mediating lipid transport and responses strongly connected to metabolic and inflammatory pathways (Hotamisligil and Bernlohr, 2015). FABP lipid chaperones are highly conserved and their basic structure features an antiparallel β-Barrel with an internal, water-filled cavity that facilitates reversible binding of hydrophobic fatty acids (Figure 2). These abundant 14-15 kDa proteins predominantly interact with saturated and unsaturated fatty acids, eicosanoids and other lipid species (Furuhashi and Hotamisligil, 2008) A diverse range of functions related to lipid homeostasis have been proposed for FABPs, including regulation fatty-acid import, storage and export as well as modulation of enzymes critical for cholesterol & lipid metabolism, transport of lipids to facilitate signaling and membrane integrity maintenance in response to fatty acid-induced damage (Hertzel and Bernlohr, 2000). They have been demonstrated to transfer fatty acids to phospholipid membranes through collisions as regulated by positively-charged lysine residues (Hotamisligil and Bernlohr, 2015). FABP4 is unique in its high affinity for saturated and unsaturated fatty acids, indicating this chaperone protein may support systemic homeostasis networks immunometabolism by facilitating signaling within and between cells and communication between organs (Hotamisligil and Bernlohr, 2015) (Figure 3).

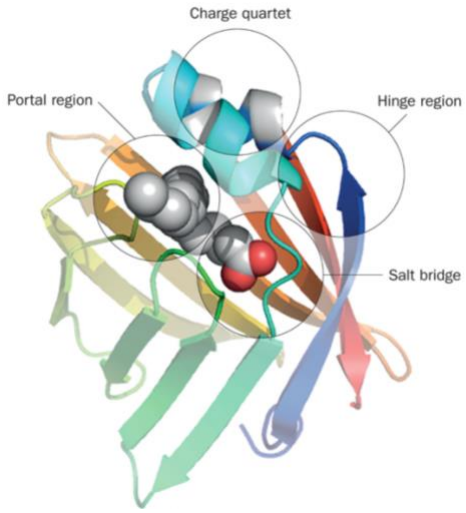


Figure 2. Ribbon and domain structure of FABP4. FABP4 binds with and has a high affinity for saturated and unsaturated fatty acids. Its structure consists of a beta-barrel with an internal cavity for fatty acid binding (adapted from Hotamisligil and Bernlohr, 2015).

Each FABP exhibits distinct patterns of tissue expression, with FABP4 predominantly observed in mature adipose tissue and macrophages as well as tissues experiencing hypoxia and/or cellular damage (Furuhashi et al. 2007). FABP4 makes up approximately 1-5% of all soluble cytosolic proteins in adipocytes, and their quantity increases follow influx of lipids into the cells (Haunerland and Spener, 2004). FABP4 is also present in the bloodstream as a secreted hormone involved in communicating energy-storage system needs to various organs in response to stressors (Prentice et al. 2019). In this manner, FABP4 functions as an interface between metabolic and inflammatory pathways in adipocytes and macrophages. Moreover, the redundant nature of the role of FABPs in regulating different metabolic and inflammatory signaling pathways supports the notion that their biological functionally is context, tissue or cell-type specific (Hotamisligil and Bernlohr, 2015).

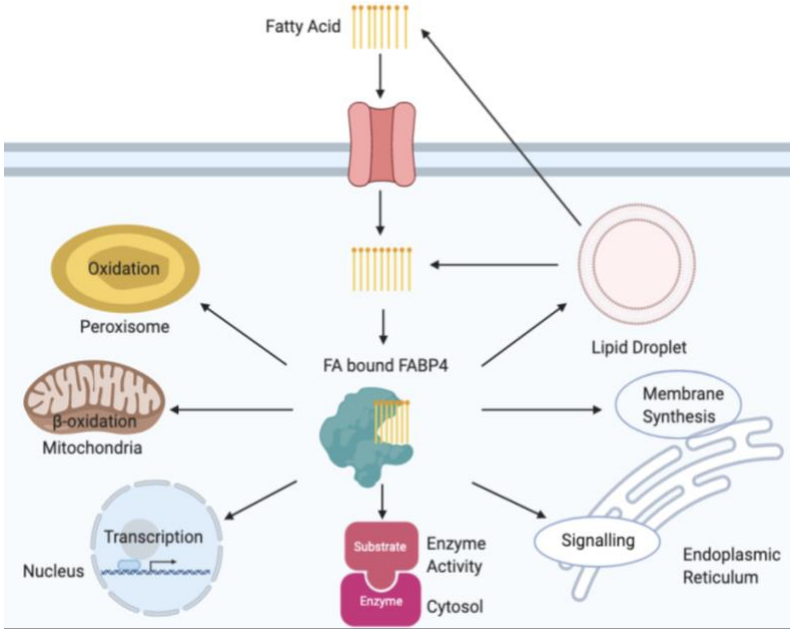


Figure 3. Roles of FABP4. FABP4 is a lipid chaperone, and as such it transports lipids to specific compartments in the cell (to lipid droplets for storage; ER for signalling, trafficking and membrane synthesis; mitochondria or peroxisome for beta-oxidation; cytosol to regulate enzyme activity; nucleus for control of lipid-mediated transcriptional programs via nuclear hormone receptors or other transcription factors; outside the cell to signal in an autocrine or paracrine manner).

However, chronic engagement of FABP4 is observed in conditions of immunometabolic stress and may worsen the disease pathogenesis in obesity, insulin resistance paired with diabetes mellitus, fatty liver disease, asthma, cancer and atherosclerosis, indicating tight regulation of the protein's expression is critical for homeostasis (Prentice et al. 2019) (Figure 4). Mice with loss of FABP4 mutations have been shown to have increased protection from obesity-induced insulin resistance and hyperglycaemia paired with enhanced lipogenesis and reduced lipolysis (Hotamisligil et al., 1996). Similarly, FABP4 is upregulated in the adipose tissue and circulation

of obese individuals compared to lean counterparts (Prentice et al., 2019). Blocking FABP4 activity also prevents atherosclerosis pathogenesis by inhibiting the accumulation of macrophages in plaques (Furuhashi and Hotamisligil, 2008). Other clinical studies have found a positive correlation between FABP4 expression and early death in patients with stroke and severe obstructive sleep apnea disorder independent of other comorbidities (Català et al., 2013). Recent investigations have focused on the proposed pro-oncogenic role of FABP4 with a specific focus on breast cancer. FABP4 is highly expressed in protumor tumor associated macrophage (TAM) and has been linked to enhancing stem cell-like phenotype, metastasis, growth and tumor progression, in part through the induction of protumor interleukin (IL)-6/STAT3 signaling (Hao et al., 2018). In ovarian tumors, FABP4 has been shown to bind FFA, providing energy necessary for tumour growth and metastasis (Nieman et al., 2011). Interestingly, endogenous FABP4 is suggested to function as a tumor suppressor whereas exogenous FABP4 is involved in cancer cell development. Low levels of FABP4 are frequently reported in cancer cells, including prostate and bladder whereas breast cancer cells have been shown to have significantly elevated FABP4 levels compared to healthy individuals (Amiri et al., 2018). The discrepancies in FABP4 levels in various types of cancer allude to a complex regulatory system to control FABP4 activity in specific cell and tissue types.

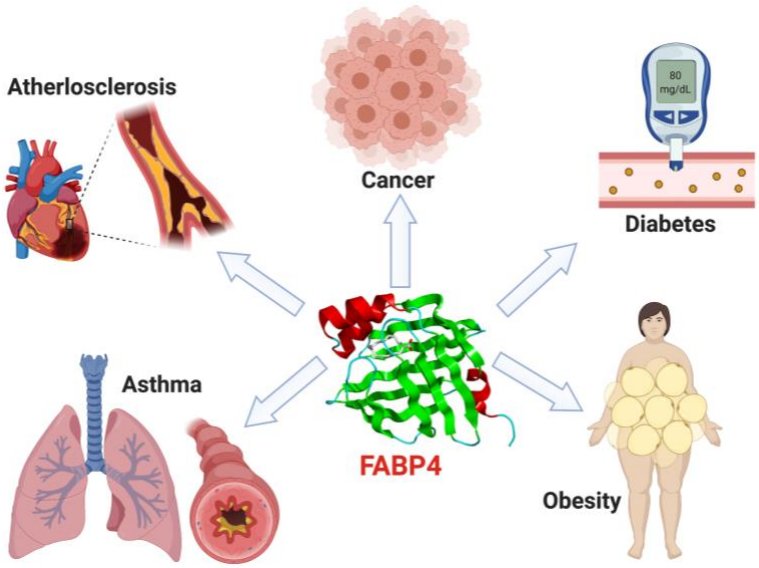


Figure 4. FABP4 in Disease Pathology. FABP4 is implicated in various immunometabolic diseases. Elevated FABP4 activity is associated with diabetes, obesity, cardiovascular disease, asthma and cancer. The mechanisms underlying the coupling of lipids to energy storage and signalling pathways in response to stressors is not well characterized and could be critical in elucidating the role of FABP4 in disease pathogenesis.

During lipolysis, FABP4 binds free fatty acids present in the cytoplasm and shuttles them out of the cell to prevent the inhibition of lipolytic enzymes (Prentice et al. 2019). Consequently, when FABP4 is constitutively active as a result of metabolic disorders, lipid homeostasis is disrupted. In macrophage, FABP4 expression is correlated with cholesterol trafficking, foam cell formation, inflammation and cellular stress and has been identified as a biomarker of diabetes and atherosclerosis (Prentice et al. 2019). The use of BMS309403, a FABP4 inhibitor that interacts with the protein's fatty acid binding pocket, has been demonstrated to reduce insulin, triglycerides and cholesterol ester levels in macrophage, implicating FABP4 as a potential therapeutic target in diabetes (Furuhashi et al. 2007). The protein is likewise speculated to be

dysfunctional in conditions of atherosclerosis and obesity, as silencing of FABP4 is also correlated with diminished inflammatory mediator concentrations and reduces hepatic glucose production. Beyond its role as a lipid chaperone, FABP4 is proposed to be critical for angiogenesis through its ability to modulate vascular endothelial growth factor signaling and cell proliferation (Prentice et al. 2019).

iii. P20K Lipocalin

The p20K lipocalin, also known as extracellular fatty acid binding protein (Ex-FABP), is a prominent GAS gene strongly upregulated during reversible growth arrest in CEF (Bédard et al. 1987). A member of the lipocalin family characterized by their high affinity for hydrophobic molecules, p20K was first discovered as a secretary protein expressed by quiescent CEF and chicken heart mesenchyme (Bédard et al. 1987; Cancedda et al., 1996). It has since been shown to be activated by various physiological and pathological circumstances of cellular stress, and is implicated in tissue development, inflammatory responses and degenerative neuromuscular disease (Cancedda et al., 1996).

P20K expression is induced in conditions of reversible growth arrest but not ER stress, as seen by the opposing accumulation patterns of p20K and CHOP, a marker of ER stress. This suggests p20K is not active in senescence or apoptotic cells due to stimulation of the UPR pathway (Erb et al. 2016). The lack of p20K expression in cells undergoing programmed death or with an inability to re-enter the cell cycle postulates p20K as a pro-survival protein (Cancedda et al. 2002). TUNEL assays conducted by the Bédard lab to analyze cell survival in conditions of p20K down-regulation have analogously found that p20K expression directly opposes apoptosis. Inhibition of p20K activation using siRNA enhances cell death in oxygen-limited environments, whereas apoptotic rates are appropriately equivalent in normoxic and hypoxic CEF when p20K

demonstrates wild-type expression. These results suggest p20K may be necessary for adaptation to inadequate oxygen conditions (Erb et al. 2016).

The importance of p20K's lipid chaperone functionality has also been investigated during reversible growth-arrest. Downregulation of p20K in hypoxia is correlated with greater lipid vesicle formation and persistence, indicating the lipocalin may shuttle excess free fatty acids out of the cell in low-oxygen environments (Erb et al. 2016). Similarly, lipid peroxidation and reactive oxygen species (ROS) release assays have shown elevated levels of lipid oxidation and ROS formation in hypoxia when p20K expression is inhibited using siRNA (Erb et al. 2016; Sriranjan, M.Sc., 2019). Taken together, these studies postulate survival during quiescence may be mediated in part by p20K through maintenance of lipid homeostasis.

iv. Hypoxia and Contact Inhibition

Several environmental stressors induce signaling pathways that lead to quiescence. Low oxygen levels (1-2% O_2), known as hypoxia, and cell-cell contact, or contact inhibition, are two primary conditions which transiently halt cellular growth (Giaccia et al., 2004). Oxygen is an essential element of aerobic respiration in mammalian cells, and cells tailor their metabolic demands to correspond to oxygen availability. While multiple mechanisms exist to regulate changes in response to oxygen levels, hypoxic expression patterns are primarily mediated by hypoxia inducible factors (HIFs) (Erb et al., 2016). Under normal physiological oxygen concentrations, hypoxia inducible factor1 alpha (HIF-1α) is degraded. When oxygen becomes depleted, HIF-1α is stabilized, allowing it to interact with hypoxia inducible factor 1 beta (HIF-1β) to form an active HIF-1 complex (Giaccia et al., 2004)(Chi et al., 2006). HIF-1 stimulates expression of several loci involved in survival in limited oxygen, permitting the cell to undergo adaptive changes related to metabolism and the inflammatory response (Majmundar et al., 2010;

Chi et al., 2006). Proliferative genes are repressed in reduced oxygen conditions, ultimately indicating hypoxia regulates factors required for cell cycle progression (Chi et al., 2006).

Elevated cell density also blocks proliferation, leading to cells with reduced protein synthesis and metabolism. The diminished growth results in a cell population predominantly exhibiting a small, vertical morphology (Leontieva et al., 2014). This reversible growth arrest ensures normal cellular function, differentiation and development patterns are maintained in an environment with limited resources and space (Erb, Msc. Thesis, 2016). Contact inhibited cells appear to display unique transcriptional profiles compared to other quiescent states. This includes downregulation of proliferating cell nuclear antigen, DNA replication factors, cell cycle progression factors, biosynthetic enzymes and splicing factors (Coller et al., 2006). Interestingly, recent gene profiling has demonstrated contact inhibition prompts expression of several hypoxia-response gene in CEF (Erb et al., 2016). It has thus been proposed confluence induces quiescence in part through inadequate oxygen availability (Erb et al., 2016).

Induction of p20K is strongly enhanced under both hypoxic conditions and contact inhibition in CEF (Fielding, Msc. Thesis, 2011; Erb et al., 2016). Despite its activity during oxygen-deprived circumstances, p20K expression is independent of HIF activity (Erb et al., 2016). Erb et al. demonstrated actively dividing CEF exposed to hypoxic environments induce HIF-1α expression after two hours of incubation, and HIF-1α levels are steadily maintained as low-oxygen conditions persisted. In contrast, p20K transcription was not observed until nearly twenty-four hours after initial hypoxic exposure, when the entire cell population was fully growth arrested. Relatedly, deletion of the entire QRU or its C/EBP binding sites abolishes activation of the p20K promoter by hypoxia, while removal of candidate hypoxia responsive elements (HRE), which HIF-1 canonically bind to, had no effect on p20K levels (Erb et al.,

2016). These results signify it is the growth arrest state triggered by reduced oxygen that ultimately activates p20K transcription, rather than a direct result of the induction of HIF-1 (Erb et al., 2016). Further supporting this model, several hypoxia-responsive genes experience upregulation in contact inhibited CEF but not cycling samples, such as FABP4, indicating oxygen availability regulates GAS gene transcription (Erb et al., 2016). Taken together, it appears the many GAS genes may play a role in an adaptive, pro-survival pathway triggered by oxygen-deprived conditions.

3. GAS Gene Regulation

i. CCAT-Enhancer Binding Protein (C/EBP) and C/EBP Homologous Protein (CHOP)

The six members of the CCATT-enhancer binding proteins (C/EBP) family function as bZIP transcription factors that regulate cellular growth, differentiation and inflammation mechanisms in a variety of tissues (Ramji and Foka, 2002; Wei et al., 2006). The protein variants C/EBPα, C/EBPβ, C/EBP γ, C/EBPε, C/EBP δ, and C/EBP ζ share highly conserved structures, comprised of a basic leucine zipper domain in their C-terminus for protein dimerization and DNA binding, and a transactivation domain at the N-terminus capable of transcriptional machinery interactions (Ramji and Foka, 2002; Nerlov, 2007). All C/EBP isoforms are activated through the formation of heterodimers or homodimers that are capable of inducing target gene transcription (Ron and Habener, 1992). Moreover, the transcriptional activation potential of C/EBP is primarily regulated through dimerization events, as these interfaces are essential to permit binding of the factor to its target DNA consensus sequence (Descombes and Schibler, 1991; Ramji and Foka, 2002)

C/EBPβ is one of the most abundant isoforms as it is ubiquitously expressed, although its induction is notably enhanced in contact inhibited CEF (Gagliardi et al., 2003). The three isoforms of C/EBPβ, liver inhibiting protein (LIP), liver activating protein (LAP) and LAP*, are products of alternative translation initiation derived from the same open reading frame. The respective abundance of each isoform is believed to govern the activity of C/EBPβ, along with the cell-type and context (Konior et al., 2013; van der Krieken et al., 2015). LIP lacks an activation domain, allowing it to act as a competitive inhibitor that hinders C/EBPβ through the formation of non-functional dimers with LAP and/or LAP* (Ramji and Foka, 2002). Beyond variations in the expression of each isoform, C/EBPβ regulation can occur at a transcriptional, translational or post-translation level. Cyclic adenosine monophosphate (cAMP), calcium and inflammatory cytokines can activate the cAMP-responsive element-binding protein (CREB) and related proteins, which then bind cAMP-responsive elements (CRE) in the promoter of C/EBPβ and induce transcription (citation). Other factors shown to modulate C/EBPβ production include NFKB, STAT3, PI3K/AKT, ERK, GSK3, PKA and CaMKII (Ichiki Toshihiro, 2006). Once C/EBPβ is present in a cell, it typically requires phosphorylation to activate it. De-phosphorylated C/EBPβ cannot bind or initiate expression of target genes as intramolecular interactions cause its inhibitory domain to block the transactivation and DNA binding domains (citation). Thus, post-translational addition of phosphate groups by PKC, MAPK and GSK3 permits the transcription factor to bind and induce gene expression.

C/EBPβ is a versatile transcription factor involved in processes such as cellular differentiation and proliferation, as well as pro-inflammatory response pathways, apoptosis and tumour formation (Nerlov, 2007). In 2003, Gagliardi et al. demonstrated C/EBPβ predominantly arrests growth in CEF as its hyperexpression severely depletes activator protein 1 (AP-1), an

initiating transcriptional factor of proliferation. The factor is also implicated in tumorigenesis. It promotes the epithelial-mesenchymal transition, invasion and metastasis by a MHCII/CD4-dependent mechanism in breast cancer, and it can be used to predict overall survival in these patients (Johansson et al., 2013; Kurzejamska et al., 2014). C/EBPβ also promotes proliferation and tumour growth in malignancies including colorectal, glioma, pancreatic and skin cancer (Sun et al., 2015). Given C/EBPβ proficiency to regulate cell survival and inflammation, recent studies have focused on a potential association between the transcription factor and oxidative stress. Emerging reports have found ROS produced by the NADPH oxidase complex (NOC) may assist in controlling terminal differentiation of adipocytes through signaling with C/EBPβ (Al-Sabbagh et al., 2011; Mussbacher et al., 2019). It is also postulated that the degree and duration of ROS signaling and NOX-dependent redox regulation of C/EBPβ may be essential for mediating cell differentiation and proliferation in diverse cell types.

CCAT-enhancer-binding protein homologous protein (CHOP), is a growth arrest DNA damage (GADD) factor that initiates apoptosis in response to endoplasmic reticulum (ER) stress through inhibition of C/EBP proteins (Zinszner et al., 1998). Similar to the C/EBP family members, it contains a conversed bZIP domain but also features a modified DNA binding domain, permitting it to dimerization with other C/EBPs, such as C/EBPβ, and control their activity. CHOP inhibits the DNA binding activity of C/EBPβ, thus preventing the transcription factor from interacting with and activating its target loci (Ubeda et al., 1996). The PERK/eI2Fα and ATF signaling pathways have been shown to induce CHOP upon the occurrence of ER stress and microbial infection, although its activity can also be regulated through post-translational modifications mediated by the p38 MAP kinase family (Hu et al., 2019). Overall, CHOP

expression and activity diametrically opposes that of GAS genes, as it functions in ER stress-induced apoptosis while GAS proteins promote cell survival and re-entry to cellular replication.

ii. P20K Regulation and the QRU

Expression of the p20K lipocalin during reversible growth arrest is predominantly controlled at the transcriptional level, through a 48 base-pair region in the promoter referred to as the *Quiescence Responsive Unit* (QRU). The regulatory region contains multiple binding sites to permit transcription factor interactions, and has been demonstrated to be both sufficient and necessary for p20K induction (Bédard et al., 1989; Kim et al., 1999; Mao et al., 1993; Gagliardi et al., 2003; Erb et al., 2016). The region houses two CCATT-enhancer binding protein (C/EBP) interaction domains (Site A and Site B) flanking extracellular regulatory kinase 2 (ERK2) consensus sequences (EBS) (Erb et al., 2016). These binding sites allow the C/EBP and MEK pathways to control the activity of the p20K lipocalin (Erb et al., 2016).

Other papers have established a role for C/EBPβ in p20K regulation through its interactions with the QRU; mutations in either one or both of the C/EBPβ binding sites within the QRU eliminated C/EBPβ interaction and consequent p20K expression (Kim et al., 1999). Interestingly, Site A displays higher activity during quiescence to induce p20K expression, while Site B is upregulated during conditions of proliferation, indicating Site A houses a quiescence responsible element (Kim et al., 1999). These results indicate C/EBPβ activates p20K expression in quiescent CEF, and both elements are necessary for this activation to occur.

Along with the two C/EBPβ interaction domains, recent findings verified the QRU contains mitogen-activated protein kinase 1 (also known as extracellular regulated kinase 2, ERK2) binding sites (Erb et al., 2016). ERK2 activity is regulated through a Ras-Raf-MEK-ERK kinase signaling pathway. This signaling mechanism is involved in regulating cellular growth

and survival, with ERK2 functioning as a transcriptional regulator in addition to its kinase function (McCubrey et al., 2007; Mebratu and Tesfaigzi, 2009). ERK2 can indirectly alter gene transcription via phosphorylation of serine and threonine residues on other transcription factors, or directly through binding events with DNA targets (Erb et al., 2016). An ERK2 localization site is present in the middle of the QRU, overlapping the two C/EBPβ binding sites. Subsequent assays found ERK2 binds to the QRU in actively dividing cells but is absent in reversibly-growth arrested CEF. Furthermore, an antagonistic relationship between C/EBPβ and ERK2 was suggested as both overexpression of C/EBPβ and inactivation of ERK2 via MEK inhibition promotes p20K transcription (Erb et al., 2016). The intersection of the ERK2 domains with the two C/EBPβ binding sites indicates the transcription factors directly compete for mutually exclusive interactions with the QRU. ERK2 binding to the QRU blocks C/EBPβ localization with the promoter, repressing p20K transcription in proliferating CEF. During quiescence, ERK2 binding is lost and interaction of C/EBPβ dimers permits p20K expression (Erb et al., 2016) (Figure 5).

CHOP-C/EBPβ dimers are prominent during ER stress and detectable in actively dividing cells, these complexes are deficient in densely-arrested CEF (Erb et al., 2016). Forced CHOP expression in quiescent and confluent CEF reduces p20K levels, while down-regulation of CHOP re-establishes p20K induction in ER stress conditions. However, forced knockdown of CHOP in cycling cells does not induce p20K, indicating additional effectors, namely ERK2, control the lipocalin (Erb et al., 2016). Overall, the strict modulation of p20K expression through a diverse group of factors indicates limiting p20K's activity to reversible growth arrested conditions is critical to maintain proper cellular function.

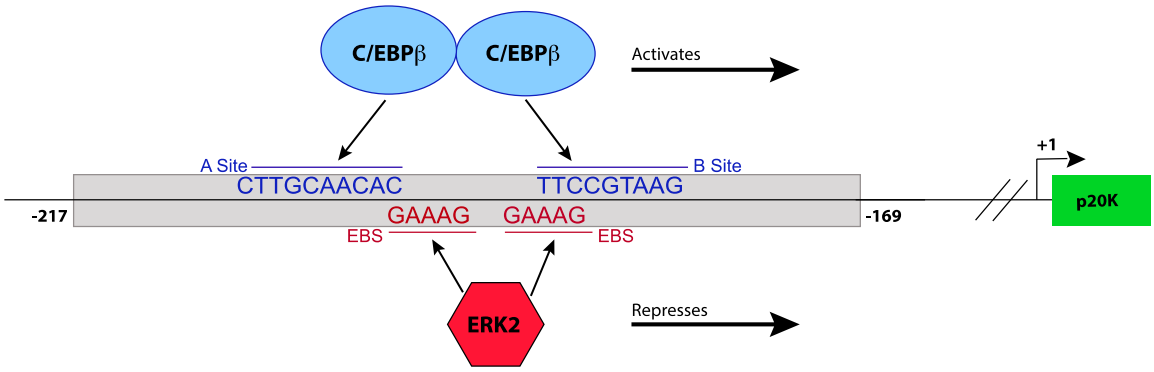


Figure 5. Transcription regulation of p20K occurs through the QRU by C/EBPβ and ERK2. The *Quiescent Responsive Unit* (QRU) is a 48-bp region (-217 to -169) within the p20K promoter that regulates the lipocalin's transcription in response to reversible growth arrest. Dimers of C/EBPβ bind specific sequence elements, denoted Site A and Site B, within the QRU, and activate p20K expression. Binding to both sites is necessary for p20K expression. ERK2 binds EBS regions of the QRU and represses p20K expression. The interaction of C/EBPβ and ERK2 with the QRU is mutually exclusive, as the binding elements of each transcription factor overlap.

iii. FABP4 Regulation

Expression of FABP4 is transcriptionally regulated by factors involved in metabolic processes such as peroxisome proliferator-activated receptor gamma protein (PPARγ), CCATT-enhancer binding protein (C/EBP), insulin, dexamethasone, fatty acids and agonists of PPARγ. The promoter region of the mammalian *FABP4* gene contains five adipocyte regulatory elements, a binding sequence for the trans-activating C/EBP and several peroxisome proliferator response elements (Graves et al., 1991; Veerkamp and Maatman, 1995).

