

GTP-BINDING PROTEIN OVEREXPRESSED IN SKELETAL MUSCLE (GEM1)

**CHARACTERIZING THE GROWTH ARREST SPECIFIC GENE, GEM1, IN
CHICKEN EMBRYO FIBROBLASTS**

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the
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Descriptive Note

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Abstract

Conditions that lead to reversible growth arrest (quiescence), promote the expression of a set of genes called growth arrest specific (GAS) genes. GAS genes play a crucial role in initiating and maintaining the entry into quiescence, while also activating stress responses to help the cell overcome the effects of the stressors. Gene profiling study examining the transcriptome has shown a vast number of genes that are upregulated during quiescence, among them is GEM1 (GTP binding protein overexpressed in skeletal muscle). GEM1 transcripts were elevated 18-fold in response to quiescence. GEM1 is a small monomeric GTPase from the Ras superfamily. It is involved in regulation of cytoskeleton reorganization, and inhibition of voltage gated calcium channels that ultimately prevents hormone secretion. A preliminary study determined that GEM1 is packaged into extracellular vesicles (EV). GEM1 is also reported to promote lipid accumulation and adipogenesis in goat pre-adipocytes. GEM1 is also reported to bind transcription factors that are involved in lipid homeostasis pathways. Thus, it is probable that GEM1 may play a major role in EV formation and/or release, and lipid homeostasis. This study examined the expression of GEM1 at the protein level and validates its candidacy as a GAS gene. We also created two GEM1-shRNA retroviral constructs capable of partially downregulating GEM1 expression which can serve as a molecular tool for further characterizing the function of GEM1 in quiescent CEF.

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Table of Contents

Abstract.....	iv
Acknowledgements	v
Table of Contents	vii
List of Tables.....	ix
List of Figures.....	x
List of Abbreviations.....	xii
Vector Acronyms	xv
Literature Review	1
1. The Cell Cycle	1
2. Growth arrest	1
3. Growth arrest specific genes	2
4. Inducing Growth Arrest	4
5. GEM-1	7
6. RasEF.....	9
7. Rhot1/Miro1.....	11
8. pRFPRNAiC and RCASBP(A).....	13
Rationale and Objectives	15
Materials and Methods.....	17
1. Tissue Culture and Sample Preparation	17
2. Collecting Cell Lysate.....	18
3. Bradford Assay.....	19
4. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) & Western Blotting	19
4.1 Antibody incubation and imaging	20
5. Reverse Transcriptase PCR to generate cDNA.....	21
6. Generating GEM1 shRNAi construct	22
6.1 Oligo design for shRNA	22
6.2 Cloning <i>GEM1</i> shRNA cassette into plasmid	24
6.3 Gel Extraction	25
6.4 Transformation and Plasmid DNA prep.....	25
7. shRNAi Vector Transfection	26

7.1 DNA precipitation.....	26
7.2 Calcium Phosphate Transfection.....	26
Results	27
Chapter 1: Characterizing the expression of GEM1 in quiescent CEF.....	27
i. GEM1.....	27
ii. RasEF.....	29
iii. Rhot1/Miro1.....	30
iv. GEM1 cDNA	31
Chapter 2: Cloning GEM1 shRNA cassette in RCASBP(A) to create a shRNA retroviral construct. ...	32
i. PCR amplification of DNA fragments of GEM1 shRNA.....	32
ii. Cloning GEM1 shRNA cassette into pRFPRNAiC (U6-)	33
iii. Cloning GEM1 shRNA cassette into RCASBP(A) expression vector.....	34
Chapter 3: Downregulating GEM1 expression in Quiescent CEF.....	35
i. Validating GEM1 shRNAi construct.....	35
Discussion.....	36
Chapter 1: Characterizing GEM1 in quiescent CEF.....	36
Chapter 2: Cloning GEM1 shRNA cassette in RCASBP(A) to create a shRNA retroviral construct. ...	43
Chapter 3: Downregulating GEM1 expression in Quiescent CEF.....	44
Experimental Figures	48
References	57

List of Tables

Materials and Methods

Table 1: Antibodies used for probing for protein of interest.....	20
Table 2: Reverse transcriptase PCR thermocycler program for generating cDNA.....	21
Table 3: Primer sets flanking <i>GEM1</i> ORF (NC_052533.1).....	22
Table 4: DNA sequence of <i>GEM1</i> shRNA.....	23
Table 5: PCR program for amplification of 59mer/58mer Target Sequence.....	24
Table 6: Primer sets flanking <i>GEM1</i> shRNA cassette in 59mer and 58mer Target Sequence.....	24

List of Figures

Introduction

Figure 1: RasEF protein structure.....	11
Figure 2: Rhot1/Miro1 protein structure.....	12
Figure 3: Schematic representation of the virus-based shRNA expression system.....	15

Experimental Figures

Chapter 1: Characterizing the expression of GEM1 in quiescent CEF.

Figure 4: GEM1 expression in cycling (Cy), contact inhibited (CI), hypoxic (Hy), serum starved (SS) CEF.....	48
Figure 5: Western blot analysis of GEM1 in extracellular vesicles of cycling cells (Cy) and contact inhibited cells (CI).....	49
Figure 6: RasEF expression in cycling (Cy), contact inhibited (CI), hypoxic (Hy), serum starved (SS) CEF.....	50
Figure 7: Rhot1/Miro1 expression in cycling (Cy), contact inhibited (CI), hypoxic (Hy), serum starved (SS) CEF.....	51
Figure 8: Agarose gel (1.0%) electrophoresis of GEM1 cDNA.....	52

Chapter 2: Cloning GEM1 shRNA cassette in RCASBP(A) to create a shRNA retroviral construct.

Figure 9: Agarose gel (1.0%) electrophoresis of PCR amplified GEM1 shRNA DNA fragment..... 52

Figure 10: Agarose gel (1.0%) electrophoresis of digested pRFPRNAiC transfer vector at NotI and ClaI restriction sites..... 53

Figure 11: Agarose gel (1.5%) electrophoresis of digested RCASBP(A) vector at NotI and ClaI restriction sites..... 54

Chapter 3: Downregulating GEM1 expression in CEF

Figure 12: Downregulating GEM1 expression in quiescent (hypoxic) CEF..... 55

List of Abbreviations

abs. EtOH	Absolute Ethanol
Amp	Ampicillin
BSA	Bovine Serum Albumin
BLAST	Basic Local Alignment Search Tool
CaM	Calmodulin
CCD	Coiled Coil Domain
CDK	Cyclin Dependent Kinase
cDNA	Complementary DNA
C/EBP β	CCAAT-Enhancer Binding Protein Beta
C/EBP α	CCAAT-Enhancer Binding Protein Alpha
CEF	Chicken Embryo Fibroblasts
CI	Contact Inhibition
CIP	Calf Intestinal Alkaline Phosphatase
Cy	Cycling / Proliferating
DMEM	Dulbeco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
EFD	EF Hand Domain
<i>Env</i>	Gene for Viral Envelope
ER	Endoplasmic Reticulum
G ₀	Growth Arrest Phase of Cell Cycle
G ₁	Growth Phase 1 of Cell Cycle
G ₂	Growth Phase 2 of Cell Cycle

<i>Gag</i>	Gene for Viral Capsid
GAS	Growth Arrest Specific
GDP	Guanosine Diphosphate
GEM1	GTP-binding Protein Overexpressed in Skeletal Muscle
GTP	Guanosine Triphosphate
HBSP	HEPES Buffered Saline Solution
HeLa	Henrietta Lacks
HIF-1	Hypoxia Inducible Factor 1
HRP	Horseradish Peroxidase
Hy	Hypoxia
LB	Lysogeny Broth
LPL	Lipoprotein Lipase
LTRs	Long Terminal Repeats
miRNA	MicroRNA
MOEC	MicroRNA Operon Expression Cassette
Miro1	Mitochondrial Rho GTPase 1
mRNA	Messenger RNA
ORF	Open Reading Frame
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PDGF α R	Platelet-Derived Growth Factor α -Receptor
<i>Pol</i>	Gene for Viral DNA-Polymerase
PPAR γ	Peroxisome Proliferator-Activated Receptor Gamma

PRD	Proline Rich Domain
Rab	Ras-Associated Binding
Ras	Rat Sarcoma
RasEF	Ras and EF-hand Domain-Containing Protein
RasEF-S	RasEF-Short
RasEF-L	RasEF-Long
RCASBP(A)	Replication Competent ALV LTR with a Splice Acceptor Bryan Polymerase
RCF	Relative Centrifugal Force
RFP	Red Fluorescence Protein
RGK	Rad/Gem/Kir
Rho	Ras Homologous
Rhot1	Alternate name for Miro1
RNA	Ribonucleic Acid
RNAi	RNA Interference
ROS	Reactive Oxygen Species
RPM	Rotations Per Minute
S	Synthesis Phase of Cell Cycle
SDS-PAGE	SDS- Polyacrylamide Gel Electrophoresis
shRNA	Short Hairpin RNA
SREBP1	Sterol Regulatory Element-Binding Protein-1
SS	Serum Starved
TBS	Tris-Buffered Saline

TPB

Tryptose Phosphate Broth

Vector Acronyms

pRFPRNAiC (U6-)	parental pRFPRNAiC missing the chicken U6 promoter and leader sequences
pRFPRNAiC – T1 GEM1	pRFPRNAiC plasmid containing GEM1 T1 shRNA cassette
pRFPRNAiC – T2 GEM1	pRFPRNAiC plasmid containing GEM1 T2 shRNA cassette
pRFPRNAiC – T3 GEM1	pRFPRNAiC plasmid containing GEM1 T3 shRNA cassette
RCASBP(A)	parental RCASBP(A) vector
RCASBP(A) – T1 GEM1	RCASBP(A) vector containing GEM1 T1 shRNA cassette
RCASBP(A) – T2 GEM1	RCASBP(A) vector containing GEM1 T1 shRNA cassette

Literature Review

1. The Cell Cycle

The eukaryotic cell cycle is a complex and fundamental process in cellular replication. It comprises two phases: interphase and mitosis. Interphase is divided into three stages: the first gap phase (G_1), DNA synthesis (S), and the second gap phase (G_2). Mitosis involves distinct stages of chromosome segregation, starting with prophase, followed by metaphase, anaphase, and finally, telophase (Schafer, 1998). G_0 phase is an additional phase that a cell can enter just before the restriction point in late G_1 . G_0 phase is also referred to as the growth arrest state, resting state, or quiescent state (Patt, H; Quastler, H, 1963). During the first growth stage, G_1 , the cell has two options. First, it can proceed with the cell cycle if favorable conditions exist, such as sufficient nutrients and the presence of appropriate cell cycle regulators. Second, if the conditions are not favourable and/or the cell is experiencing extrinsic or intrinsic stress, the cell exits the cell cycle, and it may enter the G_0 phase (Williams G, Stoeber K, 2012). Finally, the third option, where the cell has appropriate signal to terminally differentiate (Zhu et al., 1999).

2. Growth arrest

Two main types of cell cycle arrest occur in eukaryotic cells, and each is triggered by a combination of extracellular and intracellular conditions. Senescence refers to an irreversible entry into growth arrest state while quiescence is a reversible entry into growth arrest state (Blagosklonny, 2011). Senescence occurs when the cell cycle is permanently arrested due to replicative stress caused by several cycles of replication (Mombach et al., 2014). This stress is primarily induced by morphological changes, such as the accumulation of cytotoxins and

reactive oxygen species (ROS), leading to lipid peroxidation and DNA damage (Cho & Hwang, 2012). Multiple replication of DNA results in shortening of telomeres ultimately triggering permanent cell cycle arrest (Terzi et al., 2016). While some senescent cells die quickly, most cells can remain alive and contribute to their surroundings by secreting signaling molecules and compounds that affect extracellular structures. However, their contribution may not always be beneficial to surrounding healthy cells. The accumulation of senescent cells can be detrimental and cause inflammation (McHugh & Gil, 2018).

On the other hand, quiescence occurs when the cell cycle is temporarily arrested due to the lack of appropriate nutrients, growth factors, or cell cycle-regulating signals required for continued self-replication (Williams G, Stoeber K, 2012). Quiescent cells exhibit specific characteristics, including the induction of genes that inhibit growth, promote cell survival (resulting in decreased enzymatic activities, macromolecule synthesis, and DNA/lipid damage repair), and prevent the progression through the cell cycle (Erb et al., 2016). Once the cell is no longer exposed to external stresses, it can re-enter the cell cycle, provided there is appropriate signaling from cell cycle regulators (CDK and cyclins) and sufficient nutrients and growth factors to support successful self-replication (Coller et al., 2006).

3. Growth arrest specific genes

Genes known as growth arrest-specific (GAS) genes, are preferentially expressed in quiescent cells (Coccia et al., 1992). In general, these GAS genes play a crucial role in inducing and maintaining growth arrest while also preventing proliferation and apoptosis (Fleming et al., 1998). The GAS family genes, including gas1-6, were among the first genes to be identified.

Their expression was downregulated during proliferation and upregulated in response to environmental stressors which prevented proliferation (Schneider et al., 1988). GAS genes can offer cell protection against various stresses by inducing growth arrest, inhibiting DNA synthesis and apoptosis, and maintaining lipid homeostasis, as well as guarding against the effects of ROS buildup (Del Sal et al., 1992). Different types of quiescence, such as growth arrest resulting from high cell density (contact inhibition), a hypoxic environment, or serum starvation induces different GAS gene expressions (Coller et al., 2006). Among the six novel GAS genes identified by Schneider et al., 1988, *gas1* stands out as an integral plasma membrane protein that prevents the cell from entering the S phase of the cell cycle by inhibiting DNA synthesis. Although *gas1* is present at low concentrations in normal dividing cells, its overexpression restricts DNA synthesis and prevents the cell cycle from progressing into the S phase (Del Sal et al., 1992; Schneider et al., 1988). GAS genes not only induce and maintain growth arrest, but they can contribute to the cell's re-entry into the cell cycle, one such GAS gene is platelet-derived growth factor α -receptor (PDGF α R) (Lih et al., 1996).

p20K, a member of the lipocalin family, was identified as a GAS gene due to its high induction in quiescent chicken embryo fibroblast (CEF) and chicken heart mesenchymal cells (Bédard et al., 1987; Mao et al., 1993). p20K binds to hydrophobic molecules such as long chain unsaturated fatty acids, cell surface receptors, and forms macromolecule complexes (Cancedda et al., 1990, 1996; Flower, 1996). P20K is observed to be upregulated in quiescence-inducing conditions of contact inhibition and hypoxia with little to no expression in proliferating cells. Further investigations have revealed that p20K plays a vital role in cell survival. Specifically, when p20K is downregulated, there is a notable increase in apoptosis in quiescent CEF under hypoxic conditions, but not in proliferating CEF under normoxic conditions (Erb M., MSc.

