ERK2 AS A TRANSCRTIPTIONAL REPRESSOR OF QUIESCENCE GENES

CHARACTERIZATION OF ERK2 AS A TRANSCRIPTIONAL REPRESSOR OF GROWTH ARREST SPECIFIC GENES

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Abstract

The study of growth arrest specific (GAS) genes is critical for our understanding of quiescence cell states. C/EBP-β is a transcriptional activator which is central to the expression of GAS genes in growth arrested cells. C/EBP-β is involved in the activation of numerous pathways, including mitogenesis, cytokine signaling, stress response, etc. Thus, it requires signaling cues which confer specificity in terms of gene expression.

Here we used the p20K gene in chicken embryonic fibroblasts as a model system to study the control mechanisms of GAS genes. p20K is expressed in conditions such as contact inhibition mediated growth arrest and mild hypoxia. Here we explored the control mechanism mediated by ERK2 at the p20K promoter (QRU), as a mode of regulation which confers C/EBP-β binding specificity.

In this study we demonstrate that ERK2 is recruited to the QRU in proliferative cells, i.e. where p20K is repressed. Using ChIP analysis we show that ERK2 binds directly to the QRU in proliferative cell states, but not in growth arrested cell conditions. Using a similar approach we demonstrate that ERK2 binding to the QRU is lost in states of hypoxia, where p20K is strongly induced. Furthermore, we show that this interaction is specific to ERK2 and is not observed with the related ERK1 kinase. Lastly, we employed transient expression assays to illustrate that ERK2 acts as a transcriptional repressor of the QRU. Through these experiments we have illustrated that ERK2 mediated transcriptional repression is a novel control mechanism at the QRU which skews C/EBP-β mediated signaling networks in proliferating cells.

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List of Abbreviations

AP-1	Activator Protein 1
ATF	Activating transcription factor
bZIP	Basic leucine zipper domain
C/EBP	CCAAT/enhancer-binding protein
CAT	Chloramphenicol acetyltransferase
CDK	Cyclin-dependent-kinase
CEF	Chicken embryonic fibroblast
ChIP	Chromatin Immunoprecipitation
CHOP10	C/EBP homologous protein 10
CKI	CDK-inhibitory-subunit
CREB	Cyclic AMP – responsive element binding protein 1
DAPK	Death associated protein kinase
DBD	DNA binding domain
DMEM	Dulbecco’s modified Eagle's medium
EBS	ERK2 binding site
ECL	Enhanced chemiluminescent substrate
EGF	Epidermal growth factor
EMSA	Electromobility shift assay
ERK1	Extracellular signal-regulated kinase 1
ERK2	Extracellular signal-regulated kinase 2
ERK3	Extracellular signal-regulated kinase 3

ERK4	Extracellular signal-regulated kinase 4
ERK5	Extracellular signal-regulated kinase 5
ERK7	Extracellular signal-regulated kinase 7
Ex-FABP	Extracellular fatty acid binding protein
G-CSF	Granulocyte colony stimulating factor
GATE	Gamma activated transcription element
GAS	Growth arrest specific
GPCR	G-protein coupled receptor
HRP	Horse radish peroxidase
IFN-γ	Interferon gamma
IL-1	Interleukin-1
IL-6	Interleukin-6
IP	Immunoprecipitation
JAK	Janus tyrosine kinase
JNK1	c-Jun N terminal kinase 1
JNK2	c-Jun N terminal kinase 2
JNK3	c-Jun N terminal kinase 3
LAP	Liver activating protein
LIP	Liver inhibiting protein
MAPK	Mitogen-Activated Protein Kinase
MAPKAPK	MAPK-activated protein kinase
MAPKK	MAPK kinase

MAPKKK	MAPK kinase kinase
MK2	MAPK-activated protein kinase 2
MK3	MAPK-activated protein kinase 3
MK5	MAPK-activated protein kinase 5
MNK	MAPK-interacting kinase
MPF	M-Phase promoting factor
MSK	Mitogen and stress activated kinases
NEMO	NF-κB essential modulator protein
NGF	Nerve growth factor
NLK	NEMO-like kinase
ORC	Origin of replication complex
PDGF	Platelet-derived growth factor
PKA	Protein kinase A
PKC	Protein kinase C
pRB	Retinoblastoma protein
QRU	Quiescence responsive unit
RSK	p90 ribosomal S6 kinase
RTK	Receptor tyrosine kinase
SDS-PAGE	SDS polyacrylamide gel electrophoresis
shRNA	short hairpin RNA
STAT	Signal transducer and activator of transcription
TAD	Transactivation domain

TGF-β	Tumor growth factor beta
TNF-α	Tumor necrosis factor alpha
ZIP	Leucine Zipper domain

Chapter 1: INTRODUCTION

This chapter aims to provide an overview of known concepts pertaining to cellular proliferation and its regulation. This includes topics pertaining to growth arrest and the regulatory mechanisms relating to changes in proliferative/non-proliferative cell states.

1.1 Cell proliferation and the cell cycle

In 1858 Rudolf Virchow published his work describing the nature of cellular origins. In this landmark publication he popularized the term "*Omnis cellula e cellula*", which is Latin for "every cell originates from another existing cell like it."(Tan et al., 2006). This statement marks the triggering of a relentless pursuit to understand what cells are and how they come into existence. Although in Virchow's time scientists were limited to studying cells with crude microscopes, technology has come far enough to allow us to study several facets of cellular activity in rigorous detail. These technological advancements have allowed scientists to probe the inner working of replicating cells and decipher the orchestrated events which comprise cellular replication.

Somatic cells generate progeny cells by participating in the process known as the cell cycle. This is a mechanism by which an existing cell can duplicate its genetic material and then subsequently divide into two identical daughter cells (Alberts et al., 2002). This fundamental process is orchestrated by the cell cycle control system. This system regulates the progression through the cell cycle while simultaneously ensuring that cell integrity and functionality is maintained (Alberts et al., 2002). The cell cycle is divided into 2 major phases: Interphase and M phase (Alberts et al., 2002). Interphase is further broken down into G1, S, and G2 phase (Alberts et al., 2002). G1 is a growth phase

prior to S phase and subsequent to mitosis (Alberts et al., 2002). This growth phase allows the cell to establish sufficient nutrients to facilitate DNA replication (Alberts et al., 2002). This phase can vary in time because, depending on environmental cues, it is at this juncture that cells decide whether a round of cell cycle should be initiated (Alberts et al., 2002). Furthermore, this phase harbors the Restriction point, a critical commitment period for cells (Alberts et al., 2002). If the cell cycle progresses beyond the restriction point the cell is fully committed to the completion of the cell cycle and cannot revert to a non-proliferative state until the cycle is completed (Pardee, 1974; Alberts et al., 2002). This restriction point marks the transition between non-proliferative (quiescent) and proliferative states based on external signals such as nutritional availability, growth stimuli, etc (Pardee, 1974; Alberts et al., 2002). Following G1 phase is S phase. In this phase the entire genomic content of the cell is duplicated (Alberts et al., 2002). This phase typically takes half the mammalian cell cycle and is followed by another growth phase: G2 (Alberts et al., 2002). The G2 phase allows time for the cell to establish sufficient nutritional reservoirs to undergo mitosis (Alberts et al., 2002). Following G2 phase the cell enters M phase, which is marked by an abrupt nuclear and cytoplasmic division. This is encompassed within the process of mitosis, which compared to the rest of the cell cycle, is very quick and drastic. During M phase (mitosis) the cell will disassemble the nuclear envelope, condense chromosomes, align these chromosomes at the metaphase plate, and then segregates sister chromatid to opposite poles of the cell (Alberts et al., 2002). This then allows for the nuclei to reassemble around the DNA and allows for cytokinesis to occur (Alberts et al., 2002). Cytokinesis entails the division of

the cytoplasmic contents of the cell into two separate bodies, ending with a pinching of the cell membrane to encapsulate the resulting daughter cells (Alberts et al., 2002). Thus, in this fashion a single cell can reproduce to create two identical daughter cells. A schematic representation of the cell cycle phases is depicted in Figure1.

The cell cycle is choreographed by a family of serine/threonine kinases known as Cyclin-dependent-kinases (CDKs) and their regulatory sub-units, which are known as Cyclins (Morgan, 1997). Together these protein complexes drive the cell cycle forward and coordinate timing of cell cycle events such that they are observant of internal and external signaling cues (Morgan, 1997). This includes instances such as external signaling from other cells and internal responses to stress such as DNA damage (Morgan, 1997). There are approximately 10 cyclin dependent kinases, however only CDK1 and CDK2 are central to cell cycle functions while the others are shown to have secondary roles (Morgan, 1997). During the initiation of S phase CDK2 forms a complex with Cyclin E, which acts to prime the cell for DNA replication and induce DNA synthesis (Morgan, 1997). CDK2 then binds Cyclin A for the remainder of S-phase and throughout G2 phase, promoting progression of DNA replication and advancement of the cell cycle (Morgan, 1997). Towards the end of the cell cycle there is an accumulation of Cyclin B-CDK1 complex, also known as M-phase promoting factor (MPF) (Morgan, 1997). MPF prepares the cell for mitosis and advances the cell cycle through the distinct phases of mitosis until anaphase, where it is poly-ubiquitinated and broken down (Morgan, 1997). This degradation marks the onset of mitotic completion and allows for chromosomal segregation and cytokinesis to occur (Morgan, 1997).

In higher eukaryotes cell proliferation is dependent on the presence of growth factors and mitogenic signaling. It has been demonstrated that mitogenic signaling stimulates the activation of CDK4 and CDK6 by Cyclin D (Matsushime et al., Xiong et al., and Motokura et al., 1991; Matsushime et al., 1992; Meyerson et al., 1994; Sherr, 2000). This occurs in the G1 phase, prior to the Restriction Point and is fundamental to the initiation of S-phase (Sherr, 2000). During early G1 phase Retinoblastoma protein (pRb) is responsible for exerting a repressive effect on the cell cycle (Weinberg, 1991). This repression is primarily mediated by the binding of pRb to the E2F family of transcription factors, impeding their activation (Wienberg, 1991; Nevins, 1998). E2F regulates the expression of DNA synthesis associated genes and genes involved in driving the cell cycle into S-phase (Nervis, 1998). Furthermore pRb also recruits histone deacetylases and chromatin remodeling complexes to the promoters of E2F responsive genes which administers an additional repressive effect (Brehm et al., Luo et al., and Magnaghi-Jaulin et al., 1998, Zhang et al., 2000). In cells lacking mitogenic stimulation, Cyclin D is very unstable and short-lived due to its constant degradation (Sherr, 1993; Sherr 2000). However, upon mitogenic stimulation Cyclin D becomes stabilized allowing the formation of Cyclin D-CDK4/6 complexes (Sherr, 1993; Sherr 2000). These active kinase complexes phosphorylate pRb, causing it to lose its affinity for E2F (Ewen et al. and Kato et al., 1993). In doing so Cyclin D-CDK4/6 mitigates the cell cycle repression by pRb, in response to mitogenic stimulation (Ewen et al. and Kato et al., 1993). This event allows E2F to function as a transcriptional activator, facilitating the expression of several genes involved in DNA synthesis (Nervins, 1998). Additionally E2F now

activates the expression of Cyclin E, Cyclin A, and E2F itself, forming positive feedback loops (Bartek et al., Duronio et al, and Sánchez et al., 1996). Cyclin E-CDK2 collaboratively phosphorylate pRb along with cyclin D-CDK4/6 which facilitates the loss of mitogen dependence (Fang et al., 1991; Krude et al., 1997, Lundberg et al., 1998). This independence of mitogenic signals marks the progression of the cell cycle beyond the Restriction point and the commitment of the cell to cell cycle completion (Sherr, 1993; Sherr 2000). Shortly after the restriction point Cyclin A-CDK2 mediates the degradation of Cyclin E and the inactivation of E2F, while promoting progression of the cell cycle through S-phase (Morgan, 1997; Sherr, 2000) The action of Cyclin-CDK complexes is depicted in Figure 1.

The progression of the cell cycle in response to Cyclin-CDK activity is countered by CDK-inhibitory-subunits (CKIs) (Harper et al., 1996). This encompasses two families of regulatory proteins: the Cip/Kip family and the INK4 family (Harper et al., 1996; Johnson and Walker, 1999; Sherr, 2000). The Cip/Kip family of proteins includes $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ (Harper et al., 1996; Johnson and Walker, 1999; Sherr, 2000). $p21^{Cip1}$ counters the progression of the cell cycle primarily by inhibiting CDK2 although interactions with other CDKs have been reported (Morgan, 1997; Johnson and Walker, 1999; Sherr, 2000). $p21^{Cip1}$ is also capable of inhibiting DNA replication by interacting with PCNA, a DNA synthesis elongation factor (Johnson and Walker, 1999). Additionally $p21^{Cip1}$ is induced by p53 by virtue of a p53 binding site in the promoter of the gene (Johnson and Walker, 1999). Thus it is expressed in response to p53 activation and mediates stalling of the cell cycle in response to DNA damage, cellular stress, and

other p53 induced cellular events (Johnson and Walker, 1999). $p27^{Kip1}$ is also a potent CDK2 inhibitor (Morgan, 1997; Johnson and Walker, 1999; Sherr, 2000). Its expression is increased in the absence of proliferative signaling (Johnson and Walker, 1999; McDonald et al., 2001). Thus its expression results in the establishment of cell cycle stalls until such time that a proliferative cell state is signaled (McDonald et al., 2001). $p57^{Kip2}$ is another family member involved in stalling the cell cycle by inhibiting CDKs (Morgan, 1997; Johnson and Walker, 1999; Sherr, 2000). The INK4 family of proteins are specific inhibitors of Cyclin D-CDK4/6 (Morgan, 1997; Johnson and Walker, 1999; Sherr, 2000; McDonald et al., 2001). There are 4 members in this family: $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, $P19^{INK4D}$ (Morgan, 1997; Johnson and Walker, 1999; Sherr, 2000; McDonald et al., 2001). These proteins prevent the association of Cyclin D with CDK4/6 and also inhibit the activity of assembled Cyclin D–CDK4/6 complexes (Morgan, 1997; Johnson and Walker, 1999; Sherr, 2000; McDonald et al., 2001). Furthermore, the INK4 family of proteins has been implicated in displacing p21 and p27 bound to Cyclin D-CDK complexes (Morgan, 1997; Johnson and Walker, 1999; Sherr, 2000; McDonald et al., 2001). This makes them readily available to inhibit other Cyclin-CDK complexes such as Cyclin E-CDK2 (Morgan, 1997; Johnson and Walker, 1999; Sherr, 2000; McDonald et al., 2001). This amplifies the repressive role played by CKIs by an indirect displacement mechanism of the INK4 proteins (Morgan, 1997; Johnson and Walker, 1999; Sherr, 2000; McDonald et al., 2001). Thus, the INK4 family has a strong role in the establishment of growth arrested cell states.

In response to mitogenic signaling Cyclin D and E become up-regulated (Matsushime et al., Xiong et al., and Motokura et al., 1991; Matsushime et al., 1992; Meyerson et al., 1994; Sherr, 2000). As cyclin D/E accumulate active Cyclin D/E-CDK2 complexes form which act to sequester CKIs (Johnson and Walker, 1999; Sherr, 2000; McDonald et al., 2001). Furthermore, active Cyclin D/E-CDK2 complexes are capable of phosphorylating $p27^{Kip1}$(Sheaff et al., 1997; Vlach et al., 1997; Montagnoli et al., 1999; Nguyen et al., 1999). This phosphorylation allows $p27^{Kip1}$ to be recognized by an E3 ubiquitin ligase, thus resulting in its ubiquitination and subsequent degradation (Pagano et al., 1995; Vlach et al., 1997; Montagnoli et al., 1999; Nguyen et al., 1999). This contributes to the release of the cell cycle repression by CKIs and the passing of the cell cycle through the Restriction point (Morgan, 1997; Johnson and Walker, 1999; Sherr, 2000; McDonald et al., 2001). Conversely, a lack of mitogenic signaling results in decreased levels of Cyclin D/E which leads to CKIs overcoming the proliferative drive of Cyclin-CDK complexes (Morgan, 1997; Johnson and Walker, 1999; Sherr, 2000; McDonald et al., 2001). This ultimately leads to stalling of the cell cycle and growth arrest (Morgan, 1997; Johnson and Walker, 1999; Sherr, 2000; McDonald et al., 2001). The action of these CKIs is depicted in Figure 1.