PPARγ is the best characterized regulator of FABP4 activity in adipocytes, macrophage and adipose tissue. Known as the master regulator of adipocyte biology, PPARγ is a nuclear receptor that senses and interprets fatty acid signals derived from dietary lipids, pathogenic lipoproteins or essential fatty acid metabolites to regulate growth and differentiation (Varga et al., 2011). It exerts control over FABP4 induction in response to a diverse array of stimuli. Transient nuclear interactions between FABP4 and PPARγ have been noted in macrophage, where FABP4 attenuates the nuclear receptor's activity through the extraction of ligands (Makowski et al., 2005). Interestingly, some cell culture system studies have found FABP4 can translocate to the nucleus where it binds and deliver ligands to PPARγ, which may be part of a lipid sensing-response to mediate appropriate transcriptional programmes (Adida and Spener, 2006; Schroeder et al., 2008). Analysis of genetic mouse models has led to the hypothesis that PPARγ directly induces *FABP4* expression while FABP4 reduces PPARγ activity as part of a negative feedback loop (Makowski et al., 2005). The lipocalin can also stimulate proteasomal degradation of PPARγ, while FABP4-deficient macrophage exhibit enhanced PPARγ levels, suggesting FABP4 suppresses the transcriptional network modulated by the nuclear receptor (Garin-Shkolnik et al., 2014; Makowski et al., 2005).

Beyond its complex relationship with PPARγ, FABP4 collaborates with a diverse array of factors to regulate metabolic and inflammatory pathways. In adipocytes, the interaction of FABP4 with hormone-sensitive lipase (HSL) in the presence of fatty acids promotes lipolysis while inhibiting lipogenesis. Together, FABP4 and HSL form a complex on the surface of lipid droplets which is responsible for facilitating fatty acid transfer to stimulate lipid hydrolysis (Jenkins-Kruchten et al., 2003; Smith et al., 2004). In macrophages, FABP4 is detected following inflammatory activation, leading it to induce nuclear factor NF-kB and c-Jun N-

terminal kinase (JNK) pathways to further modulate inflammatory responses (Prentice et al. 2019).

PPARγ and C/EBPβ are intricately connected, as coordination of both these proteins is required to initiate the differentiation gene program necessary for the maturation of adipocytes (Lefterova et al., 2008). C/EBPβ is one of many substrates that contribute to the induction of PPARγ, which leads to subsequent activation of other transcription factors necessary for adipogenesis (Farmer, 2005). During early differentiation of stem cells into adipocytes, C/EBPβ is activated and induces expression of PPARγ and C/EBPα. This leads to increased levels of FABP4 characteristic of adipocytes and adipose tissue (Figure 6). While the exact mechanism and signal(s) required to initiate C/EBPβ-dependent induction of FABP4 in quiescence remain to be elucidated, the transcription factor may exert control over PPARγ, leading to subsequent FABP4 activation, or it may directly induce *FABP4* transcription by interacting with the promoter. Further investigations are required to characterize the regulation of FABP4 in conditions of reversible growth arrest.

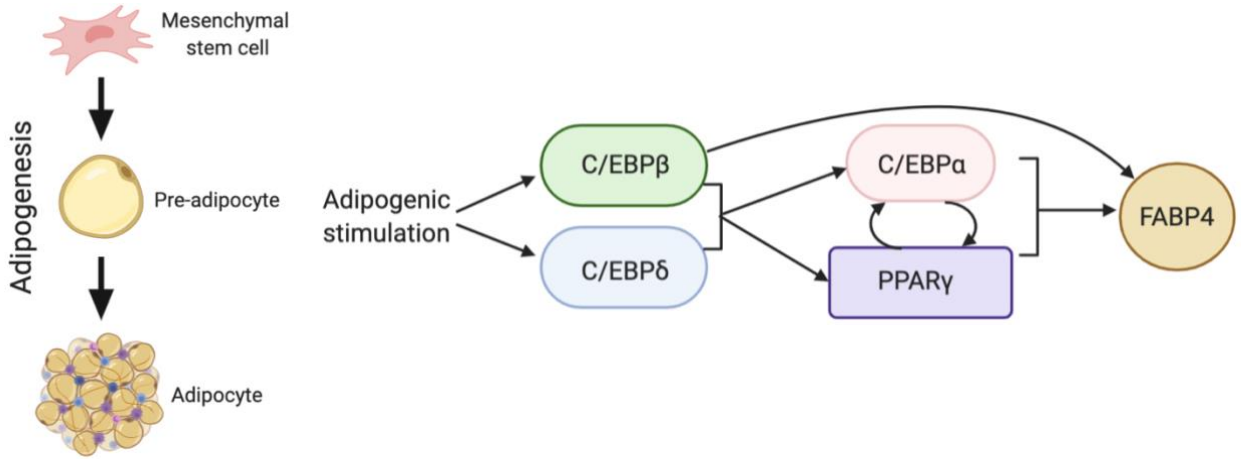


Figure 6. The proposed role of C/EBPβ and PPARγ in adipogenesis. Pluripotent mesenchymal stem cells can differentiate into adipocytes through the process of adipogenesis.

Adipogenic stimulation rapidly induces expression of C/EBPβ and C/EBPδ. These two transcription factors target a variety of genes, including key regulators of adipogenesis such as C/EBPα, PPARγ and SREBP1. PPARγ binds to the promoter of C/EBPα to activate its induction and vice versa, forming a positive-feedback loop. PPARγ and C/EBPα stimulate the expression of genes involved in lipogenesis, lipolysis and insulin sensitivity. These two factors even cooperate on multiple binding sites in promoter regions, regulating genes activated in developing and mature adipocytes. FABP4 is activated in this process, and some studies suggest C/EBPβ may also aid in directly inducing FABP4, although additional research is required.

4. Growth Arrest, Oxidative Stress & Lipid Homeostasis

i. Overview

Oxidative stress is a phenomenon characterized by the imbalance between the production/accumulation of reactive oxygen species (ROS) and the ability of the biological system to detoxify these products using free radicals (Pizzino et al., 2017). Metabolic processes normally produce ROS as a bi-product (superoxide radicals, hydrogen peroxide, hydroxyl radicals, single oxygen) and these reactive products have several physiological roles, such as cell signaling, immunity, apoptosis and differentiation (Rajendran et al., 2014). However, environmental stressors can elevate production of ROS by mitochondria and other organelles, causing ROS levels to exceed the cell's antioxidant defense systems. The reactive intermediates that result from oxidative stress can alter the composition and structure of macromolecules, triggering significant cellular dysfunction. Importantly, membrane bilayers can be modified by ROS, prompting lipid peroxidation (Repetto et al., 2012; Phaniendra et al., 2015; (Yu et al., 2017). Lipid peroxidation leads to the formation and propagation of lipid radicals along with re-

arrangements in polyunsaturated fatty acids (PUFA) (Repetto et al., 2012). Lipid peroxidation is implicated in several pathological conditions, and is particularly detrimental to biomembranes as it results in loss of membrane fluidity and permeability, disrupts ion-gradients, and modifies lipid interactions (Gaschler and Stockwell, 2017). Additionally, the destruction of lipid membranes releases cytotoxic bi-products with the capacity to alter gene expression and signal transduction pathways (Repetto et al., 2012; Phaniendra et al., 2015). The lipid peroxidases enhance toxicity as they propagate further creation of ROS, and their degradation releases compounds capable of crosslinking DNA and proteins. ER stress, calcium dysfunction, and abnormal mitochondria activity are just some of the toxic effects of lipid radical amassment (Descalzi Cancedda et al., 2000). Successive steps augmented by lipid peroxidation generates toxic conditions which may trigger apoptosis or necrosis programmed death as the damage surpasses the repair capacity of the cell (Gaschler and Stockwell, 2017) (Figure 7). Beyond lipid peroxidation, free forms of fatty acids have the potential for cellular toxicity and thus rely on proteins like FABPs and lipocalins to regulate their intracellular concentrations and behaviour.

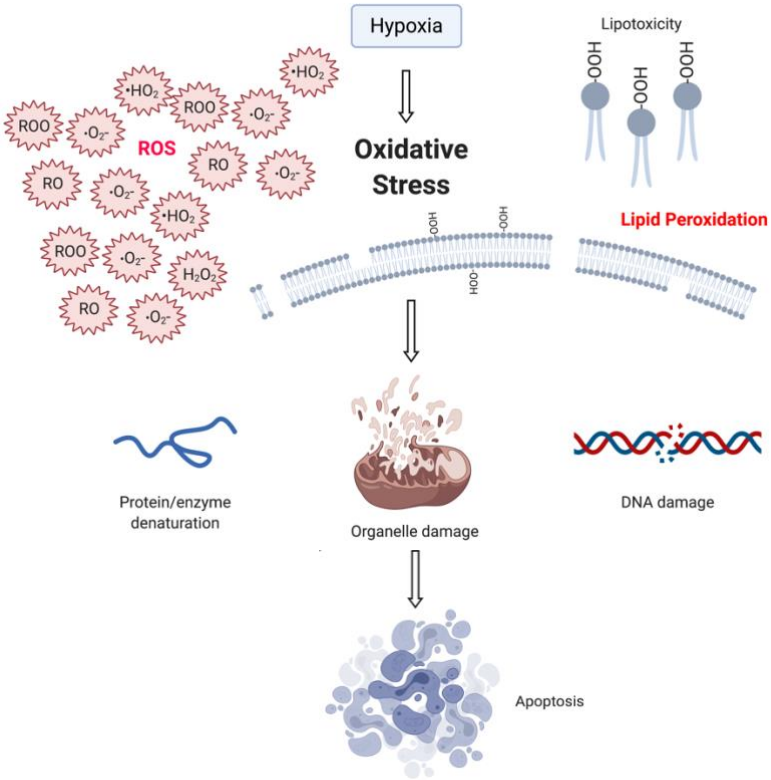


Figure 7. **Oxidative stress in response to hypoxia.** Oxygen limitations promote increased production of reactive oxygen species as mitochondrial oxidative phosphorylation is disrupted. The subsequent accumulation of free radicals can cause the oxidative degradation of lipids in a process known as lipid peroxidation. The free radicals take electrons from lipids in the cell membranes, releasing lipid peroxides into the cell. This imbalance between reactive oxygen species and antioxidants can have detrimental cellular consequences, as lipid peroxides and free radicals damage proteins, nucleic acids and organelles. Persistent oxidative stress can ultimately lead to apoptosis if the cell is unable to overcome the induced perturbations.

ii. P20K and FABP4 in Oxidative Stress & Lipotoxicity

Fatty acids perform many vital cellular functions; they act as energy sources and signals for metabolic regulation through enzymatic and transcriptional networks to modulate gene expression, growth, survival, inflammation and metabolic responses (Furuhashi and Hotamisligil, 2008). A vast network of regulators aid in the processing, shuttling, availability and disposal of lipids to maintain homeostasis.

The p20K lipocalin has been proposed to play a role in lipid homeostasis through its ability to lessen lipid peroxidation during hypoxia (Erb, Msc. Thesis, 2016). Repression of p20K in limited oxygen environments enhances lipid peroxidation compared to normoxic and p20K-expressing hypoxic CEF, as evident by elevated malondialdehyde (MDA) levels, a by-product of lipid oxidation. Enhanced 4-HNE accumulation, another by-product of lipid peroxidation and mediator of apoptosis, is also observed in hypoxic cells with diminished p20K activity (Sharma et al., 2008). P20K may also influence the production of ROS, which leads to subsequent lipid peroxidation, as inhibition of the lipocalin is associated with a significant increase in ROS levels in low oxygen conditions compared to CEF with heightened p20K expression (Sriranjan &

Donders, unpublished). Beyond its suppression of lipid peroxidation, p20K may regulate lipid homeostasis through binding of FFA and other lipids. The protein interacts with lysophosphatidic acid (LPA) isoforms, a subset of bioactive phospholipids that instigate an array of cellular responses through signaling cascades (Correnti et al., 2011). Therefore, it is postulated p20K may act as a sensor of LPA. Lipid profiling analysis has revealed lipid species including palmitate, stearate and cholesterol accumulate in hypoxia (Erb, Msc. Thesis, 2016). Such factors can induce apoptotic pathways, hence proteins like p20K may be expressed during quiescence to promote survival through lipotoxicity reduction.

Similar to p20K, FABP4 is implicated in the management of oxidative stress, although less is known about its functionality and regulation in these conditions. Murine and cell models of acute lung injury have shown that activation of FABP4 through LPS treatment results in the production of pro-inflammatory cytokines and ROS generation. FABP4 can then induce FoxM1 in a ROS-dependent manner, further stimulating inflammatory pathways (Gong et al., 2018; Wu et al., 2018). Correspondingly, FABP4-deficiency in macrophage provides protection against lipotoxcity and reduces oxidative stress normally triggered by the increased intracellular collection of FFA and monosaturated fatty acids (Xu et al., 2015).

As previously noted, one mechanism FABPs use to mediate lipid homeostasis is through the formation of cytosolic lipid storage organelles called lipid droplets (LD). LD contain triglycerides and esterified cholesterol which can be used to generate energy through lipolysis, to release signaling lipids and to function as replacement membrane components. Numerous studies have postulated a role for FABPs in fatty acid storage through the formation of LDs as well as cholesterol and phospholipid metabolism (Bensaad et al., 2014; Islam et al., 2019). Lipid droplets also protect against ROS toxicity and the concentration of LD significantly increases in

hypoxia in a time and oxygen level dependent manner (Figure 8). In hypoxic conditions, HIF-1α induces transcription of FABP3 and FABP7, and these proteins promote lipid storage through fatty acid uptake followed by formation of LDs (Bensaad et al., 2014). While additional research is still required to clarify the relationship between FABP4 and LD, it is likely that FABP4 also encourages their accumulation.

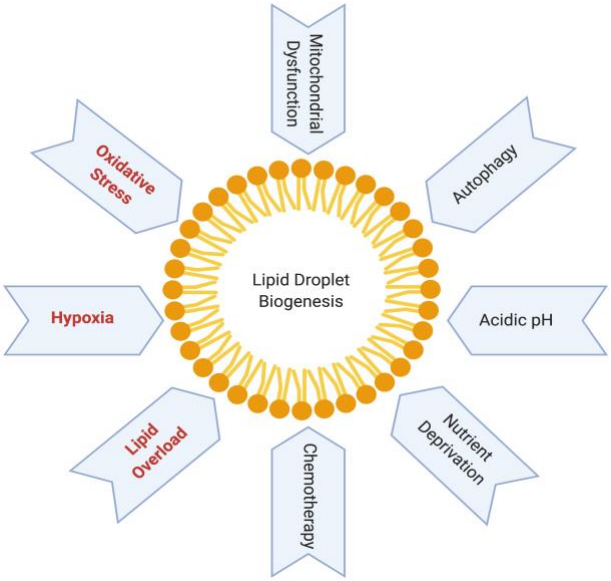


Figure 8. Conditions that stimulate lipid droplet biogenesis. Lipid droplets are lipid-rich organelles that regulaye the storage and hydrolysis of neutral lipids. They have been demonstrated to form in response to various stimuli, including oxidative stress, hypoxia, lipotoxicity, inflammation, nutrient deprivation, mitochondrial dysfunction, autophagy and in response to acidic pH and chemotherapy.

iii. Relationship between FABP4 & P20K in Lipid Homeostasis

FABP4 and p20K are both lipocalins that transport hydrophobic molecules and exhibit similar expression patterns in response to hypoxia and contact inhibition. Interestingly, the two proteins are together hypothesized to be induced by CCAT-enhancer Binding Protein Beta

(C/EBPβ) in CEF. Transcription of the p20K locus in response to quiescence is regulated by a 48 base pair region located within its promoter, known as the quiescence responsive unit (QRU). This region contains two conserved interaction domains for C/EBPβ flanking consensus binding sequences for extracellular regulatory kinase 2, ERK2 (Bédard et al. 1987). During reversible growth arrest, C/EBPβ proteins will form a dimer and bind to the A-site and B-site of the QRU. The presence and binding of C/EBP to both of these elements is required for the activation of p20K as abolishment of either region prevents p20K expression (Kim et al., 1999). Comparably, chromatin immunoprecipitation (ChIP) assays have revealed that C/EBP-ß also directly binds to the FABP4 promoter in CEF, but unlike with the QRU, this interaction is observed in both normoxic and hypoxic cells (Peragine & Bédard, unpublished results). This suggests that C/EBPβ is a regulator of FABP4 that likely works in tandem with additional factors to induce FABP4 expression during oxygen-limited conditions. The similarities of the two GAS genes in expression patterns and regulation in quiescence and their lipid trafficking functionality suggest they may exhibit analogous roles in lipid homeostasis as part of a common cellular stress response pathway.

The p20K lipocalin has been proposed to play a role in lipid homeostasis through its ability to alleviate lipid peroxidation during hypoxia (Erb, Msc. Thesis, 2016). Repression of p20K in limited oxygen environments resulted in enhanced lipid peroxidation compared to normoxic and p20K-expressing hypoxic CEF, as evident by elevated malondialdehyde (MDA) levels, a bi-product of lipid oxidation (Erb, Msc. Thesis, 2016). Interestingly, enzymes involved in polyunsaturated fatty acid (PUFA) binding and metabolism have been identified as critical determinants of ferroptosis sensitivity (Doll and Conrad, 2017).The lipocalin family and other related proteins may consequently prevent ferroptosis by stabilizing PUFAs easily attacked by

ROS to induce lipid peroxidation (Doll and Conrad, 2017). Lipid profiling analysis has revealed lipid species including palmitate, stearate and cholesterol accumulate in hypoxia (Erb, Msc. Thesis, 2016). Such factors can induce apoptotic pathways, hence proteins like p20K may be expressed during quiescence to promote survival through lipotoxicity reduction.

Rationale & Objectives

Previous research conducted by the Bédard lab has investigated the expression patterns of FABP4 in various reversible growth-arrest conditions. Contact inhibition and hypoxia were shown to induce FABP4 expression whereas actively dividing cells in normoxic and serum-deprived environments did not (Peragine, M.Sc., 2018). This suggests that FABP4 is activated in response to exit from the cell cycle mediated by oxygen limitations, as characteristic of hypoxia and high cell density, but not nutrient limitations, and thus separate from the ER stress response. Proliferation assay analysis has further indicated FABP4 is specifically regulated by inadequate oxygen supplies. FABP4 expression is predominantly stimulated in environments of 2% oxygen or lower, suggesting FABP4 may not be a primary effector of reversible growth arrest but rather hypoxia (Peragine, M.Sc., 2018). Additional functional analysis of FABP4 in conditions of quiescence is required to characterize its role in cell survival and proliferation.

A relationship appears to exist between GAS genes, such as FABP4 and p20K, and oxidative stress. FABP4 and p20K expression is induced by oxygen deprivation resulting from high cell density or hypoxia, creating conditions of cellular stress that reversibility inhibit replication, as proliferation becomes unfavourable and ROS levels increase (Erb et al., 2016). The transition from proliferation to quiescence reduces the resources allocated to growth-related processes, such as membrane biogenesis. This may lead to the intracellular accumulation of lipids and free fatty acids while ROS trigger lipid peroxidation and the release of lipid radicals. It is therefore

likely some GAS genes are activated to regulate lipid metabolism and promote survival. As lipocalins, FABP4 and p20K are crucial in the transport of fatty acids and are postulated to export damaged/excess lipids out of the cell in an attempt to restore lipid homeostasis following disruption from oxidative stress. Furthermore, preliminary western blot analysis conducted has demonstrated knockdown of p20K using RCASBP(A) p20K RNAi results in diminished FABP4 expression in conditions of contact inhibition and hypoxia (Sriranjan, M.Sc., 2019). Neither FABP4 nor p20K has been demonstrated to function as a transcription factor, and it is therefore probable that these proteins share a regulatory pathway in quiescence which is disrupted by p20K inhibition by shRNA. The regulatory mechanisms underlying FABP4 stimulation in reversible growth arrest must be defined. Given that p20K transcription activation by C/EBP-ß is well characterized in quiescent CEF, and that C/EBPβ can modulate FABP4 activity in other biological processes, the relationship between C/EBPβ and FABP4 in hypoxia should be evaluated.

Given the established expression pattern of FABP4 in reversible growth arrest conditions, the primary goal of this study is to characterize the role and regulation of FABP4 in quiescent CEF. Chapter 1 of this study will evaluate FABP4's functions in quiescence, with an emphasis on activities related to oxidative stress and lipid metabolism, through analysis of proliferation, apoptosis, reactive oxygen species release, lipid peroxidation, and lipid droplet formation in CEF with aberrant FABP4 expression. Chapter 2 of this study will then focus on describing the regulation of FABP4 induction in reversible growth arrest conditions to fill the gap in our understanding of key processes underlying exit from the cell cycle with the potential for re-entry. Based on the transcriptional mechanism established to control the p20K lipocalin activation in quiescence, the capacity for C/EBPβ to regulate FABP4 induction in low oxygen environments

will be of special interest. Finally, Chapter 3 of this study will characterize the relationship of lipocalins FABP4 and p20K based on their induction under similar circumstances of quiescence, potentially regulated by a shared pathway, and their proposed roles in lipid transport, cell survival and oxidative stress response. Taken together, the findings from this study will contribute towards understanding the functionality and regulation of GAS genes while further clarifying their relationships as part of the novel "Membrane Stress Response".

It is hypothesized that FABP4 is necessary for cell survival and maintenance of lipid homeostasis in CEF during conditions of cellular stress which induce quiescence. FABP4 is thought to be regulated in a manner analogous to the p20K lipocalin as part of a novel membrane stress response pathway.

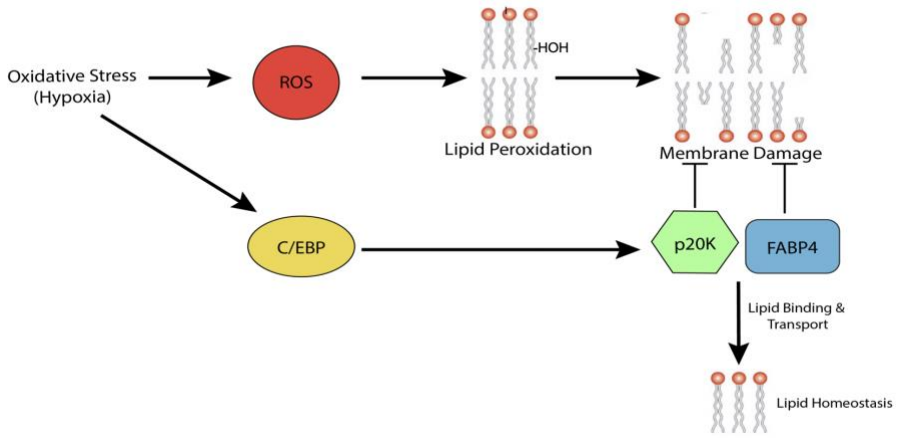


Figure 9. Hypothesis. Hypoxic conditions promote oxidative stress by stimulating the amassment of reactive oxygen species which subsequently leads to lipid peroxidation. Lipid peroxidation damages cellular membranes as lipids lose electrons and form lipid peroxides. C/EBPβ is activated in response to hypoxia (may be directly activated by reactive oxygen species) and can induce expression of p20K and FABP4 lipocalins. The two proteins can

alleviate the membrane damage through the transport of toxic lipid species out of cell, returning the cell to a state of lipid homeostasis.

Materials and Methods

1. Chicken Embryo Fibroblast Cell Culture and Culture Conditions

CEF were incubated in conditions containing 21% oxygen and 5% carbon dioxide at 41.5°C in complete medium comprised of Dulbeco's modified eagle medium (DMEM) high glucose (Sigma Life Science D649) supplemented with 5% heat-inactivated cosmic calf serum (at 57°C for 30 minutes, Hyclone AC10235353), 5% tryptose phosphate broth, 2mM of L-glutamine, 100 μg/ml of streptomycin and 100 units/ml penicillin. Near confluency, cells were split into fresh cell culture dishes using STE prepared by the Bédard lab and 0.05% Trypsin-EDTA Solution (Sigma Life Sciences #T3924).