Thesis, 2016; Moftakhari R., et al., in preparation). Interestingly, the forced expression of p20K does not increase apoptosis or alter the proliferation rate in quiescent CEF but increases saturation density. This suggests that p20K plays a crucial role in the survival of quiescent cells in oxygen-deprived conditions although the underlying mechanism for this is still unclear. Moreover, downregulation of p20K in hypoxic quiescent CEF leads to an increased formation of lipid vesicles, indicating that p20K might facilitate the transport of polyunsaturated fatty acids out of the cell. In the absence of p20K, there is also a higher accumulation of reactive oxygen species (ROS) and lipid peroxidation in hypoxic CEF. These findings indicate that p20K plays a key role in cell survival by mitigating the damaging effects of ROS and promoting lipid homeostasis in quiescent CEF (Moftakhari R., et al., in preparation)

4. Inducing Growth Arrest

It has been previously established that chicken embryo fibroblast (CEF) serves as an excellent model for investigating the expression pattern of growth-arrest specific genes in a primary cell model (Bédard et al., 1987). Under optimal conditions, CEF cells in vitro actively undergo proliferation. However, when these cells experience deviations from the optimal conditions, they arrest their cell cycle and cease proliferation, entering a reversible quiescent state (Yao, 2014). We take advantage of this behavior to induce growth arrest by subjecting the CEF culture to different stress, such as serum starvation (deprivation of bovine calf serum and Tryptose Phosphate Broth), incubation in a hypoxic environment (1.8% O₂), and promoting contact inhibition through high cell density.

Sub-confluent cells can be growth arrested when transferred to serum-free medium, a condition of growth arrest known as serum-starvation. The serum comprises several hormones and growth factors that are essential for the cell's progression through the cell cycle. Consequently, in the absence of bovine calf serum and/or fetal bovine serum, the cells cease the cell cycle and enter a state of quiescence (Pirkmajer & Chibalin, 2011). Serum is also a complete mixture of micro-nutrients required for proliferation and other biological processes. Thus, serum-starved cells experience ER-stress which leads to growth arrest.

Cells experiencing contact inhibition are growth-arrested due to a high cell density. A previous study has discovered that certain metabolic pathways, including glycolysis, the pentose phosphate pathway, the citric acid cycle, and the degradation and resynthesis of lipids and proteins, are upregulated in contact-inhibited cells compared to cycling cells (Lemons et al., 2010). This illustrates the fact that quiescent cells are still metabolically active, inducing cellular processes that help the cell adapt to the non-ideal conditions. Furthermore, contact-inhibited fibroblasts exhibit elevated levels of various extracellular matrix proteins, such as collagen 21A1, fibronectin, and laminin alpha 2, which is consistent with the observed increase in extracellular matrix in high cell density environment (Lemons et al., 2010). This increased extracellular matrix may potentially contribute to tissue repair in damaged areas. Additionally, recent studies employing transcriptome analysis have identified the upregulation of hypoxia-responsive genes in contact-inhibited CEF compared to cycling cells and serum-starved cells (Erb et al., 2016). Additionally, this paper also examined nitro reductase activity (a marker of hypoxia) in contact inhibited cells and observed increased activity compared to proliferating cells. These findings suggest that the high cell density, a characteristic of contact-inhibited CEF, creates a localized hypoxic environment, leading to the activation of GAS genes.

In hypoxic conditions cells rely more on anaerobic metabolic processes consequently an upregulation of hypoxia response genes is observed (Erb et al., 2016; Semenza, 2000). A hypoxia specific factor, hypoxia-induced factor (HIF-1), is a transcriptional factor only present in hypoxic cells which interacts with the hypoxia response element to activate the transcription of hypoxia response genes (Yamashita et al., 2001). The hypoxia response genes generally are involved in regulating cellular metabolism to help the cell adapt to decreased oxygen availability (Mathupala et al., 2001). Hypoxia response genes also regulate cell proliferation, growth arrest and apoptosis. HIF-1 also plays a role in inducing cell proliferation once the growth conditions have improved (Conrad et al., 1999; Harris, 2002). It is very important for the cell to maintain an optimum oxygen concentration because any fluctuation in the oxygen levels can produce free radicals and reactive oxygen species (ROS) which is a hallmark of oxidative stress (Zdeňka Ďuračková, 2010). Significant evidence has suggested that hypoxia response genes are involved in mitigating oxidative stress by lowering ROS (Scortegagna et al., 2003). The study by Gardiner et al., 1981 has focused on the effects of oxidative stress on fatty acids, here they observe an increase in non-esterified free fatty acids under hypoxic conditions (Gardiner et al., 1981). ROS can attack the double bond in the carbon backbone of lipids and damage the lipids, this process is called lipid peroxidation. Lipid peroxidation is a well studied process, some of the commonly targeted lipids include glycolipids, phospholipids, cholesterol, and polyunsaturated fatty acids (Ayala et al., 2014). Lipid peroxidation of phospholipid bilayer can seriously damage the cell, it can change the membrane permeability and potential which can result in ion imbalance and unstable membrane fluidity (Gutteridge, 1995). Furthermore, lipid peroxidation can impede various cellular processes such as lipid synthesis, cell signaling, metabolic pathways, vesicle formation, and even induce endoplasmic reticulum (ER) stress (Ayala et al., 2014).

5. GEM-1

GTP-binding protein overexpressed in skeletal muscle, also known as *GEMI* or *GEM* it encodes a small monomeric GTPase and a member of RGK (Rad/Gem/Kir) subgroup of Ras superfamily (Reynet & Kahn, 1993). The *GEMI* gene spans 8355bp and comprises 5 exons and 4 introns. After post-transcriptional modification, the 1989 nucleotide long mRNA encodes a 297aa protein weighing 35 kDa (Yoshizawa et al., 2004)(NIH Gene ID: 404771).

Gene profiling of growth arrested CEF showed that *GEMI* transcript was heavily expressed (18-fold increase) in contact inhibition compared to proliferating cells (Erb et al., 2016). Although the study of *GEMI* in the chicken model is limited, numerous studies have explored and characterized *GEMI* in humans and other mammalian models. GEM1, like most GTPases, contains GDP/GTP-binding domains, GTPase enzymatic activity, and a calcium channel regulator activity (UniProtID: A0A1D5PHS5_CHICK). GEM1 is observed to bind to Ca²⁺/calmodulin (CaM) at the CaM-binding site near the carboxyl-terminal. GEM1 binding to CaM and GTP is a key step for detecting an influx of Ca²⁺ which results in inhibition of L-type voltage gated calcium channel which prevents growth hormone secretion (Beguin et al., 2001; Ward et al., 2004). GEM1 also hosts 14-3-3 proteins binding domains, these proteins commonly function as intermediary that bind to kinases, phosphatases, and transmembrane receptors where they could play a post-translational regulatory role for GEM1 function (Kelly, 2005; Ward et al., 2004).

In a preliminary study, *GEMI* was found to be transiently expressed in T-cells of peripheral blood, where it was phosphorylated at tyrosine residues and translocated to the cytosolic side of the plasma membrane (Maguire et al., 1994). Although the kinase responsible for phosphorylating the Tyrosine residue was not identified, later studies have identified other

phosphorylation sites that could be responsible for regulation of GEM1 (Ward et al., 2004). Other studies have identified that GEM1 is involved in regulating cytoskeleton reorganization. Here they found that ectopic expression of GEM1 leads to significant rearrangement of actin cytoskeleton in HeLa, epithelial, fibroblast, and neuroblastoma cell models (Andrieu et al., 2014; Ward et al., 2002). GEM1 does this by binding to Rho kinase (ROK), specifically ROK β , which interferes with the downstream ROK activity resulting in loss of stress fibers and focal adhesions. ROK normally plays a crucial role in regulating actinomyosin cytoskeleton formation by phosphorylating the light chain of myosin II (MLC) which promotes stress fiber and focal adhesion formation (Kimura et al., 1996). ROK also phosphorylates myosin phosphatase that results in inhibition of disassembly of stress fibers and focal adhesions, which further promotes actinomyosin cytoskeleton formation (Kimura et al., 1996). When GEM1 interacts with ROK β , it leads to inhibition of ROK β activity resulting in loss of stress fibers and focal adhesions. ROK also regulates LIM-Kinase 1 but this function of ROK is not-affect when GEM1 binds to ROK, indicating that GEM1 may regulate ROK β by altering the substrate specificity rather than blocking its enzymatic activity (Ward et al., 2002). A missense mutation at serine residues 261 and 289 results in the loss of this function of GEM1 (cytoskeletal reorganization) and since serine residues are often phosphorylated to regulate cellular processes, suggests that these sites could be a target for post-translational phosphorylation which regulates this function of GEM1 (Ward et al., 2004).

Forced overexpression of GEM1 was also found to inhibit the growth of embryonic mouse fibroblast cell line (NIH 3T3) cells, indicating it could be a highly regulated protein with evidence pointing to it potentially involved in receptor-mediated signal transduction (Maguire et al., 1994). Recent investigations into the role of GEM1 in adipogenesis, due to its influence on

lipid metabolism in diabetic patients, revealed that knockdown of GEM1 in goat intramuscular preadipocytes suppressed lipid accumulation and inhibited adipogenesis (Xu et al., 2020). However, the exact mechanism by which GEM1 accomplishes this remains unclear. This suggests that GEM1 may play a role in lipid homeostasis, but a closer examination of this pathway is warranted. Additionally, the knockdown of GEM1 was observed to decrease the expression of various transcription factors (C/EBP α , C/EBP β , LPL, PPAR γ , and SREBP1), suggesting that GEM1 regulates the expression of adipogenic-related genes and possibly other biological processes through signaling pathways (Xu et al., 2020).

There are limited studies on GEM1 which explains why the role and mode of function of GEM1 is not fully understood and documented, especially not in the context of quiescence and CEF model. Despite that we can still speculate that GEM1 may function as a molecular switch responding to or regulated by intracellular and/or extracellular stimuli, such as changes in calcium concentration (a signal of lipid membrane damage), through its interaction with calmodulin. This interaction could ultimately regulate downstream gene expression via the previously mentioned transcription factors. Additionally, GEM1 could play a role in intracellular transport by regulating cytoskeleton reorganization. Due to the limited studies of GEM1 in growth-arresting conditions, further investigation is warranted to characterize GEM1 in quiescent CEF.

6. RasEF

When examining the expression pattern of GEM1, a high molecular weight signal with unknown origin was observed. Two promising candidates were identified that could be

responsible for that high molecular weight signal, RasEF, and Rhot1/Miro1. Ras and EF-hand domain-containing (RasEF) is a Rab GTPase. It facilitates dynein-mediated transport and dynein-dynactin motility on microtubules (Y. Wang et al., 2019). RasEF contains two EF-hand domains, several coil-coiled domains (CCD), and a C-terminus Rab GTPase domain (Figure 1) (Shintani et al., 2007). Generally, Rab GTPases have only one EF-hand domain, but RasEF has two which are known to be important for Ca²⁺ binding sites. The function of the EF-hand domain in RasEF is not fully understood but the lack of EF-hand domain results in loss of interaction with dynein–dynactin (Y. Wang et al., 2019). The CCD of RasEF is required for interacting with itself to form oligomer (Shintani et al., 2007). The GTPase domain is responsible for guanine-nucleotide binding and functions as a typical GTPase domain. Most of the large Rab GTPases have a proline rich domain, but RasEF lacks it (Shintani et al., 2007). Most of the proteins in the Rab GTPase family are small (~20-30 kDa) but RasEF is one of the few large proteins at ~83 kDa in this family (Shintani et al., 2007). Another characteristic of large Rab GTPases is that they are almost always found in two or more isoforms, RasEF is no exception, it has two isoforms, long(L) and short(S) (Shintani et al., 2007). RasEF-L has the two EF-hand domains while RasEF-S doesn't have any EF-hand domains (Figure 1) (Srikanth et al., 2017).

RasEF expression is primarily regulated by methylation of the promoter of the *RasEF* gene; increase in methylation results in lower RasEF expression while decrease in methylation results in higher expression (Li et al., 2019). Human RasEF-L is shown to be primarily localized in the Golgi apparatus and recycling endosomes (Shintani et al., 2007). RasEF-S is shown to be localized in the nucleus (Kumar et al., 2016). Most of the studies about RasEF is in the field of cancer and depending on the study, RasEF is portrayed as tumor suppressor or oncogene. In

cancer cells overexpression of RasEF results in apoptosis through activation of caspase-3 and 9, and increases phosphorylation of p38 (Nakamura et al., 2011). Patients with decreased expression of RasEF had significantly lower survival rates suggesting it acts as a tumor suppressor gene (Yu et al., 2018). Other studies have found that overexpression of RasEF promotes cell growth while deletion of RasEF gene reduces cancer cell growth, suggesting that it could be an oncogene (Oshita et al., 2013). Therefore, the action of RasEF, and its isoforms is highly context dependent.

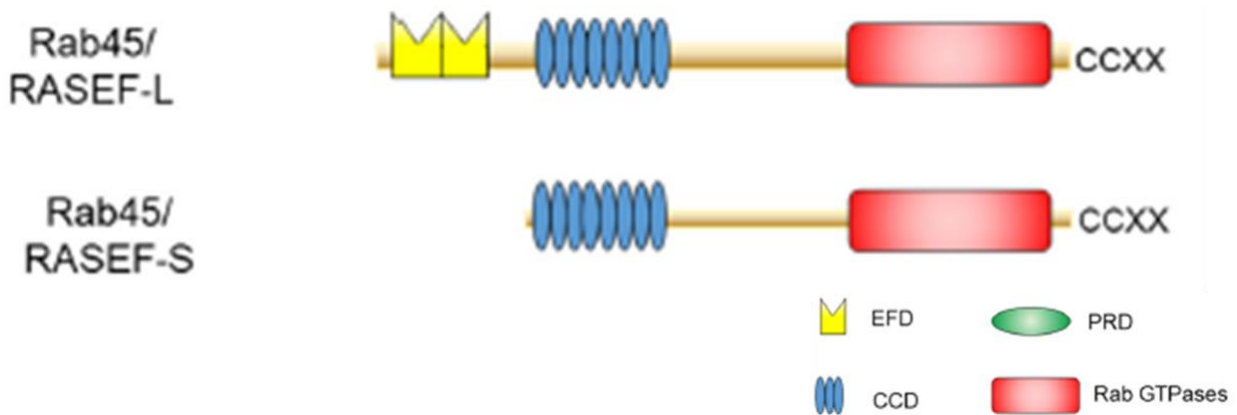


Figure 1. RasEF protein structure. RasEF-L is the longer isoform containing EF-hand domain (EFD), coil-coiled domain (CCD), and Rab GTPase domain. RasEF-S is the shorter isoform that lacks the EFD. RasEF completely lacks proline rich domain (PRD) compared to other large Rab family GTPases (Tsukuba et al., 2021).

7. Rhot1/Miro1

Rhot1, also known as Miro1 (mitochondrial Rho GTPase 1), is a member of Rho GTPase family. Miro1 is localized to the mitochondria, specifically the outer membrane of the mitochondria (Fransson et al., 2003). Miro1 interacts with adaptor protein milton, which

connects mitochondria to motor proteins that facilitates mitochondrial movement (Glater et al., 2006). This Miro1 mediated mitochondrial transport is regulated by cytosolic Ca²⁺ concentration (Chang et al., 2011; X. Wang et al., 2009). Miro1 has two GTPase domains (one at N-terminus and one at C-terminus), two EF-hand domains (EFD) between the two GTPase domains connected through linker region (Figure 2) (Kawasaki et al., 1998; Nelson & Chazin, 1998). The EFD is important for Ca²⁺ binding which plays a role in signal transduction and downstream protein regulation. The GTPase domain on the N-terminus is similar to GTPase domains in other Rho GTPases which are known to play a role in a vast variety of cellular processes but most notably some are involved in signalling pathways that regulate cell cycle progression and apoptosis (Aznar & Lacal, 2001). The role of the second GTPase domain on the C-terminus is not fully understood yet but evolutionarily it has diverged from the typical Rho GTPase domain (Fransson et al., 2003). Miro1 has many isoforms, but the general structure is maintained, it is about 600 amino acids long which weights ~70 kDa (Caldwell et al., 2005). Constitutively expressed Miro1 results in collapse of mitochondrial network into perinuclear assemblies, this also results in increased apoptosis suggesting that Miro1 is important in mitochondrial homeostasis and apoptosis (Fransson et al., 2003).

