GENE PROFILING V-SRC TRANSFORMATION IN PRIMARY AVIAN CELLS AND THE IDENTIFICATION OF THE NOVEL SRC-RESPONSIVE GENE *DAPK1* AS A MEDIATOR OF APOPTOSIS IN SRC-TRANSFORMED CELLS WITH REPRESSED AP-1 ACTIVITY

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By

BART M. MAŚLIKOWSKI, M.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirement

for the Degree

Doctor of Philosophy

McMaster University

Descriptive Note

DOCTOR OF PHILOSOPHY (2012) McMaster University

(Biology) Hamilton, Ontario

TITLE: Gene Profiling v-Src Transformation in Primary Avian Cells and the Identification of the Novel Src-Responsive Gene *DAPK1* as a Mediator of Apoptosis in Src-Transformed Cells with Repressed AP-1 Activity

AUTHOR: Bart M. Maślikowski, H.B.Sc., M.Sc. (McMaster University), B.Ed. (Brock University)

SUPERVISOR: Dr. André Bédard

NUMBER OF PAGES: xvi, 311

Abstract

Cell transformation by the Src tyrosine kinase is characterized by extensive changes in gene expression. Previous studies have indicated that many of these changes are dependent on the activity of transcription factors belonging to the AP-1, STAT and Ets families. To describe these changes in gene expression, investigators have relied extensively on the study of immortalized cell lines that may not represent the full spectrum of biological processes regulated during transformation. In this study, we took advantage of transformation deficient and temperature sensitive mutants of the Rous sarcoma virus to characterize the patterns of gene expression of two primary cell types transformed by v-Src, namely chicken embryo fibroblasts (CEF) and chicken neuroretina (CNR) cells. In these cells, v-Src transformation alters the expression of up to 6% of the protein coding genes. Among these genes, a common set of 175 genes was regulated in CEF and CNR cells implying the existence of a common program of gene expression controlled by v-Src. Comparison of this program with independent breast carcinoma data sets identified a group of 42 v-Src inducible genes associated with reduced disease-free survival. Pathway and ontological analyses of the genes differentially regulated by transformation in CEF and CNR indicated a generalized program of de-differentiation induced by Src.

To investigate the role of AP-1 in Src-mediated transformation, we conducted a gene profiling study to characterize the transcriptomes of v-Src-transformed CEF expressing the *Jun* dominant-negative allele (TAM67) or the *JunD* short-hairpin RNA (shRNA). Microarray data analysis indicated a cluster of 18 co-regulated probe-sets

activated in v-Src-transformed CEF with repressed AP-1 activity but not activated in normal CEF or CEF transformed only by v-Src. Four of these probe-sets correspond to genes involved in the interferon pathway. One gene, *death-associated protein kinase 1* (*DAPK1*), is a C/EBPβ-regulated mediator of apoptosis in IFN-γ-induced cell death. We show that inhibition of DAPK1 abrogates cell-death in v-Src-transformed CEF expressing the JunD shRNA and that expression of DAPK1 is dependent on C/EBPβ but antagonized by AP-1. Chromatin immunoprecipitation data indicated that C/EBPβ, but not JunD, is recruited to the DAPK1 promoter. We conclude that JunD promotes survival by indirectly antagonizing C/EBPβ-dependent expression of DAPK1.

Acknowledgements

Foremost I would like to thank Dr. André Bédard for taking the risk in hiring what was ostensibly "damaged goods." I thank you for the opportunity, understanding and patience and I hope that you have learned from me as I have learned from you. To my committee members, Drs. Herbert Schellhorn, Brian Golding and Peter Whyte, thank you for helping me to grow as a scientist. Special thanks go to Carlo "Maggot Master" Artieri, Steve "Krox20" Brown, Nishi Singh and Wilfried Haerty for technical advice, scientific discourse, and in maintaining a healthy sense of despair throughout the course of my PhD. I also want to thank Carlo and Wilf for elucidating the statistical dark arts of "majistix" and providing me with just enough rope with which to hang myself. Special thanks also go out to Jenny "Ghost Hunter" Wang and Benjamin Néel for stimulating discussion and their contributions to the AP-1 and Src-transformation stories. To all my friends and colleagues, past and present, especially Alicia "Dr." Pepper, Flora "The Banana" Suen, Ben "Tooth-Cracker" Fielding, Raging Romita Ghosh, Car-bomb Mo Athar, Nikol "Ex-spearmint" Piskuric, Taylor Mitchell, Lulu Vasquez Paz, Megan McKerlie, Pablo Reyes, Sam "The Godless" Garnet, Caitlin Gregory, Flore-Anne Poujade and all the Phoenix Phryday Dipsomaniacs – thanks for making grad school bearable. To my brothers in arms from Elevator 22: Curtis Smythe, Mike Durham, and Casey Reaper – thanks. We rode to battle and sometimes even won. Thanks to the Free Dive Toronto community, especially Sergei, Soren and Doug. You've done what no SSRIs have been able to do. To all the others, too numerous to name, I thank you all for your sapient conversations and stimulus. To my father Czesław and sister Julia, I thank you for your support during these trying years. To my in-laws, Fred and Janice, thanks for not judging. To my dear Sharon, without you I would never have persevered, nor eaten as well as I have, nor do I think I would have made it this far without you. This thesis is dedicated to my mother Grażyna (1946-2000), who died from the very disease I have researched for the duration of my graduate studies. I only wish that what I have learned and what I have done could have spared you this awful suffering. Finally, to those who purposely obstructed my path or deliberately sought my ruin, may your dreams go forever unfulfilled.

"Life is a terminal disease"

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List of abbreviations

4EBP-1	eukaryotic initiation factor (eIF)-4E-binding protein (4EBP-1)
AAR	amino acid response
AJ	adherens juntions
Akt	v-akt murine thymoma viral oncogene homolog
AML	acute myelogenous leukemia
AR	androgen receptor
Arp2/3	Actin related protein 2/3
ASV17	avian sarcoma virus 17
ATF	activating transcription factor
Bad	BCL2-associated agonist of cell death
BLBC	Basal-like breast carcinoma
CAT	chloramphenical acetyltransferase
Cbl-c	Cbl proto-oncogene, E3 ubiquitin protein ligase C
CEF	chicken embryo fibroblast
CKII	Casein kinase II
CML	Chronic myeloid leukemia
CRD	central regulatory domain
CRPC	castration-resistant prostate cancer
Csk	C-terminal Src kinase; official gene name: c-src tyrosine kinase
CSR	Common set of v-Src Regulated (genes)
DEF	docking site for ERK, F-X-F
DTT	Dithiothreitol
EGF	epidermal growth factor
ER	estrogen receptor
ErbB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, also known as HER2, Neu and EGFR2
Erk	extracellular regulated kinase
ER-stress	endoplasmic reticulum stress
ERα	estrogen receptor alpha
FA	focal adhesion
FBJ	Finkel-Biskis-Jinkins (FBJ) osteosarcoma virus
FOXO1	forkhead box O1
FOXO4	forkhead box O4
Fyn	oncogene related to SRC, FGR, YES
GPCR	G-protein coupled receptor
GSK3β	glycogen synthase kinase 3 beta
HEK	human endothelial kidney cells
HER2	Human EGF receptor 2
HMEC	human mammary epithelial cell
HRAS	Harvey Ras
IHC	immunohistochemical

IκB	inhibitor of NFκB
JAK	Janus kinase
JNK	Jun N-terminal kinase
kDa	kiloDalton
KRAS	Kirsten Ras
LOH	loss of heterozygosity
Maf	musculoaponeurotic fibrosarcoma
mdm2	transformed mouse 3T3 cell double minute 2
NGF	nerve growth factor
NRAS	neuroblastoma Ras
NRTK	non-receptor tyrosine kinase
N-WASp	Wiskott Aldrich Syndrome protein (N-WASp)
p21	also known as cyclin-dependent kinase inhibitor 1A or CDKN1A
PIP2	Phosphatidylinositol (3,4)-bisphosphate (PI(3,4)P2)
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P3
PR	progesterone receptor
pSRU	putative Src-responsive unit
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
Ras	Rat sarcoma
RD1	regulatory domain 1
RD2	regulatory domain 2
RNAi	RNA interference
RSV	Rous sarcoma virus
RTK	receptor tyrosine kinase
SFK	Src-family of tyrosine kinase
SH	Src-homology
shRNA	short hairpin RNA
Src	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
SRU	Src-responsive unit
TAD	tranasactivating domain
TPA	12-O-tetradecanoylphorbol-13-acetate
TR	transformation regulated (genes)
TRE	TPA responsive element
TSS	transcriptional start site
uORF	upstream open reading frame
UPR	unfolded protein response
USF-1	upstream transcription factor 1
VEGFR	vascular endothelial growth factor receptor
Yes	Yamaguchi sarcoma viral oncogene homolog 1

Definition of IUPAC* nucleotide codes

IUPAC code	Nucleotide(s)	Explanation
A	A	Adenine
C	C	Cytosine
G	G	Guanine
T	T	Thymine
U	U	Uracil
R	A or G	puRine
Y	C or T (U)	pYrimidine
M	A or C	aMino
K	G or T (U)	Keto
S	C or G	Strong (triple ‘3 H’ bonds)
W	A or T (U)	Weak (double ‘2 H’ bonds)
B	C or G or T (U)	not A
D	A or G or T (U)	not C
H	A or C or T (U)	not G
V	A or C or G	not T (U)
N	A or C or G or T (U)	aNy nucleotide

*International Union of Pure and Applied Chemistry

A note on viral strains used throughout the thesis

RCASBP or RCAS - Replication-Competent ASLV long terminal repeat (LTR) with a Splice acceptor (Bryan polymerase)
This is a virally derived vector used to express genes of interest or as a vector/virus control. The suffix A or B indicates if this is an A or B type virus.

NY315 – New York 315
This is a RSV virus strain containing a constitutively active Src allele that is myristoylation deficient and hence non-transforming. This is an A type virus

SR-A or SRA – Schmidt-Ruppin A
This is a highly transforming A-type RSV strain

NY72-4 or ts72-4 – New York 72-4
This is an A-type RSV strain containing a thermolabile Src allele. The strain is transforming at 37.5^0C but not at 41.5^0C.

LA90 – Los Angeles 90
This is a RSV strain containing a thermolabile Src allele. The strain is transforming at 37.5^0C but not at 41.5^0C. The virus used in the laboratory is actually a RCASBP(B) RSV containing the LA90 ts allele. In the text this virus is referred to as LA90.

A versus B type virus
This describes the envelope glycoprotein on RSV. A type viruses bind to the tva receptor while B-type bind to tvb. Two different viruses of the same type cannot infect the same cell.

Chapter 1: Introduction

1.1 Historical context

Prior to last century, cancer was thought to be a disease of endogenous origin. Since cancer, it was believed, was not transmissible between individuals, the germ theory of disease was not sufficient to explain its origins. In 1911, Francis Peyton Rous, working at Rockefeller Institute for Medical Research, demonstrated that cancer could be transmitted between animals. Building on the work of the Danish researchers Ellerman and Bang, who in 1908 showed the transmission of avian erythroblastosis (Ellerman & Bang, 1908), Rous showed that tumors could be transplanted between chickens and that a filterable cell-free lysate could be used to cause sarcomas (Rous, 1911). These results were met with skepticism by the scientific community and the viral cancer origin hypothesis was largely ignored until the 1930's, when work with mammalian tumor viruses such as papilloma virus (Shope & Hurst, 1933) and mouse mammary tumor virus (Bittner, 1936) gained prominence in the literature. It was not until 1958 that Rous sarcoma virus (RSV) was formally demonstrated to be the causative agent of transformation in chicken fibroblasts by Temin and Rubin (Temin & Rubin, 1958). Temin later went on to demonstrate that RSV contains virally dependent reverse-transcriptase activity (Temin & Mizutani, 1970; Baltimore, 1970). By the early 1970's the *v-Src* oncogene and its cellular homologue and progenitor, *c-Src,* were identified by molecular cloning (Stehelin *et al.*, 1976a; Stehelin *et al.*, 1976b), setting the stage for a new phase in the study of cancer genetics.

1.2 c- Src and cancer

The Src non-receptor tyrosine kinase has served as the prototypical kinase model for signaling in vertebrates. Understanding the fundamental basis for Src signaling has provided insight into both the normal function of kinase signaling in the cell as well as contributing to the understanding of human disease. c-*Src*, the cellular counterpart of the v-*Src* avian oncogene, was the first proto-oncogene cloned (Stehelin *et al.*, 1976a; Stehelin *et al.*, 1976b) and continues to serve as a target of interest in human cancer. Elevated Src activity has been observed in prostate, colon, breast, lung, pancreatic and head and neck carcinomas (Wheeler *et al.*, 2009). The activation of Src is often observed in ErbB2-induced breast tumors (Muthuswamy *et al.*, 1994), potentiates ErbB2-induced cellular transformation (Marcotte *et al.*, 2009) and is indispensable for tumor formation in the polyoma middle T mouse-mammary tumor model (Guy *et al.*, 1994). High Src activity has also been linked to poor prognosis and metastasis in breast cancers (Biscardi *et al.*, 2000) and in colon carcinomas where an activating truncation mutant of c-Src was discovered to be enriched in highly aggressive metastatic tumors (Irby & Yeatman, 2000; Irby *et al.*, 1999a).