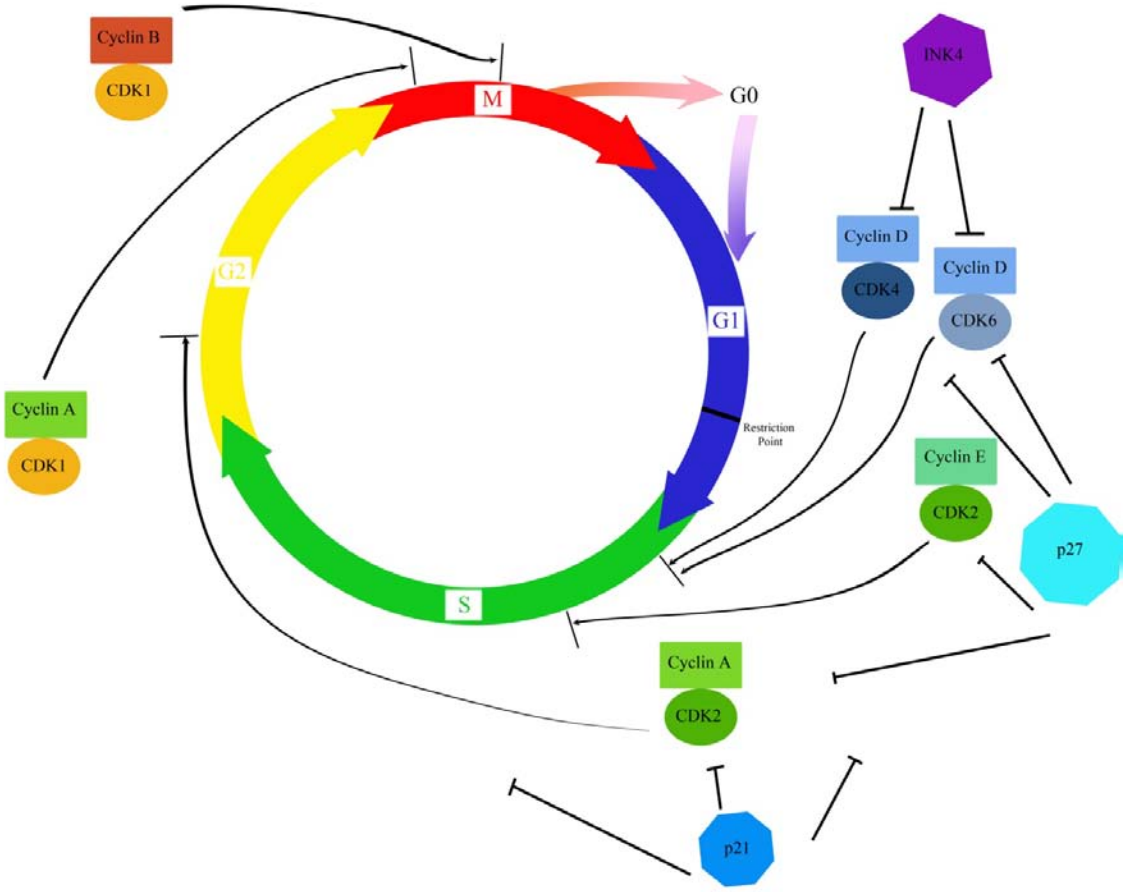


Figure 1: A depiction of the mammalian cell cycle. The 4 phases of the cell cycle are driven by the influence of CDKs and their regulatory unit, cyclins. These Cyclin-CDK complexes control the activation of proteins which promote the progression of the cell cycle through the different phases. The action of these Cyclin-CDK complexes is opposed by CKIs which act to repress the function cell cycle potentiators. Cells can also exit the cell cycle and enter a "resting" state referred to as quiescence or G_0.

The cell cycle is thus a series of coordinated phases. These phases are acted upon by the pressures of Cyclin-CDK complexes driving the cycle forward and the antagonistic pressures of CKIs which act to circumvent this progression. In doing so these processes create a regulated and controlled mechanism for eukaryotic cellular replication.

1.2 Mitogens and Mitogenic signaling

In contrast to prokaryotic cells, eukaryotic cells require growth signals in order to be stimulated into entering cellular proliferation. In this setting growth factors secreted by control cells are received at the recipient cell's surface. These growth factors activate several downstream pathways. Perhaps the most important pathway in terms of cellular proliferation is the mitogenic pathway, which stimulates cellular proliferation and survival. This mitogenic stimulation is mediated largely by Mitogen-Activated Protein Kinases (MAPKs).

MAPKs are a family of kinases which regulate several facets of cellular function including growth, proliferation, differentiation, survival, motility, stress response, apoptosis, and metabolism (Shaul et al. and Pimienta et al., 2007). There are 14 members in the MAPK family in mammals and these proteins are further categorized based on the role these proteins play in cellular regulation (Cargnello et al., 2011). These categories are as follows: extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase 1, 2, and 3 (JNK 1/2/3), p38, extracellular signal-related kinase 5 (ERK5), extracellular signal-related kinase 3 and 4 (ERK3/4), extracellular signal-related kinase 7 (ERK7), and NEMO-like kinase (NLK) (Cargnello et al., 2011). ERK1/2, JNKs, p38, and

ERK5 represent conventional MAPK proteins, as they all follow a particular pattern of activation and regulation (Chen et al., 2001; Pimienta et al., 2007). However ERK3/4, ERK7, and NLK deviate from this conventional pattern of cellular signaling and are referred to as atypical MAPKs (Coulombe et al., 2007).

Conventional MAPKs follow a conserved pattern of activation where a series of kinases form a phosphorylation cascade (Cargnello et al., 2011). Here a MAPK kinase kinase (MAPKKK) will become activated by upstream signals and phosphorylate a MAPK kinase (MAPKK) (Cargnello et al., 2011). This MAPK kinase will then become activated and phosphorylate a MAPK (Cargnello et al., 2011). Dual phosphorylation of MAPKs on a conserved Threonine-X-Tyrosine motif results in the activation of kinase activity (Robbins et al., 1993; Cargnello et al., 2011). These systematic phosphorylations occurring in response to external signaling are representative of a three-tier phosphorylation cascade which is typical of conventional MAPKs. Once activated MAPKs then phosphorylate several targets in the cytoplasm, nucleus, at the cell membrane, mitochondria, golgi, and endoplasmic reticulum (Yoon et al., 2006; Yao et al., 2009). Depending on which MAPKs are activated several classes of MAPK-activated protein kinases (MAPKAPK) become targeted for phosphorylation. This includes several types of MAPKAPKs such as p90 ribosomal S6 kinase (RSKs), Mitogen and stress activated kinases (MSKs), MAPK interacting kinases (MNKs), and MAPK-activated protein kinases2/3/5 (MK2/3/5) (Cargnello et al., 2011).

JNKs are activated in response to cellular stresses such as heat shock, ionizing/UV radiation, oxidative stress, DNA damaging agents, cytokines, and

DNA/protein synthesis inhibitors (Bogoyevitch et al., 2010). The p38 family of MAPKs are also activated in response to different stresses including oxidative stress, hypoxia, ischemia, and signaling through Interleukin-1 (IL-1) and Tumor necrosis factor alpha (TNF-α) (Cuadrado et al., 2010). ERK5 has been reported to become activated in response to growth factors, oxidative stress, and hyperosmolarity (Wang et al., 2006).

The ERK1/2 sub-family of MAPKs are perhaps the most crucial when considering cell proliferation. When these kinases were originally cloned it was found that they were strongly phosphorylated in response to growth factors (Cooper et al., 1982; Kazlauskas et al. and Ray et al., 1988; Boulton et al., 1990; Boulton et al., 1991).This includes activation by platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and nerve growth factor (NGF) (Boulton et al., 1990). ERK1/2 were also found to be activated in response to insulin stimulation (Boulton et al., 1990). These growth factors signals typically activate the canonical three-tier ERK1/2 phosphorylation cascade. This involves reception of the growth factor signal at the cell surface, mainly by receptor tyrosine kinases (RTKs) (Alberts et al., 2002). Upon ligand binding these RTKs dimerize and trans-phosphorylate one another (Alberts et al., 2002). This activation event enables the localization and binding of adaptor proteins to RTK intracellular domains (Alberts et al., 2002). These adaptor proteins become activated and initiate downstream signaling cascades (Alberts et al., 2002). In response to growth factors this downstream signaling is usually funneled through Ras, a protein kinase implicated in several different aspects of cell behavior (Alberts et al., 2002). In the case of the ERK1/2 signaling module, Ras activates the Raf family of proteins, which are MAPKKKs (Alberts et al.,

2002). These activated Raf kinases then phosphorylate the next tier of the module: MEK1/2, which are MAPKKs (Alberts et al., 2002). These in turn phosphorylate ERK1/2 (MAPKs) on the conserved Thr-Glu-Tyr motif, resulting in activation (Plotnikov et al., 2010; Cargnello et al., 2011). Other signal receptor pathways can also activate the ERK1/2 module as well. This includes G-protein coupled receptors (GPCR) and cytokine receptors (Raman et al, 2007). The ERK2 phosphorylation cascade module is depicted in Figure 2.

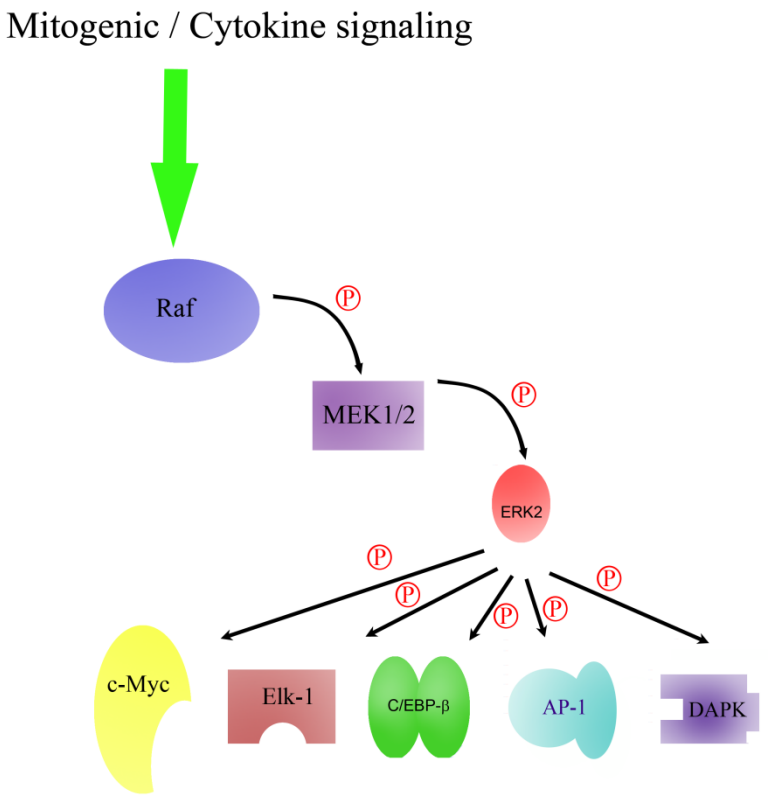


Figure 2: A depiction of the ERK1/2 phosphorylation cascade module. Typically stimuli such as mitogenic or cytokine singling result in the activation of Raf. This initiates a phosphorylation cascade, which results in the subsequent phosphorylation of MEK1/2 and ERK1/2. ERK1/2 then become activated, translocate to the nucleus, and phosphorylate hundreds of targets. Relevant ERK1/2 targets are depicted in this figure.

Once ERK1/2 become activated there are several key events which are triggered. Firstly, ERK1/2 kinase activity is initiated, allowing these proteins to phosphorylate hundreds of downstream targets (Yoon et al., 2006). Secondly, the phosphorylation of ERK1/2 results in its nuclear translocation (Chen et al., 1992; Lenormand et al., 1993). This enable ERK1/2 to activate several dormant transcription factors resulting in the alteration of cellular gene expression. The translocation and accumulation of ERK1/2 in the nucleus occurs minutes after growth factor stimulation. However, cells lacking growth factor stimulation have very little nuclear ERK1/2 (Chen et al., 1992; Lenormand et al., 1993). Some of the relevant ERK2 activation targets are depicted in Figure 2.

Activated ERK1/2 plays a central role in the initiation of the cell cycle. Studies have indicated that in order for a cell to effectively transition from G1 to S phase there must be a sufficient accumulation of active ERK1/2 (Yamamoto et al., 2006). After the G1/S transition activated ERK1/2 are no longer required (Meloche, 1995). This ERK1/2 activation requirement is one of the primary means by which MAPKs have temporal control over the cell cycle because cell cycle initiation is regulated by several events which are triggered by activated ERK1/2. Briefly, upon mitogenic stimulation activated ERK1/2 translocates to the nucleus. Once in the nucleus these kinases phosphorylate several transcription factors, one of which is Elk1 (Gille et al, 1995). It has been shown that activated Elk1 regulates the expression of several proteins. One of these Elk1 induces the expression of c-Fos, a member of the AP-1 family of transcription factors (Gille et al, 1995). Furthermore, sustained ERK1/2 activation has been shown to induce expression of other AP-1 family members such as Fra1, Fra2, c-Jun, JunD and JunB; however the exact

mechanisms behind this induction are unknown (Cook et al., 1996; Balmanno et al., Cook et al., 1999). Many of these AP-1 gene products are unstable and require direct phosphorylation by ERK1/2 in order to be stabilized (Whitmarsh et al., 1996; Murphy et al., 2002). Furthermore, JunD and Fra2 require phosphorylation of their transactivation domains by ERK1/2 in order to facilitate functional activation (Murakami et al., 1997; Vinciguerra et al., 2004). The promoter of Cyclin D1, one of the Cyclin D proteins, contains an active AP-1 binding site (Herber et al., 1994; Albanese et al., 1995). Furthermore, induction of Cyclin-D is required for the formation of active Cyclin D-CDK4/6 complexes. As previously mentioned, accumulation of these Cyclin D-CDK4/6 complexes are necessary for driving cells past the restriction point and into S-phase. Thus, by inducing and stabilizing AP-1 family members, ERK1/2 indirectly provoke the expression of Cyclin D and stimulate the cell thorough the G1/S phase boundary (Meloche et al., 2007; Plotnikov et al., and Cargnello et al., 2011).

ERK1/2 stimulate cell cycle entry by other means as well. c-Myc is a member of the Myc family of transcription factors, which regulate cell growth, proliferation, and cell death. Reports indicate that c-Myc is highly unstable. However after phosphorylation by ERK1/2; in response to mitogenic stimulation, c-Myc becomes stabilized. This stabilization allows Myc to form hetero-dimeric complexes with Max, its dimerization partner, thus forming active transcription factor complexes. These active Myc-Max hetero-dimers have been shown to induce expression of Cyclin D1 and Cdk4; and repress expression of p21 (Bouchard et al., 1999; Claassen et al., Coller et al., 2000). This further favors progression of the cell cycle and stimulates cells past the G1/S phase boundary.

Additionally ERK1/2 have roles in inhibiting repressors of the cell cycle such as p27 by phosphorylating several other transcription factors (Meloche et al., 2007; Plotnikov et al., and Cargnello et al., 2011).

The mitogenic signaling pathway is the primary mediator of cell cycle entry and transduction of growth factor signals into a measureable cell response. This cell cycle stimulation is primarily facilitated by functional roles of ERK1/2. This MAPK acts to skew protein signaling networks to favor the accumulation of factors required for cell cycle entry and inactivate cell cycle repressors. In doing so ERK1/2 are critical players in the regulation of cell cycle control and cellular proliferation.

1.3 Cell cycle regulation and cancer

Due to the clonal expansion nature of cells within a tumor, those cells which have a selective advantage in comparison to surrounding cells will grow faster. This generates a small scale natural selection process, where cells which have undergone advantageous mutations grow faster then surrounding cells. Cells which have undergone deleterious mutations die and are lost. This implies that mutations which give a tumorigenic cell a growth advantage will favor progression of the tumor. Thus, genes which regulate cell proliferation and the cell cycle are eligible candidates for being oncogenes or tumor suppressors.

It has been well documented that genes involved in cell cycle regulation play a critical role in the development and progression of cancer. Several genes in the Cyclin, CDK, and CKI families are mutated or mis-regulated in cancer cells (Johnson and Walker, 1999; Sherr, 2000; McDonald et al., 2001). Generally these mutations result in

uncontrolled cell proliferation leading to tumorigenesis. Sequential mutation of genes involved in cell cycle regulation can give rise to more aggressively dividing cells.

Cyclins and CDKs are observed to be mutated in several cancers. Cyclin D has been observed as being amplified or up-regulated in several different types of tumors (Johnson and Walker, 1999; Sherr, 2000; McDonald et al., 2001). Furthermore, animal models where Cyclin D is mis-regulated show that Cyclin D can play a strong role in tumorigenesis (Motokura et al., 1991; Lovec et al., 1994; Wang et al., 1994). Cyclin E has been found to be overexpressed in breast, colon, and leukemic cancers (Leach et al, 1993; Keyomarsi et al., 1995). Cyclin A was reported to be mutated in human hepatomas (Wang et al., 1990). CDK4 and 6 are overexpressed in several tumors and tumor cell lines (Johnson and Walker, 1999; Sherr, 2000; McDonald et al., 2001). Additionally CDK4 and 6 mutations which confer resistance to INK4 regulation have also been found in human tumors (Wolfel et al., 1995; Easton et al., 1998). These genetic changes act to drive the cell cycle in a more pronounced manner resulting in increased cell proliferation.

INK4 family members are also mutated in several different types of tumors (Kamb, 1998). Often these are missense mutations, deletion mutations, or mis-regulation of gene methylation (Kamb, 1998). Most often these mutations result in the expression of inactive INK4 proteins which can no longer inhibit Cyclin D-CDK4/6 (Kamb, 1998). The gene encoding $p57^{Kip2}$ was observed as being rearranged in two different familial cancer disorders (Polyak et al., 1994; Matsuoka et al., 1995). The gene encoding $p21^{Cip1}$ has been shown to be altered in some instances of cancer (el-Deiry and Kamb, 1998). However its role as a tumor suppressor stems mainly as a mediator of p53 dependent

growth arrest during the cell cycle (Deng et al., 1995; el-Deiry and Kamb, 1998). p53 is one of the most frequently altered genes in human cancer and its mutation often results in deactivation of the p53 response (Ko et al., 1996; Levine, 1997). This confers resistance to apoptosis and increased mutation rate, resulting in sustained cell mutations and thus cancer progression (Ko et al., 1996; Levine, 1997). The inactivation of $p27^{Kip1}$ by loss of expression and increased degradation has also been reported in certain cancers (Tan et al., and Loda et al., 1997). Lastly, pRb is mutated in numerous tumor types and is also targeted by oncogenic viruses (Ludlow, 1993). All of these mutations are involved in the inactivation of cell cycle repressors. This illustrates the importance of these proteins in control of the cell cycle and the maintenance of cellular integrity.