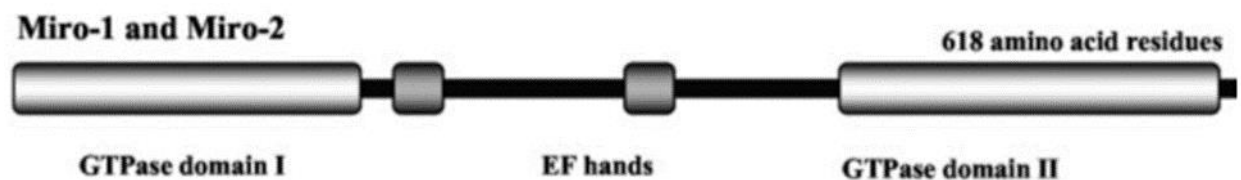


Figure 2. Rhot1/Miro1 protein structure. It contains two EF hand domains and two GTPase domains at the N-terminus and C-terminus (Fransson et al., 2003).

8. pRFPRNAiC and RCASBP(A)

Researchers have previously developed an efficient retroviral RNAi vector system, RCASBP(A) (Replication Competent ALV LTR with a Splice Acceptor Bryan Polymerase), designed for gene silencing in chicken cells (Das et al., 2006). This vector system is leveraged to express GEM1 shRNA that downregulates its expression in CEF. Cloning the DNA sequence of GEM1 shRNA into a large vector like the RCASBP(A) is inefficient and unreliable, therefore the GEM1 shRNA cassette DNA was first cloned into a smaller ‘transfer’ vector (pRFPRNAiC). Figure 3 illustrates the schematic of the pRFPRNAiC transfer vector and the RCASBP(A) retroviral vector (Das et al., 2006; L. Wang et al., 2011). The pRFPRNAiC transfer vector contains the β -actin promoter, RFP (red fluorescence protein gene), polyadenylation site, and the microRNA operon expression cassette (MOEC), which includes chicken U6 promoter, U6 leader, two miRNA sites, and strategically placed restriction sites (NheI and MluI) that easily allows for the sub-cloning of the entire MOEC into another vector (i.e., RCASBP(A)) (Das et al., 2006). We are mainly interested in MOEC for the purpose of this study. However, the MOEC used in our study has been slightly modified, it lacks the Chicken U6 promoter and U6 leader compared to conventional pRFPRNAiC vector since it was observed that the expression of the microRNA cassette was limited by the presence of the U6 promoter through transcriptional interference, where two strong promoters (viral LTR and U6 promoter) compete for co-activator complexes and limited supply of basic transcriptional machinery (L. Wang et al., 2011). Deletion of the chicken U6 promoter and leader sequence ensures that the expression of the microRNA cassette is only regulated via the strong viral LTR rather than U6 promoter (L. Wang et al., 2011). This ensures that the microRNA cassette is expressed alongside the expression and replication of the viral genome. Moreover, the pRFPRNAiC vector also harbors an ampicillin resistance gene,

which facilitates the selection of the plasmid in transformed cells. The restriction sites flanking the miRNA cassette sequence allows for the cloning of the miRNA cassette of interest, such as that containing the GEM1 shRNA, into the first miRNA cloning site in the MOEC. The MOEC has the capacity to hold two miRNA of interest however only one is required for the purpose of this study (Das et al., 2006).

Furthermore, the MOEC, containing the miRNA cassette of interest, can be directionally sub-cloned into the RCASBP(A) vector using another set of restriction sites (NotI and ClaI). The RCASBP(A) vector also contains an ampicillin resistance gene which can be used to select for it (Das et al., 2006). Additionally, the RCASBP(A) vector features flanking LTRs (long terminal repeats) upstream of the cloning site which is necessary to facilitate the expression of the downstream sequence, namely the MOEC encoding GEM1 shRNA, and proteins necessary for replication of retrovirus (*gag-pol-env*). The retroviral LTR consists of an enhancer, promoter, transcription initiation (capping), transcription terminator, and polyadenylation signal, which are sufficient for the host cell's transcriptional machinery to recognize and initiate transcription of the downstream genes (Jern & Coffin, 2008; Van De Lagemaat et al., 2003). This results in the expression of the MOEC and, subsequently, GEM1 shRNA; along with Bryan polymerase, capsid, and envelope proteins (which can assemble a retrovirus capable of infecting other cells). This will produce viruses that are unable to infect mammalian cells because ASLV envelope glycoprotein does not recognize mammalian cells surface receptors, but the newly assembled virus can infect chicken cells (i.e., CEF) and is also capable of replicating in chicken cells. This allows for rapid and efficient expression of a transgene sequence (shRNA) in a population of primary cells, such as CEF.

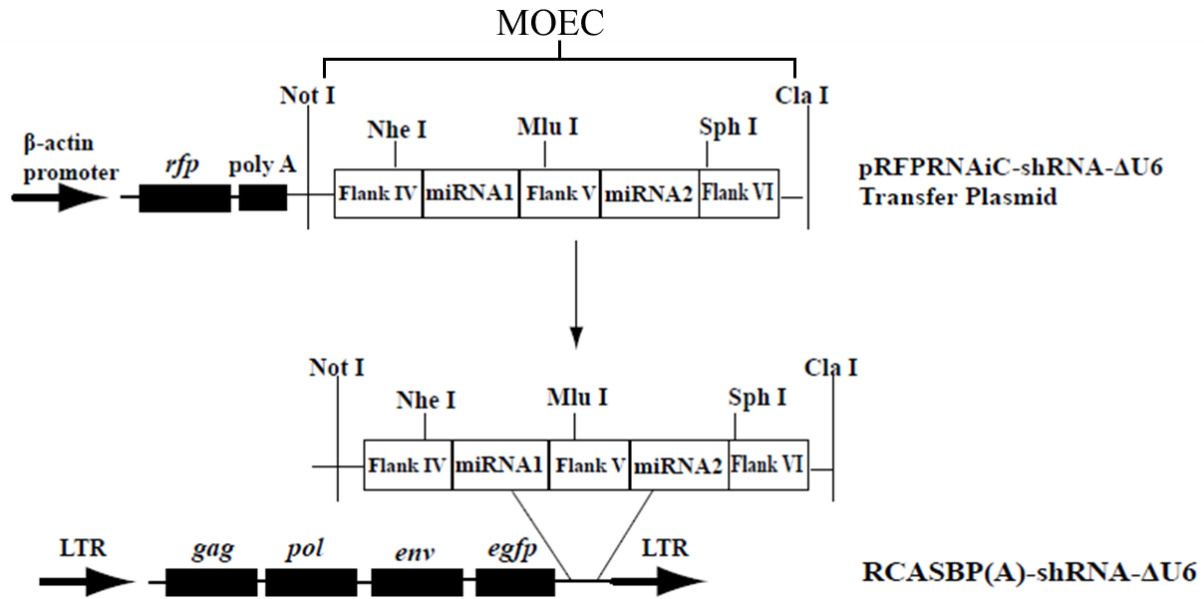


Figure 3. Schematic representation of the virus-based shRNA expression system (L. Wang et al., 2011, Supplementary Figure 6A). Sequences generating the hairpin structure are cloned at the *Nhe*I/*Mlu*I in the transfer plasmid pRFPRNAiC. The microRNA expression cassette (MOEC), harboring the hairpin sequences, is then sub-cloned in the retroviral vector RCASBP(A) at *Not*I/*Cla*I sites.

Rationale and Objectives

Cells can encounter a variety of growth conditions and environments *in vivo*, including hostile conditions that inflict stress. To cope with these challenges, cells have developed a mechanism to regulate their proliferative fate under stress. One such mechanism is the quiescence phase of the cell cycle; it can be defined as reversible growth arrest. The cells can avoid unfavorable conditions by exiting the cell cycle and re-enter the cell cycle once favorable growth conditions are restored. One defining characteristic of a quiescent cell is the upregulation

of a family of genes called GAS genes which are downregulated during active proliferation and senescence. A previous study by Bedard lab conducted gene profiling analysis to identify genes that are upregulated in response to quiescence inducing condition (i.e., contact inhibition). Among the extensive list of genes identified to be upregulated during contact inhibition, GEM1 was one of the highly expressed genes at the transcript level, with an 18-fold increase in high cell density-arrested CEF. This suggests that GEM1 is a candidate for the GAS gene family.

GEM1 is a small monomeric GTPase from the Ras superfamily. GEM1 is known to bind to calmodulin where it inhibits L-type voltage gated calcium channel which ultimately prevents growth hormone secretion. GEM1 also interacts with 14-3-3 proteins which could play a regulated role for GEM1 function. GEM1 is involved in regulating cytoskeleton reorganization, it binds to Rho kinase inhibiting its ability to recognize its cytoskeleton formation related substrates (myosin light chain, and myosin phosphatase). Downregulation of GEM1 results in suppressed lipid accumulation and inhibition of adipogenesis. Downregulation of GEM1 also results in decreased expression of various transcription factors ($C/EBP\alpha$, $C/EBP\beta$, LPL, $PPAR\gamma$, and SREBP1), suggesting that GEM1 may regulate gene expression or other biological processes through signaling pathways. The function of GEM1 could be dual nature, wherein it is reported to regulate cytoskeleton reorganization. Additionally, it may also function as a molecular switch to regulate downstream biological processes or gene expression. GEM1 is a monomeric GTPase where its function could have dramatic downstream effects. Regardless, there is little knowledge about GEM1 in the context of growth arrest.

Given that previous study by Erb M. and colleagues confirms that GEM1 transcripts are elevated in growth arrested (contact inhibited) CEF, the primary goal of this study is to evaluate the candidacy of GEM1 to be a member of the GAS gene family. Chapter 1 of this study will

examine the expression pattern of GEM1 in quiescence inducing conditions, such as contact inhibition, hypoxia, and serum starvation in CEF. Chapter 2 of this study will focus on creating a shRNA retroviral construct capable of expressing shRNA designed to target GEM1 mRNA which results in downregulation of GEM1 expression via RNAi. Finally, Chapter 3 will validate the efficacy of the shRNA retroviral construct to effectively downregulate GEM1 expression. The effectiveness of this molecular tool will determine whether it can be used to further characterize GEM1 in quiescent CEF. The findings of this study will establish a foundation to further characterize the role and regulation of GEM1 in quiescent CEF and ultimately advance our understanding of quiescence. It is hypothesized that GEM1 protein expression will be elevated in quiescent CEF (contact inhibition, hypoxia, and serum starvation).

Materials and Methods

1. Tissue Culture and Sample Preparation

Chicken embryo fibroblasts (CEF) are incubated at 41.5° Celsius in 21% oxygen, and 5% carbon dioxide. The CEFs are grown in a cell culture dish with complete medium. Complete media is Multicell DMEM (Dulbecco's Modified Eagle Medium) from Wisent Inc. (Ref. 319-005-CL) contains 5% heat-inactivated bovine calf serum, 5% TPB (Tryptose Phosphate Broth), 1% mixture of Penicillin (100 units/mL) and Streptomycin (100 µg/mL) (Sigma-Aldrich, P4458-100ML), and 2mM of L-Glutamine. Alternatively, cells were grown in the same medium supplemented with 2% fetal bovine serum (Gibco, Ref. 12483-012).

Cycling condition: The cells are lysed, and the proteins are collected when the cells are still dividing under no stress and before the cells reach more than 90% confluency.

Contact Inhibition condition: Once the cells have reached ~100% confluency, the medium is changed for a fresh complete medium. The cells can divide and reach high cell density for 16-20hrs after which they are inspected for signs of stress then lysed, and proteins are collected.

Hypoxic condition: Sub confluent (~60%) cells are used, the cell culture dish is placed in a hypoxic chamber (1.8% O₂ levels). The cells spend 24-30 hrs in the hypoxic chamber after which they are inspected for signs of stress then lysed, and proteins are collected.

Serum starvation condition: Sub confluent (~60%) cells are used; the old complete medium is removed then the cells are washed twice with serum and TPB free medium. The cells are placed in serum and TPB free medium for about 2 days. The cells are then inspected for signs of stress then lysed, and proteins are collected.

2. Collecting Cell Lysate.

Once the cells were cultured in required conditions, they were washed three times with cold 1x PBS (Phosphate-buffered saline) (pH 7.4, 137 mM NaCl, 2.7 mM KCL, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄). Using a plastic scraper, the cells were resuspended into 1x PBS. The cell suspension was centrifuged at 6500 RPM (4732 RCF) for 3 minutes at 4°C to form a soft pellet. The supernatant was removed, and the cell pellet was resuspended into 1x SDS buffer (2% Sodium dodecyl-sulfate, 10% glycerol, 5% β-mercaptoethanol, 60 mM Tris-HCL pH 6.8) containing 1% protease & phosphatase inhibitor cocktail (ThermoScientific, Ref. 78446), followed by vigorous vortex to lyse the cells. The cell sample was boiled for 2 mins followed by centrifugation for 5 minutes at 14, 800 RPM (24, 532 RCF) to eliminate any insoluble cell debris. The cell lysate was transferred to a new 1.5mL Eppendorf tube and stored at -80°C.

3. Bradford Assay

Bradford assay was performed to calculate the protein concentration in collected cell lysate. The standard curve was created using varying volumes of 1 $\mu\text{g}/\mu\text{L}$ BSA (Bovine serum albumin) (0, 1, 2, 4, 6, 8, 10, 12 μL), each containing 2 μL of SDS sample buffer (containing 1% protease inhibitor cocktail), 800 μL of Bradford reagent, and ddH₂O to bring final volume to 1 mL. The absorbance of 595nm wavelength light for the standard curve was measured to plot BSA concentration (x-axis) against 595nm absorbance (y-axis). For protein samples, two replicates were made. Each replicate contained 2 μL of respective protein sample/cell lysate, 800 μL of Bradford reagent, and ddH₂O to bring the final volume to 1 mL. The absorbance of 595nm wavelength light for protein samples was measured. The protein concentration was calculated using linear regression of the standard curve and measured absorbance value.

4. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) & Western Blotting

SDS-PAGE was performed using 10% or 14% acrylamide gel. When probing for higher molecular weight protein of interest, 10% resolution gel was used to get good separation of all proteins. 14% resolution gel is used when probing for lower molecular weight proteins. 50-75 μg of prepared protein samples [boiled for 2 mins, then centrifuged for 5 mins at 14, 800 RPM (24, 532 RCF) to remove insoluble cell debris] and Precision Plus Protein Dual Color ladder (BioRad, cat #1610374) were loaded onto the gel and resolved in 1x Running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) at 22.5mA/gel for about 1.5 hours. After the proteins are resolved on the acrylamide gel, they were transferred to the nitrocellulose blotting membrane in 1x Transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol) at 95V for 1 hour.