For normoxic, cycling conditions, cells were cultured in normoxia conditions and harvested below 90% confluency. Samples were produced by splitting the cells 16-24 hours prior to harvesting, or by changing the complete media of existing cultures and incubating for 24 hours. For contact-inhibited conditions, cells were grown in the same environment as normoxic cells to 100% confluency. 24 hours prior to lysing cells for protein sample collection, the media was changed to ensure saturation density and allowed to grow for an additional 24 hours. For oxygen-depleted conditions, cells were incubated for 6-30 hours in 1-2% oxygen.

2. SDS-PAGE and Western Blotting

2.1 Protein Sample Preparation

Following the cultivation of cells under the designated culture conditions, cells were washed three times with cold 1X phosphate-buffer saline [(PBS) - 172 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4)]. Any lingering 1X PBS was aspirated from samples preceding the addition of 1 mL of fresh 1X PBS. The plates were then scraped with a rubber scraper to suspend cells in the 1X PBS and transferred to a 1.5 mL centrifuge tube. The samples were centrifuged for 3.5 minutes at 7000 RPM in a microfuge at 4°C to aggregate the

cells, and the 1X PBS supernatant was removed. The cell pellets were re-suspended in 1X Sodium Dodecyl Sulfate (SDS) sample buffer (10% glycerol, 5% β-mercaptoethanol, 2.3 SDS, 0.75% 0.0625 M Tris-HCl, pH 6.8) with 1X Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific #1861281). The samples were then briefly vortexed and boiled for 2 minutes. Following cell lysis, the samples were centrifuged at 13000 RPM for 5 minutes to pellet any insoluble debris. The supernatant was collected in a new centrifuge tube and stored at -80°C for subsequent use.

2.2 Bradford Protein Assay

The protein concentration of sample cellular lysates was quantified using a Bradford Protein Assay. A standard curve comparing concentration (x-axis) to absorbance (y-axis) was produced by combining increasing volumes (0-15 μL) of 1X bovine serum albumin (BSA) (1 μg/μL) with 200 μL (or 200 μL – volume of BSA) of double distilled H_2O, 2 μL of sodium dodecyl sulphate (SDS), and 800 μL of Bradford reagent. The protein sample solutions featured the same composition of reagents and their respective concentrations as the standard solutions but substituted the BSA and SDS for 2 μL of cell lysate. The optical absorbance of the standards and samples was determined using an Ultraspec 2100 Pro spectrophotometer set to wavelength of 595nm. The protein concentration of each sample was obtained by comparing its absorbance to the standard curve using linear regression.

2.3 SDS-PAGE and Western Blotting

Cell protein extracts (75 μg) were run on a 12-14% denaturing DSDS-polyacrylamide gel along with a Precision Plus Protein Dual Colour ladder (Bio Rad #1610374). The gel concentration utilized was dependent on the size of the protein to be resolved. Once the proteins had been separated, the samples were blotted to a nitrocellulose blotting membrane (Schleicher

and Schuell, BA85) and then blocked in a 5% solution of powdered skim milk dissolved in 1X tris-buffered saline [(TBS} (20 mM Tris, 140 mM NaCl, pH 7.6)] at room temperature for 30-60 minutes. The nitrocellulose membrane(s) were incubated with the relevant primary antibody (see Table 1 for primary antibody concentrations) dissolved in 5% TBS milk for 12-18 hours at 4°C. The blot(s) were then subjected to a cycle of six 5-minute washes consisting of two washes of 1X TBS, two washes of 1X TBST (1X TBS containing 0.1% Tween), and two washes of 1X TBS. Incubation of the blot(s) with the corresponding secondary antibody solution (see Table 2 for secondary antibody information) conjugated with horseradish peroxidase (HRP) was conducted for 90-120 minutes at room temperature in 5% TBS milk (refer to Table 2 for secondary antibody concentrations. The membrane(s) were then exposed to the same six 1X TBS and TBST washes as previously described. The chemiluminescent signals were visualized by incubation with Luminata Forte HRP Substrate (Millipore #WBLUF0500) and imaged at various exposure times using hyperfilm (GE Healthcare #28906838) following the manufacturer's guidelines.

Table 1: Primary Antibody Dilutions

Antibody	**Dilution**
FABP4 (Bédard Lab)	1:250
P20K (601-Y, 601-Z – Bédard Lab)	1:500
P20K (565 – Bédard Lab)	1:500
C/EBP-β LIP (8582 – Bédard Lab)	1:1000
C/EBP-β (8581 - Bédard Lab)	1:1000
CHOP (Tulip – Bédard Lab)	1:1500
CUTA (Bédard Lab)	1:50
ERK2 (Millipore #05-057)	1:2000

Table 2: Secondary Antibody Dilutions

Antibody	Dilution
Anti-Rabbit IgG, HRP-Linked Antibody (Cell Signaling #7074S)	1:25000
Anti-Mouse IgG, HRP-Linked Antibody (Cell Signaling #7076)	1:25000

2.4 Western Blot Protein Quantification

Protein expression of samples was quantified for comparison using the ImageJ software available on the National Institutes of Health's (NIH) website. The exposed films were scanned, and the band intensity of each Western blot was analysed using the *ImageJ User Guide* guidelines. Target protein expression was compared to the expression of the loading control, ERK2.

3. shRNAi Vector and cDNA Transfection

Table 3: FABP4 and p20K shRNA oligonucleotide sequences

FABP4 T3 shRNA forward	5'GAGAGGTGCTGCTGAGCGCAACCATCAGATCAGAAAGTACTAGTGAAGC CACAGATGTA -3'
FABP4-T3 shRNA reverse	5'ATTCACCACCACTAGGCATAACCATCAGATCAGAAAGTACTACATCTGTG GCTTCACT -3'
FABP4-T4 shRNA forward	5'GAGAGGTGCTGCTGAGCGAAAGATGGCTGGTGTGGCCAAGTAGTGAAGC CACAGATGTA -3'
FABP4-T4 shRNA reverse	5'ATTCACCACCACTAGGCAGAAGATGGCTGGTGTGGCCAAGTACATCTGTG GCTTCACT -3'
p20K- T2 shRNA forward	5'-GAGAGGTGCTGCTGAAAGATGAAGATGGTAATGGTAGTGAAGCCAC AGATGTA-3'
P20K – T2 shRNA reverse	5'-ATTCACCACCACTAGGCAGGCAAGATGAAGATGGTAATGGTACATCT GTGGCTTCACT-3'

3.1 Plasmid Preparation

The replication competent ALV with a splice acceptor (RCAS) vectors described in Table 1 were created by within the Bédard lab for CEF transfection based on constructs used by Kim et al. (Erb et al., 2016; Kim et al., 1999). The RCAS vector system was used to express shRNA targeting FABP4, p20K or C/EBPβ to downregulate protein expression in transfected CEF. RCAS vectors were also used to express cDNA for FABP4, p20K or NFM, causing upregulated protein induction in transfected CEF. This system consists of a molecular clone of the RSV SR-A strain DNA genome which was modified to delete the *src* oncogene and insert a unique restriction site (*Cla*I) in its place (Hughes, 2004). In this study, RCAS(A) and RCAS(B) vectors were utilized. The major difference between the two vectors is the viral envelope glycoproteins expressed; the RCAS(A) vector expresses retroviral envelope A associated with the TVA receptor on the host cell surface, whereas the RCAS(B) vector expresses retroviral envelope B which interacts with the TVB receptor (Hughes, 2004) (Figure 10). The difference in the envelope genes permits effective infection of cells with multiple vectors carrying different inserted genes, allowing multiple genetic modifications to be made within one CEF.

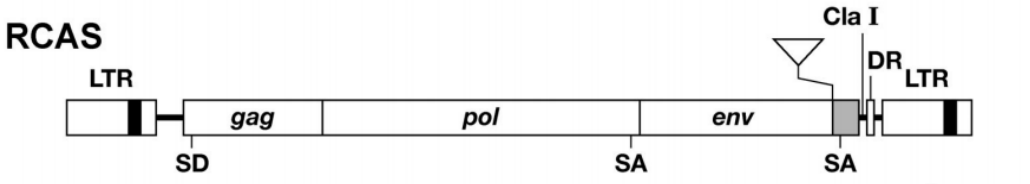


Figure 10. Schematic representation of the RCAS vector. The RCAS vector consists of three genes three genes (*gag, pol,* and *env*), long terminal repeats (LTR), splice acceptors (SA), splice donor (SD), and direct repeats (DR). This vector is a modified version of the RSV DNA genome, with most of the *src* gene deleted (retains the *src* splice acceptor) and replaced by a *Cla*I

restriction site. A target sequence can be inserted into the vector, which can then be transfected into replication competent cells. This results in the expression of the inserted sequence (Modified from Hughes, 2004).

3.2 DNA Precipitation

Prior to vector transfection, 10 μg of the plasmid construct, 20 μg of salmon sperm DNA (30 μg of total DNA per 100mm culture dish.), 0.2 M NaCl and twice the volume of 100% ethanol was combined and incubated overnight at -20°C to permit DNA precipitation.

3.3 Calcium Phosphate Transfection

The day prior to transfection, CEF were seeded on 100 mm tissue culture dishes to ensure 50-70% confluency at the time of calcium phosphate transfection. The following day, the medium from samples was aspirated and replaced with 6 ml of complete medium an hour prior to transfection. The DNA precipitate solution was centrifuged at 13,000 RPM for 15 minutes at 4°C. The supernatant was removed, and the DNA pellet was washed with 150 μl of 70% ethanol prior to re-centrifuging at 13,000 rpm for 5 minutes. The ethanol was discarded, and the DNA pellet was vacuum dried for 2 minutes. The pellet was then re-suspended in 438 μl of sterile ddH_2O and 62 μl of 2M $CaCl_2$. The sample solution was added dropwise to 500 μl of 2X HBSP (1.5mM Na2HPO4, 10mM KCl, 280mM NaCl, 12mM Glucose, 50mM Hepes) pH 7.12 while vortexed. The DNA mixture was inverted and incubated for 20-30 minutes to permit precipitation. The 1000 μl sample solution was added dropwise to cells in 100 mm culture dishes less and the plates were incubated for 4-6 hours prior to the glycerol shock. The medium was aspirated, and the cells were shocked using a glycerol shock solution (15% sterile glycerol, 50% 2X HBSP, 35% ddH_2O) for 90 seconds. The cells were washed twice with 4 ml of complete

medium and incubated in normoxic cell culture conditions overnight in 8 ml of complete medium. The cells were split for a minimum of two passages prior to experimental use.

4. Proliferation Assay

Cell proliferation and survival analysis was conducted by seeding CEF from confluent 100 mm plates into 24-well dishes. Following cell adhesion and growth in normoxic or hypoxic conditions, cells were trypsinized in 500 μL Trypsin-ETDA per well. The cell suspension was combined with 9.5 mL of Isoton II diluent (Beckman Coulter #8546719) in plastic vials compatible with the Z1 Coulter Particle Counter (Beckman Coulter), set to count particles within 12-20 μm. Cells were counted in quadruplicate after incubation of 0-, 24-, 48- and 72-hour periods. ANOVA statistical tests were performed to compare the samples.

5. TUNEL (Apoptosis Detection) Assay

Cellular apoptosis in CEF transfected was detected using TUNEL assay (TUNEL assay kit – Abcam ab66108). Cells were seeded on coverslips in 60 mm dishes and grown in normoxia or hypoxia for 30 hours. The cells were then washed with sterile 1X PBS and fixed with 4% paraformaldehyde dissolved in 1X PBS for 30 minutes at room temperature. The cells were washed with 1X PBS and permeabilized on ice 3 mL of permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate). The samples rinsed with 1X PBS followed by treatment with terminal deoxynucleotidyl Transferase (TdT) and TMR red fluorescent labeled dUTP for 1 hour at 37°C. The cells were washed with 1X PBS and stained with Hoechst 33342 (Thermo Fisher H1399) for 2 minutes. The cells were washed with 1X PBS and then sterile ddH_2O and mounted to cover slides. The slides were imaged in quadruplicated using fluorescence microscopy and 100 cells were counted per sample.

6. DCFDA Cellular ROS Detection Assay

The detection and quantification of ROS was assessed using a live cell DCFDA Cellular ROS Detection Assay Kit (Abcam ab113851). CEF were seeded in quadruplicated from confluent 100 mm dishes to a 96-well plate and incubated in normoxia or hypoxia for 30 hours. 1 hour prior to the end of the incubation period, four wells of normoxic CEF were treated with 55 μM tert-butyl hydrogen peroxide (TBHP) as a positive control. Cells were then washed with 1X Buffer and stained with 25 μM DCFDA in 1X Buffer for 45 minutes in conditions of normoxic or hypoxia. The cells were rinsed with 1X Buffer and fluorescent intensity was measured at Ex/Em=485/535 nm using a plate reader. ROS levels were calculated as a percentage of control after subtraction of background noise (subtract blank well readings from all well measurements and determine fold change from assay control). ANOVA statistical tests were performed to compare the samples.

7. Malondialdehyde (MDA) Detection Assay

Lipid peroxidation was quantified in CEF exposed to 24-26 hours of hypoxia or normoxia through the detection of malondialdehyde. Malondialdehyde quantities were measured using a MDA detection assay (Lipid Peroxidation MDA assay kit, Abcam ab118970). The cells were washed with 1X PBS, counted and centrifuged. The cells were then resuspended in 300 μl of MDA lysis buffer containing butylated hydroxytoluene (BHT). Subsequent lysis of the cells on ice was performed using a Dounce homogenizer, followed by centrifugation at 13,000 RPM for 10 minutes. 200 μl of supernatant was mixed with 600 μl of thiobarbituric acid (TBA) at 95°C for 1 hour followed by a 10-minute cooling period in an ice water bath. A spectrophotometer set to 532nm was used to collect sample measurements and from this value the MDA concentration was determined using a standard curve prepared in the same manner as described with 20-100 μM MDA standard. Once the MDA levels were calculated, comparisons were made between

samples based on average cell counts. ANOVA statistical tests were performed to compare the samples.

8. BIODIPY 493/503 Lipid Droplet Detection Assay

Lipid droplets were stained and detected in CEF incubated for 30 hours in 1.8% oxygen conditions and in normoxic cycling CEF using a lipophilic dye. Cells were plated on sterile glass coverslips in 60 mm dishes at a density that ensured they remained semi-confluent at the time of staining (12-24 hours). The cells were washed with 1X PBS the following day once they achieved a confluency of 50% and were incubated with 2 uM BODIPY 493/503 staining solution in PBS for 15 minutes at 41.5 C. Cells were rinsed with 1X PBS and then fixed with 4% paraformaldehyde for 30 minutes at room temperature. The samples were subsequently washed with 1X PBS and stained with Hoechst. The coverslips were washed with 1X PBS and mounted onto glass microscope slides. BODIPY 493/503 fluorescence was detected using fluorescent microscopy to visualize the neutral lipid droplets.

9. Transient Expression Assay

9.1 Plasmid DNA

The QRU pGluc reporter construct, was created by Michael Erb and utilized for the transient expression assay (Erb et al., 2016). The construct was cloned into a plasmid which contained a minimal promoter upstream of the target region along with an initiator element (Inr) from the beta-globin gene at the transcriptional start site (Erb et al., 2016; Kim et al., 1999) (Figure 11). The QRU construct contained the wild-type, 48-bp QRU sequence, and had previously been tested (Erb et al., 2016).

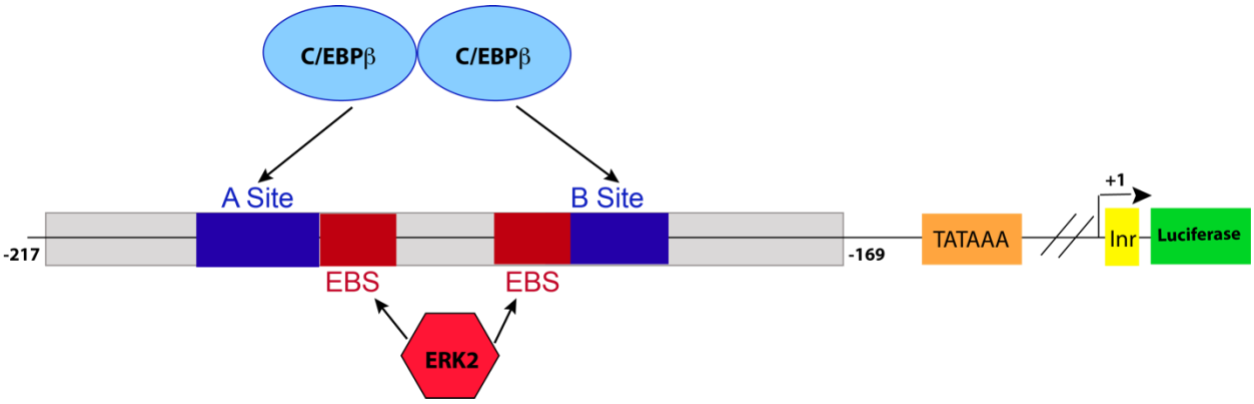


Figure 11. Luciferase Reporter Construct WT QRU. Wild-type QRU contains the 48-bp region (-217 to -169), containing two EBS elements for ERK2 binding. This construct is downstream of a minimal promoter and includes a TATAAA box (-24) along with an initiator element (Inr) from the human beta-globin gene at the transcriptional start site directly upstream of the luciferase gene.

9.2 DEAE-Dextran Transfection

DNA precipitations containing 10µg of the desired pGluc-derived reporter construct and 5µg of Rous Sarcoma Virus (RSV)-βgal control plasmid (described by Dehbi et al., 1994) were combined with salmon sperm carrier to bring the total DNA concentration to 30 µg of DNA. The DNA solution was incubated overnight at -20°C with 0.2M of NaCl and two times the total volume of cold 100% ethanol to allow DNA to precipitate. The following morning, the precipitate was centrifuged at 12,000 RPM for 10-15 minutes at 4°C prior to washing with 150 µl of cold 70% ethanol. The pellet was vacuum dried for two minutes and resuspended in 270µl of 1X TBS and 30µl ddH_2O before adding dropwise to 120µl of DEAE-Dextran (10mg/mL in 1X TBS). The solution was left for 20-30 minutes and then added to 50% confluent 100 mm plates that had previously been substituted with 6 mL of cosmic-calf serum free medium. The

plates were incubated for 5-6 hours, and then the cells were shocked with 10% dimethyl sulfoxide (DMSO) solution in 1X HBSP (7.5 mM Na_2HPO_4, 5 mM KCl, 140 mM NaCl, 6 mM glucose, 25 mM HEPES, pH 7.12). The plates were then washed twice with complete medium before being supplemented with 8 mL of complete medium. The cells were split the following day and incubated in either hypoxic or normoxic conditions for 24 hours. ANOVA statistical tests were performed to compare the samples.

9.3 Luciferase Assay

The cells were lysed in 100µl of 250 mM Tris (pH 6.8) and 1% NP-40. The BioLux *Gaussia* luciferase assay kit was used (New England BioLabs (NEB) E3300L) to measure the *Gaussia* luciferase activity in 10µl of each sample. Luminescence was quantified using an Ascent luminometer. Samples were normalized using β-galactosidase activity. The β-galactosidase reactions were conducted using 50 µl of cell lysate incubated in 3 µl of 100X magnesium buffer (100 mM Na_2HPO_4, 5 M 2-mercaptoethanol), 211 µl of 0.1 M Na_2HPO_4 pH 7.3, and 66µl of o-nitrophenyl-β-D-galactopyranoside (ONPG) at 37°C. Once a yellow colouration was observed (after approximately 30-40 minutes), the reaction was terminated using 0.5 ml of 1M Na_2CO_3. The β-galactosidase activity was analyzed colorimetrically using a spectrophotometer at an OD of 410 nm. Luciferase activity was normalized to the β-galactosidase activity.

Results

Chapter 1: Characterizing the role of FABP4 in conditions of quiescence.

i. FABP4 expression is induced by contact inhibition and hypoxia, with maximal induction after 30 hours, and can be inhibited in cells transfected with RCASBP(A) – FABP4 shRNA.

Gene profiling has shown that FABP4 is upregulated in confluent CEF and experiences enhanced expression in hypoxic conditions as oxygen deprivation is a feature of high cell density (Erb, 2016). Preliminary experiments suggest FABP4 is preferentially expressed in hypoxic and contact inhibition conditions but not by growth arrest associated with serum starvation and the ER stress response pathway (Peragine, M.Sc., 2018). Western blot analysis of cells grown in 21% O_2 normoxic, 1.8% O_2 hypoxic conditions or to maximal confluency confirmed that FABP4 is not prominent in sub-confluent normoxic cultures but is induced in response to quiescence caused by oxygen deprivation (Figure 16).

Limited oxygen observed in hypoxia and contact inhibition prompts the activation of FABP4 whereas ER-stress conditions, such as serum starvation, and "normal" proliferative environments inhibits the proteins expression (Erb et al., 2016; Peragine, M.Sc., 2018). The mis-expression of FABP4 using genetic tools permits the study of the protein's function in growth arrested cells. Retroviral RCASBP vectors were created to repress FABP4 induction regardless of the environmental or cellular state. Chicken embryo fibroblasts were infected with replication-competent viruses encoding shRNA specific to FABP4 to determine if FABP4 expression could be altered and to test cell viability (Figure 17, 18). Various vectors targeting different regions of FABP4 were tested (not shown) and ultimately two vectors (FABP4 T3 or FABP4 T4) were identified to efficiently and consistently downregulate

FABP4 in CEF. CEF were transfected with a group A retroviral vector expressing a control shRNA for GFP (RCASBP(A)- GFP RNAi) or one of two shRNA constructs for FABP4 down-regulation (RCASBP(A)- FABP4 T3 RNAi or RCASBP(A) – FABP4 T4 RNAi) and incubated in normoxia (21% O_2), hypoxia (1.8% O_2 for 24-hours and 30 hours) or to maximum cell density. Western blot analysis confirmed that FABP4 is not active in any sub-confluent cell type grown in normal oxygen levels. No FABP4 expression was observed in shRNA populations, even when they were grown in hypoxia for 24-30 hours or to contact inhibition. In contrast, control samples exhibited a slight induction of FABP4 after exposure to hypoxia for 24 hours (Figure 17A) and substantial FABP4 levels following 30-hours of oxygen deprivation or growth to maximal confluency (Figure 17B). These results confirm FABP4 can be downregulated in cells transfected with RCASBP(A) – FABP4 T3 RNAi or RCASBP(A) – FABP4 T4 RNAi and that 30-hour hypoxic exposure permits the greatest peak of FABP4 induction.

To further clarify the role of FABP4 in quiescence, cDNA was developed to force FABP4 over-expression. CEF transfected with RCASBP(B) FABP4 cDNA displayed FABP4 activity in all conditions (Figure 18 D). Meanwhile, control cells with RCASBP(B) GFP cDNA displayed negligible FABP4 expression in sub-confluent cells grown in 21% oxygen as expected. This confirms that FABP4 downregulation or upregulation can be achieved in CEF using RCASBP(A) FABP4 shRNA or FABP4 cDNA, providing an effective cellular model to study the role of FABP4 in quiescence.

ii. Cell proliferation is inhibited by the shRNA-mediated downregulation of FABP4 while over-expression of FABP4 enhances cellular growth in conditions of hypoxia.

The proliferative capacity of cells infected with replication-competent viruses encoding control GFP or FABP4 shRNA was measured to characterize the impact of FABP4 on cell growth and survival in low oxygen environments. The effect of FABP4 mis-expression was compared in conditions of normoxia and hypoxia. RCASBP(A) – GFP RNAi controls displayed exponential growth in normoxic conditions over a 72-hour time period while cells with FABP4 shRNA exhibited increasing growth at a rate analogous to- or slightly lower than the control (Figure 19 A, B). All cell types experienced reduced proliferation in low oxygen conditions when contrasted with their counterparts cultured in a normoxic environment. However, CEF experiencing downregulation of FABP4 exhibited the most striking growth arrest in hypoxic conditions, with significantly reduced population sizes observed at all time points compared to the control (Figure 19 A, B). Importantly, all cell types began with equivalent populations, indicating the differences in cell counts are not due to initial variations in seeding densities. The proliferation of FABP4 shRNA samples paralleled that of p20K shRNA cells, with both exhibiting impaired cell accumulation in conditions of hypoxia compared to the control (Figure 19 A, B).

In contrast, CEF transfected with FABP4 over-expression cDNA exhibited significantly greater cell population sizes in hypoxic conditions compared to control CEF (Figure 19 C). At 24-, 48- and 72-hour time points, the forced expression of FABP4 appreciably improved the proliferative capacity of cells incubation in perturbed oxygen concentrations when compared to control CEF. Analogous to the previous growth curves, the two cell populations displayed equivalent cell concentrations in normoxia but significant differences in low

oxygen environments. Overall, the enhanced population sizes observed in hypoxic CEF containing FABP4 cDNA, which directly diverges from the proliferative capacity of the hypoxic RCASBP(B) controls, suggests that elevated FABP4 expression may promote growth in limited oxygen environments.

iii. Increased apoptosis is induced by the shRNA-mediated down regulation of FABP4 in conditions of hypoxia.