Several different cancers harbor alterations to the mitogenic signaling pathway. For example several RTKs are mutated or up-regulated in several cancers, resulting in increased or constitutive activation (Sawyers, 2003; Gshwind et al, 2004). Ras activation mutations are observed in approximately 30% of cancers (Davies et al., 2002; Downward, 2003). These mutations result in the constitutive activation of Raf, leading to constant mitogenic signaling through ERK1/2 despite a lack of mitogenic signals (Davies et al., 2002; Downward, 2003). This constitutive activation causes cells to lose sensitivity to growth signals and harbor permanent cell cycle stimulation, resulting in cancerous growth. Similarly, several proteins controlling the MAPK signaling pathways have been mutated in cancers. This includes members such as Src, Raf, Rho, and several MAPKAPs (Kim et al., 2009; Eisinger-Mathason et al., Niault et al., 2010). All of these mutations

remove control of the cell cycle and result in cells which are being stimulated to divide regardless of external and internal cues.

Mutations in genes which regulate the cell cycle are very prominent in all types of cancers. This includes Cyclin-CDK genes which tend to incur over-activity mutations and CKI genes which incur inactivating mutations. Furthermore, genetic mutations in the mitogenic signaling pathway are observed to increase the likelihood of signaling in the absence of proper external signals. Together these mutations serve to drive the cell past the restriction point whilst ignoring cellular cues to stall the cell cycle or enter growth arrest. These mutations are fundamental to the loss of cell cycle control and illustrate how important this regulation is to the maintenance of cellular integrity.

1.4 Quiescence

Most cells within the human body are differentiated and are thus in a state where they no longer divide. The remainder of cells have proliferative potential however even these cells have the ability to exit the cell cycle, entering a resting state referred to as quiescence or G_0 (Smith et al., 1973; Martin et al., 1976). This is a form of reversible growth arrest that is a distinct cell state rather than a simple stalling of G1 phase (Smith et al., 1973; Martin et al., 1976; Zetterberg et al., 1985). Quiescence is the result of altered gene expression in response to a lack of mitogenic signaling or the stimulation of anti-growth signaling (Polyak et al., 1994). Thus there are different stimuli which can cause cells to enter this state of growth arrest. However it has been observed that there are characteristic proteins which are specifically induced by the quiescent cell state, the quiescent program if you will, and these proteins are expressed regardless of which type

of stimulus induces the cells state (Coller et al., 2006). Thus, quiescence is characterized by the activation and expression of proteins which are specific to the establishment and maintenance of the G_0 state (Schneider et al., 1988; Coppock et al., 1993; Coller et al., 2006).

It has been observed that $p27^{Kip1}$ levels are high in quiescent cells. This is consistent with reports documenting that $p27^{Kip1}$ expression is induced in response to conditions which cause cellular growth arrest (Polyak et al., 1994). This includes induction conditions such as density mediated growth arrest (contact inhibition) and anti-proliferative signaling such as Tumor Growth Factor – beta (TGF-β) signaling (Polyak et al., 1994). It is thought that $p27^{Kip1}$ is involved in regulating the establishment and maintenance of quiescent cell states until such times where cells re-enter the cell cycle. In which case $p27^{Kip1}$ is marked for degradation and its repressive functions mitigated. As previously mentioned, $p27^{Kip1}$ is phosphorylated and degraded in response to mitogenic signaling. Simultaneously Cyclin D is up-regulated in response to mitogenic signaling. This is thought to generate antagonistic pressures which allow cells to switch between cell cycle progression and growth arrest.

Additionally the establishment and maintenance of quiescence has been recently tied to factors required for DNA replication. DNA replication is triggered in early S-phase by a group of proteins known as the Origin of Replication Complex (ORC) (Sroeber et al., 2001; Blow et al., 2005). These ORC complexes, which are bound to DNA, become activated in response to S-phase Cyclin-CDK complex activity (Sroeber et al., 2001; Blow et al., 2005). CDC6 is a protein which acts as an activation factor for

ORC and is required for "firing" of ORC complexes (Blow et al., 2005). This activation is a critical initiating event for DNA replication (Blow et al., 2005). Following this firing, CDC6 is phosphorylated by late S-phase/G2 phase Cyclins causing it to become localized to the cytoplasm and degraded (Blow et al., 2005). This inactivation step is key in ensuring that only one round of DNA replication is initiated. In quiescent cells it is observed that CDC6 is down regulated (Madine et al., 2000; Stoeber et al., 2001; Eward et al., 2004; Kingsbury et al., 2005). This down regulation is more severe with longer quiescence times (Eward et al., 2004; Kingsbury et al., 2005). This repression is thought to contribute to the additional time required by quiescent cells to re-enter the cell cycle (Madine et al., 2000; Stoeber et al., 2001; Eward et al., 2004; Kingsbury et al., 2005). Furthermore, serum stimulation has been shown to stabilize CDC6 by direct phosphorylation by Cyclin E-CDK2 (Mailand et al., 2005). This mechanism has been proposed to be involved in returning cells to a proliferative state upon mitogenic stimulation. Although more work is required to decipher the finer details of CDC6 involvement in quiescence, it would appear that regulation of DNA replication activators is one method employed by quiescent cells in order to establish a non-proliferative cell state.

Thus both transcriptional and post-translational regulatory mechanisms are involved in the generation of a quiescent cell state. Some genes which are expressed solely in a quiescent cell state can be used as markers for quiescence. These genes are often referred to as Growth Arrest Specific (GAS) genes (Schneider et al., 1988; Fornace et al., 1989; Coppock et al.,1993;). The exact function of many different GAS genes is

poorly understood. However some of these genes have high affinity for lipids, are involved in repressing DNA synthesis, are proteins which regulate proliferation/survival, or proteins which enhance the cellular response to mitogens.

1.5 p20K

By studying GAS genes one can gain a better understanding of the mechanisms involved in regulating a quiescent cell state. One such GAS gene which has been well characterized in Chicken Embryonic Fibroblasts (CEFs) is p20K. p20K is also known as Extracellular Fatty Acid binding protein (Ex-FABP), Protein Ch21, and Quiescence-Specific-Protein. It is a member of the lipocalin family of proteins (Cancedda et al., 1990). Typically this family is composed of extracellular proteins which have high affinity for hydrophobic molecules such as fatty acids (Akerstrom et al., 2000). In the case of p20K, reports indicate a preferential binding to long chain unsaturated fatty acids such as linoleic acid, oleic acid, and archidonic acid (Cancedda et al., 1996). Furthermore, it has been implicated in bone development, cartilage development, heart development, myocyte development, inflammatory responses, and cell survival (Cancedda et al., 1988; Cancedda et al., 1990; Gentili et al., 1998; Cancedda et al., Cermelli et al., 2000; Di Marco et al., 2003; Gentili et al., 2005). Although perturbation of p20K has been shown to affect all of these processes, its functional role is still unclear. This 20kDa protein is shown to be a marker of quiescence and is thus a GAS gene. By using p20K expression as a model to study the regulation of GAS genes one can gain a better understanding of how cells regulate and establish a quiescent cell state.

p20K was originally discovered as a protein which was synthesized in response to cells entering quiescence as a result of high density (Bédard et al., 1987). It was further observed that p20K was repressed in conditions where cells were actively dividing (cycling), was stimulated with growth factors/insulin, or was transformed using an oncogenic form of Src (Bédard et al., 1987; Bédard et al., 1989). Additionally it was discovered that p20K is regulated transcriptionally in response to conditions inducing growth arrest (Bédard et al., 1989).

1.6 QRU

The transcriptional regulation of p20K suggested that the promoter region of this gene plays a role in the regulation of the gene expression in response to conditions which induce growth arrest. Thus, in 1993, through a series of promoter dissection experiments it was observed that a 48 bp fragment of the p20K promoter was responsible for activation of p20K in response to quiescence inducing events (Mao et al., 1993). This region spans from -217 to -169 bp from the transcriptional start site (Mao et al., 1993). It was thereafter called the Quiescence Responsive Unit (QRU) (Mao et al., 1993). The QRU has been demonstrated to confer activation in response to growth arresting inducing stimuli including serum starvation, contact inhibition, and hypoxia (Mao et al., 1993; Ben Fielding and Romita Ghosh (Unpublished Results)). A schematic representation of p20K and upstream promoter regions are depicted in Figure 3.

1.7 C/EBP-β

Although it was clearly demonstrated that the QRU was required for expression of p20K and possibly other GAS genes, it was unknown what trans-acting factors bind to

this element and regulate its activation. Until 1999 these trans-acting activators remained elusive. It was in this year that Kim et al. demonstrated that C/EBP-β binds directly to the QRU and is responsible for p20K gene expression in response to quiescent cell states (Kim et al., 1999).

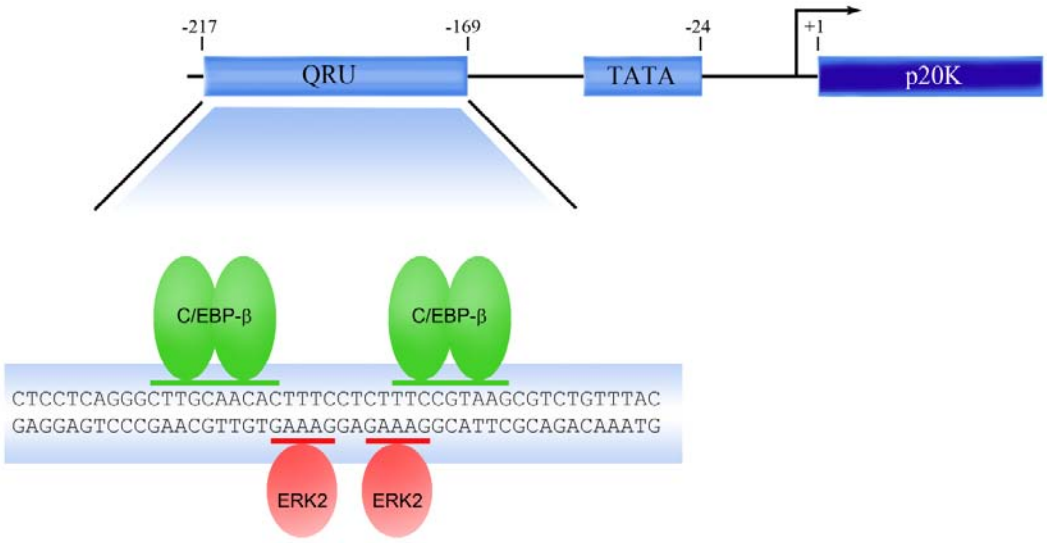


Figure 3: A schematic representation of the p20K gene and its promoter region. The promoter of p20K consist of a TATA box at the -24 bp region and a quiescence responsive unit (QRU) which spans from -217 to -169 bp of upstream of the transcriptional start site. The QRU is bound by C/EBP-β at the sites depicted in the figure. The binding of C/EBP-β is required for the transcriptional activation of p20k. Furthermore, putative ERK2 binding sites in the QRU are also shown.

The CCAAT/enhancer-binding protein (C/EBP) group of proteins is a family of transcription factors. This family of proteins has roles in many aspects of cell behavior, including cell cycle progression, extracellular signaling, tissue development, cellular differentiation, immune function, energy metabolism, adipogenesis, and viral pathogenesis (Nerlov, 2007; Tsukada et al., 2010). This family of transcription factors abides by the conventional structure of transcription factors. Typically family members contain a DNA binding domain (DBD) and a transactivation domain (TAD) (Nerlov, 2007; Nerlov, 2008; Tsukada et al., 2010). The DNA binding domain consists of a leucine zipper (ZIP) which is a region very rich in hydrophobic residues and a basic region containing several arginine and lysine residues. In the case of the C/EBP family it is called the basic leucine zipper domain (bZIP) (Nerlov, 2007; Nerlov, 2008; Tsukada et al., 2010). Upon dimerization these domains form a tertiary structure such that it favors interaction of basic residues in the bZIP domain with acidic DNA (Nerlov, 2007; Nerlov, 2008; Tsukada et al., 2010). The affinity conferred by this interaction allows C/EBP homo and hetero-dimers to bind DNA. Differences in bZIP domain sequence and thus structure allow for the recognition of different DNA binding sequences (Tsukada et al., 2010). This allows for different homo and hetero-dimers of the C/EBP family to recognize different sequences of DNA based on which dimers have been formed (Tsukada et al., 2010). The transactivation domain promotes gene transcription by means of interaction with other protein complexes which ultimately recruit and activate gene transcription (Nerlov, 2007; Nerlov, 2008; Tsukada et al., 2010). There are 6 genes in the C/EBP family: C/EBP-α, C/EBP-β, C/EBP –γ, C/EBP-δ, C/EBP-ε, and C/EBP-ζ (Nerlov,

2007; Nerlov, 2008; Tsukada et al., 2010). The expression profile of these genes varies greatly from tissue to tissue (Nerlov, 2007; Nerlov, 2008; Tsukada et al., 2010). These family members are capable of forming homo or hetero-dimers with one another. Furthermore, individual proteins can associate with other bZIP harboring transcription factors (Tsukada et al., 2010). This allows for very diverse regulation of the C/EBP factors and the resulting outcome of genes which become enhanced / repressed in response to stimuli (Tsukada et al., 2010).

C/EBP-α and C/EBP-β are the best studied genes of the family. C/EBP-β itself has 3 isoforms: Liver Activating Protein (LAP) (p33), LAP* (p38), and Liver Inhibitory Protein (LIP)(p20) (Descombes et al., 1990; Descombes et al., 1991). As the names suggest, LAP and LAP* are activating isoforms of C/EBP-β, as they contain full TADs (Descombes et al., 1990). LIP is a short isoform which contains a bZIP domain and thus is capable of dimerization (Descombes et al., 1991). However since LIP lacks a TAD, LIP containing complexes are inactive in transcriptional activation (Descombes et al., 1991). In this manner LIP can act as a suppressor of LAP and LAP* by acting like a dominant negative mutant protein which sequesters activating isoforms away from C/EBP-β binding sites (Descombes et al., 1991). C/EBP-β was originally discovered as a protein which binds to the promoter of Interleukin-6 (IL-6) and activates its expression in response to Interleukin-1 (IL-1) stimulation (Akira et al., 1990). Since then it has been demonstrated that C/EBP-β acts as an activation factor to several genes involved in numerous cellular processes. These include genes such as IL-8, IL-1, Tumor Necrosis Factor-α (TNF-α), granulocyte colony stimulating factor (G-CSF), α1-acid glycoprotein,

α2-microglobulin, and C-reactive protein, to name a few (Akira et al., 1997). The diverse and numerous targets of C/EBP-β are the result of context dependent signals and interaction with other transcription factors in response to signaling events. Levels of C/EBP-β are observed as remaining fairly stable; however there are events which can trigger a mild increase in expression (Akira et al., 1990). This includes signaling cues such as cytokine and glucose stimulation (Akira et al., 1990). Other signaling events can act to increase the transcriptional activation activity of C/EBP-β by increasing DNA binding affinity (Poli et al, 1990; Roy et al., 2000). Much of this activity is regulated by post-translational modifications, namely phosphorylation by several protein kinases. This includes protein kinase A (PKA), protein kinase C (PKC), RSK (Trautwein et al., 1993; Buck et al., 1999; Buck et al., 2001). Furthermore it has been demonstrated that ERK1/2 directly phosphorylate C/EBP-β resulting in significantly increased transactivation potential (Nakajima et al, 1993; Hanlon et al., Hu et al., 2001). This phosphorylation by ERK1/2 has been shown to be critical for activation of several C/EBP-β mediated responses (Nakajima et al, 1993; Hanlon et al., Hu et al., 2001; Cloutier et al., 2009). C/EBP-β homo-dimers bind to the consensus DNA sequence of T T/G N N G N A A C/T (Ryden et al., 1989; Osada et al., 1996). Here the doublet Adenosines and the central Guanine seem to be the most critical determinants for binding affinity. Several studies indicate that C/EBP-β homo-dimers are able to bind sequences that slightly deviate from the described consensus (Mahony et al., 2007; Tsukada et al., 2011). C/EBP-β is also capable of forming hetero-dimers with other bZIP superfamily transcription factors. This includes members of the CREB/ATF family which are involved in stress response

(Newman et al., 2003). This adds an increased element of regulation, by increasing the activating potential of C/EBP-β for other genes based on which hetero-dimers are formed (Tsukada et al., 2011). Furthermore, C/EBP-β is capable of forming hetero-dimers with C/EBP-ζ (CHOP10) (Ron et al., 1992). Hetero-dimerization of C/EBP-β and CHOP10 causes loss of binding affinity for the C/EBP-β consensus site (Ron et al., 1992). In this fashion, CHOP10 acts as a negative regulator of C/EBP-β, by generating C/EBP complexes which are unable to bind to promoter activation elements (Ron et al., 1992).

C/EBP-β plays a prominent role in mediating interferon gamma (IFN-γ) signaling. Canonically, IFN-γ signaling response genes are activated by Janus tyrosine kinase (JAK) – Signal Transducer and Activator of Transcription (STAT) signaling pathways (Stark et al., 1998; Li et al., 2007). In this signaling pathway IFN-γ binds to its ligand receptors which activate JAK1 and JAK2 signaling kinases (Stark et al., 1998; Li et al., 2007). This activation event facilitates the phosphorylation of STAT1 by JAK1 and JAK2 (Stark et al., 1998; Li et al., 2007; Shuai et al., 1993). Phosphorylated STAT1 now forms active homo-dimers, migrates to the nucleus, binds to promoter regions of IFN-γ induced genes, and activates transcription (Stark et al., 1998; Li et al., 2007; Shuai et al., 1993). Most IFN-γ induced genes are transcriptionally activated in this manner. A sub-set of IFN-γ induced genes are under the control of Gamma Activated Transcription Elements (GATE elements) (Roy et al., 2000). These 5' proximal promoter elements are involved in the induction of gene expression in response to IFN-γ stimulation (Roy et al., 2000). It was found that C/EBP-β binds to GATE elements and activates transcription in an IFN-γ signal dependent manner (Roy et al., 2000; Xiao et al., 2001). This was mediated by

C/EBP-β consensus binding motifs within GATE elements (Roy et al., 2000; Xiao et al., 2001). Furthermore, it was shown that this C/EBP-β driven response requires activation by ERK1/2 and that the ERK1/2 signaling cascade is involved in the IFN-γ response (Favata et al., 1998; Roy et al., 2002; Meng et al., 2005).