4.1 Antibody incubation and imaging

To probe for protein of interest on nitrocellulose membrane, the membrane is first blocked using 5% solution of powdered skim milk dissolved in 1X tris-buffered saline (20 mM Tris, 140 mM NaCl, pH 7.6) for 45 mins at room temperature. The membrane is then incubated in the primary antibody solution overnight at 4°C. Table 1 shows the various protein targets and their corresponding primary and secondary antibody solutions. The membrane is then washed twice with 1x TBS (Tris-buffered saline), then twice with 1x TBS + Tween, then finally twice with 1x TBS. The membrane was then incubated in the secondary antibody solution for 2 hrs at room temperature. Subsequently the membrane was washed again, as described above. Western horseradish peroxidase (HRP) substrate (Millipore, cat# WBLUF0500) was applied to the membrane which reacts with HRP bound secondary antibody to produce chemiluminescent signal, a high performance chemiluminescence film (Cytiva, cat# 28906839) was used to detect the signal.

Table 1. Antibodies used for probing for protein of interest.

Protein of interest	Primary antibody	Secondary antibody
P20K	601Y rabbit (1:400 dilution) 601X rabbit (1:400) 565 rabbit (1:100)	Antirabbit IgG, HRP-linked (1:25000 dilution)
ERK2	ERK2 (1:2000) Millipore #05-157 – Monoclonal	Antimouse IgG, HRP-linked (1:25000 dilution)
GEM-1	R02557 (1:250) – Polyclonal R02561 (1:250) – Polyclonal	Antirabbit IgG, HRP-linked (1:25000 dilution)

	Genscript – Custom antibody	
RasEF	RasEF (1:2000) – Polyclonal Proteintech cat# 11569-1-AP	Antirabbit IgG, HRP-linked (1:25000 dilution)
Rhot1/Miro1	Rhot1 (1:100) – Polyclonal Novus Biologicals, NBP1-59022	Antirabbit IgG, HRP-linked (1:25000 dilution)

5. Reverse Transcriptase PCR to generate cDNA

cDNA was generated using total RNA collected from contact inhibited CEF, and kindly provided by Ronnie Moftakhari Hajimirzaei, reverse transcriptase (NewEngland Biolabs, M0277), and primers flanking GEM1 (NC_052533.1) ORF. The cDNA was then resolved on 1.0 % agarose gel using gel electrophoresis to compare the size of cDNA and known ORF of GEM1. The PCR program in Table 2, and primer set in Table 3 was used for reverse transcription reaction and PCR amplification.

Table 2. Reverse transcriptase PCR thermocycler program for generating cDNA.

Step 1	RT reaction	48°C	30 mins	1 cycle
Step 2 (PCR)	Initial Denaturation	94°C	1 min	1 cycle
	Denaturation	94°C	15 sec	40 cycles
	Annealing	54°C	30 sec	40 cycles
	Extension	68°C	2 mins	40 cycles

	Final extension	68°C	5 mins	1 cycle
	Hold	4°C	∞	

Table 3. Primer sets flanking GEM1 ORF (NC_052533.1).

Primer Set	Primer Sequence
Set 1 Forward	5' ACG AGT ACA ATG TGC AGA AGA G 3'
Set 1 Reverse	5' CCA AAG GTG AGT GGA GAA GAA A 3'
Set 2 Forward	5' GTG CAG AAG AGA TAC ACC ATC A 3'
Set 2 Reverse	5' ATG CTT TCC CAG CAC AAT TTC 3'
Set 3 Forward	5' TAT GAC CCT CAA CAA CGT TAC C 3'
Set 3 Reverse	5' CAT GCA CTG TGC TGT TCT TAT G 3'
Set 4 Forward	5' CAT CAG CCC TGA CGA ATA CTA C 3'
Set 4 Reverse	5' CCA GCA CAA TTT CGG ATC ATT C 3'
Set 5 Forward	5' GGT CGC ACA CAA GGG TAA A 3'
Set 5 Reverse	5' TGT TCT TAT GTC AGC ACG TAT CA 3'

6. Generating GEM1 shRNAi construct

6.1 Oligo design for shRNA

Genscript website (<https://www.genscript.com/ssl-bin/app/rnai>) was used for generating potential shRNA that can downregulate GEM1 expression. Table 4 shows the top 4 DNA sequence of shRNA with the highest probability of succeeding which were used to design oligos.

The following schematic was used to design an oligo sequence for amplifying DNA sequence of *GEM1* shRNA. The 22 bp represented by N are the 21bp of shRNA sequence + 1bp extra ahead of the generated shRNA sequence. Note that the first nucleotide of 22mer in the reverse oligo stays the same, but it was changed to an incompatible nucleotide in the forward oligo (A→G, G→A, C→T, T→C).

59mer Target Sequence Forward for 1st site:

5' GAG AGG TGC TGC TGA GCG NNN NNN NNN NNN NNN NNN NNN NTA GTG AAG
CCA CAG ATG TA 3'

58mer Target Sequence Reverse for 1st site:

5' ATT CAC CAC CAC TAG GCA NNN NNN NNN NNN NNN NNN NNN NTA CAT CTG
TGG CTT CAC T 3'

The PCR program in Table 5 and primer sets in Table 6 was used to amplify the DNA sequence of *GEM1* shRNA. ProofStart DNA polymerase (Qiagen, P4218L) was used.

Table 4. DNA sequence of *GEM1* shRNA.

	DNA Sequence of <i>GEM1</i> shRNA
GEM1 T1-shRNA	CAAGTCCAAGTCTTGTCAGTAC
GEM1 T2-shRNA	GAAGGCATCGTGAGGCAAGTGC
GEM1 T3-shRNA	CAAGTCGTCACTGGCCAACATC
GEM1 T4-shRNA	GAAGCGGCTGGCTTACCAGAAA

Table 5. PCR program for amplification of 59mer/58mer Target Sequence.

Initial Denaturation	95°C	5 min	1 cycle
Denaturation	94°C	30 secs	25 cycles
Annealing	55°C	30 secs	25 cycles
Extension	72°C	45 secs	25 cycles
Final extension	72°C	10 mins	1 cycle
Hold	4°C	∞	

Table 6. Primer sets flanking *GEM1* shRNA cassette in 59mer and 58mer Target Sequence.

Primer Set	Primer Sequence
Hairpin 5' (ML18662P) pR_NheI_5	5' ATACTAGCGGCCGCATAAAGT 3'
Hairpin 3' (ML18663P) pR_MluI_3	5' GGATCCATCGATAAAAAAGCT 3'

6.2 Cloning *GEM1* shRNA cassette into plasmid

The following method for cloning is heavily influenced by Das and colleagues who first modified the following RNAi system to be effective in the chicken model (Das et al., 2006). This RNAi system was later demonstrated to be effective in CEF by Wang and colleagues (L. Wang et al., 2011). Double digestion reaction was performed to generate *GEM1* shRNA cassette DNA fragments, pRFPRNAiC(U6-) plasmid, and RCASBP(A)RNAi plasmid with sticky ends. 25µg of *GEM1* shRNA cassette DNA fragment was digested, and 3µg of pRFPRNAiC(U6-) were digested using NheI (NewEngland Biolabs, R3131L) and MluI (NewEngland Biolabs, R0198S) restriction enzymes. NotI (NewEngland Biolabs, R3189S) and ClaI (NewEngland Biolabs,

R0197S) restriction enzymes were used to digest pRFPRNAiC (U6-) and RCASBP(A)RNAi plasmid. The digestion reaction was performed at 37°C overnight. Following digestion, only the plasmids were subjected to CIP reaction to dephosphorylate 5' and 3' ends to prevent spontaneous ligation using QuickCIP enzyme (NewEngland Biolabs, M0525S). CIP reaction was performed at 37°C for 15 mins followed by heat inactivation at 80°C for 20 mins. Dephosphorylated plasmid and digested fragments were purified using QIAquick PCR purification Kit (Qiagen, cat# 28104) to remove free nucleotides and enzyme. Once the desired fragment and plasmid were digested, dephosphorylated, and purified, they were ligated using T4 DNA ligase (NewEngland Biolabs, M0202S) at 16°C overnight.

6.3 Gel Extraction

Monarch DNA Gel extraction Kit (NewEngland Biolabs, T1020S) was used to extract MOEC DNA fragment from 1.5 % agarose gel following gel electrophoresis. pRFPRNAiC (U6-) plasmid containing GEM1 shRNA cassette was double digested using NotI and ClaI restriction enzymes which excise the MOEC from the plasmid. Subsequently the DNA sample was resolved on 1.5% agarose gel to separate the MOEC fragment from the rest of the plasmid. The gel extraction kit was used to extract the MOEC DNA from the gel. The MOEC fragment was later sub-cloned into RCASBP(A)RNAi plasmid.

6.4 Transformation and Plasmid DNA prep.

Once the GEM1 shRNAi construct had been created, it was transformed into DH5 α competent cells (Invitrogen, cat# 18265-017). The DH5 α competent cells were heat shocked at 42°C for 45 secs in the presence of the shRNAi construct. Subsequently they were allowed to recover and reproduce in LB broth at 37°C then plated on LB-Ampicillin agar plates and

incubated at 37°C overnight. Ampicillin was used to select for DH5 α competent cells that were successfully transformed since the shRNAi construct hosted ampicillin resistance gene.

To collect small or large quantities of shRNAi construct plasmid-DNA, the single colonies of the transformed DH5 α competent cells were cultured in LB broth. Subsequently EZ-10 Spin column Plasmid DNA Minipreps Kit (BioBasic, BS614-250PREPS) or Plasmid Midi Kit (Qiagen, cat# 12143) was used to collect purified plasmid-DNA.

7. shRNAi Vector Transfection

7.1 DNA precipitation

A total of 30 μ g of DNA was precipitated one day before the transfection. 10 μ g of plasmid construct along with 20 μ g of salmon sperm carrier DNA was precipitated using abs. EtOH in the presence of 0.2M NaCl. The mixture was left to precipitate overnight at -20°C. The following day the mixture was centrifuged at 14, 800 RPM (24, 532 RCF) for 15 minutes at 4°C. The supernatant was discarded, and the DNA pellet was washed with 70% EtOH. The mixture was centrifuged again at 14, 800 RPM (24, 532 RCF) for 5 minutes at 4°C. The supernatant was discarded, and the DNA pellet was vacuum dried for 2 minutes. The DNA pellet was resuspended in 200 μ L of ddH₂O.

7.2 Calcium Phosphate Transfection

For transfection, CEF at 50-70% confluence were first stimulated with fresh, serum-containing medium on the day of the transfection to promote proliferation and integration of the retroviral genome prior to the transfection. Resuspended DNA was mixed with 2M CaCl₂ as per Graham & van der Eb's protocol (Graham & van der Eb, 1973). This mixture was added

dropwise to 500 μ L of 2X HBSP (1.5 mM Na₂HPO₄, 10 mM KCL, 280 mM NaCl, 12 mM Glucose, 50 mM HEPES, pH 7.12) using a low-speed vortex. The mixture was incubated for 20-30 minutes at room temperature to promote DNA precipitate. The mixture was then added dropwise onto the CEF cell culture and incubated for 4-6 hours. Meanwhile, the glycerol shock solution (15% sterile glycerol, 50% 2X HBSP, 35% ddH₂O) was prepared and warmed. Following the 4-6 hrs incubation, the cells are treated with glycerol shock solution to promote uptake of DNA. The cells were then washed twice with complete medium followed by incubation in 8mL complete medium overnight. The next day the cells were passaged. The cells were passaged a total of 3 times after transfection before being using for experiments. Downregulation of GEM1 by the shRNA retrovirus was examined by western blot analysis.

Results

Chapter 1: Characterizing the expression of GEM1 in quiescent CEF.

i. GEM1

Gene profiling of growth arrested CEF showed that *GEM1* transcripts were highly induced by contact inhibition compared to proliferating cells but GEM1 transcript levels may not reflect the expression of GEM1 at the protein level (Erb et al., 2016). So, to determine if this quiescence specific increase in GEM1 transcript is reflected at the protein level, SDS-PAGE-western blot analysis was performed. GEM1 is a small monomeric GTPase encoding a 297aa protein weighing 35 kDa (Yoshizawa et al., 2004)(NIH Gene ID: 404771). Western blot analysis of CEF cultured in normoxia (21% O₂), hypoxia (1.8% O₂), high cell density, or serum deprived medium confirms that GEM1 has greater induction in contact inhibited CEF compared to

normoxic CEF, while expression of GEM1 is much less in hypoxic and serum starved CEF compared to normoxic CEF (Figure 4A). The GEM1 antibody detects two prominent signals, ~75 kDa and ~35 kDa. The 75 kDa signal largely follows the same expression pattern as the 35 kDa where the protein has greatest induction in contact inhibited followed by hypoxic and serum starved CEF compared to normoxic CEF which has the least amount of protein expression (Figure 4B). The 35 kDa signal corresponding to GEM1 is observed to be expressed more in contact inhibited CEF compared to normoxic CEF (Figure 4B). Both hypoxic and serum starved CEF have less GEM1 expression than normoxic CEF, where there is very little expression in hypoxic CEF and little to no expression in serum starved CEF (Figure 4B). Induction of p20K expression in quiescent inducing conditions confirms that quiescence was successfully induced and that the CEF were expressing growth arrest specific genes (Figure 4A). These results along with similar replicated results (data not shown), using two different rabbit polyclonal GEM1 antibodies (R02557 & R02561), confirms that GEM1 expression is regulated as a growth arrest specific gene in CEF.

Different experiments that examined the effectiveness of GEM1 shRNA showed a different expression pattern of GEM1. Here CEF were transfected with parental RCASBP(A) vector (control virus) which should not affect the expression of GEM1. The transfected CEF were cultured in conditions of normoxia or hypoxia. Interestingly, there was a modest induction of the 35 kDa signal (GEM1) in hypoxia but little expression in normoxia (Figure 12A,B). The 75 kDa signal on the other hand had strong and equal expression in both normoxia and hypoxia (Figure 12A,B). This result is different from the previous result where the 35 kDa GEM1 signal was observed prominently in normoxia but less in hypoxia, and the only variable that changed in the

latter experiment was that the CEF were transfected with control virus which in theory should not affect the expression of GEM1.