High Src activity has also been associated with chemoresistance to various chemotherapeutic agents in numerous tumor types (Shah & Gallick, 2007). Although Src itself is generally not the causative oncogene in human cancers, its association with chemoresistance has identified Src as a potential target for combinatorial therapies in chemo-resistant cancers. Two Src-family of tyrosine kinase (SFK) inhibitors, bosutinib,

and saracatinib, are under pre-clinical and early clinical investigation (Aleshin & Finn, 2010) for use in a myriad of cancers including lung, prostrate, ovarian, pancreatic, breast, liver, and head and neck cancers. Another SFK inhibitor, dasatinib, presently used as a second line treatment for several blood cancers, is also being investigated for use in solid tumors. Early data suggest that these inhibitors are well-tolerated in single and combinatorial treatments (Aleshin & Finn, 2010). However, bosutinib, and saracatinib do not seem to be effective in single-treatment regimens. Unlike dasatinib, which cross-reacts with the non-receptor tyrosine kinase Abl, and the receptor tyrosine kinases (RTKs) cKit, PDGFR, and EphA2, both bosutinib, and saracatinib display a higher specificity with SFKs and cross-react more weakly with other kinases. The failure of these two compounds in single-treatment studies argues for the role of Src as a potentiating and not as an initiating factor in tumorigenesis. Preliminary data from combinatorial trials with saracatinib in ovarian, breast and prostate cancers is positive (see NCT00558272 and NCT00610714 at http://clinicaltrials.gov) while combinatorial trials with dasatinib show promise in so-called triple-negative breast cancers (ER-/PR-/HER2-) and in castration-resistant prostate cancer (CRPC) (Aleshin & Finn, 2010).

The ubiquitous expression of Src in all tissues and its activation over a broad range of tumor types underscore the importance this proto-oncogene plays in the development and treatment of cancer.

1.3 Src structure

Src is a 60kDa ubiquitously-expressed membrane-anchored non-receptor tyrosine kinase (NRTK) and is the prototypical member of the Src-family of kinases (SFK). Of the ten identified SFKs, three are ubiquitously-expressed (*Src*, *Fyn*, *Yes*). Of these, Src is most strongly associated with cancer (Yeatman, 2004). The structure of SFKs is highly conserved and consists of four Src-homology (SH) domains arranged SH4, SH3, SH2 and SH1 from the N-terminus to the C-terminus (Figure 1). Between the SH2 and SH1 domain, there is a linker region and on the C-terminal distal side of the SH1 domain there is a C-terminal regulatory region. The SH4 domain contains a region required for the myristoylation of Src by myristoyl-CoA (Roskoski, 2004). Conjugation of this lipid to Src is required for the association of Src to the plasma membrane and is critical for cellular transformation (Kamps *et al.*, 1986). In its inactive conformation, the Src linker region assumes a pseudo-proline heix conformation that binds to the SH3 domain while the SH2 domain binds to short phosphotyrosine-containing motifs. Interaction of SH3 and SH2 domains with their cognate ligands provides an important regulatory and signaling mechanism for Src (reviewed in (Pawson & Gish, 1992; Jin & Pawson, 2012). The SH1 domain is the kinase domain. Down-regulation of Src activity is achieved through conformational change by the phosphorylation of a C-term tyrosine (Y530, *H. sapiens*; Y527, *G. gallus*) by C-terminal Src kinase (Csk). This creates an intramolecular bond between the SH2 domain of Src and the c-terminal phosphotyrosine. This conformation is further stabilized by the interaction of a polyproline tract in the SH2-SH1 linker region with the SH3 domain. Active conformation is restored through the higher-affinity

interactions of the Src SH3 and SH2 domains with ligands on phosphoproteins such as receptor tyrosine kinases (Wheeler *et al.*, 2009). Activation is further stabilized by the dephosphorylation of Y530 by a number of cellular phosphatases such as Shp2, PTPα, PTPλ and PTP1B (Zheng *et al.*, 1992; Yeatman, 2004). In chicken, the wild-type (WT) Schmidt-Rupin v-Src strain lacks a portion of the C-terminus containing Y527, leading to constitutive activation of the v-Src protein (Roskoski, 2004). Src activity can further be potentiated by auto- or trans-phosphorylation of Y419 (Y416, *G. gallus*) of the kinase activation loop by Src or other signaling molecules such as receptor tyrosine kinases (RTKs) (Yeatman, 2004). Phosphorylated Y419 also serves as a docking site for the E3-ubiquitin ligase Cbl-c which targets Src for ubiquitin-mediated lysosomal degradation (Kim *et al.*, 2004a).

2. Src signaling

Src serves as a central nexus through which the cell can integrate various extracellular signals including those from receptors, the extracellular matrix and focal adhesions. From a cancer perspective Src has been the focus of study because it positively regulates mitogenesis, survival, adhesion, motility and angiogenesis (Figure 2). Although activating mutations in *c-Src* have been identified in breast and especially colon cancers, they are still exceedingly rare (Irby *et al.*, 1999a; Irby & Yeatman, 2000; Dehm & Bonham, 2004). By and large, high Src activity in tumors is observed because of the activation of Src through the dysregulation of positive and negative regulators of Src. In transgenic mouse models, overexpression of HER2 in the mammary gland induces high Src activity

(Muthuswamy *et al.*, 1994) while in human colon cancers, high Src activity has been associated with the activation of RTKs including EGFR, HER2 and c-Met (Irby *et al.*, 1999a). Repression of Src-regulating proteins, such as Csk and PTPα, has been observed in some hepatocellular carcinomas but is not seen universally in all tumor types, nor even in all samples with high Src activity (Masaki *et al.*, 1999; Irby & Yeatman, 2000; Urushibara *et al.*, 1998). Paradoxically, Cbl-c, a negative regulator of Src (Kim *et al.*, 2004a), is frequently overexpressed in colon carcinomas (Kim *et al.*, 1999a) indicating that the interplay between Src and Src-regulators is rather complex. Therefore, elucidating the signaling networks contributing to the activation of Src and subsequent downstream effects has been essential to understand the role of this gene in normal and transformed cells. Although Src is involved in a number of pathways and processes, including GPCR signaling, immune response, tissue differentiation, and hormone receptor signaling, I will limit my discussion to fields relevant to my thesis, namely mitogenesis, motility and survival.

2.1 Mitogenic effect of Src signaling

2.1.1 The RTK-Ras-MAPK pathway

Receptor tyrosine kinases (RTKs) are a type of membrane-bound kinases that transduce extracellular signals into the cell. The RTKs are composed of an extracellular ligand-binding domain, a hydrophobic membrane-spanning domain, a juxtamembrane dimerization domain, a tyrosine kinase domain and a C-terminal effector protein-binding

region. Structurally, the RTKs are divided into 20 classes based on the divergent extracellular ligand-biding domains. The binding of a ligand to the extracellular domain results in the homo or hetero-dimerization of the RTK and subsequent activation of the kinase by trans-phosphorylation on the activation loop on the kinase domain (most RTKs) or through allosteric activation (EGFR) (Jura *et al.*, 2011). The resultant activation allows the RTK to trans- or autophosphorylate tyrosines in the effector protein-binding region. Phosphorylation of these tyrosines creates binding sites for effector proteins containing SH2 or protein tyrosine binding (PTB) domains.

In the mid 1980's it was shown that activation of the platelet derived growth factor receptor (PDGFR) resulted in the activation of Src (Ralston & Bishop, 1985) while more recent work showed that Src and other SFKs are involved in the signaling downstream of other RTKs including EGFR, HER2/ErbB2, c-Met and the fibroblast growth factor receptor (FGFR) (Bromann *et al.*, 2004). Earlier studies revealed that Src bound to the cytoplasmic region of an activated PDGFR via its SH2 domain (Mori *et al.*, 1993) where Src is activated through the stabilization of its active conformation. While true for some RTKs, recent experiments using an EGF-inducible EGFR/ErbB2 chimera, show that Src binds to the kinase domain of HER2/ErbB2. Moreover, deletion of the SH2 and SH3 domains of Src did not affect binding suggesting that the interaction was mediated through the kinase domain of Src, possibly indicating another mechanism for Src activation in other RTKs (Marcotte *et al.*, 2009).

Critically, Src is required for growth factor mediated mitogenesis. Overexpression of c-Src increases DNA synthesis in murine fibroblasts when stimulated with EGF

(Wilson *et al.*, 1989) while over expression of myristoylation deficient, kinase-dead and SH2-domain mutants results in attenuation of this response (Wilson *et al.*, 1989). Conversely, inhibition of Src by microinjection of neutralizing antibodies or overexpression of kinase-dead mutants abrogate PDGF and colony stimulating factor (CSF)-dependent signaling *in vitro* (Bromann *et al.*, 2004). However, contrary to these findings, other groups have noted that Src is not required for DNA synthesis downstream of RTKs. Src-/- mouse embryo fibroblasts (MEFs) stimulated by FGF do not have impaired proliferation, but do exhibit aberrant motility arguing in favor of functional redundancy of the two other ubiquitously expressed SFKs, Fyn and Yes (Liu *et al.*, 1999). Indeed, in Src/Yes/Fyn null cells, FGF-dependent mitogenesis was completely inhibited but reversed with the re-expression of exogenous c-Src (Kilkenny *et al.*, 2003).

In addition to acting as a signal transducer downstream of RTKs, Src is also involved in cross talk between the receptors and other signaling molecules. Activation of Src leads to Cbl phosphorylation and its subsequent down-regulation, while activation of EGFR leads to Src phosphorylation and mono-ubiquitinylation via Cbl (Bromann *et al.*, 2004). Src antagonizes this process by phosphorylating Cbl and targeting it for degradation. Src also promotes the endosomal internalization of the receptor, thought to attenuate receptor signaling, by facilitating the formation of clathrin-coated vesicular endocytosis (Bromann *et al.*, 2004). Src has also been shown to mediate signaling between integrins and EGFR (Moro *et al.*, 2002) and to phosphorylate RTKs directly (Hansen *et al.*, 1996).

Downstream of RTKs, one of the principal pathways mediating mitogenic signaling is the Ras-MAPK pathway. Two Ras homologues were originally identified as viral oncogenes in the Harvey and Kristen murine sarcoma viruses (HRas and KRas) (Chang *et al.*, 1982). To date, the Ras subfamily comprises over two dozen members. Along with NRAS, discovered in human neuroblastoma cells (Marshall *et al.*, 1982), the HRas and KRas paralogs are of special interest because of their clinical relevance, particularly to cancer. Because these three proteins function and are regulated in a similar fashion, they will be referred to henceforth simply as Ras.

Ras is a monomeric G protein activated by the binding of GTP. Intrinsic GTPase activity converts the GTP-bound *active* form of Ras (Ras-GTP) to Ras-GDP, thereby rendering Ras inactive[1]. Activation of Ras is mediated by the guanine exchange factor (GEF) son of sevenless (SOS) while enhancement of its GTPase activity by Ras GTPase activating protein (RasGAP) inactivates Ras (Yang *et al.*, 2003b). Src in turn has been shown to inactivate RasGAP through phosphorylation (Brott *et al.*, 1991; Moran *et al.*, 1991). Ligand activation of RTKs creates docking sites for the SH2-containing adapter protein Grb2 (growth factor bound 2), which is recruited to the plasma membrane along with SOS and Ras, leading to Ras activation. Activation of Ras allows Ras effector proteins to bind Ras on the effector domain. Because Ras is anchored to the plasma membrane via farnesylation at the N-terminus of the protein, association of Ras with its effector proteins results in recruitment of these proteins to the membrane. A key Ras

[1] The association of Ras with its negative regulator, RasGAP, increases the intrinsic GTPase activity of Ras. It should be noted that the *active* form of Ras referred to herein is the GTP-bound form of Ras that activates the MAPK pathway and not the GTPase-enhanced form of Ras bound to RasGAP.

effector, the Raf serine/threonine (S/T) kinase is recruited in this way (Yang *et al.*, 2003b). Association of Raf with Ras, alleviated the autoinhibitory structure of Raf, increasing its kinase activity (Cutler *et al.*, 1998). The active conformation of Raf is further stabilized by phosphorylation by kinases PAK1 and Src (Tran & Frost, 2003). Raf in turn activates the dual specificity kinase MEK (MAPK and Erk kinase) which subsequently activates a cascade of serine/threonine MAPKs (mitogen activated protein kinases), including Erk (extracellularly regulated kinase) (Yang *et al.*, 2003b) (Figure 3). Erk regulates a number of downstream effectors including the ribosomal S6 kinase (RSK), and transcription factors such as Elk1 and Fos (Steelman *et al.*, 2008).

Early on, it was suggested that the Ras pathway is required for transformation by v-Src. Inhibiting HRas activity using neutralizing antibodies blocks transformation of fibroblasts by v-Src (Smith *et al.*, 1986). However, the expression of the HRasN17 dominant negative mutant in chicken embryo fibroblasts (CEF) did not fully abrogate transformation, nor did the inhibition of the MAPK pathway by the chemical inhibitor PD98059 (Penuel & Martin, 1999). However, inhibition of the Ras pathway and the PI3'K-mTOR pathway simultaneously inhibited v-Src transformation completely. In v-Src transformed murine pro-B cells, inhibition of Ras led to massive apoptosis while inhibition of the JAK-STAT and PI3'K pathway using dominant negative STAT3 and p85 led to decreased proliferation (Odajima *et al.*, 2000). These data show that Ras is one of several parallel pathways required for v-Src-transformation. Moreover, the role of Ras in Src signaling appears to be cell-type specific. Altogether, Src plays a role in regulating

RTK-Ras-MAPK signaling while at the same time serving as a target of regulation by the pathway itself.