1.8 Control of GAS gene expression by C/EBP-β

It is now a well established fact that C/EBP-β plays a role in the activation of GAS genes. As previously mentioned, p20K is a growth arrest specific gene, induced in conditions of contact inhibition and serum deprivation. Furthermore, this induction occurs at the transcriptional level and is dependent on the QRU. In 1999 Kim et al. demonstrated that this transcriptional activation was mediated by C/EBP-β. C/EBP-β binds to 2 distinct sites in the QRU, in a quiescent state dependent manner (Kim et al., 1999). These binding sites conform to the consensus sequence of C/EBP-β with slight deviation (Kim et al., 1999). Disruption of the C/EBP-β binding sites in the QRU or disruption of C/EBP-β levels /activity causes p20K expression to be abolished (Kim et al., 1999). C/EBP-β itself is a very rare protein in CEFs (Kim et al., 1999). Albeit there is a mild induction of C/EBP-β in a quiescent cell state, the expression levels of this protein remain relatively low (Kim et al., 1999). This induction suggests that C/EBP-β is a GAS gene itself (Kim et al., 1999). Thus the regulation of C/EBP-β plays a strong role in the whether p20K is expressed. As illustrated above, C/EBP-β has context dependent activation. It is involved in numerous processes and is regulated in several different ways. An example of this context dependent activation in CEFs is the role of C/EBP-β in growth arrested vs. proliferative cells (Gagliardi et al., 2003). In normal CEFs, C/EBP-β

represses activities associated with proliferation (Gagliardi et al., 2003). However in transformed or actively dividing CEFs, C/EBP-β potentiates processes associated with tumorigenicity (Gagliardi et al., 2003). This begs the question, what is it about growth arrested states that confer this particular behavior of C/EBP-β at the QRU? How does mitogenic stimulation cause repression of C/EBP-β dependent GAS genes and how is C/EBP-β specificity in gene activation controlled?

1.9 ERK2 as a transcriptional repressor

As previously described, ERK2 is a well established modulator of several transcriptional activators and repressors. Until 2009 it was thought that any transcriptional repression observed in response to ERK2 activation was the result of its kinase activity. However, in 2009 Hu et al. demonstrated that ERK2 functions also by direct interaction with DNA, acting as a transcriptional repressor (Hu et al., 2009). This study involved using a high throughput proteomics approach to probe the whole human DNA-protein interactome. It resulted in the profiling of 17718 direct DNA-protein interactions with 460 distinct DNA motifs (Hu et al., 2009). Many of these interactions were known or expected (Hu et al., 2009). However, an unanticipated result was the direct binding of ERK2 to a consensus DNA sequence (G/C A A A G/C), which will be referred to as a ERK2 binding site (EBS) from here onwards (Hu et al., 2009). In this study it was demonstrated that ERK2 binds directly to the EBS sequence (Hu et al., 2009). This binding is dependent on the newly characterized DNA binding domain of ERK2, which roughly spans amino acids 259-277 (Hu et al., 2009). Furthermore DNA binding activity of ERK2 is distinct from its kinase activity, i.e. ERK2 does not need to

be capable of kinase activity in order to bind DNA directly (Hu et al., 2009). Additionally Hu et al. demonstrated that ERK2 plays a physiological role in response to IFN-γ signaling (Hu et al., 2009). As previously mentioned, IFN-γ causes the induction of several genes. This induction is dependent on activation by C/EBP-β binding to GATE elements in the promoter region of these genes. Hu et al. observed that there were ERK2 consensus binding sites embedded in the GATE elements (Hu et al., 2009). There were typically two ERK2 binding sites positioned a few nucleotides apart in most of the observed GATE elements (Hu et al., 2009). Based on this observation they conducted experiments illustrating that ERK2 binds directly to EBS within the GATE elements of several IFN-γ induced genes (Hu et al., 2009). Furthermore, the binding of ERK2 conferred transcriptional repression (Hu et al., 2009). The model proposed by this work is that ERK2 maintains transcriptional repression of GATE element-regulated genes in basal conditions (Hu et al., 2009). However, C/EBP-β is phosphorylated and activated by ERK2 in response to certain stimuli such as cytokine stimulation (Roy et al., 2002). This activated C/EBP-β is able to remove ERK2 mediated repression and thereby induce transcriptional activation (Hu et al., 2009). As nuclear ERK2 levels increase, C/EBP-β is removed from GATE elements and transcriptional repression by ERK2 is reinstated, in a negative feedback fashion (Hu et al., 2009). Crosstalk between C/EBP-β and ERK2 explains the observed dynamics seen in GATE elements and is thought to underlie the regulation of IFN-γ singling response.

1.10 Rationale

Preliminary studies suggested that CHOP10 plays a repressive role at the QRU (Shi Yan (Unpublished Results)). It has been observed that in states where CHOP10 is down-regulated, there is increased formation of C/EBP-β homo-dimers, which results in the activation of p20K. Along these lines, knockdown of CHOP10 by shRNA results in super-induction and prolonged expression of p20K during serum starvation. However, depletion of CHOP10 did not result in induction of p20K in cycling cells. Therefore, we hypothesize that an additional control mechanism operates to block the expression of p20K in actively dividing cells.

In 2009, it was shown that ERK2 binds directly to a DNA consensus motif (Hu et al., 2009). These motifs consist of a direct repeat of a 5 basepair sequence, which is referred to as ERK2 binding sites (EBS) (Hu et al., 2009). Interestingly, a potential ERK2 binding motif is also present in the p20K QRU. In this context, EBS are nested in between and slightly overlapping the two C/EBP-β binding sites, which are required for activation of the p20K promoter in growth arrested cells. These binding sites are depicted in Figure 3. This observation suggests that ERK2 binds to the QRU and therefore may compete with C/EBP-β or function as a transcriptional repressor.

In this project, we investigate the potential role of ERK2 as a DNA binding protein and transcriptional repressor of the QRU during proliferative cell states.

1.11 Objectives

The primary objective of this project was to characterize the potential interaction of ERK2 with the QRU and determine the role of this kinase in the regulation of the p20K promoter.

In order to determine if ERK2 was playing a role at the QRU we first examined whether ERK2 interacted with the QRU directly, and if so whether this interaction was regulated. This was primarily addressed by Chromatin Immunoprecipitation assays (ChIP) analysis.

Finally, I sought to examine the role of ERK2 in the control of the p20K promoter by forced expression of ERK2 in combination with transient expression assays. This involved utilizing p20K promoter reporter constructs which either lacked or contained QRU regions. In doing so any influence of ERK2 on the promoter activity of p20K would be observed.

Chapter 2: Methods

2.1 Cell culture

Chicken Embryonic Fibroblasts (CEFs) were cultured in high glucose DMEM (Gibco #11995) supplemented with 5% heat inactivated cosmic calf serum (Hyclone #AUA33984), 5% Tryptose Phosphate Broth (Sigma #T8782), 1% penicillin/streptomycin (Gibco #15140) and 1% L-Glutamine (Gibco #25030). Culture conditions for these CEFs were 41.5˚C and 5% medical grade CO_2. These cells were split every 2-3 days into 10 cm plates (BD Falcon #353003) using 0.05% Trypsin-EDTA (Gibco #25300) at 1:3 dilutions. Cells were generally used for 10 passages at which point they were discarded and fresh cells were thawed.

2.2 SDS-PAGE and Western Blotting

2.2.1 Cell culture conditions

CEFs used for western blotting analysis were in either contact inhibited or cycling cell states. Contact inhibited cells were split 1:3 72 hours prior to harvesting. Cycling cells were split 1:3 40 hours prior to harvesting. Contact inhibited plates had media changed at the same time as the cycling cell plates were split. This was to avoid bias created by serum stimulation and to avoid acidosis. Cells which were to be grown in hypoxic conditions were split 1:3 and left overnight to attach and recover for cycling cell states. Following this overnight incubation cells were placed into a hypoxic chamber set at 2% O_2 for 24 h, after which they were harvested. The hypoxic chamber used for these experiments was calibrated prior to use. Normoxic cells used in parallel to hypoxic experiments were split at the same time but were left in a normal incubator (21% O_2)

overnight and then during the entire 24 h duration as well, after which they were harvested. Cells were inspected visually to ensure that they were either contact inhibited or cycling prior to harvest.

2.2.2 Cell lysate preparation

Once cells had been incubated in appropriate conditions for the designated period of time cells were collected. This was done by washing cells with 10mL of 1xPBS pH 7.4 (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4) twice. Cells were then lysed directly on the plate using 300µL of 1x SDS Sample Buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 60 mM Tris pH 6.8, Halt protease and phosphatase inhibitor cocktail (Halt – #78441)). Cell lysate was scraped off the plate, collected and heated at 100˚C for 5 min. Samples were then vortexed for 10 sec and centrifuged at maximum speed (16000 x g) for 10 min at 4˚C, to pellet cellular debris. The supernatant was transferred to a new microcentrifuge tube and lysates were stored at -80˚C until they were used.

2.2.3 SDS-PAGE and Western Blotting

Samples were thawed on ice and protein concentration was determined using a Bradford Assay. One hundred micrograms of protein lysate was aliquotted for each sample and volumes of each sample were normalized to the lowest concentration sample. These sample were then resolved on a 12% SDS-polyacrylamide gel. Gels were transferred to a nitrocellulose membrane (Mandel Scientific). Membranes were blocked with milk solution (5% skim milk powder, 0.02% Na azide, dissolved in 1xTBS pH 7.6 (50 mM Tris-HCl, 150 mM NaCl) for 1 hour at room temperature. Blots were incubated

in their respective primary antibodies in blocking buffer over night at 4˚C. The antibodies used were: rabbit α-p20K (1:2000 dilution), rabbit α-ERK1 (1:1000 dilution, Santa Cruz #sc-94), and mouse α-ERK2 (1:2000 dilution, Millipore #05-157 clone 1B3B9). After primary antibody incubations blots were washed four times with 1 x TBS pH 7.6 and incubated with their respective secondary antibodies for 1.5 hours in 5% milk solution (5% skim milk powder in 1 x TBS pH 7.6). The secondary antibodies used were: HPR conjugated α-Rabbit IgG (1:10000 dilution, Cell signaling #7074), and HPR-conjugated α-Mouse IgG (1:10000 dilution, Cell signaling #7076). Following secondary antibody incubations, blots were washed in 1 x TBS four times. Detection was then carried out using ECL (Pierce Thermo #32106) and hyperfilm (GE Healthcare #28906839) following the manufacturer's protocol.

2.3 EMSA Analysis

2.3.1 Cell culture conditions

CEFs used for Electromobility Shift Assay (EMSA) analysis were in either contact inhibited or cycling cell states. Contact inhibited cells were split 1:3 72 hours prior to harvesting. Cycling cells were split 1:3 16 hours prior to harvesting. Contact inhibited plates had media changed at the same time as the cycling cell plates were split. This was to avoid bias created by serum stimulation and to avoid acidosis.

2.3.2 Nuclear extract preparation

Cells were harvested for nuclear lysate which was to be used for interaction reactions with labeled probe. This was done by allowing cells to complete the designated time period for their respective incubations. Cells were inspected visually to ensure that

they were either contact inhibited or cycling prior to harvest. Nuclear lysate was prepared by scraping cells from plates in 1 x PBS. The cytoplasmic fraction was removed by resuspending cells in 400μL of cytoplasmic lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and Halt protease and phosphatase inhibitor cocktail). This resuspension was incubated on ice for 15 min at which time 25 μL of 10% NP-40 was added and samples vortexed for 10s. Nuclei were pelleted in a microcentrifuge at 11,000 x g for 3 min at 4˚C . Nuclei were then solublized in 50 μL of nuclear extraction buffer (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and Halt protease and phosphatase inhibitor cocktail). This resuspension was incubated on ice for 15 min, shaking vigorously. Insoluble debris was removed by centrifugation at 16,000 x g for 5 min. The supernatant was transferred to a new microcentrifuge tube. Protein concentration was then determined using Bradford assays and nuclear extracts were diluted to 2 μg/μL. Ten microgram aliquots were then frozen at -80˚C until use.

2.3.3 Probe generation and labeling

Fragments corresponding to -200 to -179 bp region of the QRU were generated by ordering oligonucleotides which correspond to the sense and anti-sense sequence of this region. These fragments had *Hind*III restriction sites placed on the ends of the coding region in case cloning into a vector was later required. For the ERK2 binding site mutant QRU probe, oligonucleotides for WT QRU was altered such that one of the alanines in the center triplet of the ERK2 consensus motif was mutated. In this case both ERK2 binding sites were mutated. This mutation was mindful of neighboring C/EBP-β binding

sites and these binding sites remained intact. The sequences for these oligonucleotides are as follows:

WT QRU F: AGCTTACACTTTCCTCTTTCCGTAAGCT,

WT QRU R: GCTTACGGAAAGAGGAAAGTGTAAGCT,

EBS Double Mutant QRU F: AGCTTACACT**G**TCCTC**G**TTCCGTAAGCT,

and EBS Double Mutant QRU R: AGCTTACGGAA**C**GAGGA**C**AGTGTAAGCT.

Refer to Figure 7 for a schematic representation of these probes. EBS mutant QRU probes where a single ERK2 binding site was mutated were also ordered, however these oligonucleotides have not been used as of yet. Oligonucleotides were ordered from Mobix. Upon arrival these oligonucleotides were diluted to 100 mM and stored at -20˚C. In order to form duplex QRU probes for EMSA these single stranded oligonucleotides were biotin labeled using a 3' end Biotin labeling kit (Pierce Thermo #89818). This was done according to the manufacturer's protocol. Following biotin end labeling, complementary oligonucleotides were annealed by mixing equal volume of single stranded complementary oligonucleotides into a single solution. This was then heated to 90˚C for 1 min and allowed to cool slowly to room temperature. Once cooled the reaction was frozen and stored at -20˚C until use.

2.3.4 Interaction reactions

Labeled probe and nuclear protein extract were incubated together in order to assess whether any complexes would form. This interaction reaction was conducted by adding 2 μg of poly dI-dC, 2 μg of nuclear protein extract, and 200 fmol of probe to a 1 x interaction buffer (25 mM HEPES pH 7.5, 50 mM KGlu, 0.1% Triton X-100, 2 mM

$MgAc_2$, 5% glycerol, 3 mM DTT, and Halt protease and phosphatase inhibitor cocktail) for a final reaction volume of 20 µL. The reaction was conducted at room temperature for 30 min and loaded on to a non-denaturing polyacrylamide gel.

2.3.5 PAGE and detection

Completed reactions were loaded onto a pre-run 4.8% non-denaturing polyacrylamide gel. This was then run in 0.5 x TBE (10x = 121.1g Tris, 55g Boric Acid, 7.4g EDTA (Sodium salt)) at 4˚C. A mock sample containing bromophenol blue was loaded beside actual reactions. Once the dye ran approximately 2/3 of the gel, electrophoresis was stopped and the gel was then transferred onto a nylon membrane (Schleicher & Schell Bioscience) in 0.5 x TBE. Following transfer, the membrane was crosslinked using a 312 nm UV lamp for 10 min. Detection was then carried out using a HPR-conjugated streptavidin Chemiluminescent kit (Peirce Thermo #89880) and hyperfilm (GE Healthcare #28906839). This was done according to the manufacturer's protocol with the exception that washes in between blocking and binding steps were increased in duration by three fold.

2.4 ChIP Analysis

2.4.1 Cell culturing conditions

CEFs used for ChIP analysis were in either contact inhibited or cycling cell states. Contact inhibited cells were split 1:3 72 hours prior to harvesting. Cycling cells were split 1:3 40 hours prior to harvesting. Contact inhibited plates had media changed at the same time as the cycling cell plates were split. This was to avoid bias created by serum stimulation and to avoid acidosis. Cells which were to be grown in hypoxic conditions

were split 1:3 and left overnight to attach and recover for cycling cell states. Following this overnight incubation cells were placed into a hypoxic chamber set at 2% O_2 for 24h, after which they were harvested. The hypoxic chamber used for these experiments was calibrated prior to use. Normoxic cells used in parallel to hypoxic experiments were split at the same time but were left in a normal incubator (21% O_2) overnight and then during the entire 24 h duration as well, after which they were harvested. Cells were inspected visually to ensure that they were either contact inhibited or cycling prior to harvest. These cells were then all harvested at roughly the same time.

2.4.2 ChIP lysate preparation

Once samples had undergone their designated experimental conditions, cells were fixed using 1% formaldehyde for 10 min. Excess formaldehyde was quenched using 10 x glycine (1.25 M glycine). Cells were then washed twice with 10 mL of cold 1 x PBS and harvested by scraping in 1 x PBS. Cell pellets were resuspended in 1 mL of SDS Lysis Buffer (1 % SDS, 10 mM EDTA, 50 mM Tris pH 8.1, Halt protease and phosphatase inhibitor cocktail). Lysates were stored at -80˚C.