FABP4 may play a role in promoting survival in reversible growth arrest conditions, contributing to the reduced cell population observed in hypoxic cells experiencing FABP4 downregulation. To evaluate cell viability in cells with aberrant FABP4 expression, TUNEL assays were performed on sub-confluent control and FABP4 shRNA CEF in normoxic and hypoxic environments. Negligible levels of apoptosis were observed in all groups grown in normal oxygen levels (Figure 20 A-E). The incidence of cell death was also low in hypoxic control cells whereas apoptosis increased markedly in FABP4 shRNA exposed to oxygen depleted conditions (Figure 20 A, C, D). An approximate 7-fold increase in apoptosis occurred when FABP4 activity is attenuated, while only about 1% of control cells experienced cell death in oxygen deprivation (Figure 20 B-D). This corresponds with the diminished cell survival noted in p20K shRNA cells grown in hypoxia, as apoptosis was elevated by 6.5-fold (Figure 20 E). TUNEL-positive cells exhibited chromatin condensation and nuclear fragmentation as shown by visualization with the Hoechst 33342 fluorescent stain. This demonstrates that FABP4 promotes cell survival in limited oxygen conditions.

iv. Elevated cellular reactive oxygen species are observed during shRNA-mediated downregulation of FABP4 in conditions of hypoxia.

Reduced oxygen availability is correlated with the development and release of reactive oxygen species and the subsequent oxidation of lipids and other important macromolecules. To examine the role of FABP4 in alleviating oxidative stress prompted by oxygen limitations, ROS discharge was analyzed in hypoxic CEF lacking FABP4 using a DCFDA/H_2DCFDA fluorogenic dye (Figure 21). This assay quantifies the presence of diverse ROS species, such as hydroxyl and peroxyl. As expected, a substantial surge in cellular ROS production was observed in all hypoxic samples compared to their respective counterparts growing in normal oxygen levels (Figure 21). CEF with aberrant FABP4 expression had significantly greater cellular ROS compared to the control samples (Figure 21). FABP4 T4 shRNA cells exhibited an approximate 2.4 factor increase in ROS emissions compared to control hypoxic CEF while FABP4 T3 shRNA and P20K shRNA displayed analogous ROS concentrations with a 1.8-fold surge in ROS compared to the control (Figure 21). Minor differences were observed in the ROS levels of normoxic FABP4 shRNA cells and control CEF. This postulates a role for FABP4 maintenance of lipid homeostasis in conditions of quiescence.

v. Elevated lipid peroxidation levels are observed with the shRNA-mediated down regulation of FABP4 while FABP4 over-expression reduces lipid peroxidation in conditions of hypoxia.

The oxidative degradation of lipids can be quantified through measuring the concentration of malondialdehyde (MDA), a reactive aldehyde by-product of lipid peroxidation. In this assay, fluorescent dye quantifies MDA levels through the formation of adducts between MDA and thiobarbituric acid, which can be measured at 532nm (Tsikas, 2017). All cells incubated in

normal oxygen conditions displayed low levels of MDA, indicating minimal lipid peroxidation was occurring with no observable differences (Figure 22 A). However, lipid oxidation was evident in cells transferred to oxygen limiting conditions for 30 hours. FABP4 shRNA experienced an approximate 1.5-fold increase in MDA concentration compared to the control, with relative MDA concentration comparable in FABP4 and p20K shRNA cells (Figure 22 A). While all samples exposed to low oxygen environments showed enhanced MDA accumulation, the significant increase in cells with FABP4 downregulation suggests that FABP4 plays a role in lipid homeostasis and mitigating the effect of low oxygen on ROS formation and lipid peroxidation.

In contrast, the over-expression of FABP4 or the p20K lipocalin reduced the release of lipid peroxidation by-products during oxygen deprivation (Figure 22 B). A significant difference was noted between MDA levels produced in control RCASBP(B)- GFP CEF in normoxic and hypoxic conditions as observed in prior trials. However, cells transfected with p20K cDNA or FABP4 cDNA did not experience a significant elevation in lipid peroxidation in response to oxygen limitations (Figure 22 B). The levels of MDA were statistically equivalent in normoxic and hypoxic samples with FABP4 cDNA or p20K cDNA. This further validates the notion that the activation of lipocalins such as FABP4 and p20K aids in mitigating cellular stressors caused by hypoxia, such as the liberation of toxic lipid radical species.

vi. Diminished lipid droplet formation is induced by the shRNA-mediated down regulation of FABP4 in conditions of hypoxia.

Lipid droplets are dynamic signaling organelles that function as the predominant unit for intracellular lipids and are critical in maintaining lipid homeostasis. Preliminary exploration of the presence of neutral lipid droplets can be conducted using fluorescent staining BIODIPY

493/501. All samples exhibited insignificant volumes of lipid droplets when grown in 21% oxygen (Figure 23). In conditions of hypoxia, control RCASBP(A) GFP shRNA cells were detected to have greater quantities of lipid vesicles compared to those incubated in normoxia (Figure 23). In contrast, FABP4 shRNA qualitatively appeared to have minimal lipid droplet formation in limited oxygen environments (Figure 23). Hypoxic p20K shRNA cells were similarly shown to have fewer lipid droplets compared to the CEF (Figure 23). The diminished presence of lipid storage units noted in FABP4 or p20K loss conditions suggests that the two proteins may play a role in lipid droplet formation in response to perturbances in lipid homeostasis caused by oxygen depletion and other cellular stressors.

Chapter 2: Characterizing the regulation of FABP4 in conditions of quiescence.

i. FABP4 expression is reduced by dominant-negative C/EBP mutant (Δ 184) and shRNA-mediated down regulation (99) of C/EBP-beta in a manner analogous to the p20K lipocalin.

C/EBPβ activity is critical for initiating the growth arrest specific response pathway, as demonstrated through its capacity to induce p20K transcription through interaction with the lipocalin's promoter in the *QRU* region (Kim et al., 1999). C/EBPβ has also been shown to be one of many regulators that control FABP4 expression, but the relationship between the two factors has yet to be elucidated in response to growth arrest in low oxygen conditions, such as hypoxia and contact inhibition. LIP, a C/EBPβ isoform that lacks a transactivating domain, is capable of binding to activating isoforms LAP and LAP* to inhibit C/EBPβ activity. Overexpression of LIP using a dominant negative C/EBPβ mutant vector, RCASBP(B) Δ184, can thus be used to determine if a reduction in active C/EBPβ influences FABP4 expression in

oxygen depleted conditions, comparable to that observed with p20K. Western blot analysis showed that FABP4 induction was lost in high density CEF and hypoxic CEF expressing LIP (Figure 24). Furthermore, the complete downregulation of C/EBPβ isoforms was achieved through transfection with C/EBPβ shRNA (RCASBP(B) 99 RNAi). Loss of C/EBPβ also resulted in the abolishment of FABP4 and p20K expression in confluent and oxygen-depleted cells (Figure 25). This suggests that hypoxic FABP4 induction is dependent on the activity of transactivating C/EBPβ isoforms.

ii. FABP4 expression is enhanced by the over-expression of C/EBP-beta (NFM overexpression mutant) in a manner analogous to the p20K lipocalin.

To further characterize the effect of C/EBPβ activity on FABP4 in reversible growth arrest conditions, FABP4 accumulation was examined in conditions of C/EBPβ over-expression. Western blot analysis displays a strong induction of FABP4 in normoxic cells exhibiting C/EBPβ overexpression, whereas the control normoxic cells lack FABP4 (Figure 26). This is comparable to the aberrant presence of p20K in cells exposed to normal oxygen levels when C/EBPβ is artificially induced (Figure 26). The direct positive correlation between C/EBPβ activity and FABP4 expression supports the notion that C/EBPβ is an upstream activator of FABP4 in response to quiescence.

iii. Reactive oxygen specific formation and release mediated by TBHP induces expression of FABP4 and the p20K lipocalin.

Tert-butyl hydroperoxide (TBHP) is an organic peroxide which can induce macromolecule oxidation and the release of ROS. To determine if reactive oxygen species and lipid oxidation levels can directly induce FABP4 and/or p20K expression, normoxic cells were treated with TBHP to enhance ROS accumulation and lipid peroxidation. Cells exposed to 220 mM TBHP

displayed p20K and FABP4 expression at early time points, whereas the controls did not induce the GAS genes' expression (Figure 27). Low FABP4 induction was observed after 1-hour of treatment with a significant increase by 3-hours of TBHP exposure (Figure 27). P20K displayed more robust expression with enhanced accumulation following 1- and 3-hours of TBHP treatment and minimal induction up to 20-hours (Figure 27). The dramatic reduction of FABP4 and p20K induction beyond 3-hours of TBHP treatment may be related to the impact of the compound on cellular proliferation and survival. TBHP is extremely toxic and the enhanced levels of apoptosis observed at time points beyond 1-hours suggest of exposure indicates the expression may have ceased as a result of a cellular switch from quiescence to apoptosis. This preliminary experiment suggests FABP4 and p20K expression can be directly activated by the accumulation of ROS.

Chapter 3: Characterizing the cross-regulation of FABP4 and P20K in quiescence.

i. Downregulation of FABP4 or the p20K lipocalin causes loss of the other by hypoxia or contact inhibition.

The p20K lipocalin is expressed in growth arrest conditions, such as hypoxia and contact inhibition, but is not observed in sub-confluent, normoxic cells. This induction pattern is akin to that of FABP4, and the apparent control of both proteins by C/EBPβ postulates a potential relationship underlying p20K and FABP4 in quiescence. Previous studies have shown an impairment of p20K in limited oxygen conditions using RNAi results in diminished levels of FABP4 (Sriranjan, M.Sc., 2019). This was verified by western blot analysis of shRNA p20K samples incubated to maximal density or in hypoxic environments. FABP4 induction is reliably lost in growth arrest conditions when p20K is inhibited (Figure 28). The inverse

situation was examined to further describe the relationship between the factors and p20K lipocalin expression is impaired by the shRNA-mediated downregulation of FABP4 in conditions of contact inhibition and hypoxia as well (Figure 28 A, B). Taken together, these results suggest that FABP4 and p20K may share a regulatory pathway in response to quiescence that is interfered with upon downregulation of one of the proteins or that dampening FABP4 or p20K activity impacts the functionality of the entire GAS gene system.

ii. The expression of CUTA, a GAS gene, is not influenced by the shRNA-mediated downregulation of FABP4 or the shRNA-mediated downregulation of the p20K lipocalin in conditions of contact inhibition and hypoxia.

To determine if downregulation of FABP4 or the p20K lipocalin using RNAi broadly interferes with GAS response pathways, the expression of other GAS genes was measured in conditions of hypoxia and contact inhibition. CUTA, a ubiquitous trimeric protein implicated in heavy metal tolerance and neurological activity, is upregulated by high cell density, serum-starvation and oxygen limitations. The mRNA experiences a 50-fold increase in its expression in CEF grown to maximal density, and featured one of the strongest reactions to contact inhibition out of 28, 418 genes analyzed (Erb et al., 2016). It is not known to share any regulatory factors associated with FABP4 and p20K, such as C/EBPβ and it is not implicated in lipid homeostasis. The vast structural and functional differences between CUTA and the two lipocalins makes it a strong candidate to function as an unrelated marker of the general growth arrest response pathway as all of the proteins are stimulated by low oxygen environments. Cells with FABP4 shRNA or p20K shRNA did not exhibit a loss of CUTA expression in conditions of hypoxia or contact inhibition (Figure 28). CUTA featured the identical induction patterns in control, FABP4 shRNA and p20K shRNA CEF for

normoxia, hypoxia and contact inhibition samples with negligible variations in protein levels (Figure 28). This shows that the downregulation of FABP4 or p20K does not impact the entire GAS response. FABP4 and p20K may share a component of their respective quiescence-mediated response pathway.

iii. Combined downregulation of FABP4 or P20K and up-regulation of the other lipocalin moderately restores cellular proliferation and reduces apoptosis and lipid peroxidation in hypoxia

Given that loss of FABP4 using shRNA causes inhibition of p20K (and vice versa), it is possible that the observed phenotype of these cells in conditions of reversible growth arrest is primarily caused by inhibition of one of the proteins or it may be by the combination of both. To evaluate if FABP4 or p20K is predominantly responsible for the pro-survival, pro-growth nature of quiescent cells, the phenotype of hypoxic cells expressing either FABP4 or P20K and exhibiting downregulation of the other was investigated using shRNA and cDNA.

Cells were transfected with both RCASBP(A) FABP4 T3 shRNA and RCASBP(B) p20K over-expression cDNA (Figure 29 A). Once the viability of these cells was confirmed, their capacity to proliferation in low oxygen environments was measured over 72 hours. As expected, CEF exhibiting FABP4 downregulation and maintained p20K expression displayed growth patterns similar to control RCASBP(A)-GFP and RCASBP(B)-GFP populations in normoxia (Figure 30 A, B). In contrast, hypoxic cells with combined FABP4 loss and p20K upregulation featured a moderate increase in proliferative capacity when compared to control groups. The population size at every time period was lesser than that of cells with p20K overexpression, but greater than groups with FABP4 knockdown. These results suggest that diminished growth observed in cells with FABP4 inhibition can be somewhat compensated

by upregulating cellular levels of p20K. Consistent with these findings, the downregulation of p20K paired with over-expression of FABP4 (Figure 29 B) also resulted in modest improvements in growth rates when compared to control cells exposed to hypoxia (Figure 30 C, D). While all populations featured similar proliferation in normoxia, cells with high levels of FABP4 displayed substantial growth at all time periods while CEF with diminished p20K activity had significantly inhibited growth. Taken together, loss of FABP4 or p20K hinders the proliferation of CEF in low oxygen while restoration of one of the lipocalins aids in moderately restoring growth in these conditions.

Similar to the effect on cell growth, the knockdown of FABP4 combined with the forced expression of p20K moderately improves cell survival in quiescence. Drastically high rates of apoptosis are detected in hypoxic FABP4 shRNA cells (Figure 31 A, D) as previously noted. In contrast, up-regulation of p20K in these conditions improves cell survival, with minimal death observed. Cells experiencing FABP4 inhibition while retaining p20K induction have reduced apoptotic rates compared to the control CEF and FABP4 shRNA cells, but there is more cell death detected than in p20K over-expression cells (Figure 31 E, F). It therefore appears that both p20K and FABP4 are required to maximize cell survival in response to low oxygen, and inhibition of one of these proteins increases the incidence of apoptosis.

Both FABP4 and p20K are implicated in maintaining lipid homeostasis during reversible growth arrest. To analyze the effect of inhibiting one of these lipocalins on the capacity to mitigate ROS release and lipid peroxidation in response to environmental stressors, an MDA assay was conducted using cells with FABP4 inhibition and p20K over-expression (Figure 32 A). In normal oxygen environments, equivalent levels of lipid peroxidation were observed in

control CEF, p20K over-expression CEF, FABP4 shRNA knockdown CEF and p20K over-expression + FABP4 shRNA knockdown CEF. However, when incubated in 1.8% oxygen for 30 hours, cells with combined p20K upregulation and FABP4 inhibition experienced an appreciable upsurge of lipid peroxidation. The elevation in MDA species detected in these cells was not as substantial as in FABP4 knockdown cells but was still significantly greater than control populations. Forced expression of p20K aids in lessening lipid peroxidation in quiescent conditions where FABP4 is unavailable. Similarly, cells with FABP4 expression but lacking p20K exhibited a significant reduction in MDA release in hypoxia compared to p20K knockdown (Figure 32 B). Interestingly, populations with FABP4 over-expression have equivalent levels of lipid peroxidation as those with forced FABP4 induction and p20K downregulation. This suggests that FABP4 can reduce the rate of lipid radical release in oxygen deprived environments without the assistance of p20K.

iv. Loss of QRU activity and inducibility is associated with the shRNA-mediated downregulation of FABP4.

The 48-bp QRU region of the p20K promoter is essential for its activation in response to reversible growth arrest. C/EBPβ binding to the QRU has been shown to be sufficient and necessary for transcriptional activation of p20K whereas CHOP and ERK2 antagonize the expression of p20K through binding to C/EBPβ and the QRU, respectively. In proliferative conditions, ERK outcompetes C/EBPβ for QRU occupancy and thus prevents p20K transcription (Erb et al., 2016). The loss of p20K in conditions of FABP4 impairment may be associated with the shared regulation of the two GAS proteins by C/EBPβ and the detailed description of the p20K promoter and its regulation provides a model to test if FABP4 downregulation impacts QRU- and C/EBPβ-dependent activation of p20K in quiescence.

The promoter activity of a construct containing the wild-type QRU was quantified using a transient expression assay where luciferase activity functioned as a marker of promoter activation. Control and FABP4 shRNA sub-confluent cells were cultivated in normoxic and hypoxic conditions for 24 hours. The average relative luciferase intensity of both sets of samples exposed to normal oxygen levels displayed insignificant differences (Figure 33). However, in hypoxia, cells with FABP4 downregulation experienced approximately 2.7 times less QRU activity than the control CEF. There were no significant distinctions in measured QRU promoter activity of FABP4 shRNA cells exposed to limited or normal oxygen environments. This is contrasted with the RCASBP(A) – GFP shRNA samples, in which hypoxia enhanced QRU activity by more than 3-fold compared to normoxia. The inhibition of FABP4 is associated with diminished QRU activity which may account for the loss of p20K accumulation observed in these conditions.

Discussion

Chapter 1: FABP4 exhibits pro-survival characteristics and is involved in lipid homeostasis in hypoxic chicken embryo fibroblasts

FABP4 is expressed in oxygen deprived conditions, and its induction patterns can be manipulated using shRNA or cDNA. Microarray analysis shows FABP4 is upregulated 30-fold in high cell densities and subsequent studies have indicated FABP4 induction is stimulated by oxygen deprived environments, such as hypoxia and contact inhibition (Erb et al, 2016; Peragine M.Sc., 2018). We verified the expression of FABP4 in response to these reversible growth arrest conditions and noted that FABP4 levels are relatively low following 24-hours of limited oxygen conditions compared to cells grown to maximal saturation density (Figure 17 A). Follow up studies revealed that FABP4 is consistently and maximally induced after 30-hours of hypoxia as opposed to the 24-hour timeframe established for related GAS genes (Figure 17 B). This suggests that FABP4 expression is strongest in response to extended exposure to minimal oxygen and has a slower activation in response to oxygen depletion. The p20K lipocalin exhibits the greatest induction after 24-hour exposure to depleted oxygen conditions, and a reduction in p20K accumulation is observed at 30 hours (Erb et al, 2016). Moreover, Erb et al found that p20K induction occurs within 6 hours of hypoxia, whereas FABP4 upregulation does not occur until 12 to 24-hours of persistent low oxygen exposure (Peragine, M.Sc., 2018). The reversible growth arrest-induced activation of FABP4 is thus slower than that of p20K. The longer onset of FABP4 induction may be related to its regulation. While the transcriptional regulation of p20K through the *QRU* region of its promoter is well-documented, the mechanisms that trigger FABP4 expression in reversible growth arrest conditions are unclear. It is possible that the process for initiating the transcription of FABP4 is more complex than that of p20K, which only relies on

two factors, ERK2 and C/EBPβ, to modulate its induction (Bedard et al., 1989; Erb, M.Sc., 2016; Kim et al., 1999). Moreover, ERK2 and C/EBPβ compete for binding to the p20K promoter, meaning to activate transcription, C/EBPβ simply needs to be stimulated to displace ERK2. In contrast, initiation of FABP4 expression may require the accumulation and binding of multiple transcription factors/substrates, such as PPARγ and C/EBPβ (which contain binding sites in FABP4 promoter), or insulin, dexamethasone, fatty acids and agonists of PPARγ (Furuhashi et al., 2015; Haunerland and Spener, 2004). One investigation by Hu et al. found hepatic expression of FABP4 is positively correlated with liver ischemia/reperfusion, a clinical condition involving hypoxia and inflammation. They show that exposure of primary hepatocytes to hypoxia or transgenic overexpression of HIF-1α is sufficient to induce FABP4 expression, establishing the protein as a transcriptional target of HIF-1α (Hu et al., 2015). P20K has been demonstrated to be activated independently of HIF-1α in oxygen deprived environments, indicating the variation in activation rate between p20K and FABP4 may be related to differences in regulation by HIF-1α (Erb, M.Sc., 2016). Alternatively, FABP4 RNA may require post-transcriptional modifications prior to protein synthesis. Studies have shown FABP4 is post-transcriptionally regulated by microRNA, in which small non-coding RNA levels are inversely correlated with FABP4 activity (Gharpure et al., 2018). Interestingly, limited oxygen downregulates microRNA that target and repress FABP4, allowing the protein to be synthesized and exert its functionality in these conditions (Gharpure et al., 2018). No investigations have found that p20K levels are controlled by microRNA, meaning the lipocalin can rapidly collect in the cell once its transcription has been activated. The drop in the concentration of p20K at the time point when FABP4 is most strongly induced could also explain some of the redundancies in the two protein's functionality. If the onset of FABP4 activity is reduced through initial

inhibition via microRNA or the need for various transcription factors to aggregate at its promoter, there is an extensive time frame where ROS can form and disrupt lipid homeostasis in its absence. The rapid activation of p20K can compensate for this delay, promoting cell survival and growth while the induction of additional GAS genes begins to ramp up in the event that the limited oxygen conditions persist. Further regulation studies of FABP4 are required to identify the mechanisms hindering its swift stimulation in reversible-growth arrest, and to analyze the length of time FABP4 remains active in cells.

FABP4 is classified as a GAS gene based on its activation in oxygen deprived states, such as maximum confluency and hypoxia, while it is not induced by ER stress conditions like serum starvation (Peragine, M.Sc., 2018; Figure 16). Despite our understanding of FABP4's expression patterns, its role in reversible growth arrest remains unknown. The development of retroviral shRNA RCASBP vectors that target FABP4 provides a molecular tool to evaluate the effects of FABP4 in reversible growth arrest. In accordance with previous research that reported repression of FABP4 in actively dividing cells (Peragine, M.Sc., 2018), control RCASBP (A) GFP shRNA and FABP4 knockdown shRNA CEF did not exhibit any FABP4 expression (Figure 17, 18 A-C). While oxygen depleted control samples displayed elevated FABP4 levels, it was not detected in FABP4 shRNA cells. Meanwhile, transfection of CEF with RCASBP(B) FABP4 cDNA had the opposite effect, as FABP4 was present in all samples, including actively proliferating cells grown in normoxia (Figure 18 D). This confirms that FABP4 downregulation can be achieved in CEF using RCASBP(A) -FABP4 shRNA while transfection with RCASBP(B) – FABP4 cDNA permits FABP4 overexpression, providing an effective cellular model to study the role of FABP4 in quiescence.

FABP4 promotes survival during reversible growth arrest. Reversible growth arrest initiates a unique gene expression profile to promote cell survival, mitigate cellular stress, prevent unnecessary metabolism and permit re-entry into a proliferative state once optimal conditions are restored. Previous studies have indicated that contact inhibited cells experience hypoxic environments in culture, and thus it appears some GAS genes, are regulated in a hypoxia specific manner (Erb et al., 2016). FABP4 has been identified as one such growth arrest specific gene, and although the protein is recognized for its abilities to mediate lipid transport, its exact role in quiescence is unclear. The fatty acid binding protein is implicated in tumorigenesis and metastasis, in part for its ability to promote tumour cell growth and survival. In bone marrow and ovarian cancers, FABP4 is constitutively active and its over-expression promotes aggressive tumour growth (Gharpure et al., 2018; Herroon et al., 2013; Nieman et al., 2011). Additional studies have found loss of FABP4 increases susceptibility to apoptosis while decreasing migration and capillary network formation in human umbilical vein endothelial cells (Elmasri et al., 2012). These findings suggest that although FABP4 activity can become pathogenic when not properly regulated, the protein may enhance cell survival in quiescence. Moreover, FABP4's ability to promote transcription may be linked to its role in facilitating membrane and lipid integrity. When measuring cell population sizes in hypoxic environments through proliferation assays, it was found that CEF expressing FABP4 downregulation shRNA were unable to accumulate at the same rate as control cells (Figure 19 A, B). In exponentially dividing cells, there we no significant differences observed between the proliferative capacity of control CEF or cells with FABP4 knockdown shRNA (Figure 19 A, B). This is expected as FABP4 is not expressed in actively dividing CEF, thus its loss should not impact cell activity. However, in cells over-expressing FABP4 using cDNA, cell accumulation occurred at a greater rate in oxygen

deprivation conditions than their control counterparts while no distinctions in growth were noted in the two cell types in normoxia (Figure 19 C). Interestingly, the most substantial difference in survival & proliferative capacity between the two groups in hypoxia was observed at the 24-hour time mark. This could be related to the finding that FABP4 expression is still relatively low, as maximal amassment occurs after 30-hours in low oxygen conditions. Thus, the greatest disparity in FABP4 concentration between the two cell populations likely occurs in the first 24-30 hours of oxygen depletion, where artificial induction of FABP4 has the opportunity to enhance survival. Of additional note, all cells incubated in minimal oxygen had lower proliferative rates than samples in normoxic conditions. This shows that oxygen availability also impacts the proliferative capacity of cells, which is anticipated as division is a metabolically taxing process and oxygen is essential to perform aerobic respiration.