2.1.2 The JAK-STAT pathway

The signal transducer and activator of transcription (STAT) family of proteins consists of seven members (STATS 1-4, 5a, 5b and 6) involved in immune response, cellular growth and differentiation (see (Silva, 2004; Steelman *et al.*, 2008) for review). Originally identified in the context of cytokine signaling, STATs were found to be activated by the JAK (Janus kinase)[2] family of membrane-bound tyrosine kinases. In the canonical pathway, activation of cytokine receptors recruits JAK, whereupon JAK phosphorylates tyrosine residues upon the receptors. STATs interact with the receptor via their SH2 domain and are subsequently phosphorylated by JAK leading to hetero- or homodimerization of the STATs and subsequent nuclear translocation. Once in the nucleus, STATs activate genes via their DNA binding and transactivating domains. It has since come to light that a number of growth factor receptors and G-protein coupled receptors (GPCRs) can activate a subset of the STAT family (STATs 1, 3, 5a, and 5b). Many of these receptors can phosphorylate STATs directly and can therefore activate them independently of JAKs (Silva, 2004). Indeed the same is true of NRTKs. DNA binding activity of STAT3 was increased by v-Src (Yu *et al.*, 1995) while dominant

[2] The JAK proteins are called such because they contain two back-to-back nearly identical kinase-like domains, much like the Roman god Janus who had two faces on either side of his head. One domain is a true kinase while the other is a pseudokinase (Wilks, 1989). Andrew F. Wilks, discoverer of JAK1 and JAK2, in disappointment, originally coined the acronym "Just Another Kinase" for JAK since the original goal of his PCR-based cloning strategy was to discover novel RTKs, which JAK1 and JAK2, were not (Wilks, 2008).

negative STAT3 inhibited v-Src-dependent transformation in murine fibroblasts (Turkson *et al.*, 1998). Both pharmacological and genetic inhibition of Src decreases EGF-dependent phosphorylation of STATs 1, 3, and 5 in cultured fibroblasts and carcinoma cells (Olayioye *et al.*, 1999). In murine xenograft models of squamous cell head and neck carcinomas (SCHNC), inhibition of STAT5b slowed tumor growth (Xi *et al.*, 2003a), while inhibition of Src in SCHNC cell lines decreased STAT3 and 5b activation (Xi *et al.*, 2003b). Inhibition of STAT3 abrogated Src-dependent angiogenesis by blocking induction of *vegf* gene expression (Niu *et al.*, 2002). Biscardi and others have shown that in mouse models of breast cancer and in human cancer cell lines, EGF and Src act synergistically to activate STATs 3, 5a and 5b. Inhibition of Src using dominant negative Src or Src inhibitors reduces STAT phosphorylation and leads to suppression of transformation, growth arrest or apoptosis (Biscardi *et al.*, 1998; Biscardi *et al.*, 1999; Garcia *et al.*, 2001; Kloth *et al.*, 2002; Kloth *et al.*, 2003; Silva, 2004). Furthermore, constitutive expression of STATs 3 and 5b have been observed in numerous solid tumors including breast, prostate, and colon and are correlated to high Src activity (Silva, 2004) suggesting an important regulatory role for Src in the regulation of gene expression via STATs.

2.2 Src promotes survival

In order for transformed cells to progress to tumorigenesis, it is crucial for cells to bypass oncogenic checkpoints leading to cell death or growth arrest. In Src-transformed cells, the PI3K-Akt pathway features prominently in promoting cell survival.

The phosphatidylinositol 3-kinases (PI3K) are a family of heteromeric lipid kinases comprised of a 110 kDa catalytic subunit and an 85 kDa regulatory subunit. PI3Ks are divided into three classes based on substrate specificity and structural similarity. Class I PI3Ks catalyze the phosphorylation of phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3). Class I PIK3s are activated by RTKs, cytokines and GPCRs and are of primary interest to Src signaling and will therefore be the focus herein (Steelman *et al.*, 2008).

Upon receptor activation, the p85 subunit of PI3K binds to the receptor via its SH2 domain whereupon the p85 subunit is phosphorylated. This leads to the activation of the p110 subunit and consequent catalysis of PIP2 to PIP3. PIP3 serves as a ligand for the pleckstrin homology (PH) domain of the Akt/PKB[3] family of serine/threonine kinases thus recruiting Akt to the membrane where phosphatidylinositols are anchored. Once at the membrane, Akt is activated by the S/T kinase PDK1 at T308 and by mTORC2 at S473. Akt activation targets numerous downstream processes regulating survival and growth. PIP2 to PIP3 conversion is antagonized by the phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome 10) (Li *et al.*, 1997; Maehama & Dixon, 1998). PTEN activity is often reduced in more advanced cancers and PTEN loss of heterozygosity (LOH) is often observed in a number of solid tumors including gastric, breast and prostate (Li *et al.*, 1997; Liu *et al.*, 2009) suggesting the role of PTEN as a

[3] Akt is the abbreviation for v-akt murine thymoma viral oncogene homolog (Staal, 1987). AKT8 is the name of the transforming virus derived from the high-leukemia AKR mouse strain. Akt was cloned independently by *Coffer* and *Woodgett* in 1991 and called PKB because of its homology to PKA and PKC (Coffer & Woodgett, 1991). When asked about the confusion regarding Akt/PKB nomenclature, Jim Woodgett replied that Akt refers to "alternate kinase terminology" (pers. comm.). The Human Genome Organisation (HUGO) nomenclature committee presently uses the abbreviation Akt.

tumor suppressor. In one study, low PTEN activity was observed in approximately 25% of breast tumors (Feilotter *et al.*, 1999). PTEN promoter hypermethylation is often seen in various leukemias resulting in lower PTEN expression (Steelman *et al.*, 2008). Activation of NFκB through the Akt pathway has been shown to suppress *PTEN* gene activation (Kim *et al.*, 2004b) as has Src-activation (Maehama, 2007). Src is known to stimulate NFκB activity (Cabannes *et al.*, 1997) and decreased PTEN mRNA expression has been observed in v-Src-transformed chicken neuroretinal cells (CNRs) (Maslikowski *et al.*, 2010).

In addition to activation by receptors, PI3K is also activated by interaction of the p85 subunit SH3 and SH2 domain to activated Src (Liu *et al.*, 1993; Haefner *et al.*, 1995) and also by direct interaction of p110 with the Ras effector loop (Rodriguez-Viciana *et al.*, 1994). Src-dependent synergistic activation of PI3K and Ras by hormone receptors including estrogen receptor α (ERα), progesterone receptor (PR), and androgen receptor (AR) has also been observed (Migliaccio *et al.*, 2007). Association and activation of these complexes can be stimulated by sex-hormone receptors in a variety of cell types, including endothelial and epithelial cells, fibroblasts and breast cancer cell lines (Migliaccio *et al.*, 2007). In turn, hormone-dependent induction of DNA synthesis can be blocked by the addition of Src-inhibitors or Src-dominant negative mutants (Migliaccio *et al.*, 2007). Therefore the coupling of Src to the PI3K-Akt pathway by synergistic activation of RTKs and hormone receptors, direct binding, and through the Ras pathway demonstrates Src's central role in promoting survival during tumorigenesis.

Activation of Akt controls a myriad of processes that promote survival and growth. Akt phosphorylates a number of substrates involved in the regulation of growth and apoptosis including Bad, procaspase 9, GSK3β, IκK, mdm2, FOXO1, FOXO4, and the CDK inhibitor p21 (Steelman *et al.*, 2008). Akt-dependent phosphorylation of Bad and procaspase 9 directly inhibits apoptosis (Datta *et al.*, 1997; Cardone *et al.*, 1998) while phosphorylation of the forkhead family transcription factors FOXO1, FOXO4 represses the expression of pro-apoptotic genes. Repression of GSK3β promotes the stabilization of myc (Sears *et al.*, 1999) and cyclinD1 (Diehl *et al.*, 1998) both directly and through the mTOR pathway (Steelman *et al.*, 2008). The phosphorylation of the p53 inhibitor mdm2 by Akt allows mdm2 to translocate to the nucleus and inhibit p53 (Mayo & Donner, 2001). Interestingly, the *PTEN* locus itself is activated by p53 and PTEN expression is required for p53-dependent apoptosis in immortalized murine fibroblasts (Stambolic *et al.*, 2001). Phosphorylation and activation of IκK, targets the NFkB inhibitor IkB for proteosomal degradation, thereby allowing NFkB to translocate to the nucleus and activate transcription. NFkB comprises a family of dimeric transcription factors involved in numerous cellular responses including inflammation, proliferation and survival. NFkB is constitutively active in most human cancers often through the dysregulation of the RTK-Ras pathway (Chaturvedi *et al.*, 2011) while Src has been shown to activate expression of NFkB genes such as *p105* and increases NFkB-dependent gene activation (Cabannes *et al.*, 1997; Bojovic *et al.*, 1996). Translocation of NFkB activates prosurvival and proliferative genes including *Bcl2*, *Bcl-XL*, the inhibitors of apoptosis genes, *IAP1*, *IAP2*, *XIAP*, and *cyclinD1* and *Myc* (Baud & Karin, 2009).

Activation of Akt also activates the mTOR (mammalian target of rapamycin[4]) pathway. The S/T kinase mTOR promotes cell growth by enhancing protein translation via the phosphorylation of a number of proteins including the eukaryotic initiation factor (eIF)-4E-binding protein (4EBP1) (Gingras *et al.*, 1998) and the ribosomal kinase p70S6K (Burnett *et al.*, 1998); eventuating in the increase in protein synthesis as well as the stabilization of growth promoting factors such as Myc and cyclinD1 (Steelman *et al.*, 2008).

2.3. The role of Src in metastasis: motility, adhesion and angiogenesis

In addition to increased proliferation and increased survival, another hallmark of cellular transformation is the ability of the cell to bypass contact-induced growth inhibition and produce foci *in vitro*. Src-transformed cell lines can also grow in an anchorage independent manner, and demonstrate increased invasive properties suggesting a role for Src in motility and adhesion. In tumors, increased Src activity is correlated to increased invasiveness and chemoresistance (Yeatman, 2004; Shah & Gallick, 2007). Src plays a major role in the control of adhesion and motility through the extracellular matrix (ECM), cell-cell adhesion, and cytoskeleton. Disruption of these processes can lead to the phenomenon known as epithelial to mesenchymal transition (EMT), a highly motile and invasive state also characterized by chemotherapeutic resistance (Shah & Gallick, 2007). In a developmental setting, EMT is reversible and occurs during processes where epithelial cells must detach from their environment and migrate to a new location, such as

[4] Rapamycin was isolated in 1975 from *Streptomyces hygroscopicus* found on Easter Island. The compound was named rapamycin in honor of the indigenous peoples' name for Easter Island, Rapa Nui (Vezina *et al.*, 1975).

during gastrulation and neural crest formation. This process appears to be hijacked by Src during transformation. EMT deserves special attention since it is required for the intra- and extravasation of tumor cells during metastasis. Associated with EMT, but a distinct process, is the angiogenesis required for increased tumor growth and effective metastasis.

2.3.1 Src regulation of adhesion to the ECM

Epithelial cells are anchored to the ECM via heterodimeric complexes composed of α and β integrins. Integrins serve to physically anchor the cells to the ECM and also act as receptors communicating the composition of the extracellular space to the cytoplasm. The cytoplasmic tail of the integrin is linked to the cytoskeleton in a complex of up to 50 proteins including adapter proteins talin, vinculin and paxillin, or a ternary complex including integrin linked kinase (ILK), pinch and parvin (Yilmaz & Christofori, 2009). ILK is important in assembling the scaffolding complex necessary for downstream signaling through the interaction of proteins including Src and the focal adhesion kinase (FAK) (Yilmaz & Christofori, 2009). Depending on the composition of integrins, the complex can transmit disparate signals including both pro- and antiproliferative signals. For example, integrins α6β4 and αvβ3 are among the mitogenic heteromers cooperating with growth factor receptors, including EGFR, HER2, and c-Met (Yilmaz & Christofori, 2009). In CEF and CNR, Src activation itself alters the expression pattern of several integrins including the up-regulation of integrin α6 and β3 (Maslikowski *et al.*, 2010) presumably augmenting mitogenic signaling by affecting integrin composition.

Activation of FAK via integrin signaling leads to the association of FAK with the SH3 domain of the mechanosensing adapter protein CAS (Crk-associated substrate) (Tachibana *et al.*, 1997). Phosphorylation of CAS by FAK along multiple tyrosines creates docking sites for the SH2 domains of SFKs (Tachibana *et al.*, 1997). Subsequent recruitment of Src via its SH2 and SH3 domains leads to its allosteric activation (Cary *et al.*, 2002). Dephosphorylation and activation of Src via phosphatases including PTP1α, PTP1B and Shp2, located at the focal adhesion (FA) is facilitated by the exposure of the terminal regulatory tyrosine, leading to full Src activation (Pallen, 2003; Yilmaz & Christofori, 2009). Counter-phosphorylation of FAK by Src leads, through a process of signaling events, to the dissolution of the focal adhesion and activation of a number of pathways including the MAPK pathway, Akt, and the activation Rho, Rac and Cdc42 GTPases, key regulators of cytoskeletal remodeling (Yilmaz & Christofori, 2009). The activation of Rac in particular requires the phosphorylation of CAS by FAK and SFKs in order to generate docking sites for adapter proteins Crk and CrkL, which in turn recruit GEFs required for the activation of Rac (Tikhmyanova *et al.*, 2010). Phosphorylation of FAK by Src on Y925 has been shown to activate Ras signaling by serving as a docking site for the Grb2 adapter protein (Schlaepfer *et al.*, 1994). Expression of FAK dominant negative mutant in cultured cancer cells lead to ECM-detachment and *anoikis* (Park *et al.*, 2004). Overexpression of activated Src in these cells rescued *anoikis*, while pharmacological inhibition of Src recapitulated the FAK dominant negative phenotype, indicating the importance of Src and FAK in maintenance of adhesion (Park *et al.*, 2004). FAK and Src nulls cells exhibit deficiencies in either motility or invasion indicating that

both proteins are necessary for the invasive phenotype seen in Src-transformed cells (Yeatman, 2004). Abrogation of FAK in murine fibroblasts leads to an accumulation of disorganized focal adhesions as does the elimination of Src kinase activity in CEF (Ilic *et al.*, 1995; Fincham & Frame, 1998). Since FA turnover is required for cellular migration, any disruption of this process antagonizes tumorigenic invasion (Destaing *et al.*, 2011).