2.4.3 Immunoprecipitations, Washes, and DNA Purification

Lysates were thawed on ice. Four hundred microliters of the original lysate was used per immunoprecipitation (IP). These 400µL aliquots were sonicated using an inverted cup horn sonicator. The sonication conditions are as follows: set to 50% output, for a total of 6 minutes, in 20 second pulses of sonication /rest. Samples were then centrifuged at maximum speed to remove insoluble debris. The entire 400 µL sample was then pre-cleared using blocked Protein A beads (Millipore #16-757). Afterwards these

beads were removed and discarded. Samples were diluted to 1mL final volume using ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCL pH 8.1, 167 mM NaCl, Halt protease and phosphatase inhibitor cocktail). At this point a 10 μL pre-immune input sample was taken, and the remainder of sample was divided into two immunoprecipitations: an IgG IP control and an experimental IP sample (~500 μL each). Experimental immunoprecipitations were conducted using the following antibodies: mouse α-ERK2 (4 μg, Millipore #05-157 clone 1B3B9) or rabbit α-ERK1 (4 μg, Santa Cruz #sc-94). IgG IP controls contains 4 μg of their respective host's IgG. The IP's were diluted further to a total volume of 1 mL. Once antibody was added immunoprecipitations were conducted overnight at 4˚C with constant rotation. Following this incubation antibody complexes were pulled down using blocked Protein A beads for 1 h. During this 1 h incubation 2 μg of rabbit α-mouse IgG (Jackson Immunoresearch #315-005-003) was added to all samples to increase mouse antibody affinity for Protein A beads. After this incubation bound antibody – protein – DNA complexes were pulled down with Protein A beads using centrifugation.

These beads and bound complexes were then placed through a series of washes: once with low salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), once with high salt immune complex wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), once with LiCl immune complex wash buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholic acid (sodium salt), 1 mM EDTA, 10 mM Tris-HCl pH 8.1), and twice with TE Buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0). All wash solutions were 4˚C. All

sample washes used 1mL of wash solution and were incubated on a nutator for 5 min, after which beads were pelleted by centrifugation and subjected to the next wash.

Following these washes, bound complexes were eluted using 100 μL of elution buffer (0.1M $NaHCO_3$, 0.005% SDS) incubated at room temperature for 15 minutes. Beads were pelleted using centrifugation, after which the supernatant was transferred to a new microcentrifuge tube. This elution was repeated once more and elution fractions were combined. These samples were then de-crosslinked over night at 65˚C. Following this incubation samples were subjected to RNA digestion using RNase A and protein degradation using Proteinase K. DNA was ethanol precipitated, pelleted, and resuspended in 20 μL 1 x TE buffer.

2.4.4 PCR Amplification of Purified DNA

PCR reactions were used to assess whether the QRU was immunoprecipitated. This entailed using ChIP purified DNA as template DNA for PCR reactions. Primers against the p20K promoter region carrying the QRU were used in order to amplify any QRU bearing genomic DNA which may have been pulled down. In addition to a PCR reaction which amplifies the QRU, a reaction containing p20K intron primers was also conducted in parallel to ensure that the QRU specifically was purified. Thus, there were 3 samples for every primer set: a pre-immune control IP, an IgG control, and an experimental IP, all three of which were done in duplicate; once for the p20K intron and once with the QRU. PCR reactions were conducted using GoTaq Green PCR mix (Promega #M712B). A typical PCR reaction was composed of 12.5 μL of 2x GoTaq Green PCR Mix, 0.25-1 μL template DNA (ChIP purified DNA), and 1 mM forward and

reverse primers. These PCR reactions were brought to a final volume of 25 μL and subjected to amplification. p20K intron primers were used at 2 mM concentration because better amplification was observed. The PCR program used was as follows: 94˚C for 5 min, (94˚C for 30 sec, 55˚C for 30 sec, 72˚C for 35 sec) x 15 cycles, after the first 15 cycles the elongation time increases by 5 sec every cycle to a total of 40 cycles, 72˚C for 10 min, 4˚C until samples are removed. Following amplification PCR reactions were resolved using agarose gel electrophoresis. This was then subjected to visualization using ethidium bromide and a UV transilluminator.

2.5 Immunoprecipitations

Immunoprecipitation experiments were performed as a control to illustrate that both ERK1 and ERK2 antibodies were capable of immunoprecipitation. Here cells were fixed, collected, lysed, and sonicated as described in the ChIP section. Following this the lysate was removed of debris, and pre-cleared with Protein A beads. A 10 μL aliquot was taken as a pre-immune input, which was empirically 1% of the amount of protein used in the IP. Following this; as was done for ChIP, samples were diluted to 1 mL with ChIP dilution buffer and placed into the IP reaction overnight with either 4 μg of ERK1 or ERK2 antibodies. The next day, washes were conducted the exact same manner as was done for ChIP. Fractions were eluted in a similar manner as described in the ChIP protocol, followed by the same de-crosslinking incubation. After de-crosslinking elutions were resuspended in 4 x Lamelli Buffer (8% SDS, 40% Glycerol, 20% β-Mercaptoethanol, 0.008% Bromophenol Blue, 0.125M Tris pH 6.8) and boiled for 5 min. This was then analyzed by SDS-PAGE and western blotting as described in section 2.2.3.

Immunoprecipitations were blotted for with the antibody used to immunoprecipitate, thus allowing us to gauge whether protein was being purified under ChIP conditions.

2.6 Cloning of ERK2 overexpression vector

Mammalian ERK2 was cloned into a transient expression vector called pCAGIG. Originally ERK2 was sent to use by the Zhu group at John Hopkins University, School of Medicine. This was in a yeast expression vector called pEGH-A-ERK2. Using GATEWAY recombination based cloning ERK2 was shuttled from this yeast expression vector into a pCAGIG which was modified with a GATEWAY Cassette cloned into the EcoRV sites. This shuttling was done according to manufacturer's protocol (Invitrogen). This entailed using a BP clonase reaction in order to shuttle ERK2 (in a GATEWAY Cassette) from pEGH-A-ERK2 to an intermediate vector called pDONR201. A LR clonase reaction was then used to shuttle ERK2 to our modified destination vector pCAGIG. This results in a vector which has ERK2 cloned into an open reading frame of the pCAGIG vector. This vector is under the control of a CAG promoter and thus should overexpress ERK2 in CEFs. The products of these cloning reactions were transformed into DH5α competent cells (Invitrogen #18265-017) and grown on LB Agar with ampicillin (100 μg/mL). This selects for positive clones only as the GATEWAY system negatively selects for clones lacking ERK2 recombination. Resulting colonies were inoculated and cultures were harvested for plasmid DNA using a mini-prep kit (Bio-Basic #BS614) according to manufacturer's protocol. The presence of ERK2 in pCAGIG was confirmed with sequencing. Maxi-preps of this vector and the empty vector control were conducted for transfections according to the manufacturer's protocol (Qiagen #12163).

2.7 Transient Expression Analysis (CAT Assays)

2.7.1 Transfections

Transient expression assays required that several different vectors be co-transfected in different combinations. In order to do this we employed the calcium phosphate method of transfection. Here we ethanol precipitated DNA, resuspended it in water and precipitated it into cell growth medium using 142.2μM $CaCl_2$ and 2 x HBSP (1.5 mM Na_2HPO_4, 10 mM KCl, 280 mM NaCl, 12 mM Glucose, 50 mM HEPES, pH 7.12). Cells were allowed to incubate with precipitated DNA for 4 hours at which point they were shocked with a 15% glycerol solution for 30 seconds, washed and incubated overnight for recovery. These cells were either split the following day or had media changed.

In the case of CAT assays we employed 2 different CAT constructs. These constructs are depicted in Figure 13. The first construct is pJFCAT-169. This contains the promoter region of p20K up to but excluding the QRU. The pJFCAT-217 construct contains the promoter region of p20K up to and including the QRU. These two constructs were transfected with pCH110. This is a vector that contains the *lacZ* gene under the control of a SV40 promoter. This promoter is constitutively active and is not subject to up regulation or down regulation based on cellular states. These constructs were also co-transfected with either pCAGIG or pCAGIG-ERK2. The transfection reactions were set up as follows: 10 μg of either pJFCAT-169 or pJFCAT-217, 5 μg of PCH110, 5 μg of either pCAGIG or pCAGIG-ERK2, and 10 μg single stranded salmon sperm DNA. Different combinations of these vectors were transfected into CEFs which were then

grown to confluency (contact inhibited) or split such that they would be cycling at the time of harvest. Transfections for each condition were conducted in triplicate in order to allow for statistical analysis. All transfections were done using plasmid DNA which was amplified in DH5α Escherichia coli and purified using a Maxi-Prep kit (Qiagen #12163).

2.7.2 Preparation of cell lysates

Cells were collected by scraping in the media in which they were grown. These cells were pelleted and resuspended in 5 mL of TEN buffer (40 mM Tris-HCl pH 7.5, 10 mM EDTA, 150 mM NaCl). Cells were pelleted again and resuspended in 150 μL of 250 mM Tris-HCl pH 8.0. These cells were then transferred to a microcentrifuge tube. In order to lyse these cells the samples were subjected to freeze thaw cycles. This entails freezing in a dry ice methanol bath for 5 min and then heating in a 37˚C water bath for 5 min. This was repeated a total of 3 times. After this, cellular debris was removed by centrifugation at maximum speed (16000 x g) for 3 min. The supernatant (lysate) was transferred to a new microcentrifuge tube and stored at -80˚C until use.

2.7.3 Normalization of lysates

Due to the fact that individual transfections can have different transfection efficiencies, we need to normalize the amount of lysate we use so that the level of transfected plasmid is equal between all of our samples. This function was fulfilled by the pCH110 plasmid. Since it has a SV40 promoter, levels are fairly consistent in different conditions. The SV40 promoter drives expression of a *lacZ* gene which is then translated into β-galactosidase. We used the level of β-galactosidase activity to normalize all of our samples such that we account for transfection efficiency. In order to do this we conducted

β-galactosidase assays for all of our samples. This entails generating a reaction containing 20 μL of lysate, 211 μL of sodium phosphate buffer (0.1 M Na_2HPO_4 pH 7.3), 3 μL of 100 x Mg^{2+} Buffer (100 mM $MgCl_2$, 5 M β-Mercaptoethanol), and 66μL ONPG (4 mg/mL o-nitrophenyl-β-D-galactopyranoside in Sodium Phosphate Buffer). These reactions were incubated at 37˚C for 20 min at which point they were stopped using 500 μL of 1 M Na_2CO_3. The absorbencies at 410 nM were measured and used as a gauge for transfection efficiencies of each sample. Lysates containing an equal level of β-galactosidase activity were then assayed for CAT activity.

2.7.4 CAT Assay

Normalized volumes of lysate based on the results of the β-galactosidase assay were used for each reaction. The final volume of each reaction was brought to 165 μl and all samples were heated at 65˚C for 10 min. Following this incubation 10 μL of a 1:1 mixture of acetyl-Co A (23.3 mg/mL) and C^{14} labeled chloramphenicol (Perkin Elmer #NEC408A250UC) was added to each reaction. These reactions were then incubated at 37˚C for 30 min. Following this another 5 μL of acetyl-Co A was added and the reaction was allowed to continue for another 30-45 min. Following this reaction, all of the C^{14} labeled chloramphenicol and acetylated products were extracted using 800 μl ethyl acetate. This extracted solution was then transferred to a new microcentrifuge tube and subjected to speed vacuum drying. Dried samples were resuspended in 20 μL of ethyl acetate. These samples were then blotted onto a thin layer silica chromatography plate. The different chloramphenicol species were then resolved by running this chromatography plate in 95:5 chloroform: methanol solvent. This plate was then dried

and exposed to a phosphor imaging screen (Molecular Dynamics) overnight. The different unacetylated and acetylated products were quantified.

2.7.5 Quantification

Unacetylated and acetylated products were quantified using Imagequant. Briefly, this entailed getting a density report on all the species of chloramphenicol present in each individual sample. This density report was then used to determine percentage conversion of chloramphenicol. That is to say that the relative amount of acetylated chloramphenicol was determined and compared to unacetylated chloramphenicol. Then the percentage conversion (acetylted : total chloramphenicol) was calculated. The percentage conversion for each reaction was determined. The mean was taken for each condition and the standard error was determined for each condition as well. These values were then graphed using Excel. Error bars represent standard error. Furthermore T-Tests were conducted on samples we wished to compare in order to determine statistical significance.

Chapter 3: RESULTS

3.1 Establishment of a set of conditions under which potential ERK2 transcriptional repression can be studied

In order to establish that ERK2 acts as a transcriptional repressor for the QRU as it does for GATE elements in interferon signaling, the conditions under which p20K is expressed or repressed must first be established. In the case of interferon signaling it was shown that there is basal repression of IFN-γ induced genes until IFN-γ signaling molecules activate downstream signaling proteins and induce gene expression (Roy et al., 2000; Xiao et al., 2001; Hu et al., 2009). This signaling event resulted in removal of ERK2 mediated transcriptional repression (Hu et al., 2009). In the case of p20K it has been previously shown that transcriptional repression occurs in proliferative cell states, that is to say when cells are cycling (Bédard et al., 1987; Bédard et al., 1989; Mao et al., 1993). In contrast, CEFs arrested by contact inhibition express copious amounts of p20K (Bédard et al., 1987; Bédard et al., 1989; Mao et al., 1993). Furthermore, preliminary work conducted by Romita Ghosh and Ben Fielding (Bédard lab members) indicated that p20K is also induced in response to moderate hypoxia (2% O_2 for 24h).

In order to establish p20K expression patterns in which possible ERK2 repression could be observed, CEFs were grown to confluency (contact inhibition) or seeded such that they were still proliferating at the time of harvesting (cycling). This was done in either normoxic (21% O_2) or hypoxic conditions (2% O_2 for 24h). Cells grown under these conditions were collected for protein lysates which were then subject to SDS-PAGE

and western blotting analysis using a p20K specific antibody. These results are shown in Figure 4.

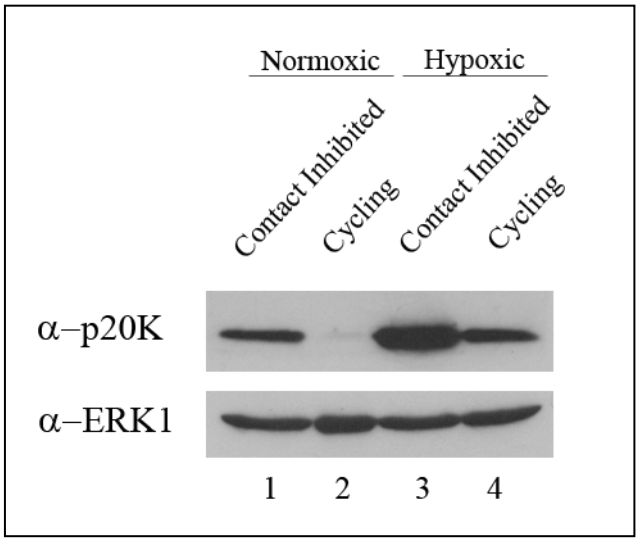


Figure 4: p20K is induced in states of growth arrest and cellular stress. Western analysis of protein lysates from CEFs grown in normoxia (21% O_2) or hypoxia (2% O_2 for 24h) in either contact inhibited or cycling cell states. Immunoblotting was conducted using an antibody specific for p20K. ERK1 was

In cycling samples which were grown in normoxia (a proliferative cell state), p20K expression was virtually undetectable. However in quiescence cell states (in this case contact inhibition induced growth arrest), there was a stark induction of p20K expression. Additionally p20K is further induced in hypoxic conditions, in both cycling and contact inhibited cell states. This provides a set of defined cell states and two different inducing conditions under which to study p20K and potential ERK2 mediated transcriptional repression.

3.2 ERK2 interacts with the QRU in states of p20K transcriptional repression

3.2.1 Analysis of ERK2 binding to the QRU by Elelctromobility Shift Assay (EMSA)

Assuming that ERK2 is playing a role in inhibiting the expression of p20K in cycling cells, we would expect that it be bound to the QRU in cycling cell states since there is strong repression of p20K in this cell state. Particularly, it would be bound to ERK2 binding sites which are nested between C/EBP-β binding sites in the QRU.

The sequence of the QRU is known, thus it is not difficult to use mutational analysis to generate QRU probes in order to assess the potential interactions of ERK2 with the QRU. This approach was used in combination with EMSA in order to assess whether we could detect ERK2 specifically binding to the QRU. Here we generated DNA duplex probes which either corresponded to the WT QRU sequence or mutant QRU probes which have a single nucleotide base pair mutation in both putative ERK2 binding sites (EBS Mutant QRU). The region used to generate these probes spans from -200 to -179 bp from the transcriptional start site. This fragment corresponds to the region of the

QRU which harbors both putative ERK2 binding sites, as well as a partial C/EBP-β binding site A and a full C/EBP-β binding site B. There is a slight overlap between C/EBP-β and ERK2 binding site consensus sequences. Therefore caution was taken when generating these mutants, such that C/EBP-β binding site sequences were not disturbed, but ERK2 binding sites were completely abolished. A schematic representation of these probes is depicted in Figure 5 and ERK2/C/EBP-β binding sites are illustrated in Figure 3. These WT and EBS mutant probes were then labeled with a biotin terminal transferase and used in interaction reactions with nuclear lysates from either contact inhibited or cycling CEFs. These interaction reactions were then subjected to non-denaturing polyacrylamide gel electrophoresis. Once this electrophoresis was completed, migrated probe and/or complexes were transferred onto a nylon membrane. This then allows for the use of streptavadin conjugated HPR to detect any biotinylated probe, and by association any shifts created by interacting protein complexes. This EMSA analysis is shown in Figure 6.