TUNEL assays were subsequently performed to analyse if the reduction in cell population noted in hypoxic FABP4 downregulation shRNA was due to diminished cell viability resulting in apoptosis. In general, apoptosis can function as an anti-tumorigenic defence mechanism that can be initiated in response to uncontrollable proliferation. It may also be stimulated as a method to remove damaged cells and maintain genomic integrity of a tissue (Kannan and Jain, 2000). Apoptosis can be induced in response to oxygen limitations through sustained activation of hypoxia inducible factor 1 (HIF-1), which stimulates expression of proapoptotic proteins and stabilizes p53 (Greijer and Wall, 2004). The intricate balance of factors that induce or prevent cell death ultimately decides the fate of cells in hypoxia. A higher incidence of cell death was observed in samples with FABP4 knockdown shRNA in hypoxia compared to the control and normoxic samples (Figure 20 A-D). Normally, CEF experience minimal apoptosis after 24- to 30-hours of exposure to limited oxygen (Figure 20 A, B), indicating these cells are equipped to

adapt and tolerate hypoxic conditions. This is further supported by the continual growth observed in normal hypoxic populations, despite the overall lower population sizes. It thus appears that FABP4 aids in maintaining cell viability in limited oxygen environments, potentially by encouraging adaptation and survival. FABP4 also appears to function similarly to p20K in terms of preventing apoptosis in quiescence, as loss of p20K produces equivalent quantities of TUNEL stained samples as those observed in FABP4 inhibition (Figure 20 A, E). This suggests both FABP4 and p20K are critical for managing cell survival in low oxygen conditions. The linkage between the two lipocalin's expression in conditions that initiate entry into G0 further supports similarities in their functionality. In hypoxia, ERK2 is lost from the *QRU,* permitting C/EBPβ to rapidly induce p20K expression (Erk et al., 2016). P20K is then able to prevent apoptosis as the cell attempts to adapt to the harsh environment. It could be that sustained exposure to low oxygen permits transcriptional regulators of the quiescence gene program (such as C/EBPβ) to initiate the expression of additional factors, such as FABP4. Thus, as the initial peak in early hypoxic factors, such as p20K, is lost, later-stage proteins with redundancies in functionality, such as FABP4, could continue to prevent apoptosis.

FABP4 is involved in mitigating the toxic effects of oxidative stress through regulation of lipid species. The generation and amassment of ROS is a strong biomarker of disrupted lipid homeostasis, as ROS may elicit free radical attacks on lipids that comprise the cellular membrane, threatening the integrity of the lipid biomembranes and overall cell survival. ROS generation is amplified in oxygen deprivation, as the mitochondria senses oxygen levels and releases ROS into the cytosol to stabilize HIF-1α and HIF-2α, thus priming the cellular response to hypoxia (Chandel et al., 1998; Guzy et al., 2005). CEF transfected with FABP4 downregulation shRNA displayed a significant upsurge in ROS levels compared to control cells

(2 to 3-fold greater) (Figure 21). An MDA colorimetric assay was then performed to directly measure the formation of lipid radicals and the chain reaction of lipid peroxidation in cells lacking FABP4. A significant increase in MDA concentration is observed CEF are exposed to hypoxia, and this is additionally augmented when FABP4 is downregulated (Figure 22 A). Notably, both ROS and MDA concentrations were extremely similar within FABP4 shRNA and p20K shRNA samples in a low oxygen environment. In circumstances of FABP4 or p20K up-regulation, the release of MDA is significantly reduced in hypoxia (Figure 22 B). While control samples display significantly greater rates of lipid peroxidation in oxygen deprived environments compared to normal conditions, cells with either forced FABP4 or p20K expression have analogous lipid peroxidation in both conditions (Figure 22 B). These findings could explain how FABP4 promotes cell survival in quiescence. Lipid peroxides readily induce apoptosis, thus FABP4's proposed ability to prevent the toxic accumulation of ROS and products of lipid oxidation would lead to enhanced cell viability (Gaschler and Stockwell, 2017). The FABP family controls lipid concentrations in the cell through their ability to bind free fatty acids. This paired with FABP4's expression at maximum cell density and in hypoxia further supports the notion that FABP4 prevents or alleviates the results of oxidative stress initiated by depleted oxygen through exportation of oxidized lipids out of the cell. Collectively, these results indicate FABP4 participates in establishing lipid homeostasis by improving the toxic effect of oxidative stress and lipid peroxidation caused by hypoxia (Figure 12).

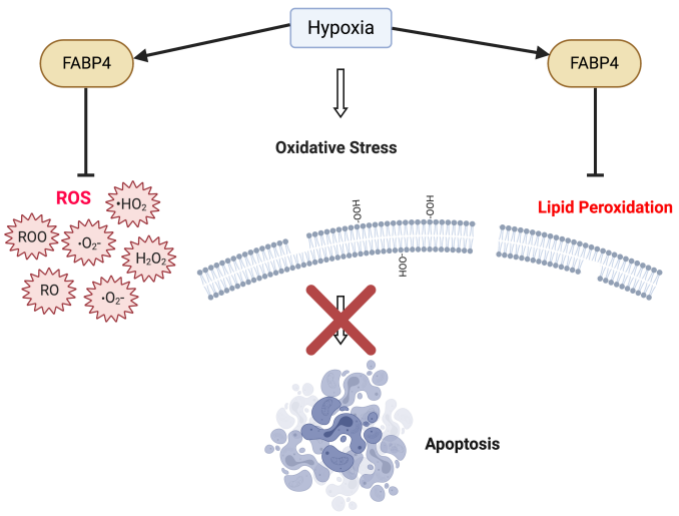


Figure 12. Proposed role of FABP4 in response to reversible growth arrest. FABP4 is critical for sustaining cell survival during oxygen deprivation by mitigating the detrimental effects of reactive oxygen species and lipid peroxidation. FABP4 mediates this through the transport and regulation of lipid species.

A relationship between FABPs and lipid droplet formation has been proposed, as FABP4 is thought to regulate enzyme and lipid storage through the establishment of lipid droplets in the cytoplasm. Its function as carrier protein for transport of free fatty acids generated by lipolysis, suggesting it may also shuttle lipid constitutions away from lipid droplets to deplete these storage units (Furuhashi et al., 2015). Interestingly, lipid droplet accumulation has been shown to be essential for cell growth and survival in response to hypoxia (Gordon et al., 1977; Koizume and Miyagi, 2016). In limiting oxygen conditions, HIF-1α is activated and induces transcription of enzymes responsible for mediating lipolysis to provide energy for cellular function (Bensaad et al., 2014; Koizume and Miyagi, 2016). Lipid droplets are crucial to this process, as they provide the triglycerides necessary to produce FFA. Lipid droplets can also act as a reservoir for toxic molecules, such as ROS species, making membrane lipids less susceptible to lipid peroxidation.

FABP4 regulates lipolysis and may therefore be involved in processes that control the storage and release of lipids during stress conditions (Bensaad et al., 2014; Koizume and Miyagi, 2016). Our findings show that loss of FABP4 dramatically reduced the formation of lipid droplets in oxygen deprived environments (Figure 23). Moreover, similar results were detected when p20K is downregulated using shRNA (Figure 23). These results are in agreement with other research as Lipocalin 2, the human homolog of p20K, has been shown to regulate intracellular lipid droplet formation in liver (Asimakopoulou et al., 2014). While fewer studies are available concerning FABP4 and lipid droplets, inhibition of FABP4 does reduce triglyceride content and intracellular lipid accumulation in human trophoblasts (Scifres et al., 2011). Furthermore, FABP7, the FABP family member chiefly expressed in the brain, has been shown to protect astrocytes from hypoxia-induced ROS toxicity through lipid droplets formation (Islam et al., 2019). It is therefore possible that FABP4 may contribute to mitigating the effects of oxidative stress and lipid peroxidation through the formation of lipid droplets in reversible growth arrest.

Chapter 2: FABP4 is regulated by C/EBPβ in a manner analogous to the p20K lipocalin in conditions of quiescence.

Control of FABP4 by C/EBPβ in reversible growth arrest conditions. C/EBPβ is implicated in diverse process within various cell types, including cell differentiation, inflammation, proliferation, survival and tumorigenesis (Nerlov, 2007). The transcription factor has been demonstrated to activate p20K expression in hypoxic environments and exerts regulatory control over FABP4 in cooperation with PPARγ in response to various stimuli (Erb et al., 2016; Furuhashi et al., 2015). To date, the exact mechanism underlying the induction of FABP4 in reversible growth arrested cells, and whether C/EBPβ is involved, is unclear. We found that the

loss of trans-activating C/EBPβ isoforms (dominant negative mutant Δ184, knockdown 99) abolishes FABP4 induction in oxygen deprived cells (Figure 24, 25). In juxtaposition to this, over-expression of C/EBPβ (NFM vector) yields a substantial increase in the expression of FABP4 during normoxic, hypoxic and maximum cell density states (Figure 26). These results show C/EBPβ expression is directly correlated with FABP4 activation, and, more specifically, C/EBPβ activation is required to stimulate FABP4 in quiescence. Despite these findings and the presence of C/EBP binding sites in the promoter of FABP4, it is possible that C/EBPβ indirectly manages FABP4 activity in hypoxia. FABP4 mRNA levels have been shown to be extensively attenuated when the LIP isoform of C/EBPβ is stably induced in mouse adipocytes. Interestingly, Esteves et al. noted that FABP4 mRNA levels were only affected after prolonged LIP activity, indicating the regulation may be indirect or that the mRNA is extremely stable (Esteves et al., 2013).

Chromatin immunoprecipitation assays coupled with whole genome tiling arrays have previously revealed FABP4 is induced in adipogenesis through binding of both PPARγ and C/EBP factors to its promoter (Lefterova et al., 2008). Given the established relationship between C/EBPβ and FABP4, it is not surprising that the lipocalin's accumulation is dependent on C/EBPβ. It is possible that proteins activated by C/EBPβ in response to oxygen limitations may be part of a feedback loop to regulate C/EBPβ activity. In hypoxia, elevated induction of p20K and FABP4 may positively feedback and promote the continued activity of C/EBPβ to ensure the *GAS* genes remain expressed to alleviate oxidative stress. Once the cell returns to normal oxygen levels, p20K and FABP4 could be downregulated by additional transcriptional regulators (such as ERK2 in the case of p20K), disrupting this positive feedback and signaling increased functionality of LAP* and LAP is no longer required. Thus, if p20K and/or FABP4

expression is prevented in hypoxic conditions using shRNAi, it may negatively impact the accumulation of C/EBPβ. This proposed model corresponds with FABP's ability to suppress activity of its transcriptional activator, PPARγ, by triggering its ubiquitination and subsequent proteasomal degradation (Garin-Shkolnik et al., 2014). Further analysis is therefore required to determine if C/EBPβ directly regulates FABP4 at a transcriptional level analogous to that of the p20K lipocalin. This could be evaluated through FABP4 promoter assays to identify the regions necessary for induction in limited oxygen conditions, and ChIP assays to determine if C/EBPβ binds to the regulatory regions of FABP4 in quiescence.

Reactive oxygen specific formation and release stimulates expression of FABP4 and the p20K lipocalin. Tert-butyl hydroperoxide (TBHP) is an exogenous inducer of oxidative stress commonly used as a model substance for evaluation of mechanisms underlying cellular alterations resulting from oxidative stress (Kučera et al., 2014). It can simulate augmented oxidative stress caused by environmental stressors, such as hypoxia, and was utilized in this study to understand the regulation of GAS lipocalins FABP4 and p20K in response to reactive oxygen species. Similar to the kinetics of the proteins in oxygen deprived states, p20K is more rapidly upregulated by TBHP compared to FABP4 (Erk, M.Sc., 2016; Peragine, M.Sc., 2019; Figure 27). These findings further confirm that p20K is readily mobilized by ROS and lipid peroxidation while FABP4 is not more robustly expressed until longer durations of cellular toxicity. Again, this may explain the redundancies observed in their low oxygen functionally, as they are maximally expressed at different time points. TBHP is extremely deleterious and its metabolism is facilitated through two cellular pathways, both of which release ROS and cause peroxidation of lipid membranes (Kučera et al., 2014). The prompt depletion of p20K and FABP4 may therefore be associated with a transition from quiescence to apoptosis, as the

majority of cells died with 3-4 hours of TBHP exposure. These results coincide with the TUNEL and proliferation assays given that expression of the lipocalins is correlated with cell survival.

The liberation of ROS by NADPH oxidase appears to stimulate terminal differentiation of adipocytes in a C/EBPβ–dependent manner (Al-Sabbagh et al., 2011). Although there are current gaps in the literature related to how signaling networks moderate C/EBPβ activity, it is likely ROS release stimulates the pro-survival role of C/EBPβ which may eventually lead to apoptosis through engagement of CHOP if toxicity persists. This suggests that FABP4 and P20K may be indirectly upregulated by ROS through the activation of C/EBPβ. However, the time delay in FABP4 induction could indicate this protein is only induced by lipid peroxidation, which occurs succeeding ROS release, or that the mechanism for its translation is more elaborate than p20K. It is more probable that FABP4 is inducible by ROS as other studies have demonstrated downregulation of FABP4 using siRNA diminishes cellular resistance to oxidative stress without altering the concentration or enzymatic activity of antioxidants (Kajimoto et al., 2014; Xu et al., 2016). FABP4 has also been established as a scavenger protein against hydrogen peroxide, implying it may function as an antioxidant protein that contributes to cytoprotection against oxidative stress (Kajimoto et al., 2014). Additional experiments to examine LAP and LAP* expression in response to TBHP and the activation of FABP4 are recommended to clarify the pathway.

Chapter 3: Cross-regulation of FABP4 and the p20K lipocalin is evident in conditions of reversible growth arrest.

FABP4 and p20K expression is connected in quiescent cells, likely through co-regulation by C/EBP, in a manner independent of the broad growth arrest specific gene program. FABP4

and p20K exhibit complementary expression patterns in reversible growth arrest conditions. Moreover, the two lipocalins promote cell survival, alleviate oxidative stress caused by ROS and lipid peroxidation and transport lipids to maintain cellular homeostasis. Given these similarities, the relationship between FABP4 and p20K in quiescence was explored in this study. Of particular interest is the observation that shRNA-targeted downregulation of one protein results in loss of the other in oxygen deprivation (Figure 28). Based on this expression pattern, it can be ruled out that either protein is an upstream regulator of the other, as loss of one always results in the inhibition of the other. P20K and FABP4 also display differing kinetics and sensitivities to oxygen levels, further implying neither protein regulates the other (Peragine, M.Sc., 2018). Conversely, forced expression of either protein in actively dividing cells is correlated with irregular accumulation of the other (Figure 18 D). Broad interference with the GAS gene network was also ruled out, as downregulation of either FABP4 or p20K did not impact the activation of CUTA in limiting oxygen environments (Figure 28). CUTA was selected for comparison as it is not involved in lipid homeostasis and has a structure vastly different than the two lipocalins, but it still induced by confluency and hypoxia. It thus appears that FABP4 and p20K share a regulatory pathway to permit activation during reversible growth arrest (Figure 13).

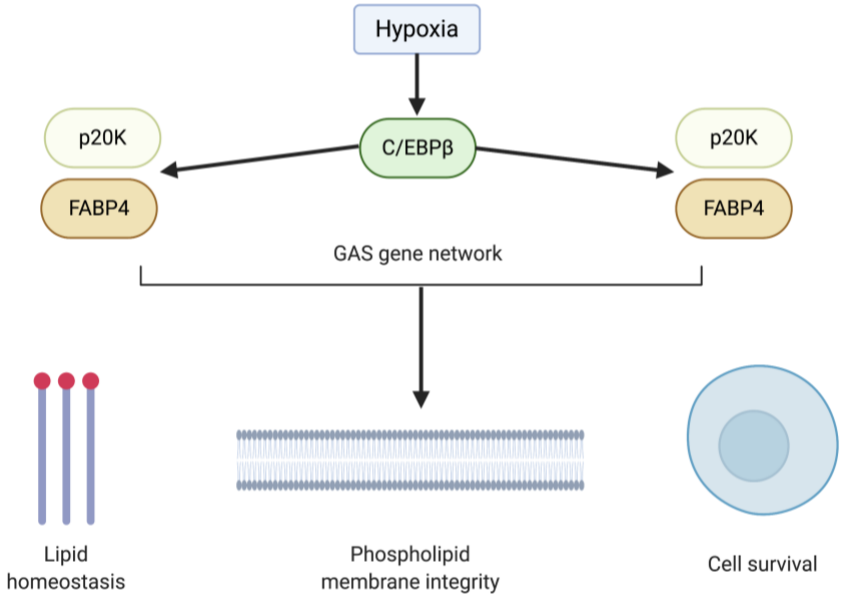


Figure 13. Proposed relationship between FABP4 and p20K in hypoxia. C/EBPβ is an upstream regulator of FABP4 and p20K in quiescence. Despite the redundancies in their functions, both p20K and FABP4 are required to maximally promote cell survival and mitigate the effects of oxidative stress and disruption of lipid homeostasis triggered by oxygen deprivation.

The transcriptional regulation of p20K through its *QRU* is well defined in quiescence as being mediated by ERK2 and C/EBPβ interacting with the promoter (Erb et al., 2016). Our findings demonstrate that FABP4 expression in hypoxia and high cellular densities is dependent on the presence of the activating isoforms of C/EBPβ (Figure 24-26). It is therefore postulated that p20K and FABP4 are co-regulated in limiting oxygen conditions by C/EBPβ. Further complicating the nature of the intricate relationship between FABP4 and p20K is the finding that inhibition of FABP4 reduces activity of the QRU by approximately 3-fold in hypoxia (Figure 33). This is significant as C/EBPβ binding to the p20K's QRU promoter region is necessary and sufficient to initiate transcription of p20K (Kim et al., 1999; Erb et al., 2016). An extensive drop

in QRU activity indicates p20K will not be efficiently expressed and could explain why inhibition of FABP4 prevents p20K accumulation. The mechanism and rationale for why loss of FABP4 lessens QRU promoter activity is unclear, but it could be related to a feedback loop between C/EBPβ and FABP4/p20K. Downregulation of the p20K lipocalin or FABP4 promotes ROS build-up in this cell, which could impair the transcriptional activity of C/EBPβ after a certain threshold. C/EBPβ can be regulated through diverse mechanisms, including enhanced protein concentration, alternative translation initiation (expression of either LAP, LAP* or LIP), nuclear translocation and increased trans-activation domain activity following post-translational modifications (Bradley et al., 2003). In avian cells, two regions within the C/EBPβ protein have been identified to repress its ability to initiate transcription of target genes, and this inhibition can be lifted through phosphorylation by MAP kinase, PKC, PKA, p90 RSK, cAMP-dependent kinase among others (Kowenz-Leutz et al., 1994). Little work has been done to characterize how C/EBPβ is activated in response to oxidative stress, but overwhelming amassment of ROS and lipid radicals could impair its activity, leading to the abolishment of both FABP4 and p20K in quiescence. Future studies should thus focus on evaluating the impact of ROS on the transcriptional, post-transcriptional, translational and post-translational regulation of C/EBPβ, as well as its ability to interact with and activate transcription of target gene promoters, to determine if gene transcription co-regulated by C/EBPβ is impacted by lipocalin mis-expression in hypoxia.

Given that shRNA-dictated loss of FABP4 results in p20K inhibition (and vice versa), the phenotype of hypoxic cells was quantified in conditions of forced expression of p20K and FABP4 downregulation (or vice versa) to determine if the observed disruption to cell survival and lipid homeostasis was primarily due to loss of p20K, FABP4 or the combination of both

(Figure 28). As demonstrated by analyses of cellular proliferation, TUNEL and lipid peroxidation analyses, up-regulation of p20K or FABP4 moderately restored the capacity for survival in limiting oxygen settings while reducing oxidation of lipids in CEF with FABP4 or p20K knockdown (Figures 29-32). However, these populations still exhibited a diminished ability to persist and prevent lipid peroxidation compared to control samples in hypoxia, suggesting that although the roles of FABP4 and p20K appear to be redundant in quiescence, they are both required for optimal cellular health. Beyond differences in their kinetics and sensitivity to oxygen concentrations, a more in-depth analysis of their roles in reversible growth arrest, and the mechanisms they used to facilitate their functionally, should be performed to identify why over-expression of one does not fully compensate for loss of the other (Peragine, M.Sc., 2018). Preliminary studies in the Bédard lab have proposed a role for p20K in aiding with intracellular iron regulation, as assessment of excessive iron can contribute to oxidative stress and may lead to ferroptosis (iron-dependent cell death caused by lethal levels of lipid peroxidation) (Dixon et al., 2012). Recent discoveries have noted an expansion in the crosstalk between oxygen metabolism and iron homeostasis, with hypoxia signaling associated with iron maintenance in erythropoiesis, pulmonary arterial hypertension and other pathologies (Renassia and Peyssonnaux, 2019; Robinson et al., 2014). P20K may be entwined with cellular responses to hypoxia, cellular proliferation and mitochondrial functions in part through control of iron homeostasis, in a manner analogous to HIF-1 and HIF-2 (Robinson et al., 2014). While the major roles of FABP4 and p20K appear to overlap when responding to attenuated oxygen availability, subtle variations in their functionality and regulation could explain the necessity of both proteins to augment cell survival and recovery from oxidative stress (Figure 14).

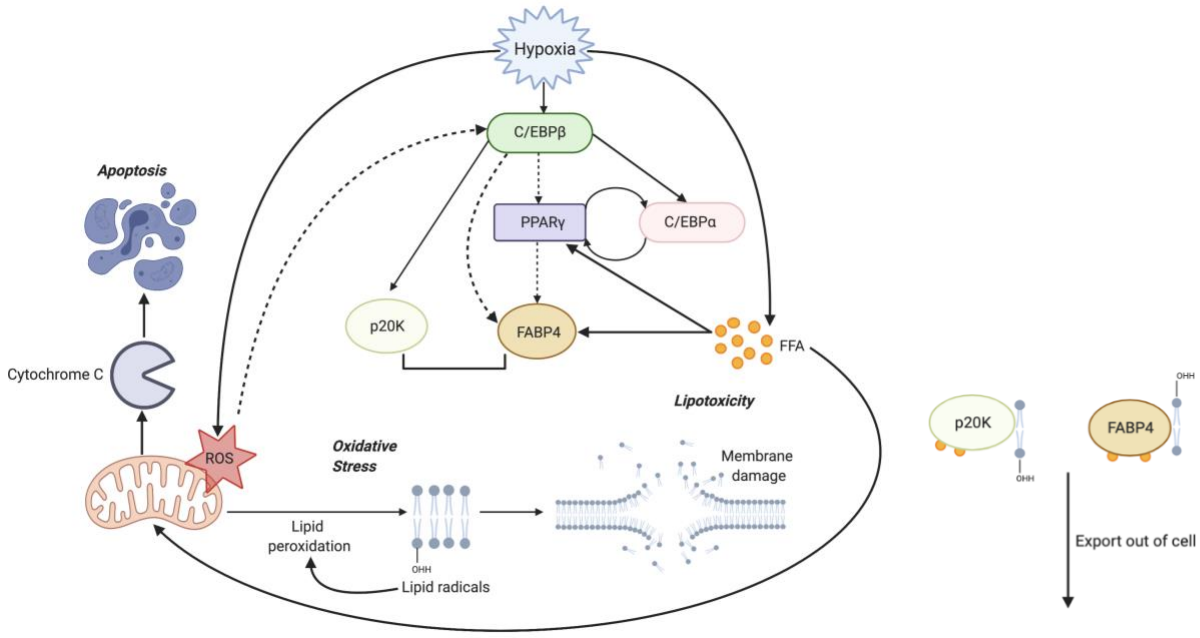


Figure 14. Proposed role of p20K and FABP4 in quiescence. C/EBPβ activates p20K and FABP4 in response to oxygen deprivation and subsequent oxidative stress. Induction of FABP4 may occur in a PPAR-γ dependent or independent manner. The lipocalins can bind and shuttle free fatty acids and other lipid species to prevent lipotoxicity and restore lipid homeostasis. FABP4 and p20K promote cell survival by mitigating the effects of ROS and lipid peroxidation.

FABP4 and p20K in novel "Membrane Stress Response".

This investigation aimed to characterize some of the proteins and processes that underlie the novel 'Membrane Stress Response'. In this proposed model of cellular survival, oxygen limitations trigger membrane stress, which the cell attempts to manage through a lipid oxidation response analogous to the unfolded protein response in the endoplasmic reticulum prompted by nutrient- and growth factor-starvation (Bédard, unpublished results) (Figure 15). In both circumstances of environmental resource restrictions, the imposed organelle stress results in a reversible growth arrest in primary fibroblasts (Bédard, unpublished results). The cell therefore

requires activation of pro-survival pathways to alleviate the strain and return to an actively proliferating state. While the unfolded protein response has been well-described, such a mechanism remains to be elucidated for the accumulation of lipid membrane damage and re-entry into the cell cycle (Chakrabarti et al., 2011).

Interestingly, both contact inhibition and hypoxia appear to reversibly inhibit proliferation through inadequate oxygen availability, as demonstrated by the expression of several hypoxia-response genes in response to these conditions (Erb et al., 2016). Oxygen limitations can stimulate the generation and accumulation of ROS, stimulating lipid peroxidation and membrane damage. Disruption of cellular membrane integrity may regulate GAS genes in an attempt to return the cell to a state of lipid homeostasis. The findings of this study contribute to our understanding of the fundamental processes activated in response to quiescence and the GAS genes that mediate the maintenance of membrane and lipid homeostasis.