Cell to cell contact is mediated by the homotypic interaction between cadherins, single span transmembrane glycoproteins, located at adherens junctions (AJ). In epithelial cells, this interaction occurs primarily through E-cadherin. E-cadherin is anchored to the cytoskeleton through interaction with α and β catenin and p120 catenin. Both β-catenin and p120 catenin play dual roles in regulating motility at the membrane and also as transcription factors when translocated to the nucleus (Yilmaz & Christofori, 2009). Overexpression of activated Src downregulates E-cadherin at the cell membrane in colon cancer cells (Avizienyte *et al.*, 2002) while repression of Src by pharmacological means represses this process (Nam *et al.*, 2002). Furthermore, in mouse tumor transplantation experiments, mice treated with the PP2 SFK inhibitor had decreased incidence of metastasis (Nam *et al.*, 2002). Activation of Src triggers the ubiquitination and degradation of E-cadherin (Fujita *et al.*, 2002) leading to the dissolution of AJs and to the accumulation of β-catenin which translocates to the nucleus where it interacts with the Tcf/Lef family of transcription factors to activate transcription of proliferative genes such as cyclinD1 and pro-invasion genes including matrix metalloproteases (MMPs) (Yilmaz & Christofori, 2009). Similarly, the release of p120 catenin allows it to translocate to the nucleus where it binds to the transcriptional repressor ZBTB33 to regulate gene

expression (Ferber *et al.*, 2008). Transcriptional repression is also mediated by a number of EMT-inducing transcription factors including Snail, Slug and Twist (Peinado *et al.*, 2007). One of the hallmarks of EMT is the "cadherin switch" whereby N-cadherin replaces E-cadherin at the AJ. Twist and STAT3 are positive regulators of N-cadherin expression (Alexander *et al.*, 2006; Lo *et al.*, 2007) while more broadly, v-Src activates the Twist and N-cadherin genes, *twist* and *cdh2* as well as a number of MMPs thereby promoting invasion (Maslikowski *et al.*, 2010; Hsia *et al.*, 2003). Both high Src activity and low E-cadherin expression are associated with poor prognosis clinically (Shah & Gallick, 2007). The expression of N-cadherin changes the cell's affinity to prefer other N-cadherin expressing cells, including, neuronal, vascular and endothelial cells (Yilmaz & Christofori, 2009). N-cadherin also potentiates PDGFR and FGFR signaling, actively sustaining the Ras- MAPK pathway.

Src is also indispensable for the formation of invadopodia. Invadopodia are cellular protrusions, similar to filopodia and lamellopodia, that facilitate migration and ECM degradation (Yilmaz & Christofori, 2009). Src phosphorylates critical proteins including N-WASP-Arp2/3 complex, and cortactin required for the nucleation of actin bundles needed for invadopodia formation (Destaing *et al.*, 2011). In CEF, Src also controls the expression of these proteins' genes, indicating parallel mechanisms of regulation during transformation among vertebrates (Maslikowski *et al.*, 2010). Furthermore, the hyaluronic acid receptor CD44, another Src-induced gene product (Maslikowski *et al.*, 2010), is thought to coordinate the formation of podosomes during invasion (Chabadel *et al.*, 2007). More generally, high CD44 expression has been used as

a criterion for identifying cancer-initiating stem cells and is used as a marker for EMT (Al-Hajj *et al.*, 2003; Blick *et al.*, 2010). High CD44 expression, along with low CD24 expression ($CD44^{high}/CD24^{low}$) has also been correlated with the most invasive basal-like or so-called triple-negative breast carcinomas (Blick *et al.*, 2010). Induction of EMT by inducible expression of Twist or Snail in human mammary epithelial cells (HMECs) induced expression of EMT markers N-cadherin, vimentin, and fibronectin, and had reduced expression of E-cadherin and CD24 (Mani *et al.*, 2008). Moreover these cells phenocopied $CD44^{high}/CD24^{low}$ putative mammary cancer-initiating stem cells morphologically exhibiting a mesenchymal phenotype and an ability to form *in vitro* mammospheres, a process requiring extensive reorganization of cell adhesion (Mani *et al.*, 2008).

Like invasion, angiogenesis is another important aspect of tumor growth and spread. Activation of STAT3, a Src target, has been shown to activate expression of angiogenic factor *vegf* (Niu *et al.*, 2002). Activation of *vegfa* and theVEGF receptor gene *vegfr/flt1* and has been observed in global expression studies in v-Src-infected CNR and CEF respectively (Maslikowski *et al.*, 2010) The vascular-endothelial factor is indispensable for tumor vascularization and growth (Grunstein *et al.*, 1999) and as such this function may be conserved among vertebrates. Expression of the pro-angiogenic chemokine IL8, has long been associated with v-Src-induced transformation (Bedard *et al.*, 1987a; Sugano *et al.*, 1987). Like its mammalian ortholog, the avian version is also a potent angiogen (Martins-Green & Hanafusa, 1997). In addition to its role in invasion, the

expression of CD44 has also been shown to be important in neovascularization in transgenic mice (Cao *et al.*, 2006).

3. Regulation of gene expression by Src

Since the identification of c-Src, functional studies have revealed numerous processes in which the proto-oncogene is involved. Significant effort has focused on understanding the signaling pathways regulated by Src. Nevertheless one of the established hallmarks of Src transformation is that it induces changes in gene expression. Early studies demonstrated the induction of genes coding for MMPs, the ECM, globins, chemokines and neuronal markers (Groudine & Weintraub, 1980; Sobel *et al.*, 1981; Crisanti-Combes *et al.*, 1982b; Bedard *et al.*, 1987a; Sugano *et al.*, 1987). Several studies also showed that blockage of Src activity, using either dominant negative mutants or inhibitory antibodies, repressed expression of the trans-activating factors *myc* and *fos* despite stimulation with mitogenic factors (Barone & Courtneidge, 1995; Simonson *et al.*, 1996). Similarly, genetic or pharmacological inhibition of transcription factors including those of the AP-1, Ets, and STAT families, as well as Myc, were able to block some or all of the characteristics of v-Src induced transformation (Wasylyk *et al.*, 1994; Turkson *et al.*, 1998; Suzuki *et al.*, 1994; Lloyd *et al.*, 1991; Barone & Courtneidge, 1995; Wang *et al.*, 2011a). Furthermore, the specific activation of v-Src-induced genes has been shown to be dependent on the activation of specific transcription factors. The v-Src-transformation associated avian *il8* gene is regulated by a 80 nucleotide stretch of DNA proximal to the promoter called the Src-responsive unit (SRU) (Bedard *et al.*, 1987a; Dehbi *et al.*, 1992). This SRU contains

binding sites for AP-1, NFκB and C/EBPβ transcription factors (Dehbi & Bedard, 1992). Mutation of the binding sites on the SRU or inhibition of individual factors alone, or in combination, attenuates or abrogates *il8* transcription illustrating the importance of specific transcription factors in mediating Src-dependent gene expression (Dehbi & Bedard, 1992; Dehbi *et al.*, 1992; Gagliardi *et al.*, 2001).

The post-genomic era brought new high throughput methodologies to address genome-wide transcriptional changes in response to, among other things, oncogenes. Global expression studies of Src-induced gene expression signatures using immortalized cell lines, tumor cells, and primary cells have shown profound changes in gene expression ranging from a few hundred genes to several thousand, representing up to six percent of the protein-coding transcriptome (Malek *et al.*, 2002; Paz *et al.*, 2004; Bild *et al.*, 2006; Masker *et al.*, 2007; Maslikowski *et al.*, 2010). In Src-transformed CEF, repression of different AP-1 family members by short-hairpin RNA-mediated RNA interference (shRNA mediated RNAi) affect v-Src-transformed CEF in different ways including induction of senescence, apoptosis and adipogenesis (Wang *et al.*, 2011a) demonstrating that different transcription factors mediate, even within the same family of factors, different responses to Src-activation. Repression of AP-1 activity in v-Src transformed CEF via JunD shRNA or c-Jun dominant negative dysregulates the expression of approximately 1100 annotated genes suggesting that the regulation of transformation via AP-1 is crucial for Src (see Chapter 3). Repression of AP-1 in these cells induces apoptosis (Wang *et al.*, 2011a) and the expression of the C/EBPβ-regulated *death-associated protein kinase 1* (*DAPK1*; (Gade *et al.*, 2008)) gene (see Chapter 3). Previous

studies have shown that although C/EBPβ is required for the expression of the v-Src-transformation marker IL8, its repression using dominant negative mutants does not antagonize transformation *in vitro* (Gagliardi *et al.*, 2001). Indeed, expression of the dominant negative mutant Δ184 enhances expression of AP-1 members c-Jun, JunD, and Fra2 and enhances proliferation (Gagliardi *et al.*, 2003) suggesting an antagonistic relationship between AP-1 and C/EBPβ. Indeed, C/EBPβ has been implicated in a number of roles promoting both proliferative and antiproliferative activity (Sebastian & Johnson, 2006). Given the importance of AP-1 in v-Src-transformation and the possible antagonism between AP-1 and C/EBPβ, the next sections will focus on the structures and function of these two factors.

3.1 AP-1

The activator protein-1 (AP-1) members are dimeric transcription factors that regulate the expression of genes involved in proliferation, survival, differentiation and transformation (Eferl & Wagner, 2003; Zenz & Wagner, 2006). The AP-1 family encompasses the Jun, Fos, ATF (activating transcription factor) and Maf (musculoaponeurotic fibrosarcoma) protein families. Homo- or heterodimerization of the different members gives rise to a myriad of AP-1 dimers all with different DNA-binding specificities and transactivating potentials (Eferl & Wagner, 2003; Milde-Langosch, 2005). Structurally, all AP-1 members possess a basic-leucine zipper domain (bZIP) required for factor dimerization and DNA binding. The leucine-rich motif allows for dimerization with other bZIP-containing proteins, a prerequisite for DNA-binding mediated through the basic domain (Eferl & Wagner, 2003). Although Jun, Fos and ATF members bind to the 12-O-

tetradecanoylphorbol-13-acetate responsive element (TPA responsive element, or TRE)[5] and the cyclic AMP responsive element (CRE)[6], Jun and Fos members bind preferentially to the TRE while ATF members bind preferentially to the CRE (Eferl & Wagner, 2003). Maf members bind altogether different elements called MAF-recognition elements (MAREs) and the antioxidant-response elements (AREs) (Eferl & Wagner, 2003) and will not be discussed herein. Both the *jun* and *fos* genes were established oncogenes originally isolated from avian and murine tumor viruses (Maki *et al.*, 1987; Curran & Teich, 1982). Because of their role in promoting cancer, this review will focus primarily on the Jun and Fos families.

3.1.1 Structure and function of the Jun family

The Jun family of AP-1 transcription factors comprises Jun (also called c-Jun), JunB and JunD (Eferl & Wagner, 2003). *Jun* is the prototypical member and was isolated as the cellular counterpart of the avian sarcoma virus 17 *v-Jun* oncogene (Maki *et al.*, 1987)[7]. Although structurally similar, the Jun members regulate different biological processes. Jun is critical for cellular proliferation, while both JunB and JunD are involved in other aspects of development (Eferl & Wagner, 2003; Hess *et al.*, 2004). Mice nullizygous for Jun or JunB are embryonically lethal (Hilberg *et al.*, 1993; Johnson *et al.*, 1993; Schorpp-Kistner *et al.*, 1999) while JunD -/- animals survive to adulthood, though they exhibit retarded growth and male reproductive defects (Thepot *et al.*, 2000). Both *Jun* and *JunB*

[5] 5' TGAG/CTCA 3'
[6] 5' TGACGTC/AA 3'
[7] Jun is a truncation of ju-nana, the word for 17 in Japanese.

are immediate early genes responding rapidly to serum activation in MEFs (Eferl & Wagner, 2003). *Jun* expression is regulated through a positive feedback loop acting on CRE sites located in the *Jun* promoter (Angel *et al.*, 1988). Runaway stimulation of *Jun* activity is controlled both by decreased mRNA stability through an AU-rich destabilizing element in the 3' UTR (Peng *et al.*, 1996) and by post-translation regulation. Although experiments in immortalized fibroblasts showed that *JunD* was not stimulated by serum and was constitutively expressed in quiescent cells (Pfarr *et al.*, 1994), experiments in other cell types have shown differing results. Studies by different groups using immortalized cell lines have shown transient activation of *JunD* upon serum stimulation (Wang *et al.*, 1996; Toualbi-Abed *et al.*, 2008) while experiments in primary CEFs showed that full length JunD expression was unchanged in dividing cells versus quiescent or serum-starved cells; however, the expression of a truncated JunD was increased in the latter two conditions (Gagliardi *et al.*, 2003) whereas both JunD isoforms were shown to accumulate in conditions of Src-transformation (Wang *et al.*, 2011a).

The presence of an active TRE along with a number of different binding sites argues for a more complex context-dependent regulation of *JunD* (Hernandez *et al.*, 2008). Indeed, the *JunD* transcript generates two different isoforms, one of them, ΔJunD, arises from an alternate AUG downstream of the full length AUG resulting in an N-terminal truncated JunD thought to be weaker in its transcriptional activation (Yazgan & Pfarr, 2002). Furthermore, JunD protein levels appear to be controlled by translational regulation through the 5' UTR (Hernandez *et al.*, 2008). Although much effort has been devoted to understanding the mechanistic and functional relevance of these features, at

present very little is known about the function of either (Short & Pfarr, 2002; Hernandez *et al.*, 2008).