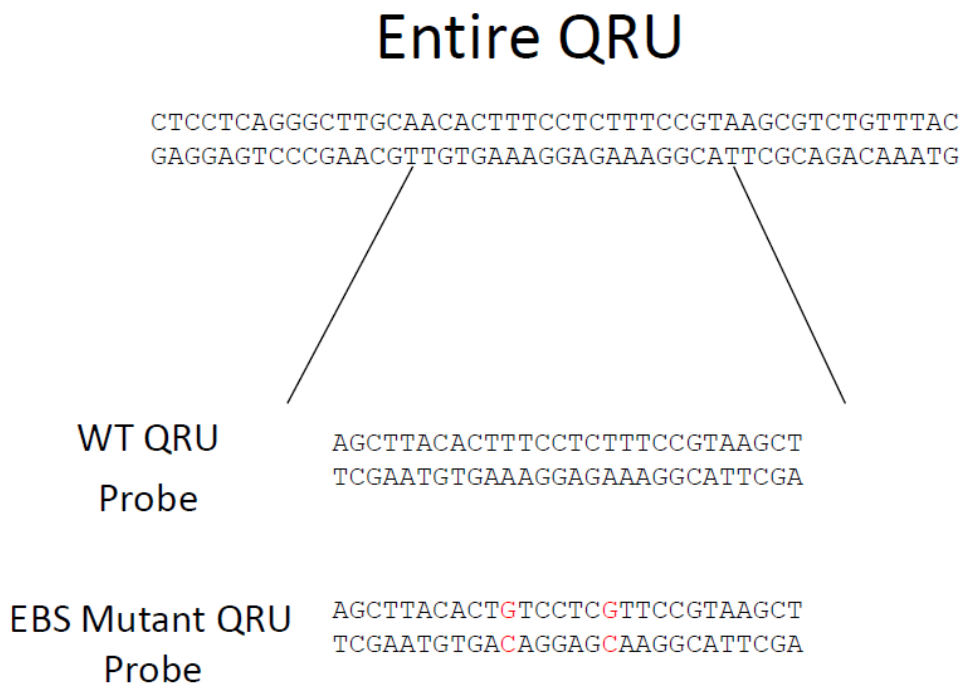


Figure 5: Schematic representation of WT and EBS mutant probes used for EMSA analysis. Two different probes were used to assess whether ERK2 binds to the QRU via the putative ERK2 binging motifs. WT QRU probe consist a duplex DNA fragment which corresponds to -200 to -179 bp of the QRU. Another probe was generated such that the consensus ERK2 binding motifs were destroyed by mutating one of the alanines in the central triplet. Caution was taken in generating these mutations such that C/EBP-β binding sites were not destroyed. These probes were then used in interaction reactions using nuclear lysate from CEFs, and were subjected to EMSA analysis in order to detect any possible complexes.

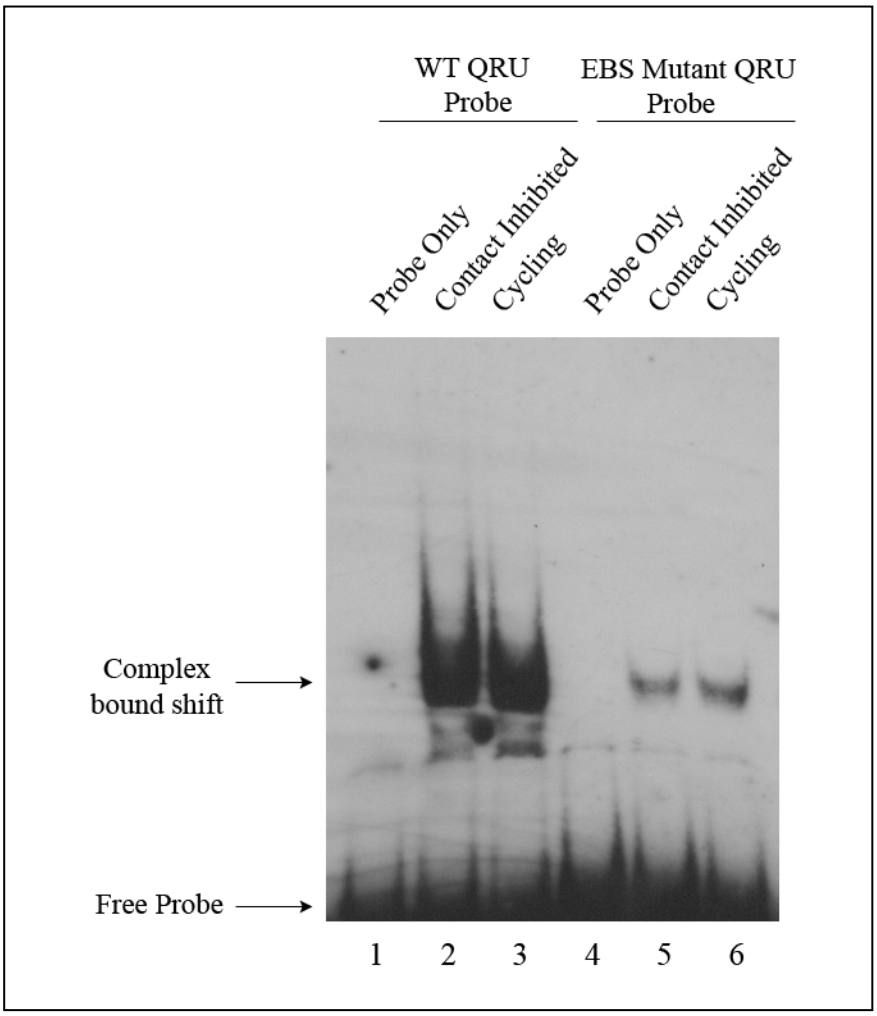


Figure 6: Complex formation on the EBS of the QRU. EMSA analysis of WT QRU Probe or Probe which has had both ERK2 binding sites destroyed with single nucleotide mutations (Mutant EBS probe). These mutations destroy the consensus sites for ERK2 but leave the partial C/EBP-β sites at the extreme ends of the probe, intact. These probes were incubated in an interaction reaction with nuclear lysates from either contact inhibited or cycling CEFs. These reactions were then subject to non-denaturing gel electrophoresis and subsequent detection procedures.

WT QRU probes bind to a very prominent and distinct complex generating an obvious shift. This shift occurs in both contact inhibited and cycling cell state samples. This complex was greatly diminished when the ERK2 binding sites in the QRU were mutated in both contact inhibited and cycling cell samples.

This EMSA analysis was ultimately unsuccessful at showing that ERK2 binds to the QRU *in vitro*. We deem this experiment unsuccessful in the sense that we did not see any regulated difference between contact inhibited and cycling lysate samples. This would indicate that the observed shift is not controlled by cell state. Furthermore, there is no evidence definitively illustrating that the observed shift is an ERK2 complex. The C/EBP-β binding site is still intact in these probes and thus the observed complex may be a C/EBP-β containing complex, however this is unlikely. Super-shift experiments; where we attempted to shift this observed complex using an ERK2 specific antibody, were conducted however these experiment were un-successful at producing ERK2 antibody bound shifts. We attribute the failure of this specific experiment to the fact that EMSA is an *in vitro* approach to studying this binding event and is subject to several caveats and technical limitations.

3.2.2 Analysis of ERK2 binding to the QRU by Chromatin Immunoprecipitation (ChIP)

Chromatin Immunoprecipitation (ChIP) analysis is a very sensitive method of detecting DNA – protein interactions *in vivo*. This assay involves using an antibody to purify DNA sequences bound by a specific protein and then using traditional DNA analysis tools to study purified DNA directly. This *in vivo* approach allows us to

determine if a protein is binding to a specific DNA sequence within cells. Thus, it is very sensitive and is also an extremely biologically relevant method of studying DNA-protein interactions. In order to study a potential ERK2 interaction with the QRU we employed ChIP analysis. Briefly, this experiment entailed taking either contact inhibited or cycling CEFs and crosslinking all DNA and proteins using formaldehyde. We then lysed these fixed cells, sonicated the lysates to break the chromatin into approximately 200-600 bp fragments. We removed a pre-immune input sample and placed the remainder into an immunoprecipitation reaction with α-ERK2 antibody or IgG (IP negative control). Once these immunoprecipitations were completed antibody bound ERK2 was captured using Protein A beads. These beads were processed through a series of washes to remove unbound and non-specific cellular matter and then de-crosslinked. The protein and RNA was removed from these samples, leaving only DNA which was bound, and thus purified by ERK2. These purified DNA samples were then used as template DNA in PCR reactions using primers specific for the QRU. Thus, if ERK2 binds to the QRU we would expect to see it being precipitated and then amplified in PCR reactions. To ensure that only the QRU was being immunoprecipitated a parallel PCR reaction was carried out using primers against a p20K intron sequence. This essentially acts as a specificity control, ensuring that ERK2 is specifically bound to the QRU and not a distant DNA site which gets precipitated when we purify ERK2 bound sequences. By conducting this experiment we assayed for ERK2 interacting with the QRU in conditions where p20K is transcriptionally repressed. The results for this ChIP/PCR analysis are shown in Figure 7.

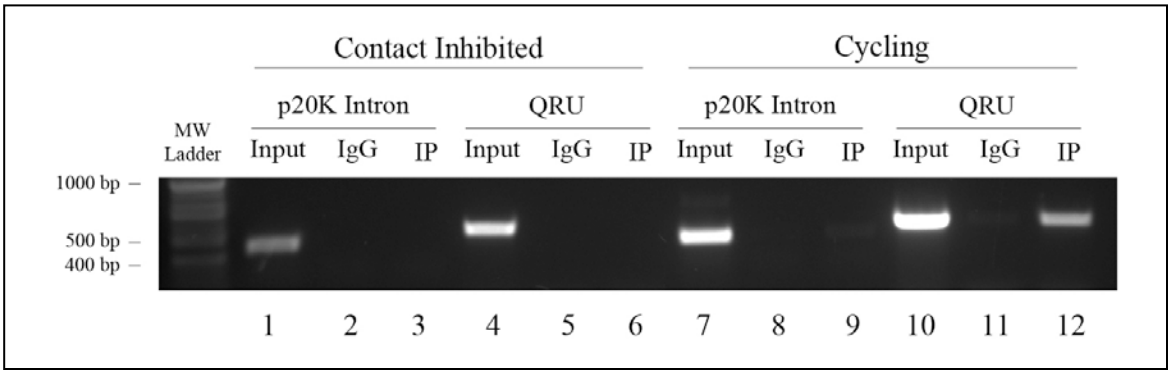


Figure 7: ERK2 binds to the QRU in cycling but not contact inhibited cells. ChIP analysis of contact inhibited and cycling CEFs. Lysates were immunoprecipitated using an α-ERK2 monoclonal antibody. IgG was used as an IP negative control. Precipitated DNA was used in PCR reactions containing primers which amplify the QRU. As a negative control reactions with primers against a p20K intron were carried out in parallel to ensure that the QRU was being specifically pulled down.

Figure 7 clearly shows that ERK2 immnoprecipitates with the QRU in cycling cells but not in contact inhibited cells. This strongly indicates that ERK2 interacts with the QRU in proliferative cell states (where p20K is repressed). However in quiescent cells states such as contact inhibition (where p20K is expressed) there is no detectable ERK2 bound. This lends support to the notion that; like with GATE elements, ERK2 plays a transcriptional repressive role at the QRU during proliferative cell states.

In order to further substantiate this observation we conducted similar ChIP analysis using an antibody against ERK1. This serves to demonstrate that this phenomenon is specific to ERK2. Seeing as how Hu et al. did not observe ERK1 binding to DNA in their proteomics study it would seem like a strong candidate for using as a specificity control. Additionally, ERK1 lacks critical amino acids in the region analogous to the DNA binding domain, which are present in its ERK2 counterpart. This may mitigate ERK1's DNA binding ability. Thus the same ChIP assay was conducted using ERK1 and ERK2 antibodies in parallel samples in order to IP these ERK proteins and possibly the QRU. Similarly ChIP purified DNA samples were used in PCR reactions which utilized QRU amplifying primers. Simultaneously, p20K intron primers were used as a negative control. The results of this assay are in Figure 8.

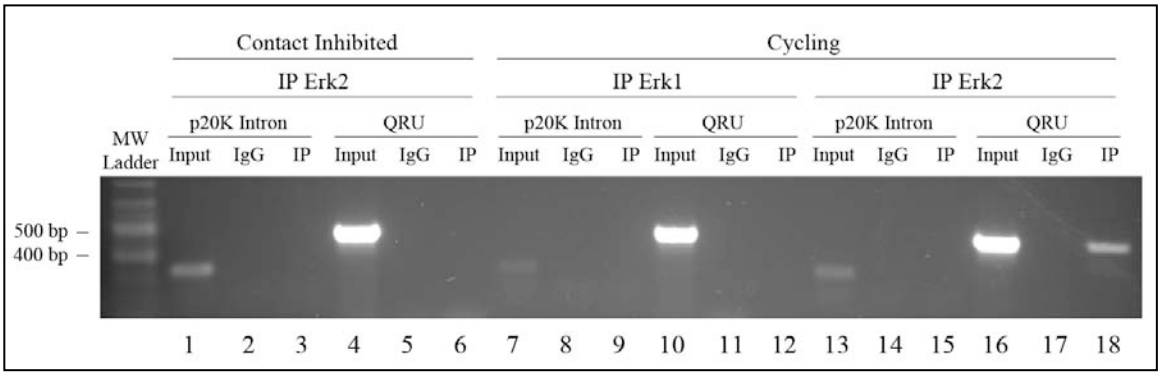


Figure 8: ERK1 does not bind to the QRU in proliferative cell states. ChIP analysis of contact inhibited and cycling CEFs using ERK2 or ERK1 antibodies. IgG was used as an IP negative control for α-Erk2 and α-Erk1. PCR reactions were carried out with primers against the QRU. Negative control reactions with primers against a p20K intron were carried out in parallel.

Figure 8 illustrates that the QRU is only immunoprecipitated in cycling cells using α-ERK2 but not by using α-ERK1. In this manner ERK1 serves as a valid negative control for testing the physiological relevance of ERK2 binding to the QRU. Furthermore, this abides by the results predicted by our putative model, further lending support that this is a genuine phenomenon.

As a control experiment we ensured that both antibodies were capable of immunoprecipitating their respective proteins under ChIP conditions. This entails subjecting samples to the same harvesting, immunoprecipitating, and washing conditions as done in the actual ChIP assays. However, in this case we test whether the antibodies are immunoprecipitating the protein of interest by subjecting the samples to western blotting analysis following ChIP washes. In this way we can ensure that the observed lack of ERK1 interaction with the QRU is not the results of ERK1 antibody's inability to immunoprecipitated ERK1. This control immunoprecipitation assays are shown in Figure 9.

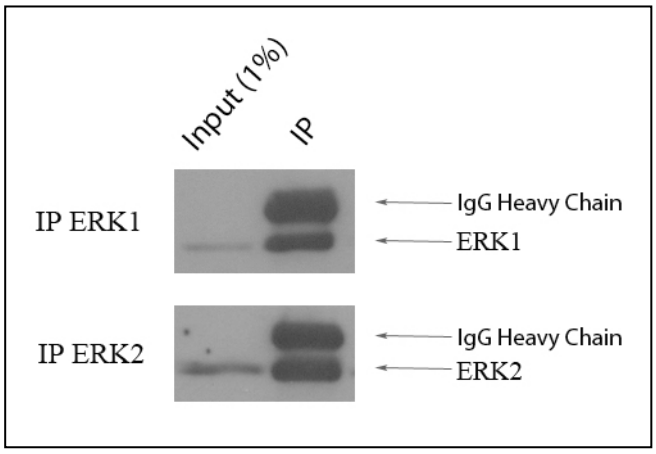


Figure 9: ERK1 and ERK2 antibodies are capable of immunoprecipitating under ChIP conditions. Erk1 and Erk2 immunoprecipitations were conducted using conditions used for ChIP assay analysis. These samples were subject to western analysis following standard ChIP washes. Each IP was blotted for with its respective antibody.

Here we can clearly see that both ERK1 and ERK2 antibodies are immunoprecipitating their respective proteins under ChIP assay conditions. This solidifies the results obtained in Figure 7 and 8 because any shortcoming of the antibody in terms of immunoprecipitation can be ruled out.

3.2.3 ERK2 binds to the QRU in conditions of normoxia by not hypoxia

Following these results we sought to lend additional support to this phenomenon. This was done by using ChIP analysis once again. As you may recall from Figure 4, there is a stark induction of p20k in hypoxic conditions. This implies that in hypoxia CEFs will express p20K, even if they are cycling. Our model suggests that if ERK2 is acting as a transcriptional repressor of the QRU we would be unable to immunoprecipitate the QRU in hypoxic conditions, just as was the case for contact inhibited cells. Thus we conducted an experiment using hypoxic conditions as an additional means to assess the interaction of ERK2 with the QRU. In this experiment we utilized either contact inhibited or cycling CEFs in normoxic (21% O_2) or hypoxic (2% O_2 for 24h) conditions. These cells were then collected as was described for standard ChIP analysis. Chromatin immunoprecipitations were conducted using ERK2 antibody and IgG was used as a negative control. Purified DNA was then subject to PCR amplification using either primers for the QRU or for a p20K intron as a negative control. The results of this analysis are shown in Figure 10.