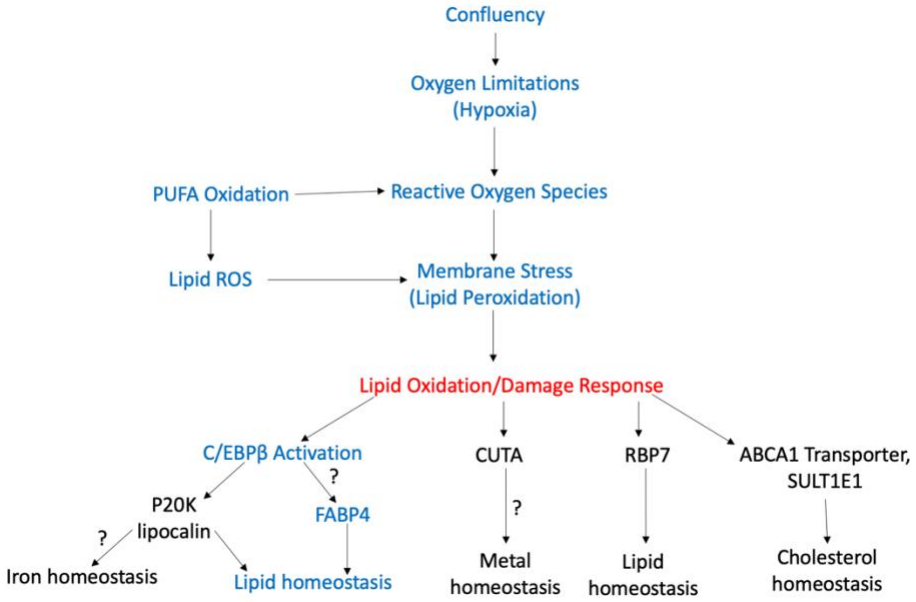


Figure 15. Proposed Model of GAS gene network in Lipid Membrane Response.

Experimental Figures

Chapter 1

A.

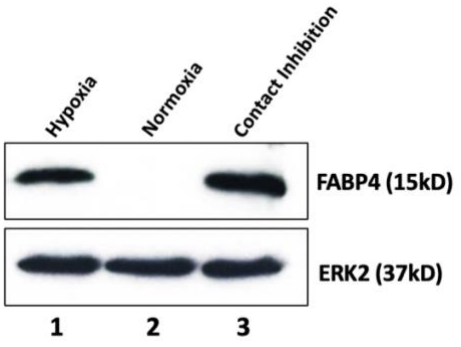
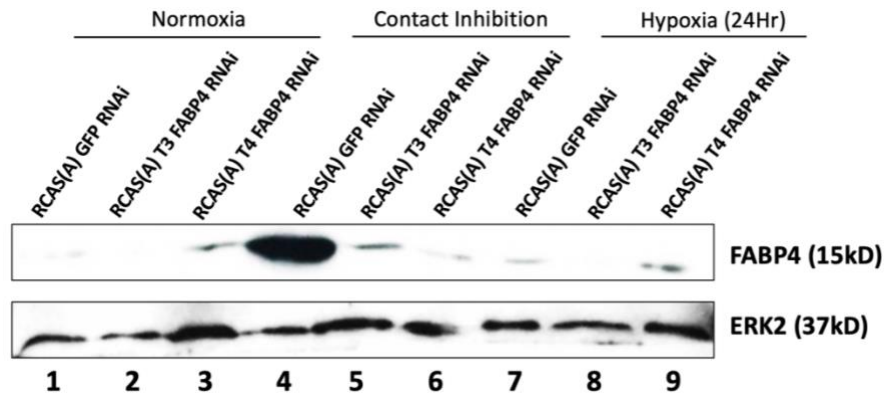
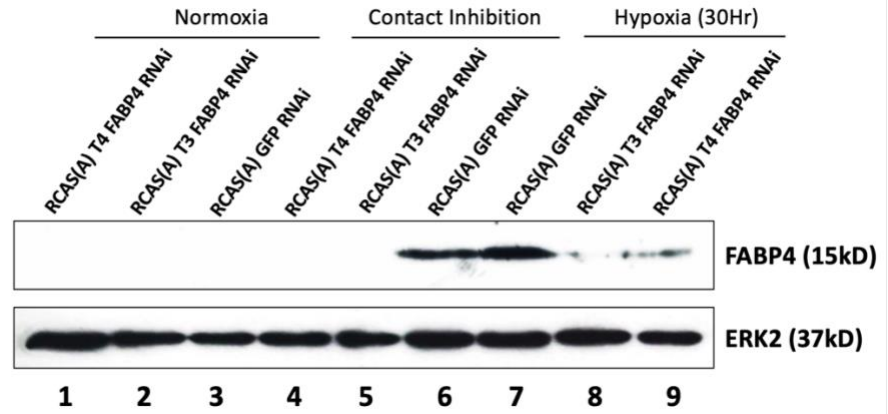


Figure 16. Western blot analysis of FABP4 expression in CEF incubated in normoxic (21% O2), hypoxic (1.8% O2) conditions or to maximal confluency. ERK2 expression was used as a loading control.



A.



B.

Figure 17. Western blot analysis of FABP4 in CEF transfected with group A retroviral vector expressing a control shRNA for GFP (RCASBP(A) – GFP RNAi) or an shRNA for FABP4 downregulation (RCASBP(A) – FABP4 T3 RNAi or RCASBP(A) – FABP4 T4 RNAi) incubated in normoxic (21% O_2), hypoxic (1.8% O_2) or conditions or to maximal confluency. ERK2 expression was used as a loading control. A) CEF exposed to hypoxic conditions for 24 hours. B) CEF exposed to hypoxic conditions for 30 hours.

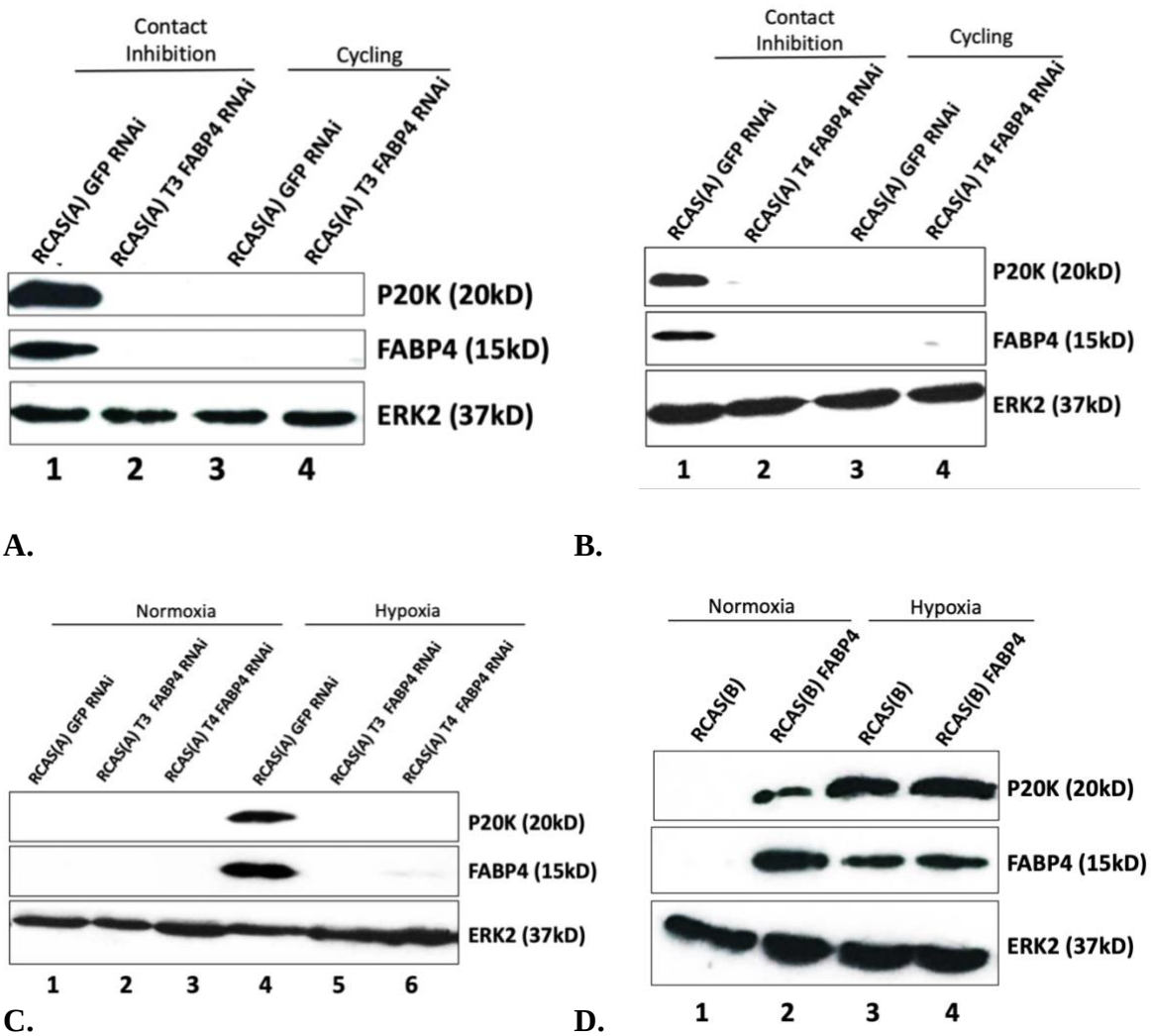
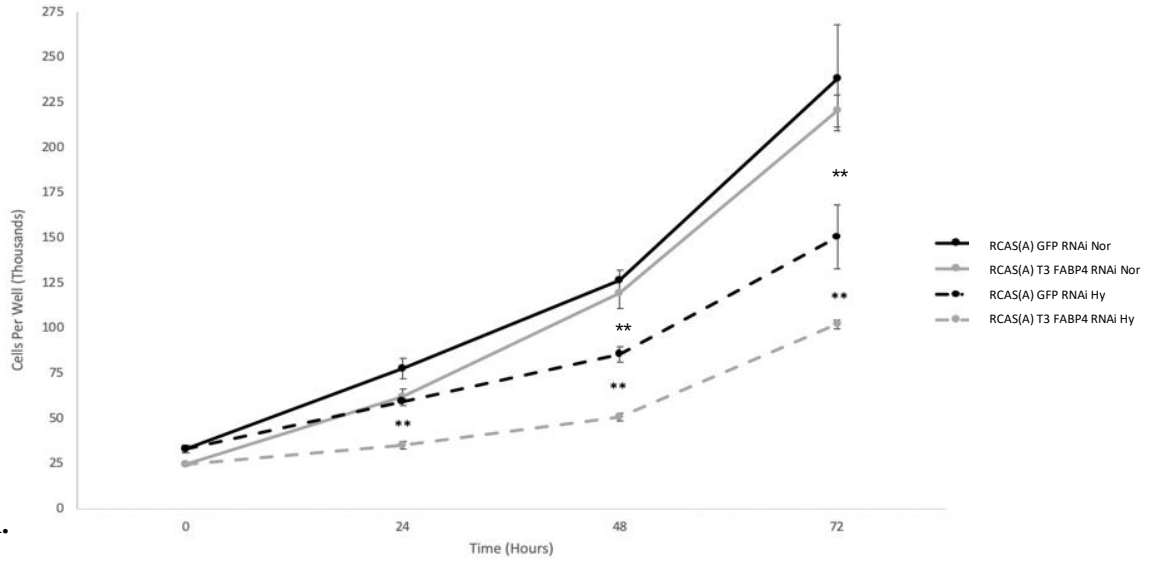
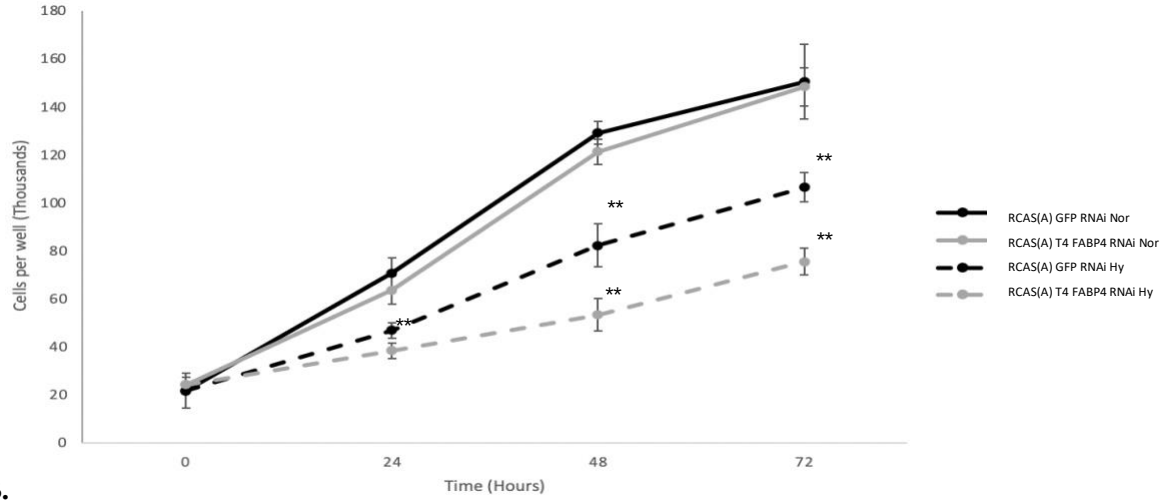


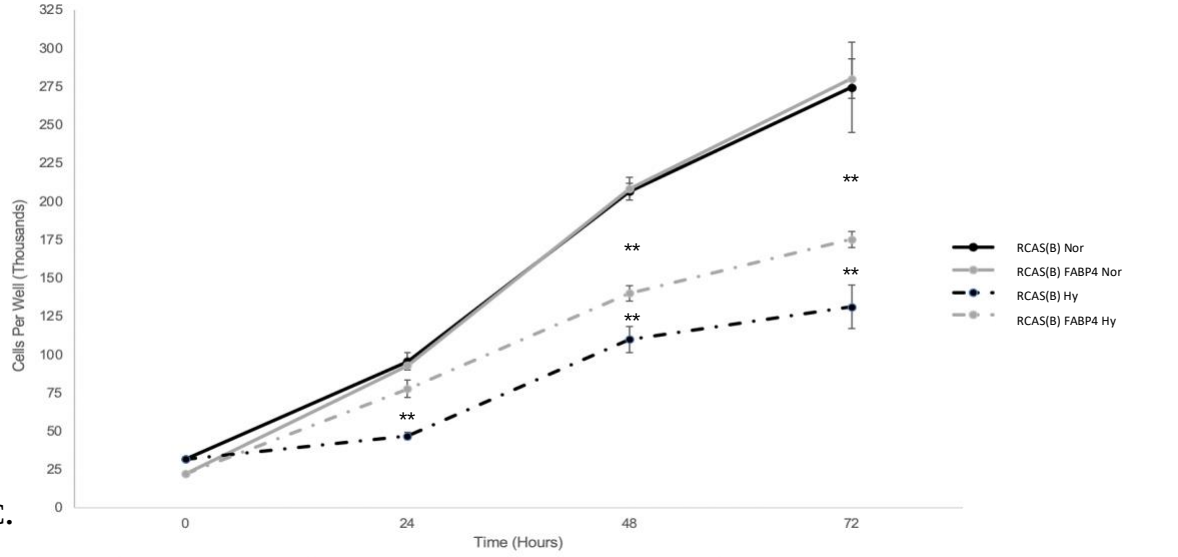
Figure 18. Western blot analysis of FABP4 and p20K in CEF transfected with group A retroviral vector expressing a control shRNA for GFP (RCASBP(A) – GFP RNAi) or an shRNA for FABP4 downregulation [**A)** RCASBP(A) – FABP4 T3 RNAi, **B)** RCASBP(A) – FABP4 T4 RNAi] in cells grown in normoxia (21% O_2), to maximal confluency or in **C)** hypoxia. ERK2 expression was used as a loading control. **D)** Western blot analysis of FABP4 in CEF transfected with group B retroviral vector (RCASBP(B)) or group B retroviral vector with cDNA for FABP4 over-expression (RCASBP(B) – FABP4 cDNA).


Cells Per Well (Thousands)
Time (Hours)
RCAS(A) GFP RNAi Nor
RCAS(A) T3 FABP4 RNAi Nor
RCAS(A) GFP RNAi Hy
RCAS(A) T3 FABP4 RNAi Hy

A.

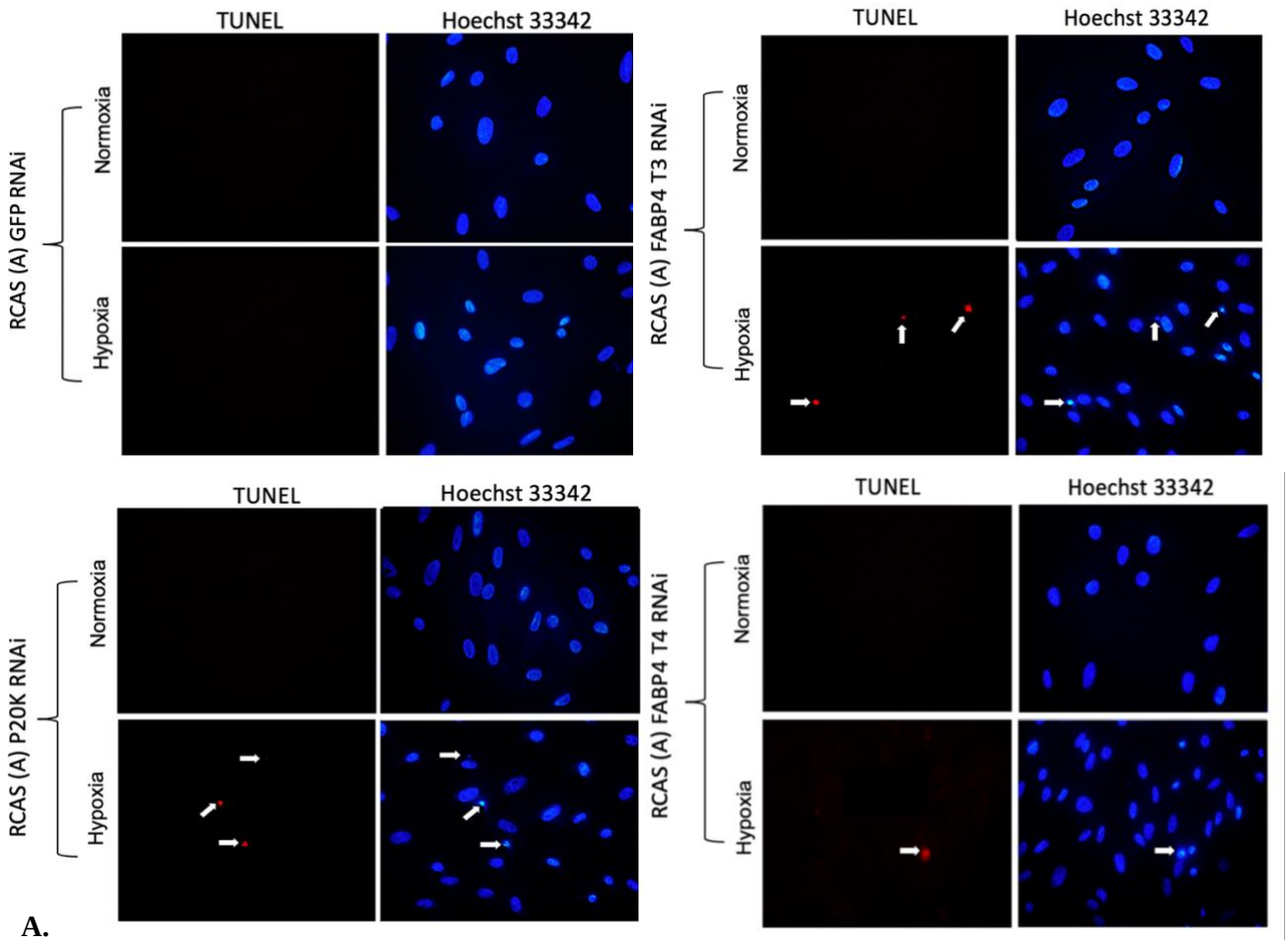

Cells per well (Thousands)
Time (Hours)
RCAS(A) GFP RNAi Nor
RCAS(A) T4 FABP4 RNAi Nor
RCAS(A) GFP RNAi Hy
RCAS(A) T4 FABP4 RNAi Hy

B.

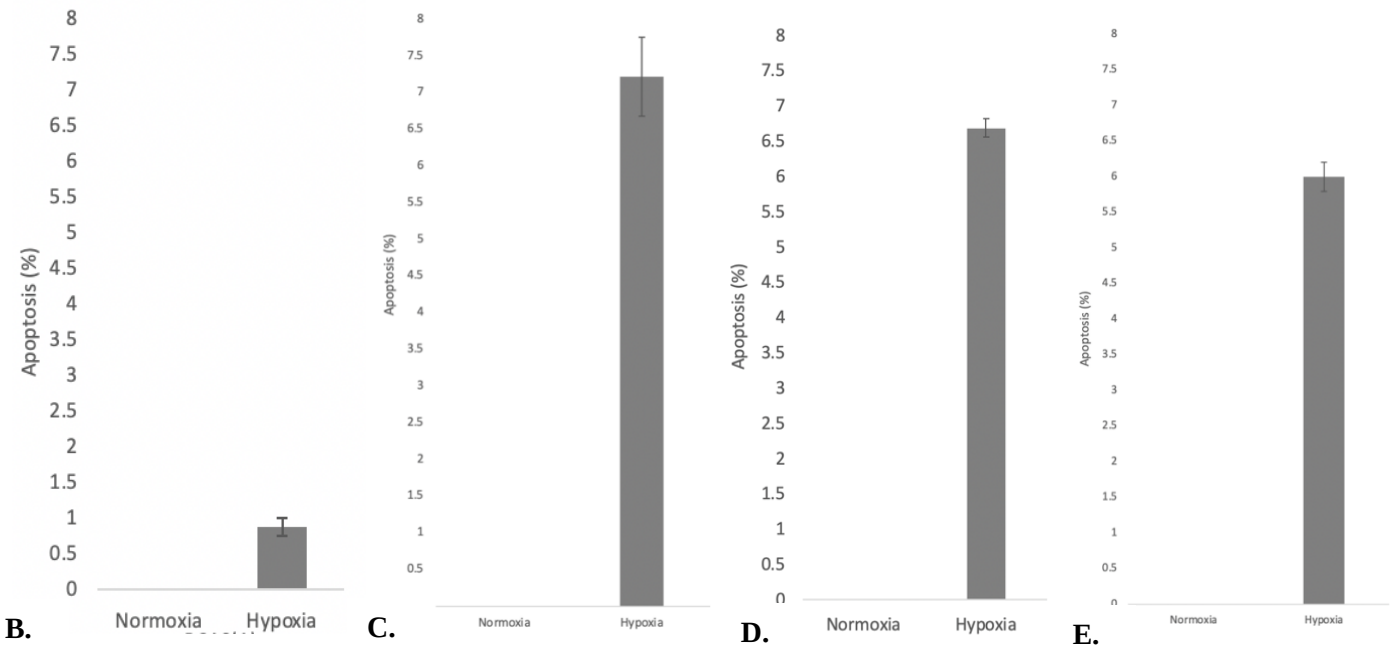

Cells Per Well (Thousands)
Time (Hours)
RCAS(B) Nor
RCAS(B) FABP4 Nor
RCAS(B) Hy
RCAS(B) FABP4 Hy

C.

Figure 19. A) and B) Proliferation of CEF transfected with a group A retroviral vector expressing a control shRNA for GFP (RCASBP(A) – GFP RNAi) or a shRNA for FABP4 [**A)** RCASBP(A) – T3 FABP4 RNAi, **B)** RCASBP (A) T4 FABP4 RNAi] incubated in normoxic (21% O2) or hypoxic (1.8% O2) conditions. Time 0 represents the cell count taken prior to hypoxic incubation. Cell counts were taken at 24-hour intervals after incubation in normoxia or hypoxia. Error bars represent the mean standard deviation of cell counts measured in quadruplicates. Asterisks represent significance differences between samples, where * indicates $P<0.05$ and ** indicates $P<0.01$. C) Proliferation of CEF transfected with a group B retroviral vector control (RCASBP(B)) or an over-expression cDNA for FABP4 (RCASBP(B) –FABP4) incubated in normoxic (21% O2) or hypoxic (1.8% O2) conditions. Time 0 represents the cell count taken prior to hypoxic incubation. Cell counts were taken at 24-hour intervals after incubation in normoxia or hypoxia. Error bars represent the mean standard deviation of cell counts measured in quadruplicates. T-tests were performed, and asterisks represent significance between samples within the same conditions, where * indicates $P\leq0.05$ and ** P indicates ≤0.01.


TUNEL
Hoechst 33342
RCAS (A) GFP RNAi
Normoxia
Hypoxia
RCAS (A) FABP4 T3 RNAi
RCAS (A) P20K RNAi
RCAS (A) FABP4 T4 RNAi

A.


Apoptosis (%)
Normoxia
Hypoxia

B. C. D. E.

Figure 20. A) Fluorescent staining with TUNEL assay. CEF transfected with a group A retroviral vector expressing a control shRNA for GFP (RCASBP(A) – GFP RNAi), a shRNA for p20K (RCASBP(A) – p20K RNAi) or a shRNA for FABP4 (RCASBP(A) – T3 FABP4 RNAi or RCASBP (A) T4 FABP4 RNAi) were incubated in normoxic (21% O2) or hypoxic (1.8% O2) conditions for 30 hours. The left panel represents cells stained with TdT and TMR red-fluorescent labelled dUTP. The right panel represents cells stained with Hoechst 33342. Arrows on the left panel point to TUNEL-positive (apoptotic cells), and arrows on the right panel point to cells undergoing chromatin condensation and nuclear fragmentation. B) The levels of apoptosis in RCASBP (A) GFP – RNAi, C) RCASBP (A) p20K – RNAi, D) RCASBP (A) T3 FABP4– RNAi and E) RCASBP (A) T4 FABP4 – RNAi were quantified. Error bars represent the mean standard deviation of the levels of apoptosis per 400 cells measured in quadruplicate.