In addition to the bZIP domain, Jun members share a partially conserved transactivating domain (TAD) at the N-terminus. The pleiotropic action of the various AP-1 members is in part due to the differing structure of the TAD (Hess *et al.*, 2004). Activation of the Jun TAD occurs through the phosphorylation of S63 and S73 by JNK (Pulverer *et al.*, 1991; Davis, 2000). The interaction between JNK and Jun is mediated through the δ-domain (AA 34-60) (Eferl & Wagner, 2003). Deletion of the δ-domain in v-Jun increases transcriptional activity of Jun (Bohmann & Tjian, 1989) and also renders it transforming, presumably by decoupling JNK signaling from the activation of Jun and by the elimination of a poly-ubiquitinylation site therein (Treier *et al.*, 1994). Deletion of the δ-domain, and hence the poly-ubiquitinylation site, restores Jun stability and increases its transcriptional activity (Treier *et al.*, 1994). Phosphorylation of Jun by casein kinase II (CKII) and GSK3β on S243 and S247, adjacent to the basic domain, attenuates DNA-binding activity (Lin *et al.*, 1992; Boyle *et al.*, 1991) and primes Jun for ubiquitination-mediated degradation by the Fbw7 ubiquitin ligase (Laine & Ronai, 2005; Wei *et al.*, 2005). Conversely, inactivation of GSK3β through serum stimulation leads to further stabilization of Jun (Wei *et al.*, 2005).

Significantly, JunB and JunD, differ structurally from Jun at the N-terminus. JunB retains the JNK docking site, but lacks the phosphoacceptor amino acids required for efficient transactivation (Eferl & Wagner, 2003). In contrast, JunD possesses the conserved serines (S90 and S100 in mammals) but lacks the JNK-binding domain

resulting in weakened transactivation by JNK unless heterodimerized with a docking domain-containing partner such as Jun (Kallunki *et al.*, 1996; Eferl *et al.*, 2003; Hess *et al.*, 2004). Instead, JunD is preferentially phosphorylated by ERK on S100 (Gallo *et al.*, 2002; Vinciguerra *et al.*, 2004). The affinity of ERK for JunD is determined by the presence of two short N-terminal motifs dubbed the docking domain (D-domain)[8] and DEF motif[9]. In immortalized human embryonic kidney (HEK) cells, mutation of the DEF motif eliminated ERK-dependent phosphorylation of JunD while mutation of the D domain only attenuated this response (Vinciguerra *et al.*, 2004). Consistent with this, introduction of a DEF motif into Jun rendered it inducible by ERK illustrating that the DEF motif is the primary determinant for ERK phosphorylation of JunD (Vinciguerra *et al.*, 2004). A conserved D domain is also found with the δ-domain of Jun and is required for the phosphorylation of Jun by JNK (Derijard *et al.*, 1994). An important negative regulator of junD activation is the tumor suppressor *multiple endocrine neoplasia type I* (*MEN1*) encoded gene product menin (Hernandez *et al.*, 2008). Menin inhibits JunD activity both by abrogating Erk2- and JNK-mediated phosphorylation of JunD and by the recruitment of the mSin3A-histone deacetylase complex (Gobl *et al.*, 1999; Gallo *et al.*, 2002; Kim *et al.*, 2003). Curiously, experiments using menin deletion mutants show that suppression of Erk and JNK occurs independently of each other through different structural mechanisms (Gallo *et al.*, 2002). Together, these data indicate multiple

[8] The D domain is not an autonomous protein domain *per se* but rather a short oligomeric motif defined as R/K-K-X_{4}-L-X-L (Vinciguerra *et al.*, 2004).

[9] DEF stands for **d**ocking site for **E**RK, **F**-X-F. The canonical DEF motif is F/W-X-F/Y/W with a P preferred in the fourth position (Sheridan *et al.*, 2008). The DEP motif in JunD is FLYP (Vinciguerra *et al.*, 2004)

activating pathways controlling Jun and JunD activity and illustrate how interaction with different AP-1 dimers can confer pleiotropic effects.

3.1.1 Structure and function of the Fos family

Similar to the Jun family, the Fos family prototype was isolated from a sarcoma virus. *Fos* (also *c-fos*) is the cellular progenitor of the murine Finkel-Biskis-Jinkins (**F**BJ) **o**steo**s**arcoma virus oncogene *v-Fos* (Curran & Teich, 1982). The Fos family comprises Fos, FosB, Fra1, and Fra2[10] (Eferl & Wagner, 2003). In addition to the bZIP domain, Fos and FosB, but not Fra1 nor Fra2, contain strong transactivating domains near the C-terminus of the proteins (Wisdon & Verma, 1993). Due to a series of negatively charged residues adjacent to the leucine zipper, strong electrostatic repulsion prevents the formation of Fos homodimers (Halazonetis *et al.*, 1988). However, Fos proteins can dimerize with other bZIP members, preferring the Jun family of factors, with a strong preference for Jun-Fos dimers (Halazonetis *et al.*, 1988; Ryseck & Bravo, 1991). Similar to *Jun* and *JunB*, *Fos* and *FosB* are immediate early genes expressed rapidly and transiently upon serum stimulation (Greenberg & Ziff, 1984; Kovary & Bravo, 1991). In contrast, early studies conducted in immortalized fibroblasts showed both *Fosl1* and *Fosl2* were expressed constitutively at low levels but were also activated by serum, albeit with delayed kinetics compared to *Fos* and *FosB* (Kovary & Bravo, 1991; Milde-

[10] Fra is short for fos-related antigen, while the gene names for mammalian Fra1 and Fra2 are *fosl1* and *fosl2* for *Fos-like antigen1* and *2*.

Langosch, 2005). In CEF, serum activation of *Fosl2*[11] is delayed but persists longer than *Fos* (Sonobe *et al.*, 1995). Subsequent promoter analysis experiments showed that the activation was sustained by an AP-1 positive feedback loop mediated primarily by Jun/Fos heterodimers binding to the *Fosl2* promoter switching to Jun/Fra2 dimers in the latter phase of activation (Sonobe *et al.*, 1995). Consistent with this, experiments in murine and avian cells showed that full activation of both the *Fosl1* and *Fosl2* required a TRE and SRE (serum response element) (Adiseshaiah *et al.*, 2005; Sonobe *et al.*, 1995).

Post-translational activation of Fos is biphasic, initially requiring phosphorylation of S374 and T362 by Erk and RSK respectively (Milde-Langosch, 2005). This stabilizes Fos and creates a docking site for Erk. Full activation occurs through phosphorylation of T232, T325 and T331 (Pellegrino & Stork, 2006). Phosphorylation of S374 and T362 stabilizes a C-terminal degron in both Fos and FosB. Turnover of Fos is mediated through ubiquitin-independent proteolysis (Milde-Langosch, 2005). In v-Fos, a frameshift deletion in the C-terminus eliminates the degron, increasing the stability of v-Fos. Similarly, in Fra1 stabilization occurs through the phosphorylation of S252 and S265 (Basbous *et al.*, 2008); both conserved residues in Fra2. Dimerization of Fos and Jun family members prevents the nuclear export and degradation of Fos, thus posing another mechanism of AP-1 regulation (Malnou *et al.*, 2007).

Overexpression of Fos and FosB but not Fra1 or Fra2 is transforming in rat fibroblasts (Wisdon & Verma, 1993). The lack of a transactivating domain in Fra1 and

[11] Chicken *Fosl1*/Fra1 has not yet been identified. As of this writing, there is no known *Fosl1* ortholog as identified by reciprocal BLAST analysis (Remm *et al.*, 2001; Ostlund *et al.*, 2010) or by traditional biochemical methods.

Fra2 have implicated both these proteins in the negative regulation of gene expression and hence transformation (Wisdon & Verma, 1993). However, early experiments in primary cells also showed that Fra2 is a major component of AP-1 activity in v-Src transformed cells and that AP-1 activity is critical for Src transformation (Sonobe *et al.*, 1995; Murakami *et al.*, 1997; Murakami *et al.*, 1999). A more recent study showed that inhibition of Fra2 eliminates anchorage-independent growth in v-Src-transformed CEF (Wang *et al.*, 2011a). Although it is thought that Fra1 and Fra2 act as transcriptional repressors by directly binding to DNA or by out-competing the formation of more active AP-1 dimers (Lopez-Bergami *et al.*, 2010), a recent study using a cell-free *in vitro* reconstituted HPV genome showed that Jun-Fra1 and Jun-Fra2 dimers have comparable activity to Jun-Jun and Jun-FosB dimers. The same study also showed that Jun-Jun and different Jun-Fos-family members bind various TRE-like elements with different affinities and specificities (Wang *et al.*, 2011b) suggesting that the pleiotropy of AP-1-dependent gene expression may be attributable to differences in positive as well as negative gene regulation by different Jun-Fos dimers.

3.1.2 Role of AP-1 in oncogenesis

The formation of Jun family homo- and heterodimers, as well as the formation of heterodimers with other bZIP members contributes to the pleiotropy of AP-1 activity in the organism. Tissue and cell cycle-specific differences in AP-1 factor expression and activation are thought to account for some of the apparently contradictory functions of specific AP-1-members under different circumstances (Hernandez *et al.*, 2008).

Activation of AP-1 results in the dysregulation of genes involved in cell cycle, survival, and in phenomena associated with tumor progression. Importantly, every Jun and Fos member has been shown to be dysregulated in some type of cancer (Eferl & Wagner, 2003; Milde-Langosch, 2005; Hernandez *et al.*, 2008; Lopez-Bergami *et al.*, 2010) indicating the significant role played by AP-1 in transformation and tumorigenesis.

3.1.2.1 Role of AP-1 in proliferation and transformation

Both the viral orthologs of Jun and Fos were isolated based on their transforming potential (Maki *et al.*, 1987; Curran & Teich, 1982) and accordingly, overexpression of both Jun and Fos can transform fibroblasts (Castellazzi *et al.*, 1990; Miller *et al.*, 1984). While Fos overexpression induces osteosarcomas in transgenic mice (Grigoriadis *et al.*, 1993), Jun overexpression does not lead to tumor formation (Vogt, 2001). Jun has been shown to activate the *cyclinD1* promoter in cell culture thereby promoting G1-S transition (Albanese *et al.*, 1995). In contrast, deletion of Jun in MEFs results in potentiated *p53* expression and retarded cell proliferation. *Jun*/*p53* double-null MEFs do not exhibit this phenotype (Schreiber *et al.*, 1999). *Jun-/-* MEFs cannot be transformed by Ras^{V12} or v-Src, however, subcutaneous injection of these cells did result in latent tumor formation in nude mice (Johnson *et al.*, 1996). Recovery of these tumors showed up-regulated AP-1 activity resulting from the overexpression of JunD (Johnson *et al.*, 1996). Silencing of *Jun* in v-Src-transformed CEF results in premature senescence and a moderate increase in apoptosis (Wang *et al.*, 2011a).

Similar to Jun, overexpression of *Fos* in transgenic mice induces *cyclinD1* expression in chondrocytes and osteoblasts (Sunters *et al.*, 1998). However, inducible expression of Fos in cultured osteoblasts resulted in high levels of cyclin A, and cyclin E while osteoblasts derived from Fos-overexpressing mice exhibited only abnormal cyclin A expression (Sunters *et al.*, 2004). MEFs overexpressing Fos were aberrant in neither cyclin A nor E expression (Sunters *et al.*, 2004) indicating cell type-specific differences in the promotion of cell cycle by Fos. Interestingly, *fosl1* knock-in mice rescue the lethality phenotype in *fos*-/- mice indicating functional redundancy between some fos members (Fleischmann *et al.*, 2000). Since Fra1 is thought to lack the transactivation domain that Fos possesses, this suggests that Fra1 activity is mediated through other AP-1 factors such as Jun or that Fra1 has heretofore uncharacterized transactivating potential.

In contrast to Jun, the role of JunB and JunD in proliferation and transformation is less clear. JunB overexpressing fibroblasts exhibit reduced proliferation from reduced cyclinD activity and are resistant to Src and Ras-induced transformation (Passegue & Wagner, 2000). This study also showed that delayed G1 entry was due to activation of the $p16^{INK4A}$ gene by JunB. Previously, the same group showed that repression of *JunB* in murine myeloid cells phenocopied human chronic myelogenous leukemia (CML) (Jochum *et al.*, 1999). Consistent with this, hypermethylation of the *JunB* promoter and concomitant repression of *JunB* expression seen in human CML suggests that JunB is a tumor suppressor (Yang *et al.*, 2003a). Nevertheless, overexpression of *JunB* rescues the lethality phenotype in *Jun*-/- mice. In cultured *Jun* null MEFs, JunB overexpression

rescues cyclinD1 while decreasing p53 and p21 levels similar to that of *wt* MEFs indicating functional redundancy among Jun members (Passegue *et al.*, 2002) akin to Fos.

In an influential study, Pfarr *et al.* showed that JunB and JunD overexpressing murine 3T3 cells grow more slowly and are refractory to Ras-transformation (Pfarr *et al.*, 1994). Similarly, it was shown that immortalized *JunD*-/- fibroblasts grow faster than their wild-type counterparts (Weitzman *et al.*, 2000) suggesting a negative role for JunD in proliferation. However, *JunD* null primary MEFs display premature senescence in culture and a higher sensitivity to p53-mediated apoptosis (Weitzman *et al.*, 2000). Ectopic expression of JunB and JunD can partially recover colony formation in Ras^{V12}-transformed MEFs nullizygous for *Jun* (Johnson *et al.*, 1996). JunD overexpressing CEF exhibit accelerated growth and grow to higher density compared to *wt*, while inhibition of JunD in v-Src-transformed cells abrogates anchorage independent colony formation (Wang *et al.*, 2011a). Again, as in JunB, overexpression of JunD in *Jun*-/- mice rescues the embryonic lethality phenotype, albeit only until birth (Eferl & Wagner, 2003). Taken together, these data show a more complex role that JunB and JunD play in the regulation of proliferation and survival in the cell.