A separate experiment conducted by Emmerson Teas, who examined extracellular vesicles (EV) (Figure 5). Here she detected a protein (~25 kDa) using GEM1 antibody that is packaged more in EVs of contact inhibited than normoxic CEF. There was also a secondary signal (~30 kDa) which had a strong basal level in EVs of normoxic CEF and slightly more protein in EVs of contact inhibited CEF (Teas E., BSc. Thesis, 2022). Other conditions of quiescence (hypoxia or serum starvation) were not examined. Additionally, a loading control was not used since there is no previously established loading control for EVs in chicken embryo fibroblasts.

ii. RasEF

The antibodies used to probe for GEM1 were custom made using a GEM1 peptide as the antigen. The peptide (antigen) was analyzed carefully to avoid using a conserved sequence that shares similarity with other proteins, ensuring that the antigen used was specific to GEM1. Additionally, Genscript immune-purified both of the GEM1 antibodies to further ensure the antibodies are specific to GEM1. Nevertheless, it is always possible that the GEM1 antibody may recognize a similar epitope in other proteins which could be why we observed a secondary signal at higher molecular weight (~75 kDa). One of the approaches we took to identify the 75 kDa protein was to examine other monomeric GTPases that share structural similarity with GEM1. GEM1 is a GTPase so the antibody may recognize conserved regions in other GTPases, therefore, to investigate the origins of that unknown higher molecular weight signal, we examined a known large GTPase (RasEF). RasEF is a large Rab GTPase which facilitates dynein-mediated transport and dynein-dynactin motility on microtubules (Y. Wang et al., 2019). It contains two EF-hand domains, several coil-coiled domains (CCD), and a C-terminus Rab

GTPase domain (Figure 1) (Shintani et al., 2007). RasEF is one of the few large proteins, at ~83 kDa, in this family. It is observed to have a doublet signal due to the two isoforms (RasEF-long and RasEF-short) with roughly the same size, similar to the unknown signal (Shintani et al., 2007). Western blot analysis of RasEF expression in CEF cultured in normoxia (21% O₂), hypoxia (1.8% O₂), high cell density, or serum deprived medium confirmed that RasEF is expressed constitutively in normoxia and quiescence inducing conditions (Figure 6A). Two prominent signals can be identified, ~150 kDa and ~250 kDa. Both of these signals have the greatest expression in normoxic, followed by contact inhibited, hypoxic, then serum starved CEF (Figure 6B). Induction of p20K expression in quiescence inducing conditions confirmed that quiescence was successfully induced and that the CEF were expressing growth arrest specific genes (Figure 6A). The expression pattern of RasEF does not match that of a growth arrest specific gene, confirming that RasEF is not a GAS gene, and it is certainly not the high molecular weight GEM1 protein detected by our polyclonal antibodies.

iii. Rhot1/Miro1

After discovering that the unknown signal in GEM1 was not RasEF, we examined another large GTPase (Miro1). Miro1 is about 600aa long and observed at 70 kDa which also has similar structures as GEM1. Miro1 is a Rho GTPase. It has two GTPase domains (one at N-terminus and one at C-terminus), two EF-hand domains (EFD) (Figure 2) (Kawasaki et al., 1998; Nelson & Chazin, 1998). Miro1 interacts with adaptor protein Milton, which connects mitochondria to motor proteins that facilitates mitochondrial movement (Glater et al., 2006). Miro1 is also regulated by cytosolic Ca²⁺ concentration (Chang et al., 2011; X. Wang et al., 2009). Western blot analysis of Miro1 expression in CEF cultured in normoxia (21% O₂), hypoxia (1.8% O₂), high cell density, or serum deprived medium showed that Miro1 is expressed in normoxia and

serum starvation but not in hypoxia or contact inhibition (Figure 7A). The dominant signal is present at ~30 kDa, with the most expression in normoxic and contact inhibited CEF, with less but still prominent expression in hypoxic and serum starved CEF (Figure 7B). The 30 kDa signal is a result of a previously reported non-specific binding of the Miro1 antibody (Figure 7A) (NovusBio, NBP1-59022, 2023). The Miro1 signal at 70 kDa is only observed in normoxic and serum starved CEF with nearly the same level of expression, while there is zero expression in contact inhibited and hypoxic CEF (Figure 7A). Normoxic CEF has the greatest induction of Miro1 closely followed by serum starved CEF (Figure 7B). Induction of p20K expression in quiescence inducing conditions confirmed that quiescence was successfully induced and that the CEF were expressing growth arrest specific genes (Figure 7A). Analyzing the expression pattern of Miro1, it is evident that Miro1 does not follow the expected expression pattern of a GAS gene, nor does it match the expression pattern of the higher molecular weight signal in GEM1.

iv. GEM1 cDNA

Analyzing the expression pattern of GEM1 showed two prominent signals (75 kDa and 35 kDa), although only the 35 kDa signal was expected to be present based on GEM1 protein and ORF. The 75 kDa signal could potentially be a GEM1 isoform, a result of alternative splicing. Therefore, identifying isoforms of GEM1 could prove to be beneficial in determining the origins of the higher molecular weight signal. One of the simplest methods to identify isoforms is to analyze the cDNA generated from mRNAs. It was assumed that alternatively spliced transcripts of GEM1 would share the same 5'UTR (including the translational start site), and 3'UTR, so primers were designed to target that region. Reverse transcriptase PCR with the use of primers targeting the 5' and 3' UTR of GEM1 mRNA was performed on total RNA collected from CEF. The resulting cDNA was resolved on agarose gel (Figure 8). Five different primer sets (lanes 2-

6) were used, each showing the cDNA that is roughly 1kb in size. No other signal was observed which confirms that there is only one isoform of GEM determined solely based on size which matches with the known size of the GEM1 ORF.

Chapter 2: Cloning GEM1 shRNA cassette in RCASBP(A) to create a shRNA retroviral construct.

i. PCR amplification of DNA fragments of GEM1 shRNA

The shRNA targets were determined using Genscript siRNA target finder (<https://www.genscript.com/ssl-bin/app/rnai>). The shRNA target sequences were used to design short DNA sequences which encoded shRNA sequences flanked by primer binding sites to amplify the shRNA sequences. The top four siRNA targets were selected for further experimentation. A common primer set (forward and reverse) was used to amplify the shRNA containing DNA sequences in addition to the flanking regions containing NheI and MluI restriction sites. The shRNA sequences will then be cloned into a microRNA cassette in the pRFPRNAiC vector at the NheI/MluI cloning sites. The microRNA cassette (containing the shRNA sequence) will then be sub-cloned into the retroviral vector, RCASBP(A), at the NotI/ClaI cloning site. The first step was to amplify the shRNA sequences prior to cloning it into microRNA cassette. The amplified shRNA sequences were resolved on 1.0% agarose gel (Figure 9). Three out of four candidate shRNA (lanes 1-3) were successfully amplified as evident by the ~200bp signal. These amplified DNA sequences (containing shRNA) will be used to clone GEM1 shRNA into the transfer vector (pRFPRNAiC U6-). It should be noted that the *GEM1* T4 shRNA target failed to amplify (data not shown).

ii. Cloning GEM1 shRNA cassette into pRFPRNAiC (U6-)

GEM1 shRNA DNA fragments need to be cloned into the retroviral vector RCASBP(A) so that when the CEFs are transfected with the vector, they will express the GEM1 shRNA from within retroviruses capable of infecting other cells. When cloning PCR DNA fragments into a large vector like the RCASBP(A), the cloning efficiency is dramatically decreased. Therefore, to overcome this barrier, the GEM1 shRNA DNA sequence is first cloned into a smaller transfer vector pRFPRNAiC (U6-). It is a relatively small plasmid compared to RCASBP(A). pRFPRNAiC (U6-) contains β -actin promoter, *RFP* gene, polyadenylation site, and the microRNA operon expression cassette (MOEC). The MOEC contains two unique cloning sites (only one is required for this study) under the control of chicken U6 promoter (Das et al., 2006). Although, our lab has modified the vector to remove the U6 promoter, instead the expression of MOEC will be controlled by retroviral LTR when subcloned into RCASBP(A) (L. Wang et al., 2011). To clone DNA fragments into pRFPRNAiC (U6-), *GEM1* T1, T2, and T3 shRNA DNA fragments, along with the pRFPRNAiC (U6-) transfer vector, underwent digestion reaction using *NheI* and *MluI* restriction enzymes. Following digestion reaction, the fragments and plasmid have compatible sticky ends allowing for directional cloning into the pRFPRNAiC (U6-) vector. The digested pRFPRNAiC (U6-) plasmid then underwent a CIP reaction to dephosphorylate the 5' and 3' ends of DNA, preventing re-ligation of the linearized plasmid. Subsequently, the digested *GEM1* shRNA DNA fragments and the digested-CIP pRFPRNAiC (U6-) plasmid were ligated with T4 DNA ligase. The ligated pRFPRNAiC-T1/2/3 transfer vectors were then transformed into DH5 α competent cells.

Plasmid DNA was isolated from the colonies of the transformed DH5 α cells. The isolated plasmid DNA was then digested again, but now with *NotI* and *ClaI* restriction enzymes which

flank the microRNA operon expression cassette (MOEC) which encodes for GEM1 shRNA and resolved it on a 1.0% agarose gel (Figure 10). Lanes 1-6 show digested plasmid DNA isolated from six different colonies of DH5 α cells transformed with pRFPRNAiC-T1 GEM1 transfer vector. Lane 7 shows digested DNA of the parental pRFPRNAiC (U6-) transfer vector. Lanes 8-11 represent digested plasmid DNA isolated from four different colonies of DH5 α cells transformed with pRFPRNAiC-T2 GEM1 transfer vector.

The DNA fragment (~250bp) seen after digesting pRFPRNAiC-T1/T2 GEM1 with NotI and ClaI is larger than the fragment (~150) from parental pRFPRNAiC (U6-) (control). This provides evidence that the *GEM1* T1/T2 shRNA cassette was successfully cloned into the pRFPRNAiC (U6-) transfer vector. It should be noted that DH5 α competent cells transformed with pRFPRNAiC-T3 GEM1 failed to yield any colonies.

iii. Cloning GEM1 shRNA cassette into RCASBP(A) expression vector.

Once the GEM1 shRNA DNA fragment is cloned into MOEC of the transfer vector pRFPRNAiC (U6-), it is now ready to be sub-cloned into the retroviral vector RCASBP(A). DH5 α competent cells transformed with pRFPRNAiC-T1/2 GEM1 were cultured in LB to collect large amounts of plasmid DNA using Qiagen plasmid Midi kit. Subsequently, the plasmid is digested using NotI and ClaI restriction enzymes to excise the MOEC (encoding *GEM1* T1/2 shRNA) from the pRFPRNAiC (U6-) transfer plasmid. The resulting digested product is then resolved on 1.5% agarose gel. The MOEC fragment (~250bp) is excised and purified from the agarose gel using the Monarch DNA gel extraction kit (NewEngland Biolabs). Meanwhile, the RCASBP(A) plasmid is also digested using NotI and ClaI restriction enzymes, followed by a CIP reaction. Same as before, digesting at two different restriction sites results in compatible sticky ends for the plasmid and DNA fragments. Next, the digested-CIP RCASBP(A) plasmid and the

digested MOEC fragment were ligated (sticky end) together using T4 DNA ligase. The ligated RCASBP(A)-T1/2 GEM1 RNAi plasmid is then transformed into DH5 α competent cells.

Plasmid DNA is isolated from the colonies of the transformed DH5 α cells. The isolated plasmid DNA is subsequently digested again with NotI and ClaI restriction enzymes and resolved on a 1.5% agarose gel (Figure 11). Lanes 1-5 represent digested plasmid DNA isolated from five different colonies of DH5 α cells transformed with RCASBP(A)-T1 GEM1 RNAi plasmid. Lane 6 shows digested DNA of the parental RCASBP(A) plasmid (control). Lanes 7-11 correspond to digested DNA isolated from five different colonies of DH5 α cells transformed with RCASBP(A)-T2 GEM1 RNAi plasmid.

The DNA fragment (~250bp) seen after digesting RCASBP(A)-T1/T2 GEM1 RNAi with NotI and ClaI is larger than the fragment (~150bp) from the control parental RCASBP(A) vector. This result provides evidence that the MOEC containing *GEM1* T1/T2 shRNA sequence was successfully cloned into the retroviral RCASBP(A) vector.

Chapter 3: Downregulating GEM1 expression in Quiescent CEF

i. Validating GEM1 shRNAi construct

Once the GEM1 shRNAi construct has been created, it is utilized to transfect CEF using Calcium Phosphate transfection technique (Graham & van der Eb, 1973). The transfected CEF are then exposed to quiescence inducing condition, hypoxia, to induce GAS gene expression. Western blot analysis of GEM1 expression in transfected CEF cultured in hypoxia and normoxia reveals that both GEM1 shRNA (T1 and T2) are capable of downregulating GEM1 expression in hypoxic CEFs, although not completely (Figure 12). GEM1-T1 shRNA is capable of

downregulating GEM1 expression by a factor of 3 compared to control (parental RCASBP(A)) viruses while GEM1-T2 shRNA is capable of downregulating by a factor of 2 compared to control (Figure 12B). GEM1 expression was also examined in normoxic CEF transfected with GEM1 shRNAi construct. It is interesting to note that the ~35 kDa signal of GEM1 was expressed in hypoxia only, while the ~75 kDa signal was expressed in normoxia and hypoxia. Additionally, both GEM1 shRNA were only able to downregulate the 35 kDa signal (GEM1) while having no effect on the 75 kDa signal, suggesting that the 75 kDa protein is not GEM1 or that the mRNA corresponding to that signal does not contain the sequences targeted by either of the shRNA. It may share an epitope with GEM1, but it is likely not related to GEM1. This provides evidence that the higher molecular weight signal does not behave like a GAS gene and further strengthens that GEM1 (35 kDa) is a GAS gene. It further provides evidence that the GEM1 shRNA (T1,T2) are capable of downregulating GEM1 expression to a certain degree. Induction of p20K expression in hypoxia confirms that quiescence was successfully induced and that the CEF were expressing growth arrest specific genes (Figure 12A).

Discussion

Chapter 1: Characterizing GEM1 in quiescent CEF.

Microarray analysis of quiescent CEF has shown that *GEM1* is upregulated 18-fold at the RNA level in condition of high cell density compared to proliferating CEF (Erb et al., 2016). These data suggest that *GEM1* is a GAS gene. While analyzing gene expression at the transcriptional level provides valuable insights into which genes are upregulated in response to a stressor, it falls short of capturing the intricate regulatory mechanisms and dynamic processes that shape the proteome. A comprehensive understanding of gene function necessitates the

integration of transcriptional, post-transcriptional, and post-translational data. Examining the expression of GEM1 at the protein level provides better understanding of how GEM1 is involved in quiescence inducing conditions. Western blot analysis confirmed that GEM1 (35 kDa) has increased expression in quiescence inducing conditions of contact inhibition compared to proliferating CEF (Figure 4). It was noted that GEM1 has a basal level expression in proliferating CEF; accounting for that, GEM1 still has increased expression in contact inhibited CEF, and more modest expression in hypoxic and serum starved CEF. The expression pattern of GEM1 is similar to other well characterized GAS genes, p20K and FABP4, in quiescent CEF (Erb et al., 2016). This evidence supports *GEM1* candidacy as a GAS gene.

Most notably there is an unexpected doublet signal consistently observed at higher molecular weight (75 kDa) (Figure 4A). This signal was observed to have increased expression in quiescence inducing conditions of contact inhibition, hypoxia, and serum starvation (Figure 4B). This shows that the unidentified signal follows a gene expression pattern similar to that of a GAS gene. The origin of this signal still remains to be resolved but identifying it could contribute to characterizing GEM1 or discovering a new member of the GAS gene family. The GEM1 antibody used is a polyclonal antibody which was custom made for the purpose of this study. Two GEM1 antibodies were made, R02557 and R02561, each with a different set of GEM1 peptides as an antigen. The antigens sequences were examined prior to making the antibody to ensure that the peptides don't share similarities with proteins other than GEM1. Additionally, the antibodies were immuno-purified to select for antibodies specific to GEM1 peptides. Each GEM1 antibody produced the same result, albeit R02557 had more non-specific binding i.e., background noise, than R02561. One of the simplest explanations for the unknown signal could be that there is non-specific binding of the GEM1 antibody which recognizes a

different protein altogether. The polyclonal nature of the GEM1 antibodies has the potential to recognize multiple epitopes on the target antigen. It's plausible that some of these epitopes may share structural similarities with other proteins. As a consequence, polyclonal antibodies might exhibit cross-reactivity, recognizing similar epitopes present in unrelated proteins.