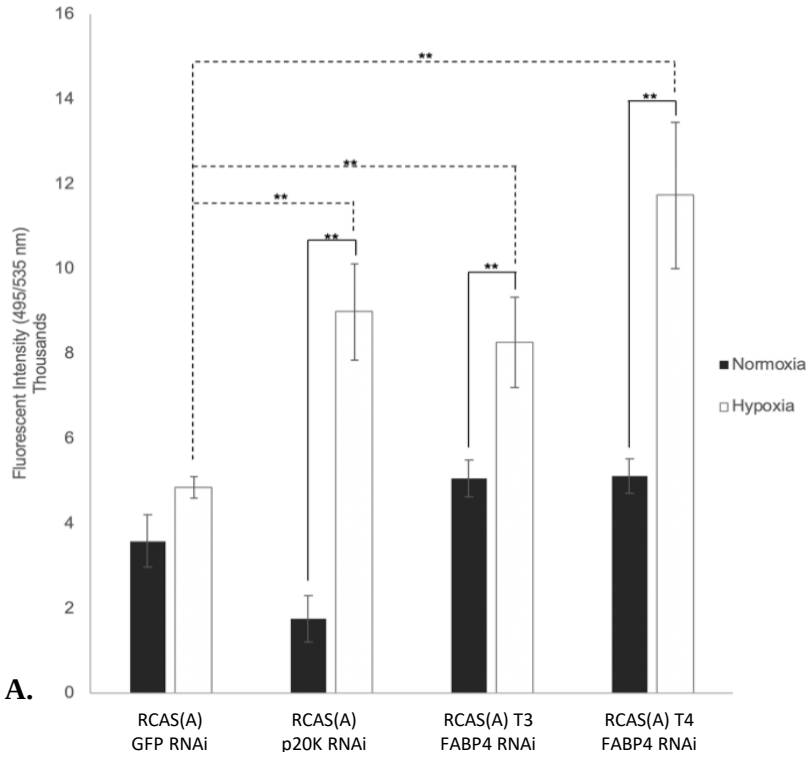


Figure 21. Quantitation of cellular reactive oxygen species (ROS) with DCFDA reactive oxygen species assay. CEF transfected with a group A retroviral vector expressing a control shRNA for GFP (RCASBP (A) - GFP RNAi), a shRNA for p20K (RCASBP (A) – p20K RNAi) or a shRNA for FABP4 (RCASBP(A) – T3 FABP4 RNAi or RCASBP (A) T4 FABP4 RNAi) were incubated in normoxic (21% O2) and hypoxic (1.8% O2) conditions for 30 hours. Error bars represent the mean standard deviation of the fluorescence intensity measured in quadruplicate. ANOVA tests were conducted to compare MDA levels within and between the cell populations. Asterisks represent significance between samples within the same conditions, where * indicates $P \leq 0.05$ and ** P indicates ≤ 0.01.

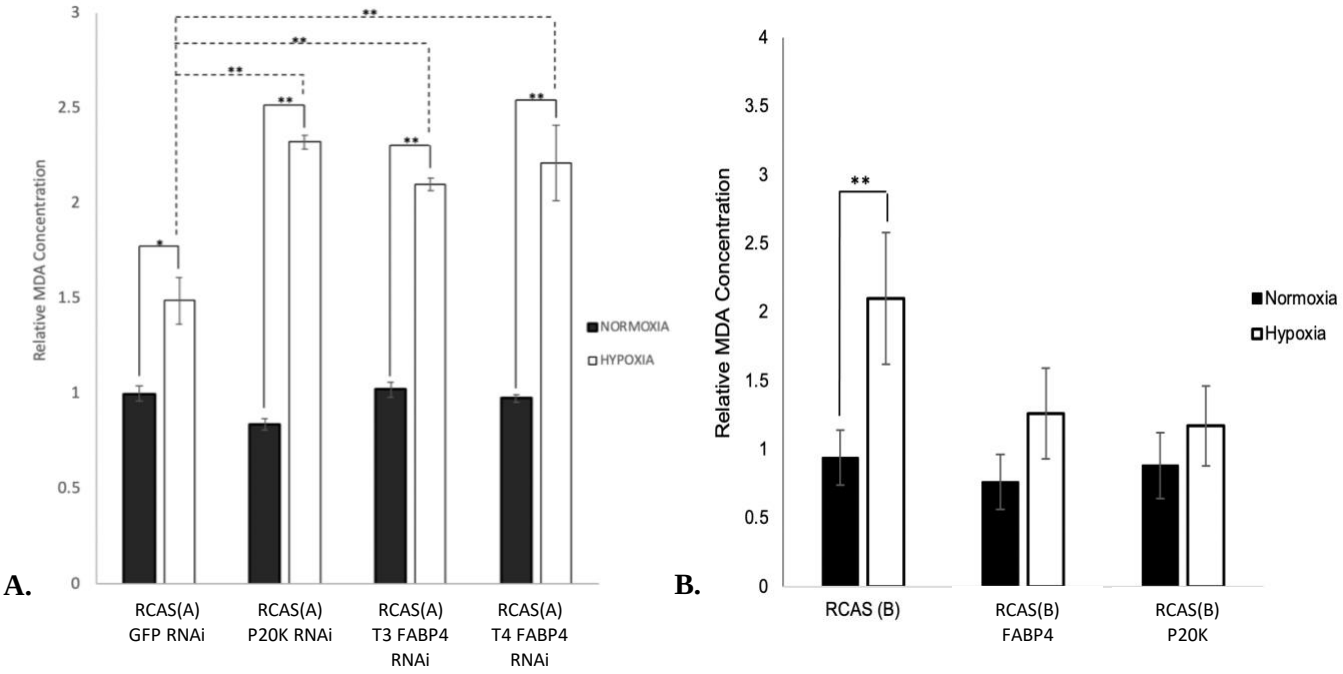
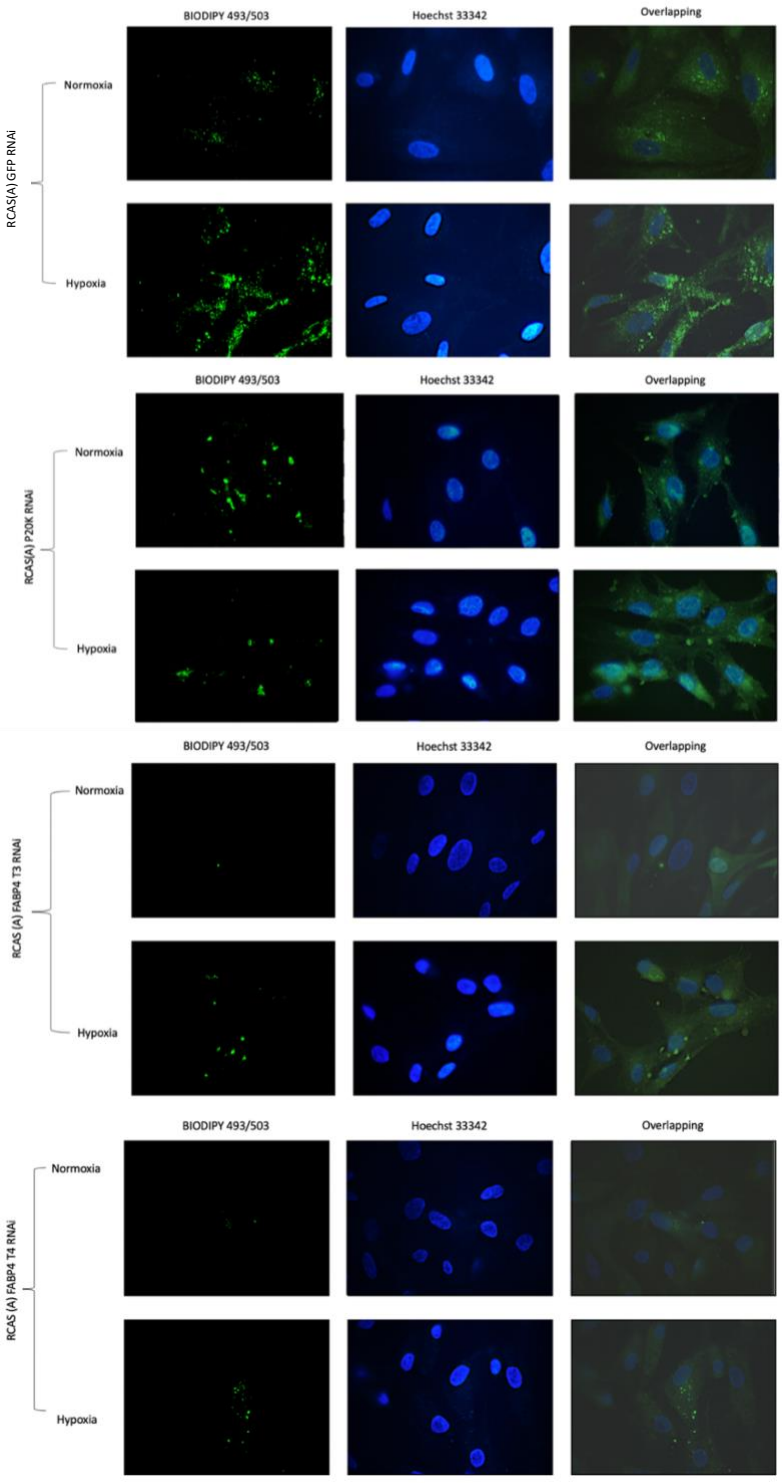


Figure 22. Quantitation of malondialdehyde (MDA), a by-product of lipid peroxidation, with MDA assay. **A.** CEF transfected with a group A retroviral vector expressing a control shRNA for GFP (RCASBP (A) - GFP RNAi), a shRNA for p20K (RCASBP (A) – p20K RNAi) or a shRNA for FABP4 (RCASBP(A) – T3 FABP4 RNAi or RCASBP (A) T4 FABP4 RNAi) were incubated in normoxic (21% O2) and hypoxic (1.8% O2) conditions for 30 hours. **B.** CEF transfected with a group B retroviral vector control (RCASBP (B)), cDNA for FABP4 (RCASBP (B) - FABP4), or cDNA for p20K (RCASBP (B) – p20K) were incubated in normoxic and hypoxia conditions for 30 hours. Error bars represent the mean standard deviation of the fluorescence intensity measured in quadruplicate. ANOVA tests were conducted to compare MDA levels within and between the cell populations. Asterisks represent significance between samples within the same conditions, where * indicates $P \leq 0.05$ and ** P indicates ≤ 0.01.



A.

Figure 23. A) BIODIPY 493/503 fluorescent staining with Lipid Droplet Detection assay. CEF transfected with a group A retroviral vector expressing a control shRNA for GFP (RCASBP(A) –

GFP RNAi), a shRNA for p20K (RCASBP(A) – p20K RNAi) or a shRNA for FABP4 (RCASBP(A) – T3 FABP4 RNAi or RCASBP (A) T4 FABP4 RNAi) were incubated in normoxic (21% O2) or hypoxic (1.8% O2) conditions for 30 hours. The left panel represents cells stained with Biodipy 493/503 (lipid droplets). The middle panel represents cells stained with Hoechst 33342. The right panel represents the overlay of Biodipy 493/503 and Hoechst 33342 staining to identify the location of lipid droplets in relation to cells.

A.

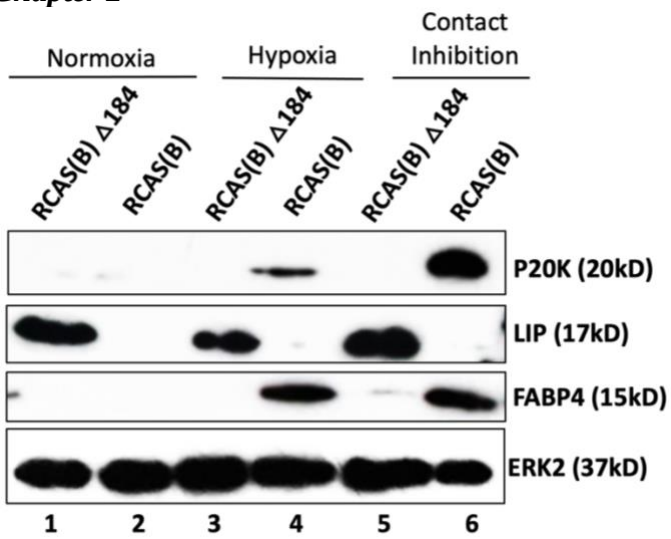


Figure 24. A) Western blot analysis of p20K, FABP4 and LIP (inhibitory isoform of C/EBP-β) in CEF transfected with a group B retroviral vector expressing control (RCASBP(B) or dominant negative C/EBP-β mutant (RCASBP(B) - Δ184 LIP over-expression cDNA) incubated in normoxia (21% O2), hypoxia (1.8% O2) or high-density conditions. The blot was probed for ERK2 (loading control).

A.

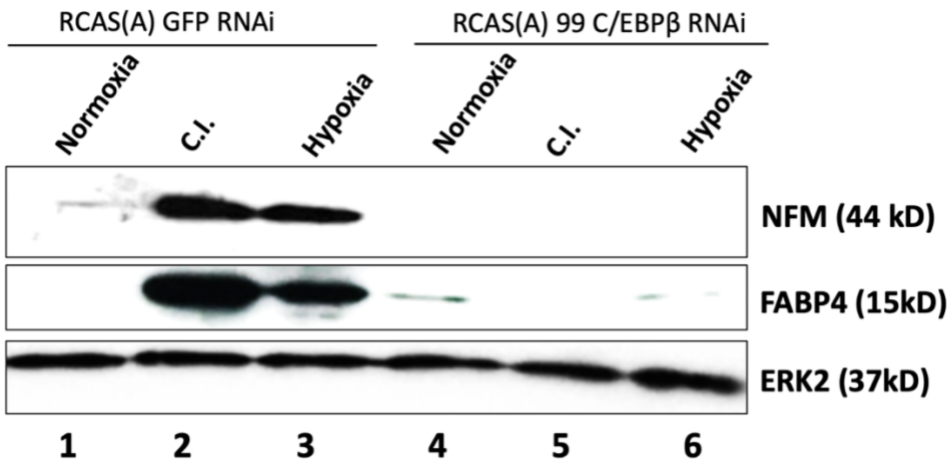


Figure 25. A) Western blot analysis of FABP4 in CEF transfected with a group A retroviral vector expressing a control shRNA for GFP (RCASBP(A) – GFP RNAi) or shRNA for C/EBP-β (RCASBP(A) - 99 C/EBP-β RNAi) incubated in normoxia (21% O2), hypoxia (1.8% O2) or in high density conditions. The blot was probed for ERK2 (loading control).

A.

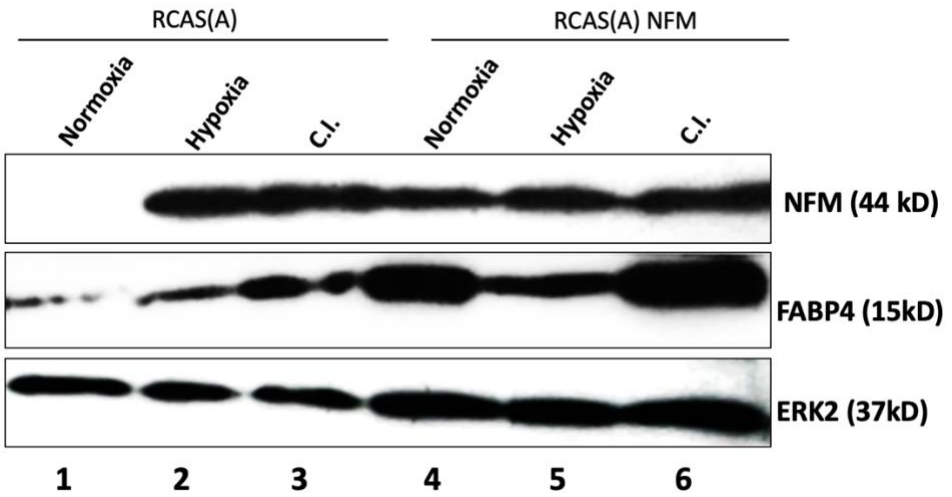


Figure 26. A) Western blot analysis of FABP4 in CEF transfected with a group A retroviral vector expressing a control (RCASBP(A)) or cDNA for NFM over-expression (RCASBP(A) - NFM cDNA) incubated in normoxia (21% O2), hypoxia (1.8% O2) or in high density conditions. The blot was probed for ERK2 (loading control).

A.

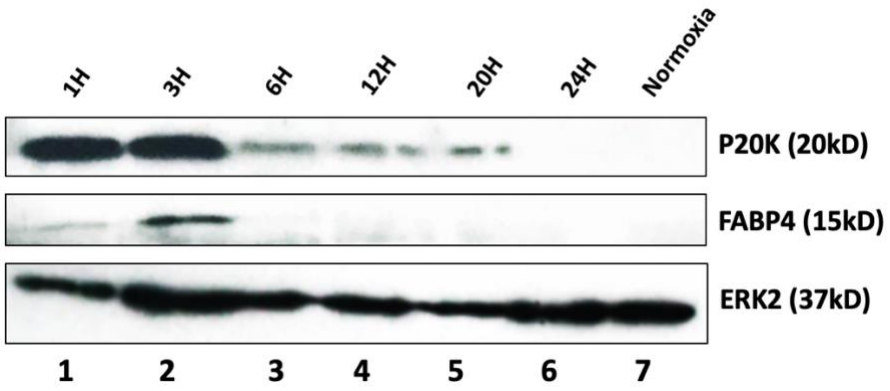
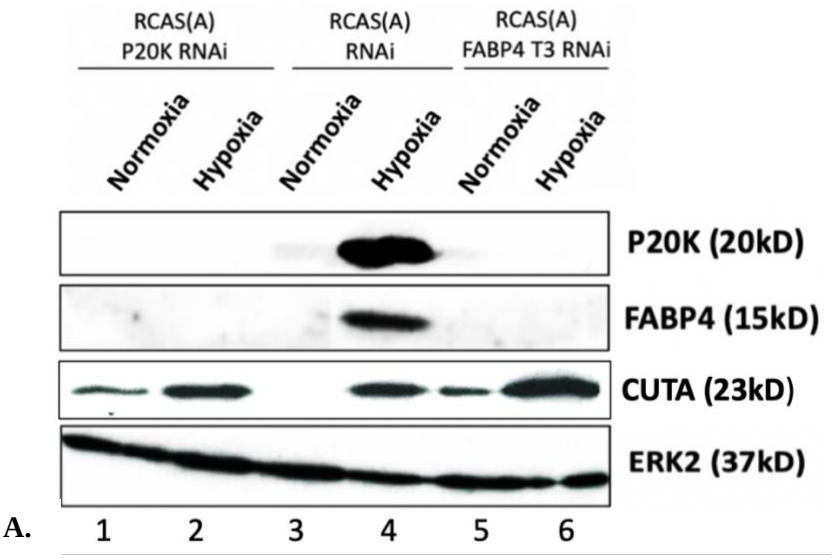
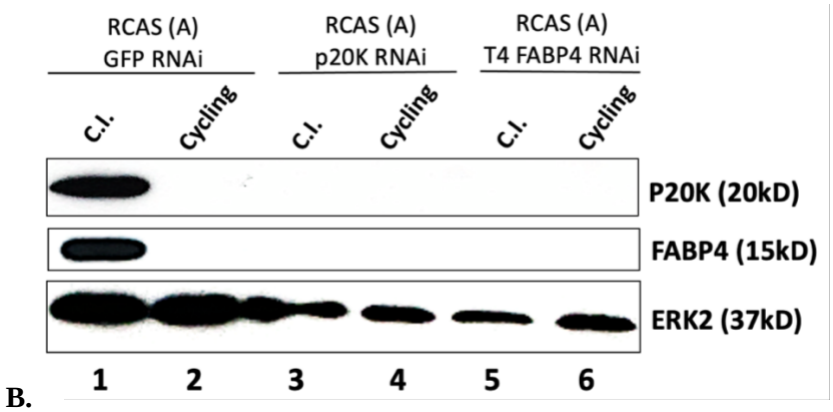


Figure 27. A) Western blot analysis of p20K and FABP4 in CEF incubated with 500μM TBHP for 1-24 hours or in normoxia. The blot was probed for ERK2 (loading control).



A.



B.

Figure 28. A) Western blot analysis of p20K, FABP4 and CUTA in CEF incubated in normoxia (21% O2) or hypoxia (1.8% O2). The blot was probed for ERK2 (loading control). B) Western blot analysis of p20K and FABP4 in CEF incubated in normoxic or maximal cell density conditions. The blot was probed for ERK2 (loading control).

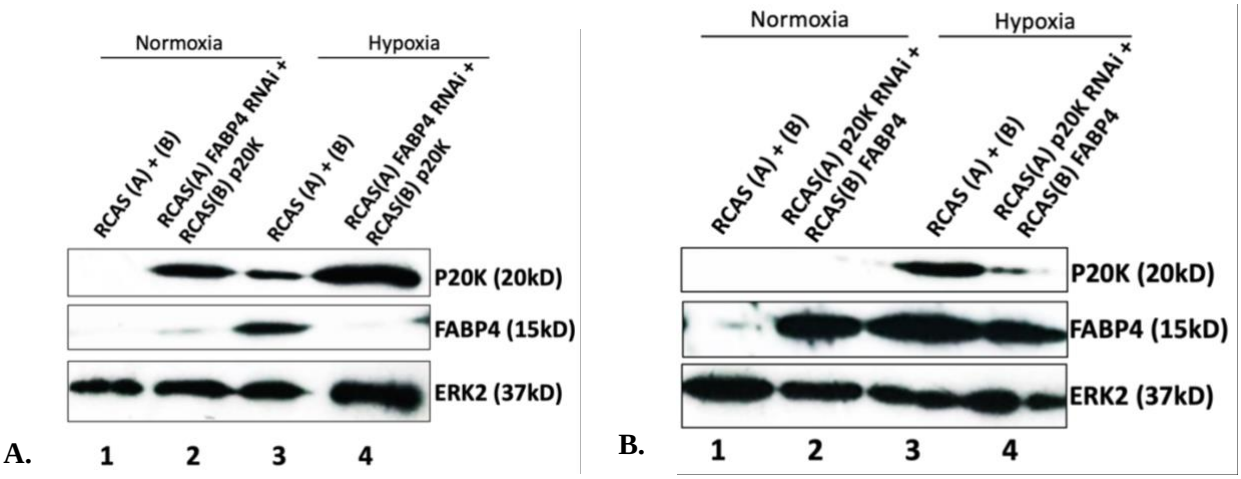
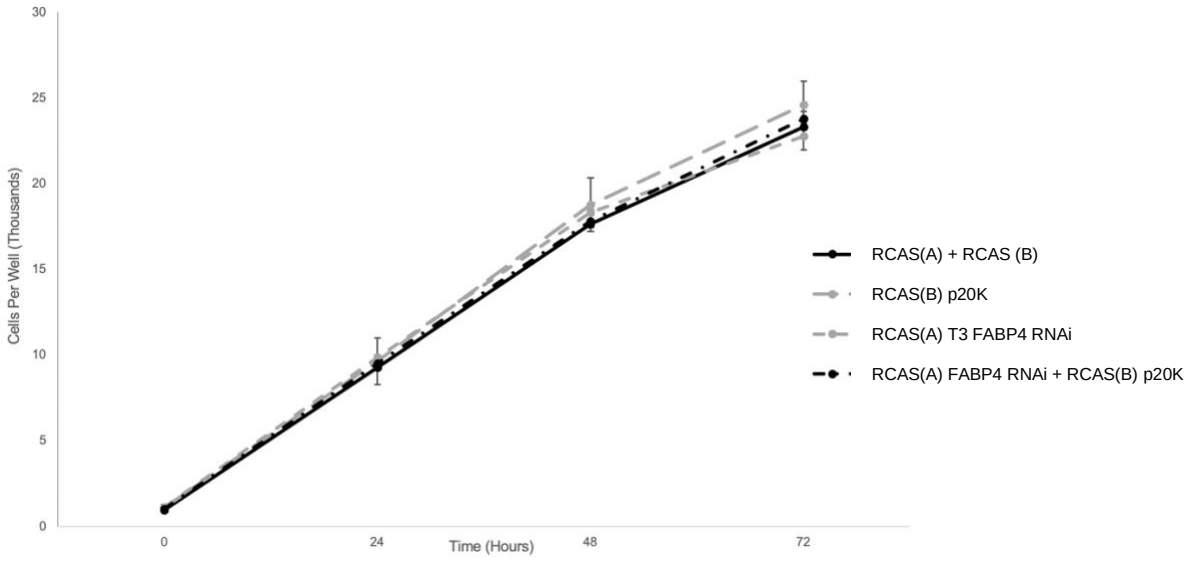
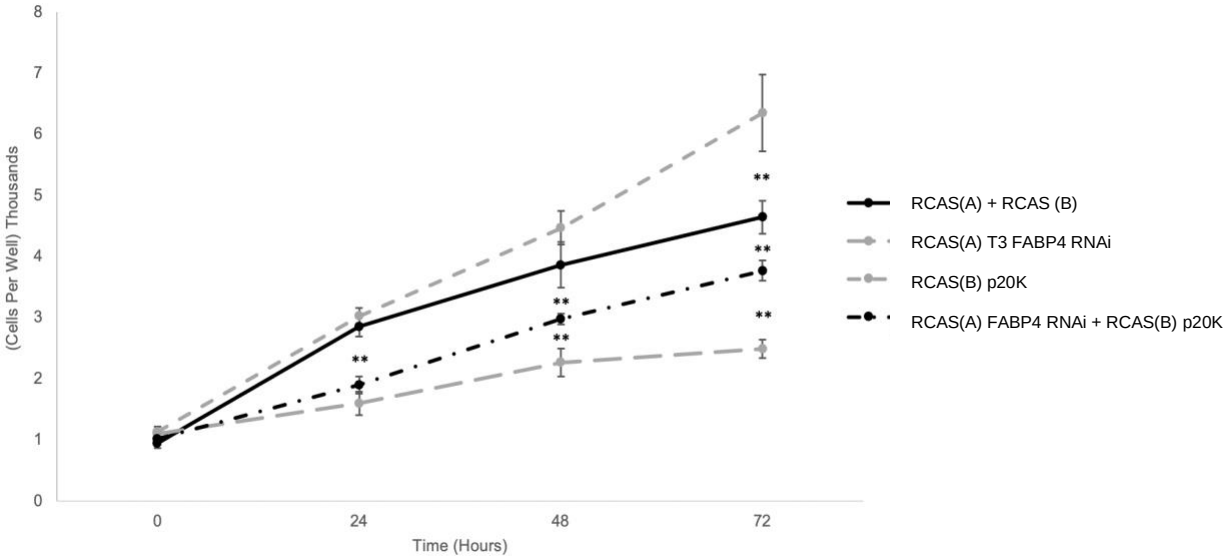