3.1.2.2 Role of AP-1 in cell survival

The pro- and anti-survival role of AP-1 is determined largely by the specific AP-1 dimers and the cellular context in which AP-1 activity is observed. As a case in point, overexpression of Jun in undifferentiated PC12 cells induces differentiation, whereas Jun expression in nutrient starved differentiated PC12 cells induces apoptosis (Leppa *et al.*,

2001). Overexpression of Jun has been shown to induce apoptosis in numerous cell types including neurons, fibroblasts and endothelial cells (Ham *et al.*, 1995; Bossy-Wetzel *et al.*, 1997; Wang *et al.*, 1999). Furthermore, the JNK-Jun pathway has been shown to be involved in FasL and other death receptor ligand-induced apoptosis (Hess *et al.*, 2004). Both the *FasL* and *TNFα* promoters contain AP-1 sites and can be activated by Jun (Hess *et al.*, 2004). Cultured thymocytes from *JunAA* (Jun S63A and S73A substitution mutants) knock-in mice were shown to be resistant to TNFα-induced apoptosis, indicating the requirement of Jun phosphorylation by JNK in cell death (Behrens *et al.*, 2001). In cultured neurons, NGF-withdrawal-induced apoptosis is rescued by expression of TAM67 (Whitfield *et al.*, 2001). Furthermore, overexpression of TAM67 was shown to decrease expression of the pro-apoptotic Bcl member *Bim* (Whitfield *et al.*, 2001). Inhibition of JNK2 using a dominant negative allele represses expression of Bim (Eminel *et al.*, 2004), and consistently, pharmacological inhibition of JNK in cultured neurons represses Jun phosphorylation and kainate[12]-induced apoptosis (Zhao *et al.*, 2011). Therefore, at least under some conditions and in certain cell types, Jun functions in a pro-apoptotic fashion.

JunB also has a positive role in apoptosis, primarily in the context of the hematopoietic cell lineage. Absence of JunB in murine myeloid cells induces a CML-like phenotype (Jochum *et al.*, 1999). Mice lacking JunB during myeloproliferation exhibit aberrant apoptosis, overexpression of the survival genes *bcl-2* and *bcl-x,* and repression of $p16^{INK4A}$ (Passegue *et al.*, 2001).

[12] Kainate is a neuroexcitoxin derived from seaweed used to model certain neurodegenrative disorders.

Nevertheless, Jun has also been characterized in the context of promoting survival. In MEFs, overexpression of Jun represses the AP-1 target and Akt-pathway antagonist, *PTEN* (Hettinger *et al.*, 2007). Consistent with this, *Jun*-/- MEFs are sensitive to apoptosis in conditions of nutrient starvation and repression of PTEN by siRNA recovers this phenotype (Hettinger *et al.*, 2007). In mice with a targeted disruption of *Jun* in the liver, hematopoietic cells and hepatoblasts underwent increased apoptosis (Eferl *et al.*, 1999). Cultured hepatocytes from these mice displayed reduced growth rates and increased apoptosis (Eferl *et al.*, 1999). Partial hepatectomies of mice with a perinatal liver-specific deletion of *Jun* showed aberrant liver regeneration (Behrens *et al.*, 2002). This regeneration was accompanied by both reduced hepatocyte proliferation and increased apoptosis (Behrens *et al.*, 2002). Interestingly, *JunAA* knock-in mice did not display this phenotype, suggesting that JNK-dependent phosphorylation of Jun is not required to maintain proliferation and survival in these cells. *Jun*-/- hepatocytes also show higher levels of p53 expression and are sensitized to TNFα-induced apoptosis (Eferl *et al.*, 2003). Deletion of p53 or p21 rescued the *Jun*-/- induced hepatocellular proliferation defects indicating the dependence of p53 in *Jun* null-induced apoptosis (Stepniak *et al.*, 2006). Similarly, in *JunD* deficient MEFs, cells undergo p53-dependent apoptosis in response to irradiation and *JunD* null mice are sensitized to TNFα-induced hepatitis (Weitzman *et al.*, 2000). Cardiomyocytes from *JunD* null mice also exhibit high levels of apoptosis and overexpress p53 (Hilfiker-Kleiner *et al.*, 2005) while overexpression of JunD in MEFs can attenuate UV and H_2O_2-induced apoptosis (Zhou *et al.*, 2007).

Inhibition of AP-1 by TAM67 in v-Src-transformed CEF induces apoptosis and senescence, while reducing proliferation (Wang *et al.*, 2011a). Targeted repression of *Jun*, *JunD* and *Fosl2* by shRNA shows that JunD is the primary determinant of survival in v-Src-transformed CEF (Wang *et al.*, 2011a). Although in CNR v-Src transformation is associated with a decrease in *PTEN* expression, this is not the case in CEF (Wang *et al.*, 2011a). Down-regulation of *JunD* in CEF on the other hand is accompanied by activation of the pro-apoptotic gene *dapk1* (see chapter 3 and Discussion) suggesting different mechanisms regulating survival in different cell types.

The role of Fos proteins in survival appears to be mainly pro-apoptotic. Overexpression of a Fos dominant negative mutant can block *myc*-induced apoptosis in cultured hepatoma cells (Kalra & Kumar, 2004). Sustained expression of *Fos* in TGFβ-Ras-transformed hepatocytes has been shown to lead to reduced proliferation and apoptosis (Mikula *et al.*, 2003). Interestingly, in this model of hepatocarcinoma, *Fos* is induced initially but then is gradually lost as the cell acquires a metastatic phenotype suggesting a role for Fos in tumor initiation but not progression (Mikula *et al.*, 2003). Nevertheless, Fos displays pro survival characteristics as well. While FosB has been shown to work cooperatively with Jun to promote expression of Fas (Baumann *et al.*, 2003) in cultured lymphoma cells, Fos does the opposite (Lasham *et al.*, 2000). Curiously, while some groups reported increased apoptosis in *Fos* deficient lymphoid, neuronal and retinal cell lineages (Hess *et al.*, 2004; Oshitari *et al.*, 2002), others reported no differences in apoptosis in developing tissues of *Fos* null mice (Roffler-Tarlov *et al.*, 1996). Other Fos members have been implicated in survival as well. Both the murine and

human *ARF* promoters are targets of Fra1 and its overexpression in cultured cells leads to increased ARF levels and growth arrest (Ameyar-Zazoua *et al.*, 2005). Similarly, overexpression of Fra1 in cultured glioblastoma cells inhibited proliferation and induced differentiation while high Fra1 expression sensitized cells to chemotherapeutically-induced apoptosis (Shirsat & Shaikh, 2003).

3.1.2.3 Role of AP-1 in angiogenesis and tumor expansion

One of the phenomena associated with increased tumor invasiveness and growth is the dysregulation of genes involved in motility, the degradation of the ECM and angiogenesis. One of the early observations in Src-transformed cells was the induction of the pro-angiogenic chemokine IL8, suggestive of a role for Src in promoting tumor vascularization (Bedard *et al.*, 1987a; Sugano *et al.*, 1987). v-Src-transformation and expression of IL8 is wholly dependent on AP-1 activity illustrating the importance of AP-1 in expressing this factor (Dehbi & Bedard, 1992). Indeed, both Jun and Fos are associated with the induction of other proangiogenic genes (Orlandini *et al.*, 1996; Hossain *et al.*, 2000). DNAzyme-mediated[13] inhibition of Jun blocks proliferation and tubule formation in human endothelial cells *in vitro*. It also abrogates VEGF-induced neovascularization in rat cornea and melanoma progression in mice (Zhang *et al.*, 2004). Activation of Jun is detected in the invasive front of breast carcinomas and is associated with increased VEGFD expression and hyperphosphorylation of Rb. Furthermore, Jun

[13] The authors used synthetic single-stranded DNA catalysts (DNAzymes) engineered to bind to their complementary sequence in order to cleave specific mRNA species (Santoro & Joyce, 1997).

activation was correlated with poor survival outcome in these patients (Vleugel *et al.*, 2006). JunB may also play a cooperative role with Jun in the promotion of angiogenesis. Both Jun and JunB are up-regulated in mouse models of fibrosarcoma and haven't been associated with increased vascularization (Bossy-Wetzel *et al.*, 1992). In JunB null mice, embryonic lethality was shown to arise from defects in placentation. Disruption of JunB resulted in defective placental vascularization from repression of the pro-angiogenic factors proliferin and flt-1, and the proteases MMP9 and urokinase plasminogen activator (PLAU) (Schorpp-Kistner *et al.*, 1999). Similarly, the pro-angiogenic factors VEGFD[14] was shown to be a target of Fos activation in fibroblasts (Orlandini *et al.*, 1996). Treatment of human umbilical cord vein endothelial cells (HUVECs) with VEGFD induced cell growth and when injected ocularly induced vascularization in rabbit corneas (Marconcini *et al.*, 1999).

In addition to the contribution of AP-1 to angiogenesis, tumor progression also requires the ability to spread via extravasation/intravasation, requiring changes to genes regulating motility and the remodeling of the ECM. The fact that high AP-1 activity correlates with poor prognosis in numerous cancers suggests that AP-1 plays a role in metastasis (Ozanne *et al.*, 2007). Early studies showed that transformation of fibroblasts with v-Fos and v-Jun increases their invasive capacity in culture and that this is associated with expression of pro-invasive gene products such as the MMP1, MMP3, MMP9, and CD44 (Taniguchi *et al.*, 1989; Lamb *et al.*, 1997; Bos *et al.*, 1999). *In vitro*, both Jun- and Fos-estrogen receptor fusion proteins (JunER and FosER) can induce estrogen-dependent

[14] VEGFD is also known as c-fos induced growth factor and its official gene symbol is FIGF.

reversible EMT in murine mammary epithelial cells indicating that AP-1 plays a direct role in tumor progression and not only initiation (Reichmann *et al.*, 1992; Fialka *et al.*, 1996). The role of the different AP-1 members in invasion however is largely disease dependent and there is considerable overlap in function. Though, what appears to be common is that there is usually a rearrangement of AP-1 dimers in cells with a less invasive to a more invasive phenotype. In mammary adenocarcimoma cell lines, expression of Fos and FosB was detected in less aggressive cells while Fra1 and Fra2 were seen in more aggressive cells (Kustikova *et al.*, 1998). Interestingly, while Fos, Fra1 and Fra2 all induced expression of pro-metastasis genes CD44, osteopontin, and thrombospondin, only Fos and Fra1 regulated genes in the urokinase pathway (Andersen *et al.*, 2002). In both breast cancer cell lines and tissues, FosB expression was correlated with positive hormone receptor status and a highly differentiated tumor type whereas Fra1 expression was negatively correlated with both FosB and prognostic markers including low grade and positive receptor status (Bamberger *et al.*, 1999). Consistent with this, loss of Fos expression was correlated with poor prognosis in patients with ovarian cancer (Mahner *et al.*, 2008). In support of this, several studies showed that a Fos switch, from Fos and FosB to Fra2, and particularly Fra1, is associated with an invasive cancer phenotype (Milde-Langosch, 2005). Interestingly, Fra2 appears to block differentiation in v-Src transformed cells. Indeed, inhibition of Fra2 in v-Src-transformed CEF leads to adipogenesis (Wang *et al.*, 2011a). Fra2 is also a known inhibitor of differentiation antagonizing Pax6, a master regulator of eye development (Cvekl *et al.*, 1994).

3.2 C/EBPβ

The activation of the Src-transformation marker *IL8* is dependent on the activity of AP-1 NFκB and C/EBPβ. Mutation of the binding sites in the Src-responsive unit of the *IL8* promoter or inhibition of the factors themselves abrogated *IL8* promoter activity (Dehbi *et al.*, 1992; Cabannes *et al.*, 1997; Gagliardi *et al.*, 2001). Despite this, inhibition of C/EBPβ using the Δ184 C/EBPβ dominant negative mutant did not block transformation. Overexpression of Δ184 in CEF increased proliferation and activated expression of cyclinD1 while C/EBPβ null murine fibroblasts proliferated better than WT fibroblasts (Gagliardi *et al.*, 2003). Normal CEF entering G_0 downregulate AP-1 activity and activate the expression of the C/EBPβ-dependent growth arrest specific (GAS) marker *p20k* (Gagliardi *et al.*, 2001; Gagliardi *et al.*, 2003). Conversely, overexpression of Δ184 in CEF enhances Jun, JunD and Fra2 expression while overexpression of C/EBPβ antagonizes the expression of AP-1 and enhances *p20k* expression (Gagliardi *et al.*, 2003) illustrating that AP-1 and C/EBPβ play opposing roles in the expression of GAS genes and the control of CEF proliferation.

3.2.1 Structure and regulation of C/EBPβ

The CAAT/enhancer-binding proteins comprise a family of bZIP transcription factors involved in the regulation of proliferation, differentiation, inflammation and metabolism. The family comprises six factors named, in order of their discovery, C/EBPα, C/EBPβ,

C/EBPγ, C/EBPδ, C/EBPε and C/EBPζ[15] with C/EBPα, C/EBPβ being the best studied. All factors in the family can hetero- and homodimerize and bind to the IUPAC[16] consensus sequence RTTGCGYAAY, with the exception of CHOP (Osada *et al.*, 1996). CHOP is unable to homodimerize, requiring the presence of other bZIP factors for dimerization. Heterodimerization of C/EBPβ with CHOP reduces affinity for the C/EBPβ consensus site indicating that CHOP can function in a transdominant fashion to attenuate C/EBPβ activity (Ron & Habener, 1992). However, under conditions of lipopolysaccharide (LPS)-induced stress, CHOP was shown to bind the sequence RRRTGCAATMCCC as a CHOP-C/EBP heterodimer and induce reporter gene expression (Ubeda *et al.*, 1996). Significant pleiotropy is also derived from the interaction of C/EBP with other members of the family or unrelated bZIP proteins binding to distinct regulatory elements. For instance, the regulation of *IL1B* gene is regulated by C/EBP-CREB dimers binding a C/EBP-CREB composite site consisting of a one C/EBP and one CRE half site (Tsukada *et al.*, 1994).