The presence of GEM1 in extracellular vesicles (EV) is a novel discovery (Figure 5) (Teas E., BSc. Thesis, 2022). Interestingly, the higher molecular weight protein (75 kDa) was not observed in the EVs, but there was a secondary protein at ~25 kDa along with the GEM1 protein (~30 kDa). The 25 kDa protein exhibited a stronger quiescence specific expression compared to the 30 kDa protein. The migration of proteins on SDS-PAGE can vary based on the post-translational modifications of the proteins, so the discrepancy in the observed size of GEM1 can be explained but it also makes it difficult to distinguish the true GEM1 signal from the two bands that were observed. Assuming that the ~30 kDa signal corresponds to GEM1, it raises the question of the origin of the other ~25 kDa protein. This ~25 kDa protein was never previously observed in the cell lysate yet it exhibits an expression pattern similar to GAS genes, one possible explanation could be that proteins associated with EVs have post-translational modifications, or it is a product of degradation associated with the EV isolation procedure. Additionally, an important factor to consider is that a loading control was not used for this experiment. ERK2 is a well-established loading control for cell lysate samples in CEF but there is no appropriate loading control for EVs in CEF. This raises the question whether or not the samples used by Teas E. were loaded evenly for normoxia and contact inhibited samples. Despite using Bradford assay to measure protein concentration and ensure the proteins are evenly loaded, there is still a possibility where the proteins were not evenly loaded on the gel and the differential “packaging” of GEM1 in EVs is just an artifact. Knowing that GEM1 is packaged into EVs, it is

possible that when we analyzed GEM1 in protein lysate, and due to the nature of the procedure for collecting protein lysate, we would have missed a significant fraction of GEM1 which was packaged into the EVs. This raises the question whether the observed expression of GEM1 in protein lysate is an accurate representation of the total expression of GEM1.

GEM1 is known to play a role in regulating cytoskeleton reorganization, which could be why we observe GEM1 in EVs (Ward et al., 2002). Cytoskeleton reorganization is crucial for the formation and release of EVs (Chen X. and Williams J.A., 2004). Given that GEM1 regulates cytoskeleton reorganization, and it is present in EVs, suggests that it may play an indirect role in regulating EV formation and/or release (P. Beguin et al., 2001). Although a closer examination of GEM1 in EV formation and release is required to better understand the mechanism by which it does that. A future experiment could examine whether the release of EVs is impacted by the knockdown of GEM1. There are several proteins identified in our lab that are exclusively observed in EVs (ANGPTL5, Osteocrin, PKC η , etc), thus they can be used as markers for EVs. If these markers are observed to accumulate within the cell upon GEM1 downregulation, it would suggest that GEM1 plays an important role in release of EVs.

To identify the 75 kDa protein, two main experimental strategies were utilized. First, reverse transcriptase – PCR was used to identify isoforms of GEM1 that could potentially contribute to the 75 kDa protein. The second strategy was along the line of “educated guess”, where we identified two proteins that could account for the higher molecular weight signal. They were also structurally similar to GEM1 and were regulated by Ca²⁺/Calmodulin (similar to GEM1).

One of the explanations for observing a secondary signal in GEM1 could be that GEM1 has multiple isoforms caused by alternative splicing or other post-transcriptional and/or post-translational modification. There are no known isoforms of GEM1 in the current literature, albeit there is no literature about GEM1 in the context of growth arrest. It is likely that induction of growth arrest that increases expression of GEM1 may also contribute to other isoforms of GEM1 via post-transcriptional and/or post-translational modification. To test whether there are isoforms of GEM1 due to alternative splicing, we generated GEM1 cDNA and analyzed it on agarose gel (Figure 8). Five different sets of primers were used, which flanked the known ORF of *GEM1*. Using primer sets that target the 5' and 3' UTR ensures that these primer sets are capable of detecting any potential mRNAs of GEM1 isoforms. This assumed that the 5' and 3' UTR are shared among all potential mRNAs of GEM1 isoforms, where the only difference is the arrangement of exons as a result of alternative splicing. For all five primer sets only one isoform of GEM1 was detected, as evident by the presence of only one band. The size of the cDNA detected was about 1 kb which is similar to the known size of GEM1 coding sequence (893 bp). The small discrepancy between the size of the GEM1 coding sequence and the cDNA is due to the primer sets targeting the 5'/3' UTR and not directly targeting the ORF, which accounts for additional nucleotides. The generated cDNA was not sequenced since its size matched the known size of GEM1 coding sequence, so it was assumed that this cDNA sequence would be the same as the known sequence of GEM1. Nevertheless, only one isoform of GEM1 was detected, and based on its size it could only encode for the 35 kDa protein of GEM1. This limits the possibility that the higher molecular weight protein observed could have been from an isoform of GEM1. One of the limitations of this experiment is that there is always a possibility that there is additional exon/s upstream of the 5' primers or downstream of the 3' primers that contributes to

alternatively spliced isoform. The last exon of the 3' end is generally the same in different isoforms of most proteins, thus the primers targeting the 3' UTR may have been sufficient to encompass most of the possible isoforms. While the primers targeting the 5' UTR could have missed additional potential exon/s upstream of their targets. Additional primer pairs targeting the upstream regions could have been used to address this question.

For the second approach to identify the 75 kDa protein, RasEF was one of the proteins identified to be similar to GEM1. RasEF is a large Rab GTPase (~83 kDa) which is implicated in dynein-mediated transport (Y. Wang et al., 2019). RasEF contains a GTPase domain, two EF-hand domains, and several coil-coiled domains (Shintani et al., 2007). The GTPase domain has a typical function, similar to other GTPases. The EF-hand domains are involved in Ca²⁺ binding which facilitates interaction with dynein-dynactin, crucial for dynein-mediated transport (Y. Wang et al., 2019). Although GEM1 is not entirely similar to RasEF, it does have some similarity such as a GTPase domain, and it is known to bind to Ca²⁺/Calmodulin. Comparing the protein sequence of GEM1 and RasEF using BLAST, it can be noted that there is 29% similarity between them within their respective GTPase domain, with an Expect value (E-value) of 2e-18. Additionally, RasEF is known to be expressed as two isoforms, RasEF-S and RasEF-L (Shintani et al., 2007). Given this similarity between GEM1 and RasEF, and the expression pattern of RasEF in the literature matching the expression pattern of the unknown higher molecular weight signal, it can be suggested that the GEM1 antibodies may recognize RasEF. Western blot analysis of RasEF using commercially available RasEF polyclonal antibody illustrates that it is expressed in quiescent (contact inhibited, hypoxic, and serum starved) CEF as well as proliferating CEF. However, RasEF has the highest expression in proliferating CEF followed by contact inhibited, hypoxic, and serum starved cells (Figure 6). It is also observed at a higher molecular weight

(150-250 kDa) than the unknown signal, which is observed around 75 kDa. This suggests that RasEF is not a GAS gene, because it doesn't match the expression pattern and size of the unknown signal. Despite the promising similarity between RasEF and GEM1, this rules out the possibility that RasEF is the identity of the unknown signal, prompting further exploration to identify the origin of the unknown signal.

Additionally, Miro1 was another protein identified to be similar to GEM1. Miro1 is a large Rho GTPase (~70 kDa) that helps facilitate mitochondrial movement using motor proteins (Glater et al., 2006). Miro1 has two GTPase domains and two EF-hand domains. The N-terminal GTPase domain is conserved and similar to other GTPase domains in Rho GTPases, while the C-terminal GTPase domain has diverged, and its role is not fully understood (Fransson et al., 2003; Kawasaki et al., 1998; Nelson & Chazin, 1998). The EF-hand domains are known to bind Ca²⁺ ions which plays a regulatory role in mitochondrial transport (Chang et al., 2011; X. Wang et al., 2009). Much like the resemblances between GEM1 and RasEF, there are also structural similarities between GEM1 and Miro1. Comparing the protein sequence of GEM1 and Miro1, it can be noted that there is 31% similarity between them within a small region. The GTPase domain of GEM1 aligns well with the N-terminal GTPase domain of Miro1 with an E-value of 3e-10. Additionally, the commercially available antibody for Miro1 by Novus Biologicals (NBP1-59022), has some non-specific binding around 35 kDa which is suspiciously similar to the GEM1 signal observed using GEM1 antibody (Figure 4A). Given the similarities between GEM1 and Miro1, the unknown signal observed could be caused by non-specific binding of GEM1 antibody to Miro1. Western blot analysis of Miro1 showed that the most prominent signal observed was the ~35 kDa (lower) signal while the Miro1 signal (~75 kDa) was barely observed in proliferating, and serum starved CEF only (Figure 7). Meanwhile, the 35 kDa signal was

strongly observed in proliferating, and quiescent CEF, with the highest expression in proliferation CEF followed by contact inhibited, hypoxic, and serum starved CEF. This mismatch between the expression pattern of 75 kDa and 35 kDa signal in Miro1 and GEM1 confirms that the unknown high molecular weight signal in GEM1 is not a result of GEM1 antibody recognizing Miro1, ruling out Miro1 as a candidate for the high molecular weight signal in GEM1. Additionally, this experiment also confirms that Miro1 is not a GAS gene since its expression pattern does not match with the expected expression of a GAS gene.

Chapter 2: Cloning GEM1 shRNA cassette in RCASBP(A) to create a shRNA retroviral construct.

Based on the increased expression of GEM1 in quiescence inducing condition, such as contact inhibition, it is characterized as a GAS gene. The specific role that GEM1 plays in quiescence is still unknown. Constructing a retroviral vector (RCASBP(A)) that expresses GEM1 shRNA which downregulates GEM1 expression would be a valuable molecular tool to investigate the role of GEM1 in quiescent CEF. The short DNA fragments encoding GEM1 shRNA were first cloned into a transfer plasmid (pRFPRNAiC) prior to sub-cloning into the retroviral vector (RCASBP(A)). Specifically, the GEM1 shRNA DNA fragments were first directionally cloned into the MOEC within the pRFPRNAiC plasmid. The MOEC was then directionally sub-cloned into the retroviral RCASBP(A) vector downstream of the viral LTR region using NotI & ClaI. The benefits of first cloning into a transfer vector have few advantages. First, it is easier to manipulate the smaller vector than the larger RCASBP(A) vector. Second, it allows for directional cloning of the shRNA sequence into the microRNA cassette at cloning site NheI/MluI. Third, it provides cloning sites, NotI/ClaI, for directional cloning of the

microRNA cassette into RCASBP(A), which only have these two cloning sites. Cloning the shRNA sequence in the context of a microRNA cassette allows for a more efficient expression/processing of small RNAs, it also allows for a more efficient expression of the shRNA by RNAP II recruited by the strong viral promoter/enhancer since microRNA genes are expressed/transcribed by RNAP II. The MOEC was originally designed to be expressed under the control of chicken U6 promoter but for the purpose of this study that promoter along with the U6 leader sequence was deleted. This was done due to the concern that the regulation of the MOEC via U6 promoter may interfere with the regulation of the MOEC via viral LTR (L. Wang et al., 2011). Since the goal for this study is to ectopically express the MOEC hence expressing the GEM1 shRNA, the control provided by the U6 promoter is not necessary. The host cellular machinery will recognize viral LTR upstream of the MOEC and strongly express the MOEC from a sub-genomic transcript, consequently expressing GEM1 shRNA (Jern & Coffin, 2008; Van De Lagemaat et al., 2003). This level of expression is sufficient for downregulating GEM1.

Chapter 3: Downregulating GEM1 expression in Quiescent CEF

After creating a shRNA retroviral construct for GEM1, it was assessed to check whether it can downregulate GEM1 expression (Figure 12). From the two different targets of GEM1 shRNA, target 1 (T1) showed the most efficient downregulation of GEM1, while target 2 (T2) showed a more modest downregulation. Although T1 shRNA displayed nearly 3-fold decrease in GEM1 expression compared to control (parental RCASBP(A)), it was not able to fully downregulate GEM1 expression. Similarly, T2 shRNA displayed about 2-fold decrease in GEM1 expression. The exact cause of why the shRNA were unable to fully knockdown GEM1 is unknown but one possible explanation could be that we are selecting for cells not infected by the

retrovirus, i.e. that downregulating GEM1 is somewhat deleterious to the cells, even in proliferating condition. For instance, if GEM1 is essential for cell survival, then downregulating it could result in cell death, therefore the cells that survive are not infected by the retrovirus thus not expressing the shRNA. This experiment was repeated multiple times, each time the cells were passaged for a different amount of time following transfection. It was noted that cells passaged once post-transfection, did not display any downregulation of GEM1. Similarly, cells passaged four times also did not display any downregulation of GEM1. It was only the cells that were passaged twice that showed the mentioned partial knockdown of GEM1. This may explain the partial downregulation of GEM1, where passage-1 cells did not have all of the cells infected by the retrovirus, and passage-4 cells may have been selected for ones not infected by the retrovirus.

Interestingly, in this experiment it was clear that only the 35 kDa protein (GEM1) was induced by quiescence in hypoxia, the 75 kDa protein was expressed in normoxia and hypoxia (Figure 12A). It should be noted that this result is different from the earlier experiment where both the 35 kDa and 75 kDa proteins were exhibiting GAS gene-like expression. The only difference in this experiment is that the cells were transfected with either the parental RCASBP(A) retroviral construct (control) or RCASBP(A)-GEM1 T1/2 retroviral construct. Ignoring the cells expressing GEM1 shRNA, and only examining the cells infected with the control viral construct (Figure 12A, Lanes 1 and 4) which does not affect GEM1 expression. It is apparent that the expression of the 75 kDa protein is not affected by induction of quiescence while the 35 kDa protein (GEM1) is only expressed in quiescent CEF. This result was observed in multiple biological replicates (data not shown). This suggests that the 75 kDa protein is not a GAS gene and it is unrelated to GEM1. Additionally, both of the GEM1 shRNA had no effect on

the higher molecular weight signal (75 kDa) in normoxia and hypoxia. This further suggests that the higher molecular weight protein is unrelated to GEM1.

An important confounding factor to consider is that GEM1 is mostly expressed in contact inhibited CEF, and only modestly expressed in hypoxia, which is corroborated by other members of our lab. We assumed that like other GAS genes studied in our lab, hypoxia would be a good inducer of GEM1, but that is not the case. This makes it a unique and more interesting GAS gene since it is mostly inducible by contact inhibition and little by hypoxia. So, examining the effects of downregulation of GEM1 in hypoxic conditions may not be ideal for assessing the function of GEM1, rather downregulation should be done in contact inhibited cells. Another confounding factor to consider is that GEM1 is packaged into EVs as evident by the work done by Emmerson Teas, but when we examined GEM1 expression in contact inhibited and hypoxic cells, we only looked at the protein lysate. So, the strong expression in contact inhibition and modest expression in hypoxic cells may not be an accurate representation of the total expression of GEM1 by the cells.