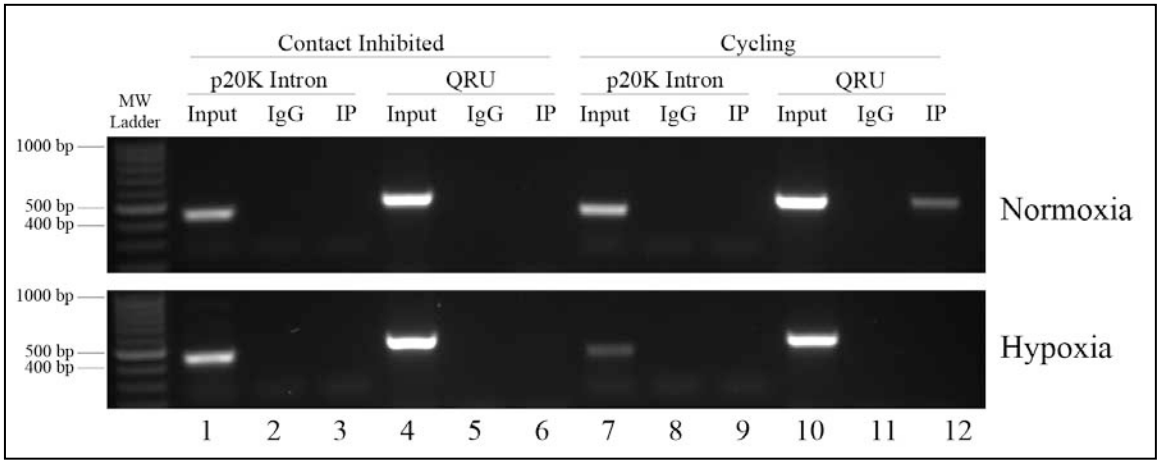


Figure 10: ERK2 does not interact with the QRU in conditions of hypoxia. ChIP analysis of contact inhibited or cycling CEFs in normoxic and hypoxic conditions was conducted. CEFs were grown in normoxic conditions (21% O_2) or hypoxic conditions (2% O_2) and seeded such that they were contact inhibited or cycling in either condition. Lysates were immunoprecipitated using an α-Erk2 monoclonal antibody. IgG was used as an IP negative control. Precipitated DNA was used in PCR reactions where primers amplifying the QRU were utilized. Negative control reactions with primers against a p20K intron were carried out in parallel.

This experiment clearly demonstrates that in normoxic states ERK2 is only bound to the QRU in cycling cells, whereas this interaction is lost in normoxic contact inhibited cells or cells in hypoxic conditions. This datum supports that in states where p20K is expressed, such as in response to contact inhibition or hypoxia, ERK2 is no longer interacting with the QRU. However in states where p20K expression is repressed ERK2 interacts with the QRU. This experiment further supports the notion that ERK2 dependent transcriptional repression at the QRU is a significant mechanism for p20K regulation.

3.3 ERK2 acts as a transcriptional repressor of the QRU

The evidence we have presented until now has illustrated that ERK2 interacts with the QRU in states where p20K is repressed. That is to say that in growth arrested or hypoxic cell states p20K is expressed. Simultaneously, ERK2 has not been detected interacting with the QRU in these quiescent cell states *in vivo*. Whereas in proliferative cell states; where p20K is repressed ERK2 has been detected as being bound to the QRU *in vivo*. Although this is circumstantial evidence of ERK2 acting as a transcriptional repression of p20K, in order to truly determine if ERK2 is functionally active at the QRU, functional assays are required. In order to demonstrate that ERK2 acts functionally as a transcriptional repressor of the QRU we undertook transient expression assays in order to investigate its functional role.

Briefly, this entailed conducting transient expression assays which utilized a reporter gene downstream of a promoter element. Here we utilized the chloramphenicol acetyltransferase (CAT) system. CAT is an enzyme which acetylates chloramphenicol,

using acetyl – Coenzyme A as an acetyl donor. Expression vectors placing this gene downstream of promoter elements can be used to control the expression of CAT. CAT activity is directly proportional to the levels of CAT and thus the activity of the promoters which drive its expression. Thus, this CAT system can be used to assess whether certain promoter elements activate or repress gene expression. Different promoter elements are tested for transcriptional activation in different conditions by cloning these elements upstream of the CAT gene. These constructs are then transfected in cells and CAT activity of the resulting constructs in a given condition is indicative of promoter activation. The level of CAT activity is assessed by conducting CAT assays with lysates which are normalized for transfection efficiency. The amount of acetylated chloramphenicol is detected using thin layer chromatography. The percentage conversion (acetylated species) is calculated and used to gauge activation by promoter elements in different conditions.

Using this system, we utilized two different CAT constructs: One of these construct contains a fragment of the p20K promoter which excludes the QRU (-169) and the other construct contains a fragment of the p20K promoter which contains the QRU (-217) (Marc et al., 1993). A schematic diagram of these two constructs is depicted in Figure 11.

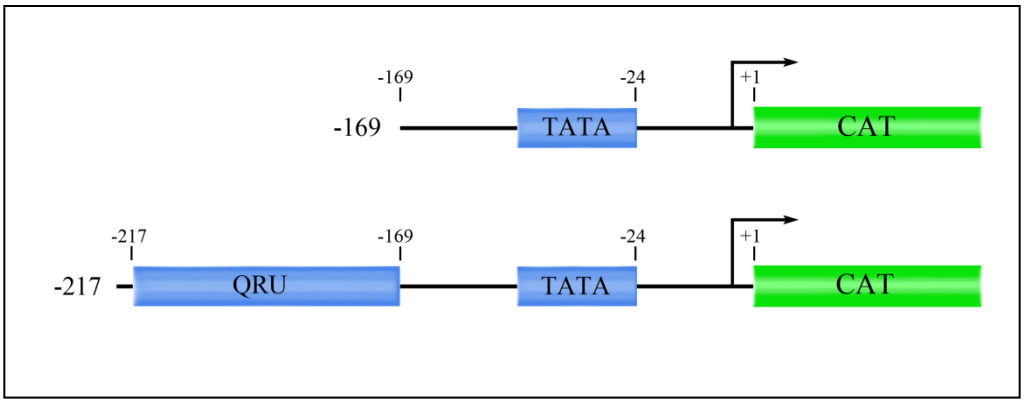


Figure 11: **Schematic representation of CAT constructs used to study transcriptional repression of the QRU**. The -169 CAT construct bears the p20K promoter up to but excluding the QRU. The -217 construct bears the entire p20K promoter region including the QRU. Both of these promoter fragments were placed upstream of the CAT reporter gene. These constructs were expressed in CEFs and used to assess transcriptional activation or repression by measuring CAT activity.

The -169 construct is missing the QRU and the -217 construct contains everything that is present in the -169 construct in addition to the QRU. Thus, we would expect the -169 construct to have no quiescence responsive activity. Conversely, we expect the -217 construct to be strongly induced in response to a quiescent cell state, such as contact inhibition. Since the -217 construct contains the QRU we can use this as a tool to gauge whether there is transcriptional repression of the QRU in response to ERK2 overexpression.

In order to assess whether ERK2 is acting as a transcriptional repressor at the QRU we transfected the -217 CAT construct into CEFs which were either harvested in contact inhibited or cycling conditions. Furthermore, these -217 CAT constructs were co-transfected with an ERK2 overexpression vector or an empty vector control. Additionally the -169 CAT construct was used in parallel to the -217 CAT transfection as an activation dead negative control. Thus, if ERK2 is actually acting as a transcriptional repressor of the QRU we expect that samples where ERK2 is overexpressed to have reduced CAT activity in comparison to -217 constructs which were co-transfected with an empty vector control. The QRU confers quiescence dependent activation of p20K, thus we expect strong activation in contact inhibited samples in comparison to cycling samples. However repression by ERK2 should be observed in both cell states. Lastly, we expect to -169 to have virtually no transcriptional activation, as the QRU is required for transcriptional activation of the p20K and thus CAT in this context. The results of this transient expression assay experiment are shown in Figure 12.

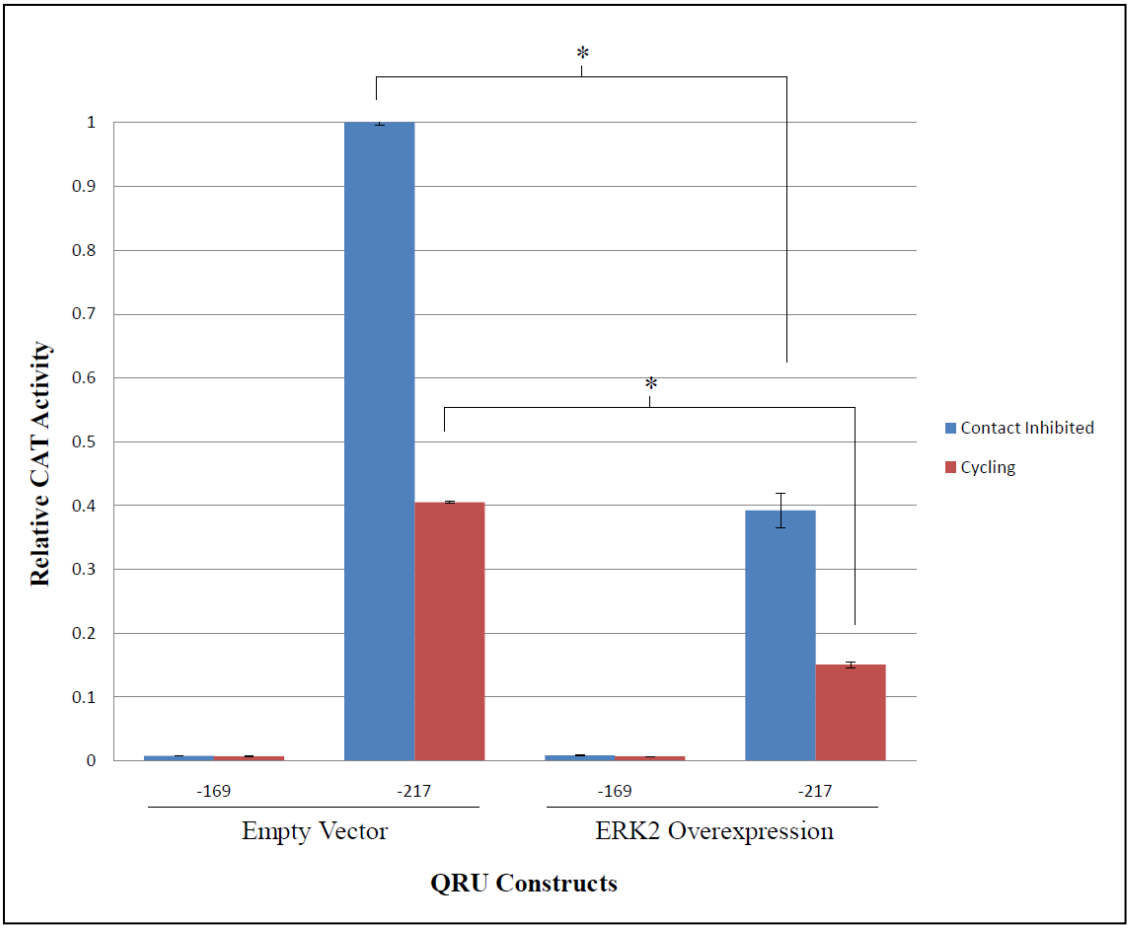


Figure 12: ERK2 acts as a transcriptional repressor of the QRU. Transient expression assays using the CAT system were conducted in CEFs. CEFs were transfected with either an activation dead p20K promoter construct (-169) or a QRU bearing construct of the p20K promoter (-217). These CAT vectors were co-transfected with either an ERK2 overexpression vector or an empty vector control. Once transfected CEFs were seeded such that they would be contact inhibited or cycling at the time of harvest. Lysates were normalized and CAT activity was gauged by separating acetylated chloramphenicol by thin layer chromatography. The acetylated and non-acetylated species were quantified and percentage conversion was calculated. This was then normalized to the -217 – contact inhibited – empty vector control sample. Statistical analysis confirms significance to $p < 0.01$. (* = $p < 0.01$).

Based on Figure 12 we can deduce that ERK2 acts as a transcriptional repressor of the QRU. This is based on the observation that in both contact inhibited and cycling conditions there is a significant reduction in CAT activity when ERK2 is overexpressed. There is an observed 2.5 fold and a 2.7 fold reduction of relative CAT activity in contact inhibited and cycling cells respectively, when ERK2 is overexpressed. These observed reductions are statistically significant ($p < 0.01$). Additionally we see a strong reduction of CAT activity between contact inhibited and cycling cells in both ERK2 overexpression and empty vector control transfected cells. This is expected as there is induction of p20K in contact inhibited cells and whereas p20K is repressed in cycling cells. Furthermore, both sets of -169 constructs in both assayed conditions have minimal CAT activity. This is expected because the activation of p20K; and thus CAT in our vectors, is dependent on the QRU. Seeing as how -169 CAT constructs lack the QRU, we expect to see very minimal induction, and thus no CAT activity.

Chapter 4: Discussion

4.1 p20K expression and transcriptional repression by ERK2

Preliminary data suggested that CHOP10 plays a repressive role at the QRU (Shi Yan). Here it was observed that overexpression of CHOP10 caused a decreased induction of p20K in quiescent cells states (Shi Yan (Unpublished Results)). Furthermore, inhibition of CHOP10 by the expression of shRNA caused super-induction of p20K in quiescence cells states (Shi Yan (Unpublished Results)). However in these experiments the disruption of CHOP10 never resulted in the expression of p20K in cycling cells. This suggested the existence of an additional mechanism regulating the repression of p20K in proliferative cells states. In 2009, it was shown that ERK2 binds to a DNA consensus motif directly (Hu et al., 2009). These motifs usually consisted of two ERK2 binding sites separated by 2-3 nucleotides (Hu et al., 2009). It was then that we noticed the presence of this ERK2 binding motif nested between the C/EBP-β binding sites of the QRU (Fig 3). Additionally, Hu et al. demonstrated that ERK2 acts as a transcriptional repressor at these binding sites in GATE elements, which are elements bound by C/EBP-β/ATF hetero-dimers. Therefore, these observations suggested that ERK2 functions as a repressor of the QRU when cells are actively dividing. Additionally, we know that C/EBP-β can become activated by several different conditions and mediate transcriptional induction of genes in numerous pathways (Akira et al., 1997; Tsukada et al., 2011). Therefore, there must be additional factors and mechanisms which provide specificity in the pattern of C/EBP-β dependent gene expression. One suspected mechanism is competition of C/EBP-β and other transcription factors for binding and

recruitment of a limited number of transactional co-activators. Another mechanism providing specificity in the C/EBP-β dependent expression profile may thus be transcriptional repression by ERK2.

The rationale supporting this potential phenomenon generated a model where ERK2 acts as a transcriptional repressor of quiescence specific genes. This mechanism dictates that during proliferation, cells are constantly receiving and processing mitogenic signals. This results in ERK2 activation and subsequent localization to the nucleus. Once ERK2 is localized to the nucleus it activates several transcription factors. One of these transcription factors is AP-1 (Wang et al., 2011). Interestingly AP-1 plays a repressive role of GAS genes such as p20K (Gagliardi et al., 2003). We now know that ERK2 also acts as a transcriptional repressor via direct interaction and repression of promoter elements (Hu et al., 2009). Thus, it would seem consistent for ERK2 to act as a transcriptional repressor of quiescence specific genes in proliferative cell states because ERK2 acts as critical mediating factor for the mitogenic response. Additionally, the entry of G_0 is associated with a reduction of ERK1/2 activity and induction of C/EBP-β. A corresponding decrease in CHOP10 levels would promote the formation of potent C/EBP-β homo-dimers, removal of ERK2 on the QRU, and subsequent expression of p20K would follow.

In the experiments described in chapter 3.1 we confirmed that the quiescence specific protein p20K was strongly induced in contact inhibited cells. However in cycling cells, which are in a proliferative cell state, this induction was abolished. Furthermore, there was a very strong induction of p20K in hypoxic conditions in both contact inhibited

and cycling cells. The expression profile of p20K allowed us to predict which conditions may harbor potential p20K repression by ERK2 and when this potential repression would not be observed.

Hypoxic growth conditions may be a stress for cells and thus result in decreased proliferation, which can lead to a reduction in ERK1/2 activation. This questions whether our hypoxic cycling cell samples were actually still dividing. Preliminary studies conducted by Yudi Camacho (Bédard lab) have shown that CEFs growing in hypoxia have no alteration to proliferation rates when compared to cells grown in normoxia. This indicates that cycling cells growing in hypoxia are not expressing p20K due to growth arrest *per se*, because these cells grow at the same rate. Rather, the observed induction of p20K is the results of the hypoxia signaling networks activating GAS gene expression in actively dividing and contact inhibited cells. Although it may seem counter intuitive that a growth arrest specific gene (p20K) is being induced in cells which are actively dividing, preliminary studies indicate that contact inhibited cells carry a hypoxic gene expression signature (Bart Maslikowski and Romita Ghosh (Unpublished results)). Consistently, recent paradigm shifts in the hypoxia field support that hypoxia is a physiologically common phenomenon, and plays a strong role in regulating cell states. This reasons that hypoxia and growth arrest are not mutually exclusive conditions, rather they are an intertwined signaling network.

4.2 ERK2 binds to the QRU in states of transcriptional repression

Through a series of DNA interaction experiments we demonstrated that ERK2 is recruited to the QRU. Furthermore, this interaction was contingent upon proliferative cell

states only. In nearly all cases binding of ERK2 to the QRU in a quiescent cell state was not observed.