In mammals, C/EBPα, C/EBPβ, C/EBPγ and C/EBPδ are coded by intronless genes while C/EBPε and CHOP contain introns. C/EBPε codes for several proteins arising from splice isoforms (Zahnow, 2009). C/EBPα and C/EBPβ each code for three isoforms derived through alternate translational start codon usage and are regulated dynamically by an out-of-frame upstream open reading frame (uORF) (Wethmar *et al.*,

[15] C/EBPζ was originally cloned as C/EBPβ-homologous protein clone 10 (CHOP10) or simply CHOP (Ron & Habener, 1992) and is most frequently referred to in that manner in the literature and will therefore be referred to as such herein. It should be noted that the official gene name for CHOP is DNA-damage-inducible transcript 3 (DDIT3) and is also referred to as growth arrest and DNA damage-inducible protein (GADD153).

[16] International Union of Pure and Applied Chemistry

2010). C/EBPβ is expressed in three isoforms referred to as LAP1, LAP2, and LIP (also called LAP*, LAP and LIP respectively for liver enriched transcriptional activator protein and liver enriched transcriptional inhibitor protein) (Descombes *et al.*, 1990; Descombes & Schibler, 1991). Structurally, full length LAP1 contains an N-terminal activation domain, two adjacent regulatory domains, a DNA-binding basic domain and leucine zipper dimerization domain (Zahnow, 2009) (Figure 4). LAP2 differs from LAP1 by lacking the first 21 amino acids of the activation domain while LIP is devoid of any activation domain suggesting that the protein acts in a dominant-negative fashion (Descombes & Schibler, 1991). The N-terminal activation domain of LAP1 has been shown to interact with the SWI/SNF chromatin remodeling complex to activate gene expression, and as such, LAP1 is a more potent transactivator than LAP2 (Kowenz-Leutz & Leutz, 1999).

A key control of the expression of C/EBPβ isoforms resides with the mTOR pathway. Activation of mTOR signaling leads to enhanced global translation and increased availability of eukaryotic initiation factors. Treatment of rat preadipocytes with rapamycin favored expression of LAP isoforms while treatment of cells with the PKR[17] inhibitor 2-aminopurine favored expression of LIP (Calkhoven *et al.*, 2000). Overexpression of eukaryotic initiating factors eIF4E and eIF2α also favoured LIP expression. Mutation of the initiating ATG in the uORF however, strongly abrogated LIP expression in favor of LAP isoforms, illustrating the importance of translational initiation control in the expression of C/EBPβ variants (Calkhoven *et al.*, 2000). The modulation of

[17] Activation of PKR leads to a general suppression of translation by phosphorylating and thus inactivating eIF2α (Meurs *et al.*, 1990).

the LAP/LIP ratio has in turn been implicated in regulation of various processes including cell fate and proliferation in adipocytes and osteoclasts (Calkhoven *et al.*, 2000; Smink *et al.*, 2009) as well as in other normal and transformed cells (see below).

C/EBPβ is also regulated by post-translational modification and conformational regulation. Constitutive inactivation of C/EBPβ occurs through the occlusion of the transactivating and DNA-binding domains by regulatory domains one (RD1) and two (RD2) respectively (Kowenz-Leutz *et al.*, 1994; Williams *et al.*, 1995). Alleviation of stearic inhibition is mediated by phosphorylation of RD1 and RD2 by the Ras-MAPK pathway (Williams *et al.*, 1995). Furthermore, the serine and threonine-rich central regulatory domain (CRD) comprising RD1 and RD2 can be regulated by other kinases and pathways including growth hormones, GSK3β, IFNγ, and RSK (Zhu *et al.*, 2002; Piwien-Pilipuk *et al.*, 2001; Tang *et al.*, 2005; Li *et al.*, 2007; Hanlon *et al.*, 2001). In adipocyte differentiation, phosphorylation of T188 (mouse) by ERK in the regulatory domain alleviates stearic inhibition of the activation domain while phosphorylation of S184 and T179 (mouse) by GSK3β potentiates DNA binding activity of C/EBPβ through the disinhibition of the DNA binding domain (Tang *et al.*, 2005). Both these phosphorylation events are required for activation of C/EBPβ and its subsequent activation of the *C/EBPα* and *PPARγ* genes (peroxisome proliferator-activated receptor γ) required for adipogenesis and terminal differentiation (Tang *et al.*, 2005).

In addition to phosphorylation, other post-translational modifications have been shown to regulate C/EBPβ activity. Acetylation of the conserved K39 residue within the activation domain has been shown to potentiate LAP1 and LAP2 (Cesena *et al.*, 2008)

while methylation at this position is associated with reduced activity (Pless *et al.*, 2008). Sumoylation of LAP1 but not LAP2 on K173 in human C/EBPβ has also been shown to inhibit transcriptional activity of LAP1 and subsequent activation of *cyclinD1* in mammary epithelial cells (Eaton *et al.*, 2001; Eaton & Sealy, 2003). Disulfide bond formation between cysteines 11 and 33 has been shown to attenuate transcriptional activity of LAP1 but not LAP2 since it is missing C11 and cannot form a disulphide bridge at this position. Disruption of these bonds under reducing conditions potentiates LAP1 activity and induces LPS-dependent *IL6* expression in cultured macrophages (Su *et al.*, 2003). Interestingly, DNA binding of C/EBPβ was increased under conditions of oxidative stress in adipocyte differentiation (Lee *et al.*, 2009). Furthermore, treatment of pre-adipocytes with anti-oxidants slowed mitotic clonal expansion of adipocytes and reduced C/EBPβ DNA binding activity and homodimer formation (Lee *et al.*, 2009). In a previous study, oxidation of cysteines 143 and 296 potentiated DNA binding and C/EBPβ/LAP dimer formation while mutation of these residues or treatment with reducing agents attenuated that activity *in vitro* (Kim *et al.*, 2007). The authors hypothesized that enhanced dimer formation was mediated through disulfide bridges formed between LAP dimers on the two cysteines which bracket the DNA binding domain of C/EBPβ. However, phosphorylation of the CRD by ERK and GSK3β was still a necessary prerequisite for C/EBPβ activation and the hypothesized disulfide bond formation only served to potentiate DNA-binding activity of an already active C/EBPβ dimer (Kim *et al.*, 2007). Nevertheless, these data suggest that C/EBPβ can act as a redox

sensing transcription factor through the regulation of both DNA-binding activity and transactivating potential.

3.2.2 Dual role of C/EBPβ in the regulation of proliferation and differentiation

Studies with C/EBPβ knock-out mice suggest an important role for C/EBPβ in differentiation, proliferation and metabolism. Approximately half *C/EBPβ*-/- mice die perinatally from hypoglycemia while surviving littermates exhibit impaired energy metabolism, and defective hepatic regeneration and adipogenesis (Croniger *et al.*, 2001; Greenbaum *et al.*, 1998). However, *C/EBPβ* null MEFs proliferate faster than their WT counterparts and in CEF, overexpression of C/EBPβ suppresses AP-1 expression and reduces cell proliferation (Gagliardi *et al.*, 2003). Serum starvation or contact inhibition also induces C/EBPβ expression and the activation of growth arrest specific genes such as p20K (Kim *et al.*, 1999b). Ras^{V12} expressing C/EBPβ null MEFs do not undergo oncogene induced senescence despite intact Arf and p53 expression. However, overexpression of C/EBPβ in C/EBPβ null MEFs is sufficient to induce senescence even in the absence of Arf and p53 (Sebastian *et al.*, 2005).

Nevertheless, C/EBPβ has also been shown to promote survival. Phosphorylation of mouse C/EBPβ on T217 by RSK is required for the survival and regeneration of hepatocytes following carbon tetrachloride induced hepatotoxicity (Buck *et al.*, 2001). Increased rates of apoptosis were also found in C/EBPβ null mice subjected to chemically induced skin tumors (Zhu *et al.*, 2002). These data indicate that in hepatocytes and keratinocytes, C/EBPβ promotes survival. Indeed, C/EBPβ has been found to be

correlated with high cyclinD1 expression in mammary tumors (Lamb *et al.*, 2003a). However, these studies did not address the role of C/EBPβ isoforms in the promotion of survival. It has been argued that the relative LAP/LIP ratio is a determinant of pro-survival activity of C/EBPβ (Zahnow, 2009). For instance, LAP2 and LIP activity, but not LAP1 has been associated with cyclinD1 expression in mammary epithelial cells (Eaton *et al.*, 2001) while aggressive breast tumors as well as Hodgkin's and anaplastic large cell lymphoma have been shown to favor a high LIP/LAP ratio (Zahnow, 2009; Jundt *et al.*, 2005). In support of this, mammary gland-specific expression of LIP in transgenic mice was sufficient to induce neoplasias and non-invasive carcinomas (Zahnow *et al.*, 2001). To test the role of the C/EBPβ uORF in the regulation of LAP and LIP *in vivo*, Achim Leutz's group generated knock-in mice carrying a C/EBPβ allele with a mutation in the start codon of the uORF ($C/EBP\beta^{\Delta uORF}$). These mice were shown to be deficient in LIP expression which resulted in prolonged repression of the cell cycle, delayed S-phase entry and a concomitant repression of E2F target genes in hepatocytes following partial hepatectomy (Wethmar *et al.*, 2010). Furthermore, osteoclast differentiation was impaired and MEFs derived from these mice displayed reduced proliferation compared to WT MEFs (Wethmar *et al.*, 2010). In knock-in transgenic mice expressing LIP only, hepatocyte regeneration was normal while osteoclast differentiation was enhanced (Wethmar *et al.*, 2010). Together these data show that the LAP/LIP ratio and its regulation are important for both control of proliferation and cell fate determination. However, the ability of C/EBPβ to dimerize with other bZIP factors under different physiological condition also contributes to the pleiotropy of C/EBPβ.

3.2.3 Interaction of C/EBPβ with other factors in the regulation of proliferation and differentiation

The somewhat paradoxical role of C/EBPβ in both promoting and opposing survival and differentiation can be better understood not only in the light of the function of different C/EBPβ isoforms but also through the association of C/EBPβ with other transcription factors. Early studies showed that C/EBPβ interacts with different factors in order to regulate both survival and differentiation. C/EBPβ was shown to act cooperatively with NFκB proteins p50, p65 and Rel in the activation of a reporter gene fused to a concatenated *fos* serum response element[18] or to a core C/EBPβ binding site (Stein *et al.*, 1993). Furthermore, mutational analyses indicated that C/EBPβ interacts with NFκB through the interaction of the C/EBPβ bZIP domain and NFκB Rel domain even in the absence of DNA. Interestingly, C/EBPβ repressed activation of a reporter fused to a native NFκB binding site in a dose dependent manner indicating that C/EBPβ can act both as transcriptional activator and repressor depending on the composition of the transcription factor binding site and the composition of the dimer (Stein *et al.*, 1993). Similarly, stress-induced activation of *CHOP* using tunicamycin led to a dimer switch wherein C/EBPβ homodimers were replaced by C/EBPβ-CHOP dimers (Ubeda *et al.*, 1996). These dimers could not bind canonical C/EBPβ-binding sites but instead bound a novel DNA sequence. When fused to a reporter construct, the C/EBPβ-CHOP dimer was shown to be active (Ubeda *et al.*, 1996). Both C/EBPβ and C/EBPδ have been shown to

[18] The rat fos SRE contains a core C/EBP-binding site (Metz & Ziff, 1991b; Metz & Ziff, 1991a).

be important in adipogenesis *in vivo* (Tanaka *et al.*, 1997) and overexpression of C/EBPβ in pre-adipocytes is sufficient to induce differentiation that is blocked by overexpression of CHOP (Darlington *et al.*, 1998). Consistent with this, CHOP expression is repressed during adipogenesis in mice (Darlington *et al.*, 1998). In T-lymphocytes, the interaction of C/EBPβ and Jun is crucial for the differentiation of B-cells. Activation of the IL4 gene is required for the commitment of naive T-cells to the B-cell lineage. Chemically induced B-cell differentiation was shown to require C/EBPβ and Jun for IL4 expression (Li-Weber *et al.*, 1997).

In addition to the role played in differentiation, C/EBPβ has also been shown to regulate proliferation through interaction with other transcription factors. Previously, it was shown that C/EBPβ synergizes with AP-1 and NFκB to activate the pro-angiogenic cytokine IL8 in Src-transformed cells (Gagliardi *et al.*, 2001). In a similar fashion, the expression of the FGF-BP is regulated by both AP-1 and C/EBPβ in response to EGF stimulation (Kagan *et al.*, 2003). In breast cancer, elevated expression of FGF-BP is associated with poor prognosis (Kagan *et al.*, 2003). Secretion of FGF-BD is known to potentiate FGF signaling stimulating mitosis and angiogenesis. In cultured breast carcinoma cells, deletion of C/EBPβ or AP-1 regulatory elements in the FGF-BD promoter abrogates EGF-dependent reporter activity (Kagan *et al.*, 2003). In another example, the expression of the lymphocyte mitogen IL1B requires the binding of a C/EBP-CRE composite site by C/EBPβ-CREB dimer (Tsukada *et al.*, 1994). Therefore under some conditions of mitogenic stimulation, C/EBPβ can synergize with other transcription factors to promote proliferation.