Ideally having a shRNA retroviral construct that can fully downregulate GEM1 expression would be greatly useful in characterizing the function of GEM1 in quiescence, but even a partial knockdown of GEM1 could prove to be useful in characterizing GEM1. Knowing that the GEM1-shRNA retroviral construct created in this study is capable of partially downregulating GEM1 expression, it can be used in a variety of experiments that can help characterize the function of GEM1. One such experiment is the cell proliferation assay which compares the proliferation of cells with wildtype GEM1 or downregulated GEM1 expression in quiescent and proliferation CEF. This experiment can determine if GEM1 is necessary for cell survival, more specifically does GEM1 downregulation have a detrimental effect on cells which

can be inferred by measuring the total number of cells and their rate of proliferation. Previous studies have shown that GEM1 knockdown in goat preadipocytes suppresses lipid accumulation and inhibits adipogenesis (Xu et al., 2020). A similar experiment can be conducted in quiescent CEF with wildtype GEM1 or downregulated GEM1 expression, that uses Oil red O staining to quantify lipid accumulation. This experiment can help determine if GEM1 is involved in lipid homeostasis. Immunofluorescence (IF) microscopy is another experiment that does not require the use of GEM1-shRNA retroviral construct but can still help characterize GEM1, specifically determining the localization of GEM1 within the cell. In fact, our lab had conducted some preliminary test with IF-microscopy and noted that in proliferating cells, GEM1 signal is observed to be diffused throughout the cell. While GEM1 in hypoxic cells formed small clusters throughout the cell. Although it should be noted that the GEM1 antibodies used in this study have non-specific binding to the 75 kDa protein, distinguishing GEM1 from this non-specific binding would prove to be difficult in immunofluorescence microscopy. Perhaps a fluorescence fusion protein could help mitigate this problem but that too could introduce its own set of problems. Nevertheless, the finding of this study provides evidence that GEM1 is a GAS gene, and it can serve as a proof-of-concept and a starting point for other students to utilize the GEM1-shRNA retroviral construct created in this study to further characterize GEM1.

Experimental Figures

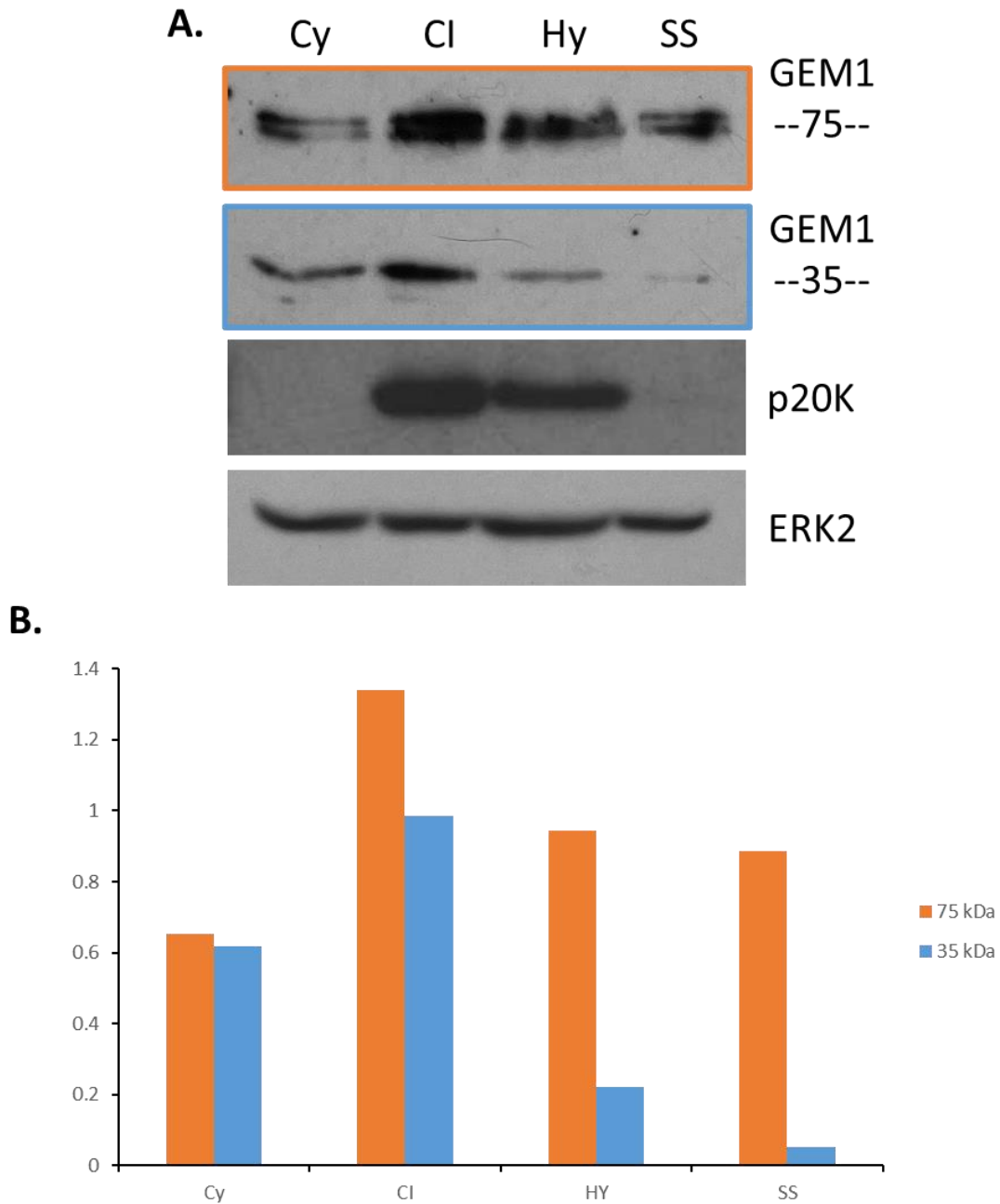


Figure 4. GEM1 expression in cycling (Cy), contact inhibited (CI), hypoxic (Hy), serum starved (SS) CEF. A) Western blot analysis highlighting GEM1 expression using GEM1 R02561

antibody. p20K expression was analyzed to confirm induction of quiescence in CEF. ERK2 was used as a loading control. B) Quantification analysis of GEM1 expression on western blot, analyzing the 75 kDa and 35 kDa bands. GEM1 expression level was normalized to ERK2.

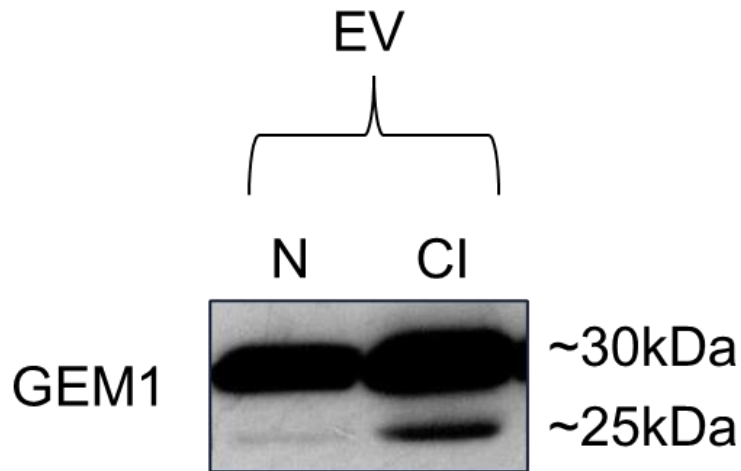


Figure 5. Western blot analysis of GEM1 in extracellular vesicles of cycling cells (Cy) and contact inhibited cells (CI) (Teas E., BSc. Thesis, 2022). Western blot was probed with GEM1 (R02561) antibody.

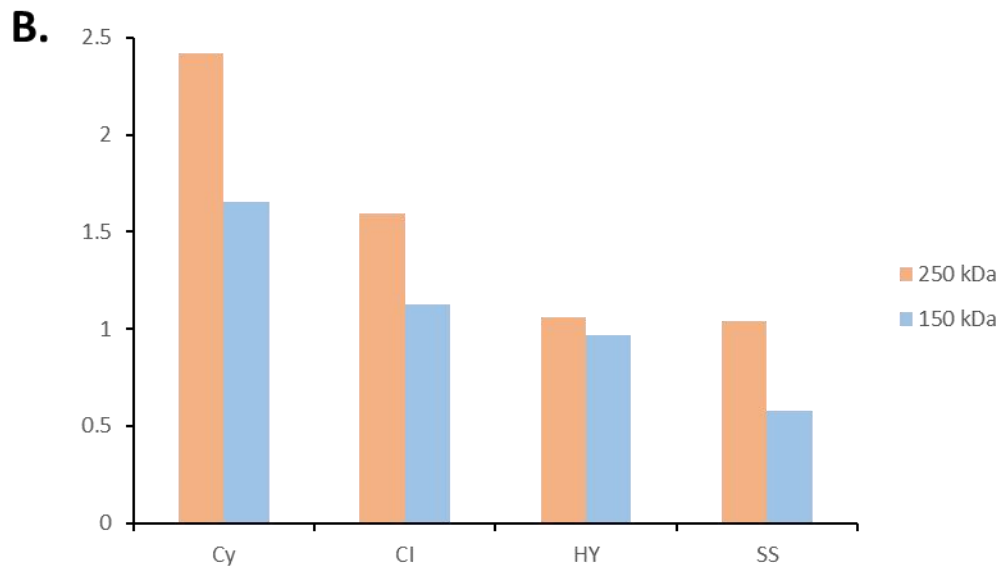
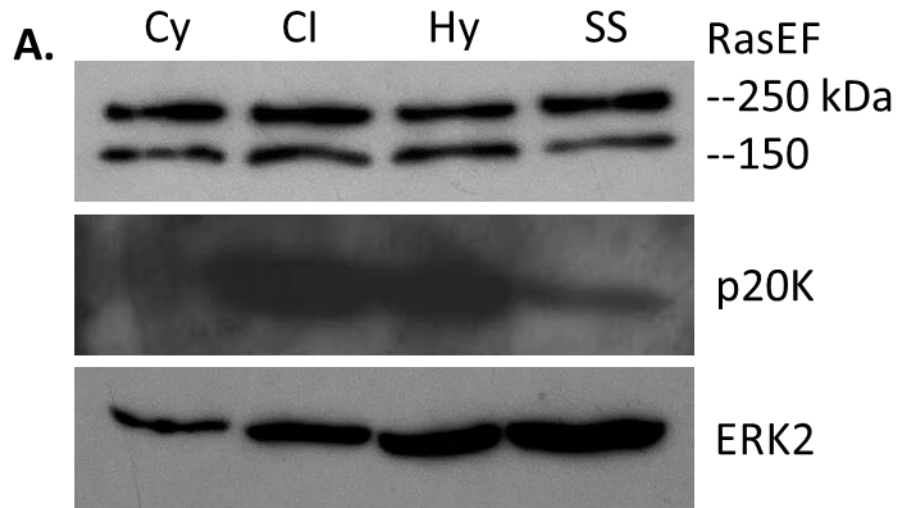


Figure 6. RasEF expression in cycling (Cy), contact inhibited (CI), hypoxic (Hy), serum starved (SS) CEF. A) Western blot analysis highlighting RasEF expression using RasEF antibody. p20K expression was analyzed to confirm induction of quiescence in CEF. ERK2 was used as a loading control. B) Quantification analysis of RasEF expression on western blot, analyzing the 250 kDa and 150 kDa bands. RasEF expression level was normalized to ERK2.

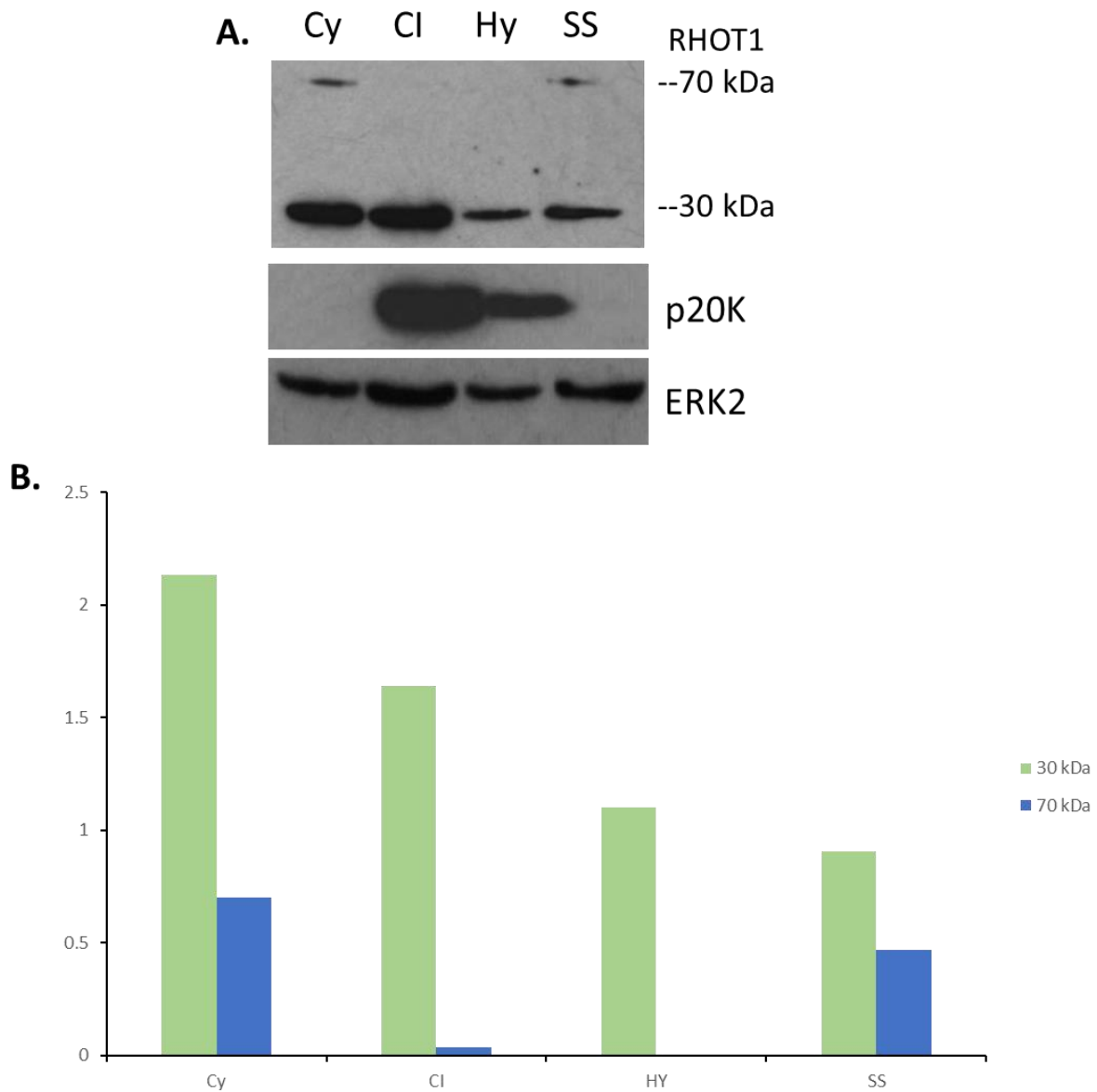


Figure 7. Rhot1/Miro expression in cycling (Cy), contact inhibited (CI), hypoxic (Hy), serum starved (SS) CEF. A) Western blot analysis highlighting Miro1 expression using Rhot1/Miro1 antibody. p20K expression was analyzed to confirm induction of quiescence in CEF. ERK2 was used as a loading control. B) Quantification analysis of MIRO1 expression on western blot, analyzing the 70 kDa, and 30 kDa bands. MIRO1 expression level was normalized to ERK2.