Although our EMSA analysis was unsuccessful, using ChIP analysis demonstrated that ERK2 is recruited to the QRU *in vivo*. Furthermore, we illustrate that this binding occurs only in conditions where p20K is transcriptionally repressed. Figure 9 clearly illustrates that ERK2 interacts with the QRU in cycling cells but not in contact inhibited cells. Thus, ERK2 is bound the QRU in states where p20K is repressed. This phenomenon was further validated when a similar ChIP analysis was conducted using hypoxic conditions. Figure 12 clearly illustrates that ERK2 binds to the QRU in cycling cells, where p20K is repressed. However ERK2 was undetectable at the QRU contact inhibited cells or in conditions of mild hypoxia, where there is strong induction of p20K. These results convincingly demonstrate that ERK2 binds to the QRU in proliferative cells states. Additionally, we used ERK1 as a control to illustrate that this phenomenon is specific to ERK2. Due to the fact that ERK1 was not described as having any DNA binding affinity, it is a valid control for ensuring that this is a real phenomenon. Figure 10 illustrates that ERK2 was specifically immunoprecipitated with the QRU, but ERK1 did not. ERK1 and 2 are very closely related. Having 83% identity and 89% similarity, one would expect these proteins to have very similar regulations and activities. However structural analysis by Hu et al. (2009) revealed that ERK2 includes a DNA binding domain which is missing in ERK1. Our results further substantiate that ERK2 is recruited to the p20K promoter, whereas ERK1 is not. All of this ChIP analysis illustrates that ERK2 specifically interacts with the QRU in proliferative cell states. However this is

only circumstantial evidence that ERK2 acts as a repressor of the QRU during conditions where p20K expression is inhibited.

4.3 ERK2 acts as a transcriptional repressor of the QRU

ChIP analysis provided circumstantial evidence for ERK2 playing a transcriptional repression role at the QRU. This was deduced from the fact that ERK2 was only detected at the QRU when p20K was transcriptionally repressed. In order to further validate this phenomenon functional evidence was required. To address this issue we employed transient expression assays which utilized an ERK2 overexpression approach. The results of this experiment clearly indicated that ERK2 overexpression caused the repression of the QRU. This functional data in combination with the ChIP analysis strongly suggest that ERK2 acts as a transcriptional repressor of the QRU in proliferative cell states.

These data support a model where mitogenic stimulation causes the activation of ERK2. Once activated, ERK2 binds to the QRU and represses transcription. C/EBP-β may be inhibited by direct contact or by steric hindrance with ERK2 when it occupies the QRU. Since C/EBP-β binding activity increased in contact inhibited CEF; as determined by EMSA analysis, competition between C/EBP-β and ERK2 for interaction with the QRU is a more likely mechanism for regulation control of the QRU (Kim et al., 1999). Either way C/EBP-β is unable to activate transcription of p20K due to this mitogenic stimulation and subsequent activation of ERK2. Once cells exit the cell cycle; either by contact inhibition, serum starvation, external / internal signaling cues, etc., signaling networks favor the activation of the QRU. This is in part through activation of C/EBP-β

at the QRU. Simultaneously, mitogenic signaling pathways are dampened, implying that nuclear ERK2 may become scarce or is regulated such that it loses efficacy as a transcriptional repressor. This allows for the expression of quiescence specific genes such as the p20K. This model is depicted in Figure 13.

Originally this model was proposed as a mechanism for the observed repression of p20K in cycling cells. Previous studies have suggested that CHOP10 plays an important role in the regulation of p20K expression in growth arrested cell states (Shi Yan, Romita Ghosh and Ben Fielding, Unpublished Results). However it was observed that the down regulation of CHOP10 by shRNA did not induce the expression of p20K in cycling cells, as was initially expected. This led us to believe that there was an additional mechanism for p20K repression. The study conducted in 2009 by Hu et al. directed us to the putative ERK2 binding sites and the possibility that ERK2 may act as a repressor at the QRU. The work presented here suggests that ERK2 binding is a significant mechanism in the control of quiescence specific gene expression. Furthermore, this may serve as a specificity mechanism controlling the expression profile of C/EBP-β dependent gene activation. Indeed, v-Src leads to activation of C/EBP-β in CEFs, a process which is required for the activity of IL-8 during transformation (Gagliardi et al., 2001). However; despite the activation of C/EBP-β, these conditions do not lead to p20K expression. Interestingly, v-Src activates ERK1/2 kinases through the Ras pathway. Thus, ERK2 may provide specificity in the pattern of C/EBP-β dependent gene expression in proliferating and v-Src transformed CEFs. This illustrates that p20K expression is under the control of several regulatory mechanisms. Thus, control of quiescence specific genes is multi-

faceted and contains redundant modes of regulation. Interestingly, ERK2 transcriptional repression was coupled with C/EBP-β mediated regulation of GATE elements. Here we have shown that this same coupling exists for regulation of the QRU. Thus, this may hint that the interplay between the ERK2 transcriptional repressor and the C/EBP-β activator is a reoccurring theme in transcriptional regulation of many pathways. It also suggests that there must be a complex feedback mechanism regulating the activation / inactivation of these transcription factors depending on signaling cues.

There are still aspects of this model which remain unclear. Firstly, C/EBP-β is phosphorylated and activated by ERK2 in response to certain stimuli such as IFN-γ stimulation (Roy et al., 2002). How this interaction plays a role in the regulation of the QRU is still unclear. It may be that ERK2 dependent phosphorylation of C/EBP-β plays a greater role in response to mitogenic or cytokine signaling than in the case of quiescence specific gene regulation. Interestingly, DAPK; a Ser/Thr kinase involved in apoptosis and autophagy, is a gene product induced by IFN-γ (Deiss et al., 1995, Lin et al., 2010). It contains a GATE element in its promoter, and the induction of DAPK is dependent on C/EBP-β (Gade et al., 2008). It was observed that phosphorylation of C/EBP-β by ERK2 is a requirement for the induction of DAPK upon IFN-γ stimulation (Gade et al., 2008). Additionally, when DAPK is induced it binds to and sequesters ERK2 in the cytoplasm (Chen et al., 2005). This results in attenuation of ERK2 activity, thus functioning as a negative feedback loop (Chen et al., 2005). Such negative feedback mechanisms may exists in the regulation of the QRU as well, although how exactly this would function is unclear. Secondly, the dynamics of how ERK2 and C/EBP-β interact at the QRU and the

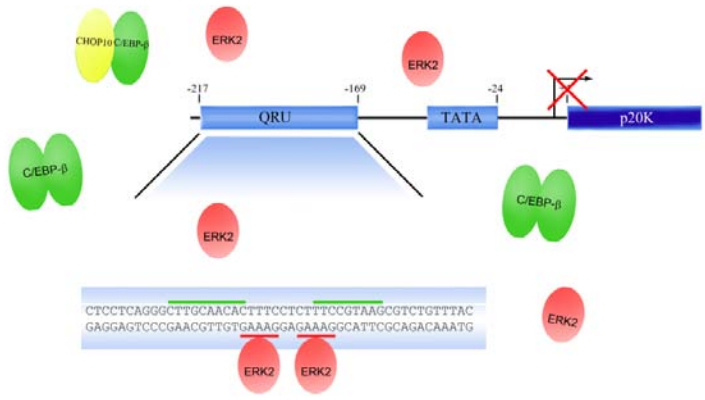
resulting effect on gene expression is obscure. During mitogenic signaling there is a massive influx of ERK2 into the nucleus. This may compete any C/EBP-β molecules away from the QRU. Once bound ERK2 remains stably bound and represses transcription until such a time where nuclear ERK2 becomes scarce; such as in times of lack of mitogenic signaling or growth arrest. Furthermore, a quiescence cell context favors C/EBP-β activation at the QRU. Such control mechanisms may involve inactivation / removal of ERK2 which would allow C/EBP-β to activate transcription of p20K. Thirdly, it is known that other factors play an active role at the QRU. As previously mentioned, CHOP10 plays a repressive role at the QRU in quiescent cell states. How CHOP10 and ERK2 collaboratively regulate p20K gene expression in response to external/internal cues is not entirely clear. Lastly, it is well established that ERK1/2 induces, stabilizes, and activates the AP-1 family of transcription factors (Cook et al., Whitmarsh et al., 1996; Murakami et al., 1997; Balmanno et al., Cook et al., 1999; Murphy et al., 2002; Vinciguerra et al., 2004). This family has been shown to oppose the roles of C/EBP-β in growth arrested CEFs and repress the expression of p20K (Gagliardi et al., 2003). This suggests that ERK2 functions as a transcriptional repressor directly (by interacting with the QRU) and indirectly (activation of AP-1) to repress GAS gene expression.

p20K was identified as a quiescence specific protein in 1987. Since then many aspects of its regulation have been divulged. We know more about the dynamic processes which modulate the expression of quiescence specific genes than ever before. p20K and the QRU have been a strong model in studying the regulation of not just p20K specifically but quiescence genes in general. The regulation of p20K can be generalized

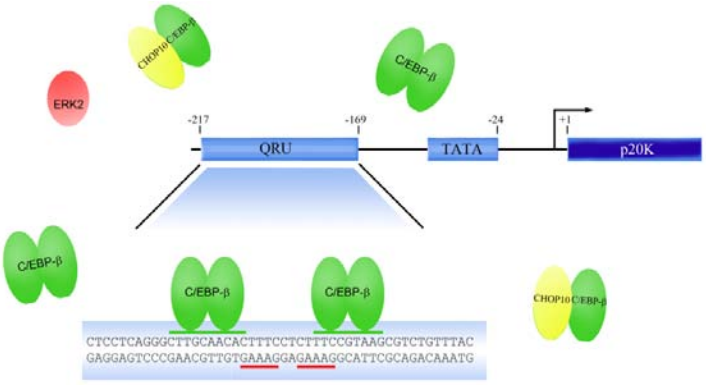
to the mechanisms which are involved in regulating signaling networks which control cell proliferation and growth arrest. By gaining a better understanding of how quiescence specific genes are regulated, we are one step closer to understanding what constitutes the cellular pressures which drive proliferation or growth arrest. The regulation of these processes is critical for our understanding of several proliferative diseases such as cancer.

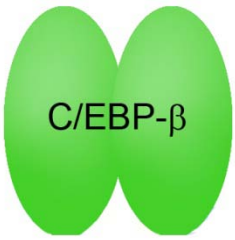
Figure 13: A schematic model of QRU transcriptional control in CEFs. Evidence presented in this thesis supports the notion that ERK2 acts as a transcriptional repressor of the QRU in proliferative cell states. Actively dividing cells are observed to have consistent activation of ERK2. Thus, in these states, ERK2 acts as a transcriptional repressor of GAS genes such as p20K. As cells enter growth arrest there is a loss of ERK2 activation, which results in the ability of C/EBP-β to bind to the QRU and induce p20K expression. In response to ER stress or apoptosis there is up-regulation of CHOP10. This results in the formation of CHOP10 and C/EBP-β hetero-dimers. This causes loss of C/EBP-β dependent gene expression, and leads to the repression of p20K. There is a potential CHOP10-C/EBP-β hetero-dimer binding site in the QRU, but whether binding actually occurs and if it serves a function is still unclear.

Proliferative cell state

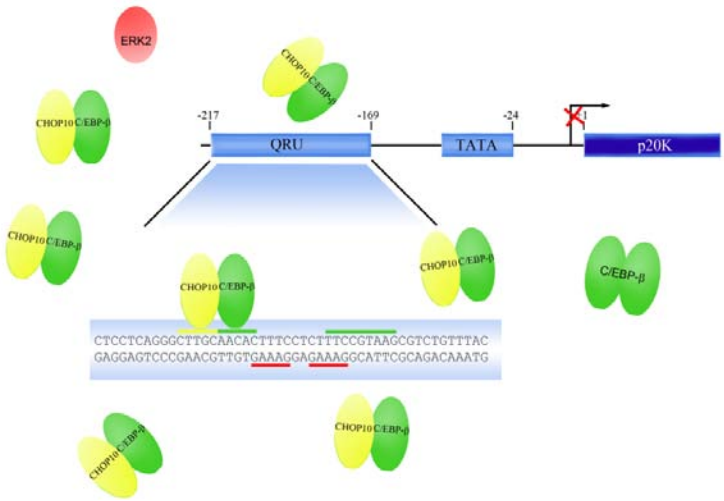


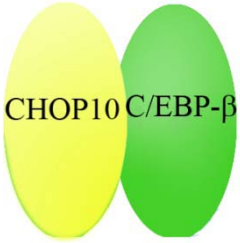
Growth arrested cell state or hypoxic conditions





ER stress or apoptosis





4.4 Future directions

Additional experiments can be conducted in order to generate evidence in support of ERK2 mediated transcriptional repression of QRU. These primarily utilize other techniques and approaches in order to confirm that this is a genuine aspect of GAS gene regulation.

Kinetics experiments where quiescent CEFs are serum stimulated for differing amounts of time and then assessed for p20K expression would be useful. If an inverse relationship between ERK2 activation and p20K expression is observed then this lends support to the idea that ERK2 plays a repressive role at the QRU. Furthermore this may support a regulatory model where competition is involved. Since there is a massive influx of ERK2 when cells are serum stimulated, the sudden increase in ERK2 concentration in the nucleus may be sufficient in competing off C/EBP-β. If there is a sharp decrease in p20K expression in response to serum stimulation one can use serum stimulation in conjunction with ChIP analysis to confirm that the observed repression is a results in ERK2 binding to the QRU. This would affirm the repressive role of ERK2 at the QRU and support a competition based mode of regulation.

Overexpression experiments can be conducted to establish whether there is increased repression of p20K in both proliferative and quiescent CEFs in response to increased levels of ERK2. This may provide additional evidence that ERK2 plays a repressive role at the QRU. However these experiments may be difficult as ERK2 regulates hundred of factors, one of which is C/EBP-β. The fact that ERK2 is so

promiscuous in terms of the number of pathways that get activated in response to stimulation, may complicate whether we see a clear cut result at the QRU.

Knockdown experiments where ERK2 levels are decreased using shRNA vectors may also be a useful approach to studying this transcriptional repression role. Although this may be complicated for the same reasons listed for overexpression studies, there is still the potential that one would see the release of transcriptional repression of the QRU in response to ERK2 knockdown. Previous studies have reported that knockdown of ERK2 results in increased expression of IFN-γ induced genes (Huang et al., 2008). We now realize that this was the result of the loss of ERK2 mediated transcriptional repression of GATE elements (Huang et al., 2008). By knocking down ERK2 we may observe similar effects on GAS genes, namely p20K. This would provide additional support that GAS genes are under the control of ERK2 mediated transcriptional repression.

Additional transient expression assays can be conducted using either CAT or Luciferase reporter assays as tools to gauge repression of the QRU by ERK2. Here we have illustrated that ERK2 represses the QRU when overexpressed. Additional transient expression assays can be conducted in order follow up on this observation. Firstly, repression by ERK2 overexpression can be confirmed in hypoxic conditions. This would lend support to the notion that ERK2 mediated repression is a genuine mechanism working in different cellular contexts. Secondly, transient expression assays which overexpress ERK1 instead of ERK2 can be conducted as well. This would serve to reiterate that this repression phenomenon is specific to ERK2. This would be functional

data supporting ERK2's specific repressive role at the QRU. Thirdly, experiments where the WT QRU and ERK2 binding site mutants are placed into reporter constructs would be very useful. With these expression vectors we would be able to assess whether disrupting these ERK2 binding sites has any effect on QRU activation in cycling cells. As we believe that ERK2 functions as a transcriptional repressor, the results of these experiments may support the notion that ERK2 specifically represses the QRU by binding to these sites in cycling cells. As CHOP10 down-regulation is not sufficient to induce the expression of p20K, ERK2 would provide the 'fail safe" mechanism to repress GAS gene expression in actively proliferating cells. We predict that ERK2 binding site mutants will be insensitive to ERK2 overexpression.

Additional EMSAs can be conducted to further affirm the interaction of ERK2 with QRU. Although we have *in vivo* evidence of ERK2 binding to the QRU during states of transcriptional repression, EMSA analysis can provide *in vitro* support, which is still additional evidence that this interaction exists. Furthermore EMSA analysis presents an alternative method of study binding to ERK2 to the QRU when putative ERK2 binding sites are mutated. This would require that we be able show ERK2 specific shifts, which entails doing "super-shift" experiments where an ERK2 bound shift is super-shifted by using an ERK2 specific antibody. This would show that the observed shift is a complex containing ERK2. Furthermore, competition reactions can be conducted using cold probes in the reaction to compete off the binding of ERK2 to the hot QRU probe. Here we would use cold WT probe to try to abolish the observed shift, whereas when we use cold EBS mutant probe we would expect that this shift will be unaltered. This

experiment would support that ERK2 binds specifically to the putative ERK2 binding sites in the QRU. Perhaps the most definitive and effective method of illustrating that ERK2 binds the QRU *in vitro* is by purifying recombinant ERK2 and using this recombinant protein in EMSA interaction reactions to illustrate that ERK2 directly binds to the QRU. Previous studies have used this approach with some success (Hu et al., 2009).

Our data suggest that ERK2 is a novel mechanism of QRU repression in proliferative cell states. Further insight into the dynamics of this mechanism would be useful. Experiments studying how ERK2 interacts with C/EBP-β specifically at the QRU would be interesting. Whether this is simply a competition based model or whether there is an underlying mode of regulation present would be an interesting facet to study.

4.5 Conclusions

Through a series of ChIP analysis we have demonstrated that ERK2 specifically binds to the QRU during states of p20K transcriptional repression. Furthermore, ERK2 is functionally associated with transcriptional repression of the QRU. Thus it can be inferred that ERK2 acts as a transcriptional repressor of the QRU in proliferative cell states. Additionally we have illustrated a novel instance of ERK2 mediated transcriptional repression of a C/EBP-β controlled gene. Originally the control of GATE elements was the only reported occurrence of this control mechanism. However with this we have shown that this occurs for GAS genes as well. Thus, the coupling of ERK2 mediated transcriptional repression and C/EBP-β transcriptional activation occurs in more than one occasion and may be a mode of regulation for several inducible pathways.

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