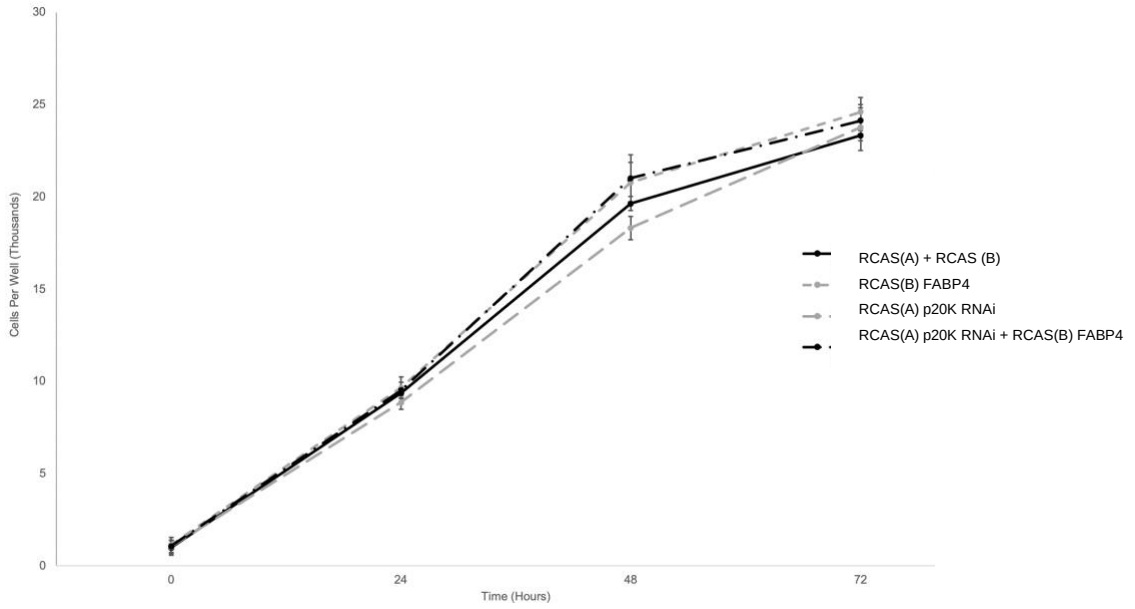
Figure 29. Western blot analysis of FABP4 and p20K in CEF transfected with a combination of group A retroviral vector expressing a control shRNA for GFP and group B retroviral vector expressing a control shRNA for GFP (RCASBP(A) – GFP RNAi + RCASBP(B) – GFP RNAi), **A)** a combination of shRNA for FABP4 and P20K cDNA (RCASBP(A) – FABP4 T3 RNAi + RCASBP(B) – p20K OE cDNA), or **B)** a combination of shRNA for p20K and FABP4 cDNA (RCASBP(A) – p20K RNAi + RCASBP(B) – FABP4 OE cDNA incubated in normoxic (21% O2) or hypoxic (1.8% O2) conditions. The blot was probed for ERK2 (loading control).



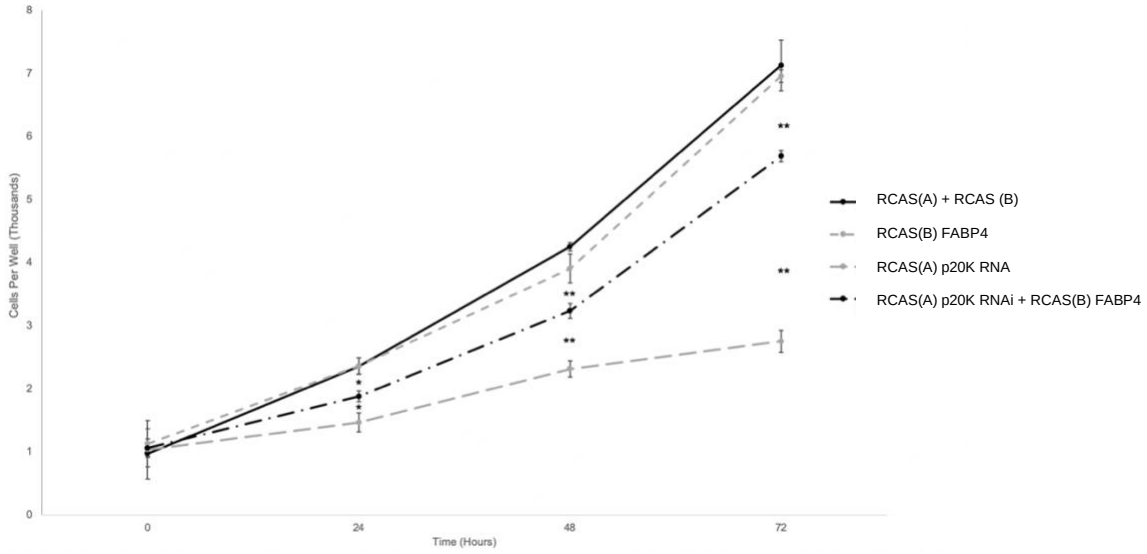
A. FABP4 shRNA + p20K cDNA Normoxia



B. FABP4 shRNA + p20K cDNA Hypoxia



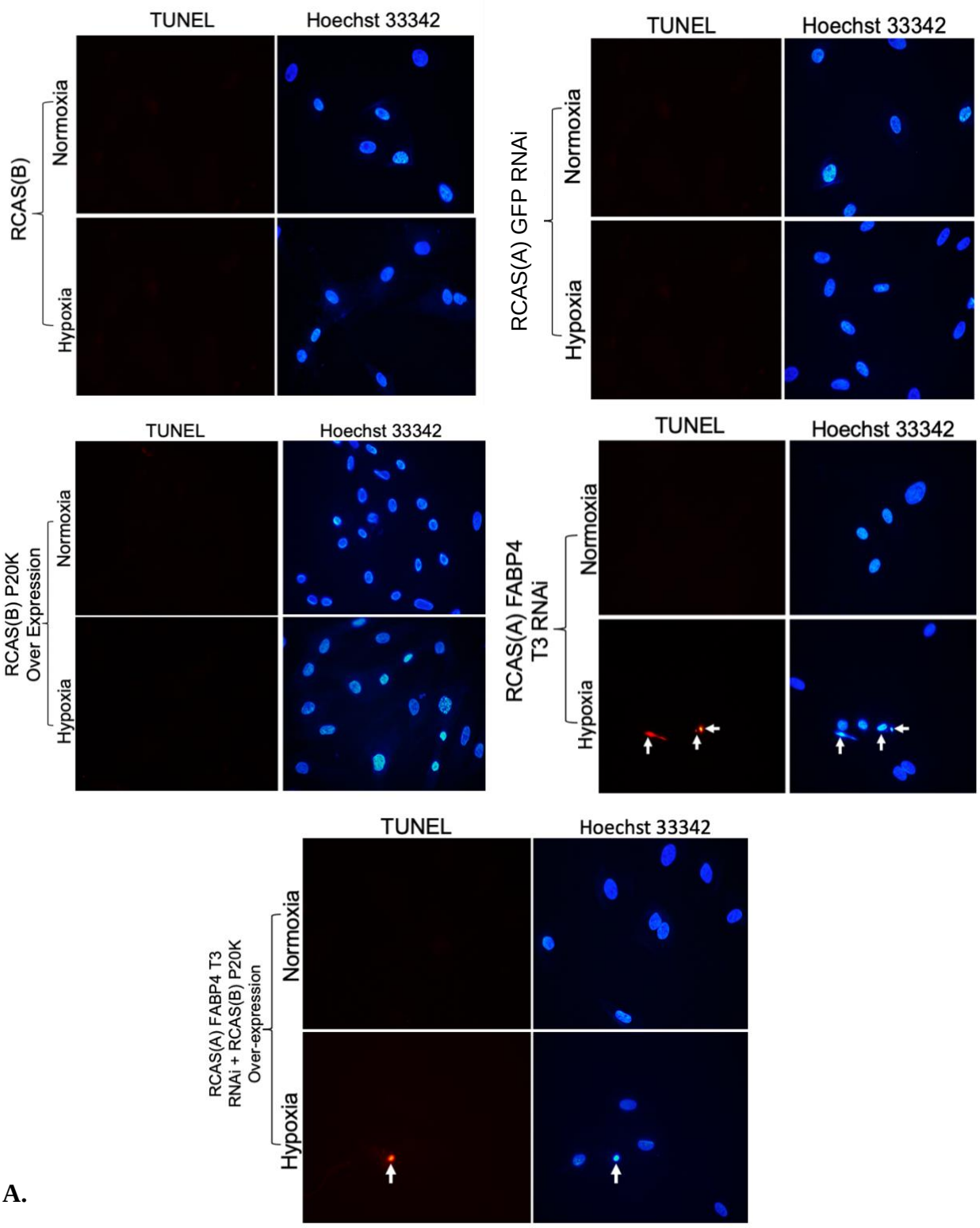
C. FABP4 cDNA + p20K shRNA Normoxia



D. FABP4 cDNA + p20K shRNA Hypoxia

Figure 30. Proliferation of CEF transfected with a group A retroviral vector and a group B retroviral vector expressing a control shRNA for GFP (RCASBP(A) – GFP RNAi + RCASBP(B) – GFP RNAi) or a combined shRNA for FABP4 and cDNA for p20K (RCASBP(A) – T3 FABP4 RNAi, RCASBP (B) p20K OE cDNA) incubated in A) normoxic

(21% O2) or B) hypoxic (1.8% O2) conditions, or a combined shRNA for p20K and cDNA for FABP4 (RCASBP(A) – p20K RNAi + RCASBP (B) FABP4 OE cDNA) incubated in C) normoxic (21% O2) or D) hypoxic (1.8% O2) conditions. Time 0 represents the cell count taken prior to hypoxic incubation. Cell counts were taken at 24-hour intervals after incubation in normoxia or hypoxia. Error bars represent the mean standard deviation of cell counts measured in quadruplicates. T-tests were performed, and asterisks represent significance between samples within the same conditions, where * indicates $P \leq 0.05$ and ** P indicates ≤ 0.01.


TUNEL
Hoechst 33342
RCAS(B)
Normoxia
Hypoxia
RCAS(A) GFP RNAi
RCAS(B) P20K
Over Expression
RCAS(A) FABP4
T3 RNAi
RCAS(A) FABP4 T3
RNAi + RCAS(B) P20K
Over-expression

A.

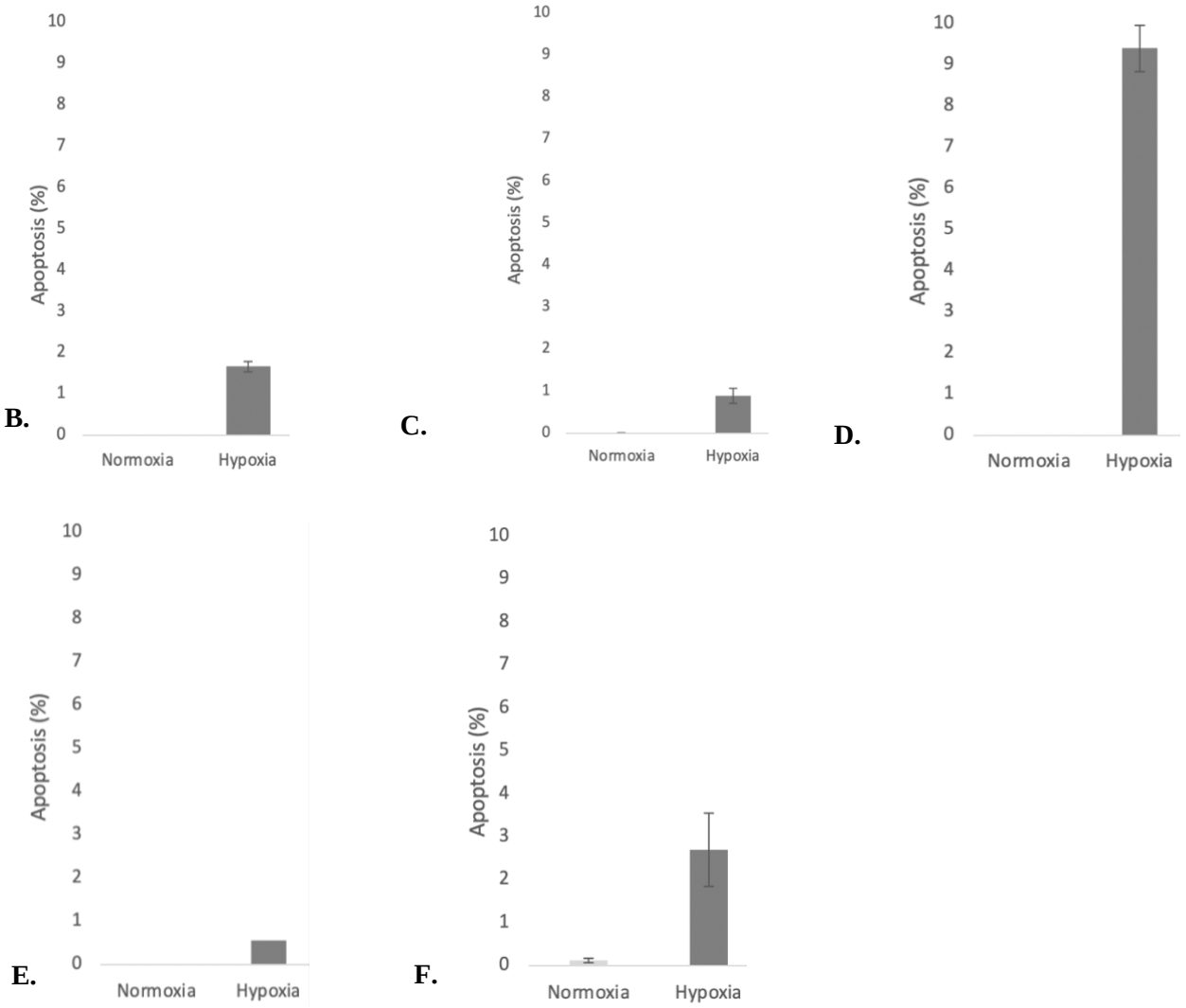
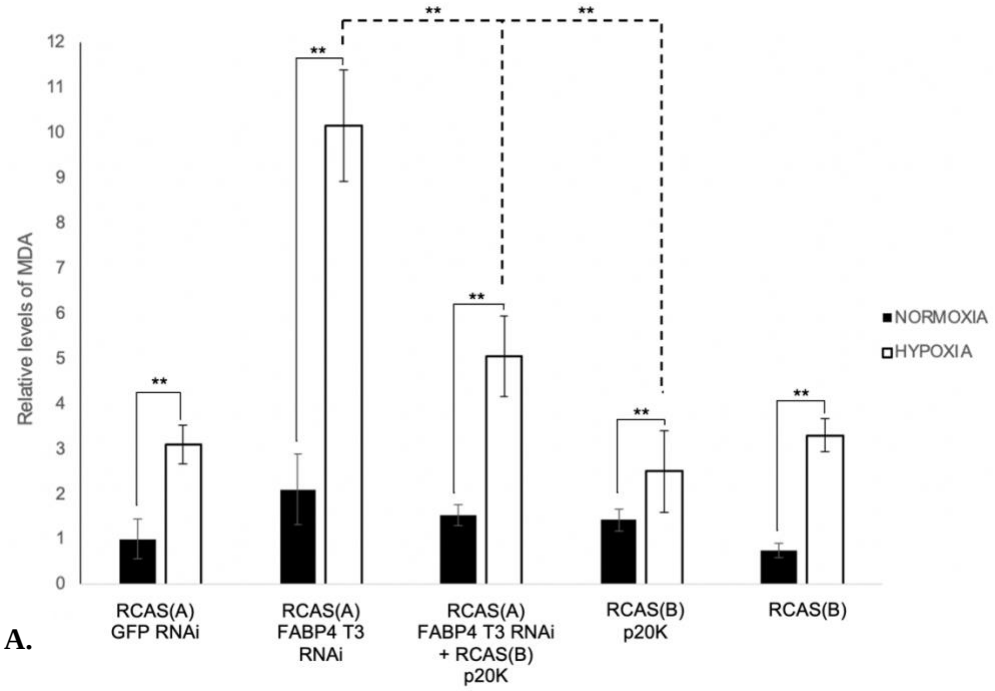
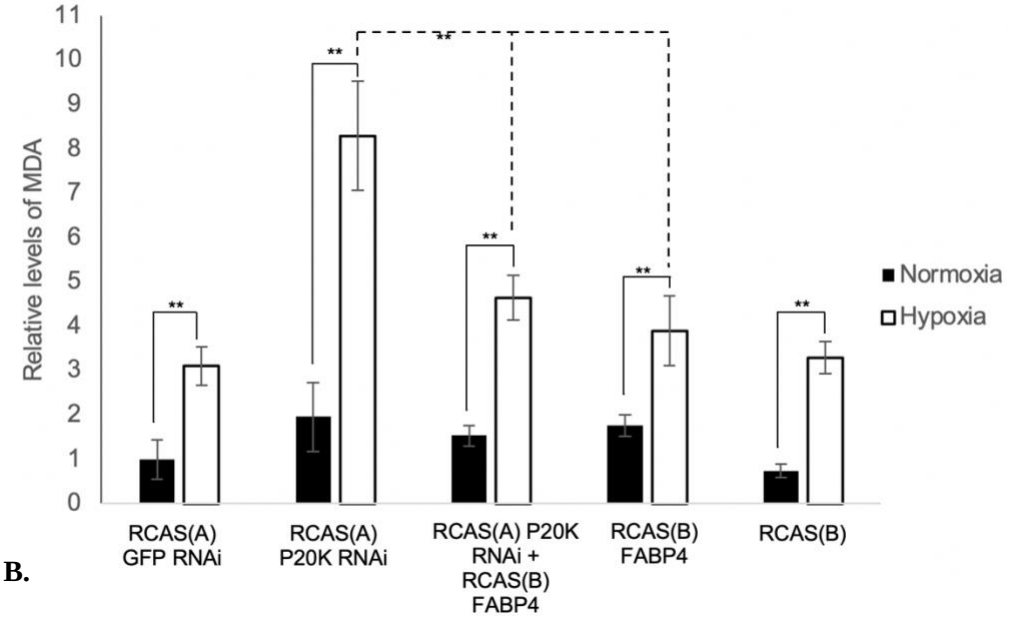


Figure 31. A) Fluorescent staining with TUNEL assay. CEF transfected with a group A retroviral vector expressing a control shRNA for GFP (RCASBP(A) – GFP RNAi), a group B retroviral vector expressing a control shRNA for GFP (RCASBP(B) – GFP RNAi), a shRNA for FABP4 (RCASBP(A) – T3 FABP4 RNAi), a cDNA for p20K (RCASBP(B) – p20K OE cDNA) or a combination of shRNA for FABP4 and cDNA for p20K (RCASBP(A) - T3 FABP4 RNAi + RCASBP(B) p20K OE cDNA) were incubated in normoxic (21% O2) or hypoxic (1.8% O2) conditions for 30 hours. The left panel represents cells stained with TdT and TMR red-fluorescent labelled dUTP. The right panel represents cells stained with Hoechst 33342. Arrows

on the left panel point to TUNEL-positive (apoptotic cells), and arrows on the right panel point to cells undergoing chromatin condensation and nuclear fragmentation. B) The levels of apoptosis in RCASBP (A) GFP – RNAi, C) RCASBP (B) GFP – RNAi, D) RCASBP (A) T3 FABP4– RNAi, E) RCASBP (B) p20K OE cDNA and F) RCASBP (A) T3 FABP4 – RNAi + RCASBP (B) p20K OE cDNA were quantified. Error bars represent the mean standard deviation of the levels of apoptosis per 400 cells measured in quadruplicate.



A.



B.

Figure 32. Quantitation of malondialdehyde (MDA), a by-product of lipid peroxidation, with MDA assay. CEF transfected with a group A retroviral vector expressing a control shRNA for GFP (RCASBP (A) - GFP RNAi), group B retroviral vector empty vector (RCASBP (B)). ANOVA tests were conducted to compare MDA levels within and between the cell populations.

Asterisks represent significance between samples within the same conditions, where * indicates P≤0.05 and ** P indicates ≤0.01.

A) Depicts MDA levels of CEF transfected with group A retroviral vector expressing shRNA for FABP4 (RCASBP(A) T3 FABP4 RNAi), group B retroviral vector expressing cDNA for p20K (RCASBP(B) p20K) or a combination of vectors for FABP4 knockdown and p20K over-expression (RCASBP(A) T3 FABP4 RNAi + RCASBP(B) p20K).

B) Depicts MDA levels of CEF transfected with group A retroviral vector expressing shRNA for p20K (RCASBP(A) p20K RNAi), group B retroviral vector expressing cDNA for FABP4 (RCASBP(B) FABP4) or a combination of vectors for p20K knockdown and FABP4 over-expression (RCASBP(A) p20K RNAi + RCASBP(B) FABP4).

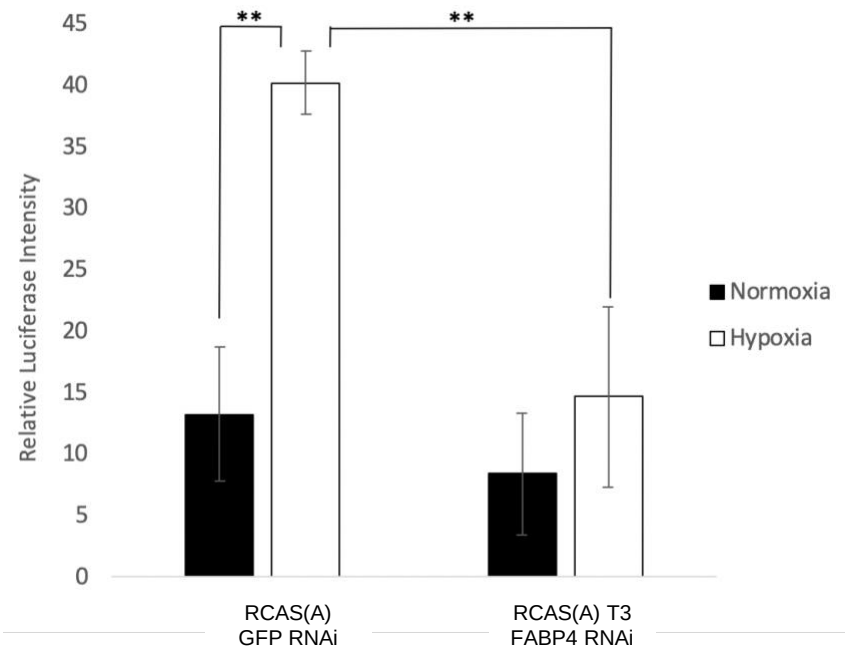


Figure 33. Promoter activity in CEF containing the wild-type QRU and group A retroviral vector expressing a control shRNA for GFP (RCASBP(A) – GFP RNAi) or a shRNA for FABP4 (RCASBP(A) – T3 FABP4 RNAi) were analyzed by transient expression assays in conditions of normoxia (21% O2) or hypoxia (1.8% O2) for 24 hours. ANOVA tests were conducted to compare QRU promoter activity within and between the cell populations. Asterisks represent significance between samples within the same conditions, where * indicates $P \leq 0.05$ and ** P indicates ≤ 0.01.

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