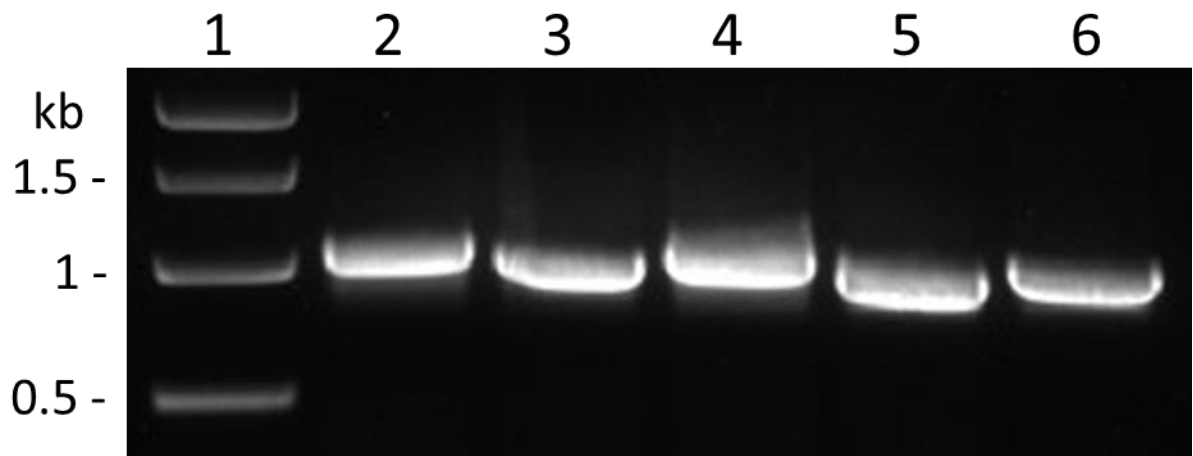


Figure 8. Agarose gel (1.0%) electrophoresis of GEM1 cDNA. Reverse transcriptase PCR was performed using total RNA and primer sets flanking GEM1 ORF. Lane 1 is the 1kb DNA ladder. Lane 2-6 is GEM1 cDNA generated using primer sets 1-5.

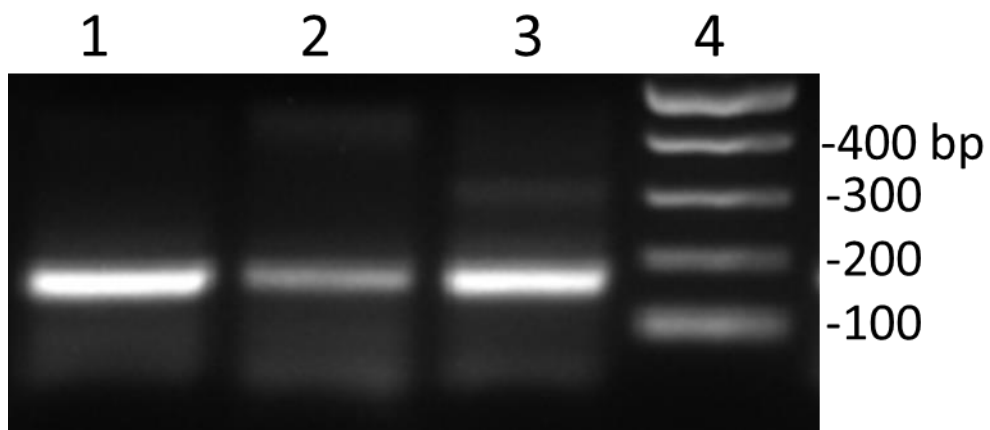


Figure 9. Agarose gel (1.0%) electrophoresis of PCR amplified *GEM1* shRNA. Lanes 1 represents *GEM1* T1 shRNA. Lanes 2 represents *GEM1* T2 shRNA. Lanes 3 represents *GEM1* T3 shRNA. Lane 4 is 100bp DNA size marker.

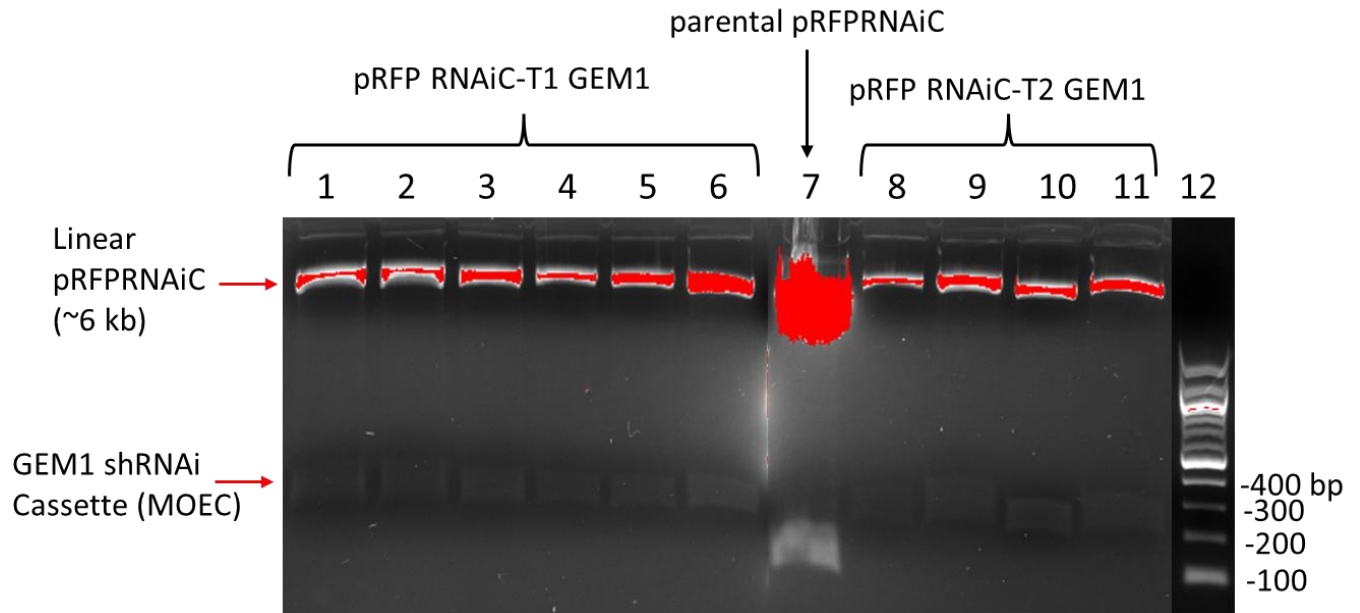


Figure 10. Agarose gel (1.0%) electrophoresis of digested pRFPRNAiC transfer vector at NotI and ClaI restriction sites. Lanes 1-6 represents pRFP RNAiC-T1 GEM1 transfer vector isolated from six different colonies of transformed DH5a competent cells. Lane 7 represents blank pRFP RNAiC-transfer vector. Lanes 8-11 represents pRFP RNAiC-T2 GEM1 transfer vector isolated from four different colonies of transformed DH5a competent cells. Lane 12 is 100bp DNA size marker.

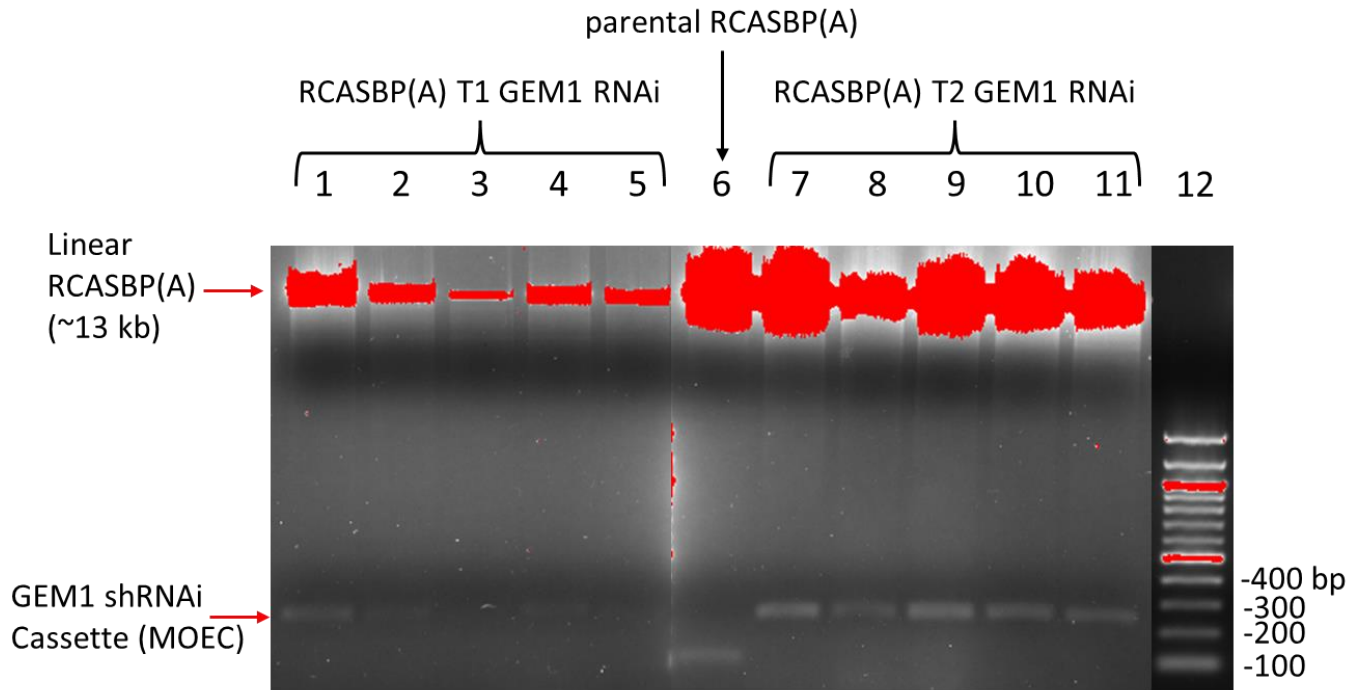


Figure 11. Agarose gel (1.5%) electrophoresis of digested RCASBP(A) vector at NotI and ClaI restriction sites. Lanes 1-5 represents RCASBP(A) T1 *GEM1* RNAi vector isolated from five different colonies of transformed DH5a competent cells. Lane 6 represents blank RCASBP(A) RNAi-vector. Lanes 7-11 represents RCASBP(A) T2 *GEM1* RNAi vector isolated from five different colonies of transformed DH5a competent cells. Lane 12 is 100bp DNA size marker.

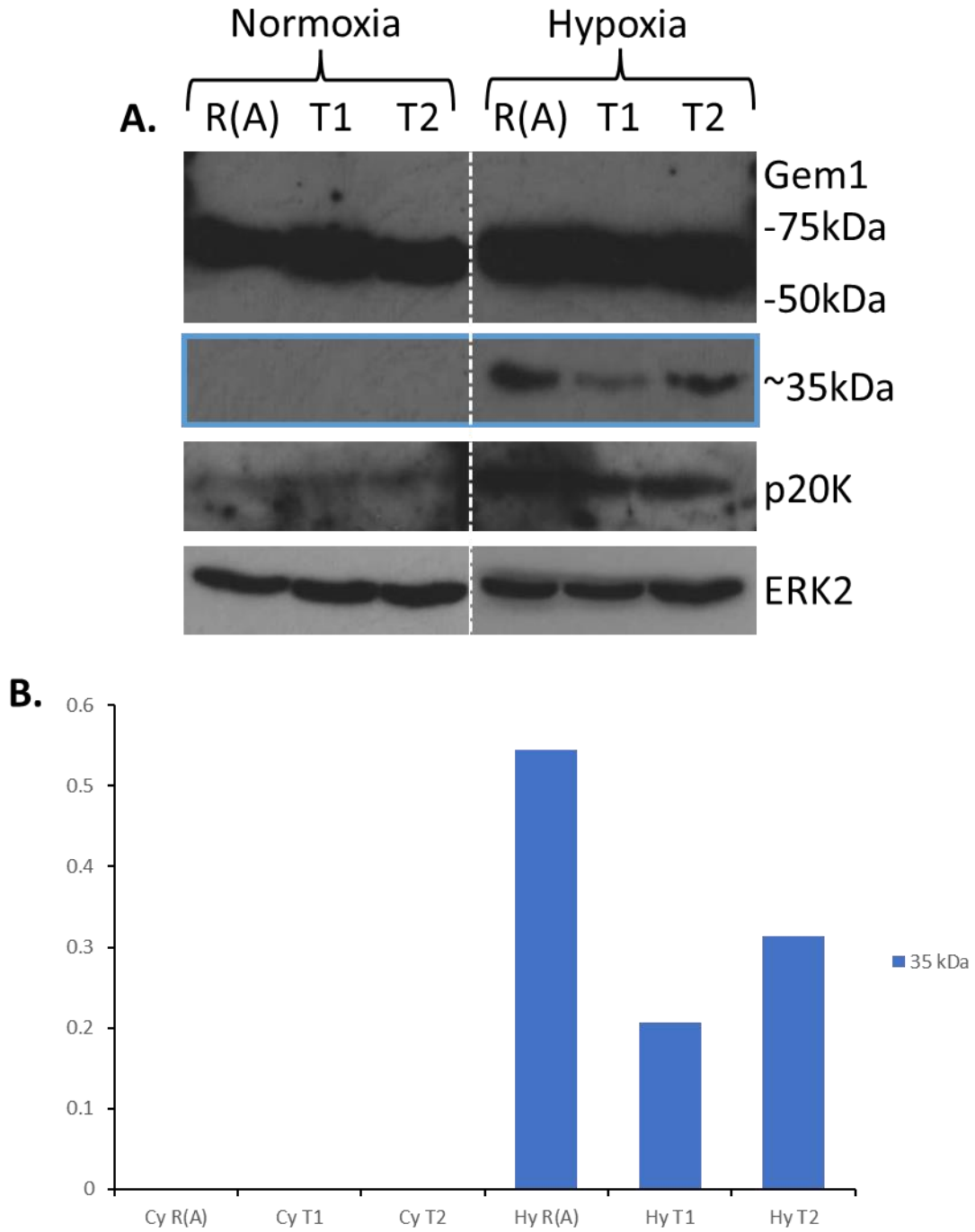


Figure 12. Downregulating GEM1 expression in quiescent (hypoxic) CEF. GEM1 T1 and T2 shRNA was used to downregulate GEM1 expression, empty RCASBP(A) was used as a knockdown control. A) Western blot analysis highlighting GEM1 expression using GEM1 R02557 antibody following GEM1 downregulation. Downregulated GEM1 expression was

analyzed in normoxic condition as a control. p20K expression was analyzed to confirm induction of quiescence in CEF. ERK2 was used as a loading control. B) Quantification analysis of GEM1 expression on western blot, analyzing the 35 kDa band. GEM1 expression level was normalized to ERK2.

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