Under conditions of stress, such as amino acid depletion, C/EBPβ expression can be induced by the up-regulation of ATF4 in response to low nutrients (Kilberg *et al.*, 2009). Upregulation of C/EBPβ leads to the activation of genes containing composite C/EBP-ATF sites known as CAREs (CCAAT-enhancer binding protein-activating transcription factor response elements). ATF4 also activates *ATF3* and *CHOP* (Kilberg *et al.*, 2009). Following an initial acute phase of activation, after prolonged amino acid depletion both ATF3 and CHOP can interact with ATF4 and C/EBPβ to attenuate the amino acid response (Kilberg *et al.*, 2009). In macrophages, C/EBPβ has been shown to activate the expression of the pro-apoptotic gene *death associated protein kinase 1* (*DAPK1*) in response to gamma interferon (IFNγ) (Gade *et al.*, 2008). In mouse, induction of *DAPK1* was dependent of the presence of a C/EBP site and a CRE. Reporter constructs containing only the CRE could be activated by IFNγ in normal MEFs but not in C/EBPβ$^{-/-}$ MEFs indicating that C/EBPβ was required for the activation of *DAPK1* at the CRE. Furthermore, since C/EBPβ cannot bind CRE as a homodimer, it is likely that C/EBPβ is binding to the CRE as a C/EBPβ-ATF heterodimer (Gade *et al.*, 2008). Together these data indicate that the function of C/EBPβ in the regulation of proliferation and differentiation is dependent on different physiological conditions that regulate C/EBPβ isoforms expression and also the dimerization partners with which C/EBPβ interacts. As such, C/EBPβ can act as a stress sensor that interact with different transcription factors to either potentiate or attenuate their activity in a cell-context dependent manner.

4. Application of DNA microarrays to high-throughput screening of gene expression

Over the last several decades, the development of high-throughput technologies has allowed for the rapid multidimensional[19] interrogation of numerous biological problems. The development of the DNA microarray permitted the investigator to probe changes in gene expression of hundreds or thousands of genes simultaneously. An important driver of high-throughput gene profiling was the understanding of how global gene expression changes influence disease. Early reports of nascent pre-microarray technology sought to characterize changes in gene expression between normal and transformed cells (Augenlicht & Kobrin, 1982; Augenlicht *et al.*, 1987; Augenlicht *et al.*, 1991) and in cells treated with anti-cancer therapy (Kulesh *et al.*, 1987). The emergence of the understanding of human cancer as a heterogeneous disease that varies as much between individuals as between cancer types set the stage for investigation into the molecular processes that define cancer types and prognoses. Since 1977 the American Joint Committee on Cancer has used the histopathological TNM staging regimen for the classification of tumors as a guideline for diagnosis, prognosis and treatment response (Beahrs *et al.*, 1977). Numerous solid cancer types including breast, lung and colon come under the TNM diagnostic and treatment guidelines (2010). The three letter system refers to **t**umor size, and lymph **n**ode and **m**etastasis status. A further classification of *grade* refers to the differentiation status of the tumor with I being the most differentiated and IV being the least differentiated (anaplastic). Similar staging regimens have been adopted across the world by different organizations such as the Union for International Cancer

[19] Whole genome sequencing can, for example, generate data with numerous dimensions such as sequence, copy-number, loss of heterozygosity, methylation status, gene expression, etc.

Control (http://www.uicc.org/tnm). Nevertheless, a certain subjectivity with regard to tumor staging creates an ambiguity with respect to diagnosing and treating intermediate cancers, a problem that could potentially be ameliorated using molecular diagnostics (Sotiriou & Piccart, 2007). Certainly, since the 1980's, it has been shown that specific molecular markers correlate with poor prognosis in various cancers. Both HER2 amplification and high Src activity correlate with poor prognosis in breast carcinoma (Slamon *et al.*, 1987; Rosen *et al.*, 1986; Biscardi *et al.*, 1998). Investigators have used both model systems and primary tumors to analyze patterns of gene expression to better understand tumor development and prognosis *in vivo*. This section will focus on some of the developments in recent years in gene profiling predominantly as it pertains to Src, and breast cancer, but also in some other cancers associated with high Src activity.

4.1 Profiling of Src-responsive genes

Since the discovery that transcription factors can drive transformation, it has been understood that transformation necessarily comes with changes in gene expression (Bohmann & Tjian, 1989; Vogt, 2001). Knowing that certain oncogenes are associated with more advanced stages of disease, investigators over the past decade have used bottom up hypothesis-driven approaches at resecting gene expression patterns associated with disease. A number of studies over the last decade have sought to identify patterns of Src-induced gene expression using different *in vitro* models largely with different results. Several different studies using cultured fibroblasts or tumor cells derived numerous Src-expression signatures that differed not only in their gene complement but also in the size

of the sets identified, ranging from as few as 24 to as many as 645 unique genes (Malek *et al.*, 2002; Paz *et al.*, 2004; Irby *et al.*, 2005; Liu *et al.*, 2006). Other than general trends seen in changes to genes involved in cell cycle, cytoskeletal rearrangement, metalloproteases, and apoptosis, few genes were seen commonly expressed between two or more of these studies, with the exception of some previously characterized proto-oncogenes such as *cyclin D1* and *FOSL1* (Malek *et al.*, 2002; Liu *et al.*, 2006) Nevertheless, it should be stated that methodology and the objectives between these studies differed. For example, the Paz and Liu studies sought to identify patterns of gene expression in Src-transformed fibroblasts. Comparing expression profiles of cells overexpressing the oncoproteins v-Src, Ras or STAT3, three proteins involved in Src signaling, Paz *et al*. were able to show an overlapping signature of 35 unique genes co-regulated by these three oncogenes (Paz *et al.*, 2004). Furthermore, they described three unique signatures regulated by Src, Ras and STAT3 and showed the variations in the kinetics of gene activation of genes common to the three oncoproteins (Paz *et al.*, 2004). Using similar cell lines, Liu *et al*. revealed a smaller non-overlapping Src-responsive gene set (24 genes). Inhibition of Src-transformation by the re-expression of the normally Src-repressed tumor suppressor AKAP12 reversed transformation in v-Src expressing cells (Liu *et al.*, 2006). Furthermore, the expression pattern of sixteen genes including the cell cycle proteins Cdkn2d, Cdc2a and the transcription factor Hmgb3 were also reversed, illustrating a potential mechanism of tumor suppression by AKAP12 (Liu *et al.*, 2006).

In an earlier and perhaps more ambitious study, Malek and colleagues used stably transfected rat fibroblasts expressing either *wt Src* or one of two transforming *Src* alleles

to model transformation (Malek *et al.*, 2002). In total they found 645 unique genes whose expression patterns were associated with different transformational indices in cell culture. By comparing these genes with orthologous genes from staged colon carcinomas they identified that less than 20% of the orthologous genes had a corresponding expression pattern that correlated with the transformational indices as seen in their fibroblast models. Nevertheless, these genes appeared to correspond to genes associated with late stage colon cancer (Malek *et al.*, 2002). Among coregulated genes, Malek *et al*. identified those coding for cell cycle control, RNA processing and cytoskeletal remodeling illustrating that invasion is a pleiotropic process requiring the coordination of multiple facets of cell biology (Malek *et al.*, 2002).

In a similar study, Irby *et al*. phenotypically defined a set of high Src-expressing colon carcinoma cell lines as progressively invasive using invasion assays. After defining a high-invasive gene expression set, they used an iterative RNAi strategy to reveal other downstream genes associated with invasive colon carcinomas (Irby *et al.*, 2005). Their study identified *ADAM21*, *CD53* and *CCR9*, genes that code for a metalloprotease, an adhesion molecule and a pro-inflammatory cytokine receptor respectively (Irby *et al.*, 2005). Knock-down of each of these gene products repressed migration in Matrigel assays indicating that these three genes may play a role in invasion. By gene profiling subsequent knock down cell lines, Irby *et al*. identified a second tier of Src-responsive genes downstream of *ADAM21*, *CD53* and *CCR9* potentially involved in transformation. Interestingly, the signature identified by Irby *et al*. revealed a dearth of proliferation or survival genes unlike the fibroblast studies. Since Irby *et al*. used already transformed and

immortalized cell lines, their profiling study likely enriched for genes related directly to invasion and not proliferation or survival *per se*.

None of these groups however showed a direct attempt at prognostication using a bottom up approach. An important study out of Joseph Nevins lab at Duke University tested whether *in vitro* models of transformation could be used for predicting outcome in cancer patients. Bild *et al*. used primary HMECs infected with adenovirus coding for either GFP or the oncogenes Myc, Ras, E2F3, Src or β-catenin. These models were assayed for changes in gene expression and were used to define sets of deregulated genes associated with transformation in the HMECs (Bild *et al.*, 2006). External validation of the pathway signatures using transgenic mouse models showed a concordance of pathway dysregulation in the relevant genes sets. For example, a high correlation of gene expression associated with Myc was found in mouse tumors from MMTV-Myc mice versus MMTV-HER2 mice (Bild *et al.*, 2006). These signatures were subsequently used to classify tumor expression patterns with respect to the five oncogene expression "pathways". What Bild *et al*. showed was that different tumors exhibited different enrichment patterns for the deregulated pathways. High Ras, Src and β-catenin activity was associated with poor disease-free survival in lung tumors whereas only high Src and β-catenin activity was found to be associated with poor prognosis in ovarian cancers. Paradoxically there was no strong association with prognosis and pathway signature in the breast cancer tumors tested despite using HMECs as a model for transformation. Considering this, the authors noted a number of confounding variables such as hormone receptor and HER2 status that were not taken into account when looking at the breast

tumor samples (Bild *et al.*, 2006). Finally Bild *et al*. profiled a number of tumor cell lines and showed that cells expressing a high Ras or Src signature reacted in a dose dependent manner to their corresponding inhibitors showing that their signature analysis could be used to classify tumors by prognosis and to predict drug response (Bild *et al.*, 2006). It should be noted that Nevins' group used a number of activated oncogene alleles for their work but did not use activated Src. Since it is high Src activity, and not merely high Src expression that is associated with poor prognosis and triple negative status in breast cancer (Anbalagan *et al.*, 2012), the relevance of over expressing Src is not clear. Furthermore, over-expression of c-Src is not transforming on its own. Indeed, comparison of their gene signature to the aggressive genes signature published by our group shows a greater degree of similarity to their activated Ras signature than their c-Src signature (Bild *et al.*, 2006; Maslikowski *et al.*, 2010) (see Discussion). Interestingly, a following study sought to devise a method for predicting response to dasatinib therapy in breast cancer based on some of the data from Bild *et al*. (Moulder *et al.*, 2010). This study used three gene indices to predict dasatinib response. This included a target index corresponding to a weighted measure of dasatinib sensitivities of 19 kinases, a modified dasatinib-sensitivity index corresponding to a gene profile derived from dasatinib sensitive versus insensitive cell lines and a Src index taken from Bild *et al*. (Bild *et al.*, 2006). Although the authors were able to show a modest predictive response to dasatinib, the Src index performed the poorest in predicting dasatinib sensitivity in biopsies and was least reproducible (Moulder *et al.*, 2010).

4.2 *De novo* tumor gene signature development

In addition to model-based, bottom up approaches to developing tumor signatures, several successful attempts have been made at developing both bottom up (hypothesis driven) and top down (outcome driven) approaches with respect to tumor prognostication. Earlier last decade, Perou *et al*. characterized a heterogeneous cohort of tumors to devise a set of 496 intrinsic genes that successfully classified tumor types into one of four histopathological subtypes (Sorlie *et al.*, 2001). This classification scheme divides tumors into basal-like[20], luminal A, luminal B and HER2 positive categories (Figure 5). These distinctions are still used today and in many respects have aided the targeting of specific therapeutics to cancer patients (Sotiriou & Pusztai, 2009). Using supervised top down approaches two European groups defined gene expression sets with strong prognostic ability (van 't Veer *et al.*, 2002; Wang *et al.*, 2005). Both the 70-gene[21] (van 't Veer *et al.*, 2002) and 76-gene (Wang *et al.*, 2005) tumor signatures discriminated between ER positive and negative tumors in retrospective external validation studies and have been shown to accurately predict distal metastases better than immunohistochemical (IHC) means (Foekens *et al.*, 2006; van de Vijver *et al.*, 2002; Buyse *et al.*, 2006). Since inter-observational discrepancy is often an issue with pathological assessment of tumor grade, in a more recent study, investigators defined a 97-gene Genomic Grade Index (GGI) in order to discriminate between intermediate grade tumors (Sotiriou *et al.*, 2006). Using a bottom up approach using well-defined grade tumor samples, Sotiriou *et al*. were able to

[20] Basal-like breast carcinoma (BLBC) frequently overlaps with so-called triple negative tumors, tumors that are negative for HER2, estrogen receptor and progesterone receptor.

[21] The 70-gene signature has been commercialized into a prognostic microarray test marketed as Mammaprint by Agendia (Sotiriou & Piccart, 2007)

define a gene set that consistently discriminated between low and high grade tumors irrespective of receptor or HER2 status (Sotiriou *et al.*, 2006). Interestingly, the 70-gene, 76-gene and GGI signatures all have similar ability to predict disease-free survival, time to distant metastasis and overall survival (Wirapati *et al.*, 2008). Despite little overlap in genes between the three signatures it was shown that the 70-gene, 76-gene, GGI and other tested signatures shared genes predominantly involved in proliferation and differentiation (Haibe-Kains *et al.*, 2008a).

Other hypothesis-driven approaches like the 21 gene recurrence score[22] and the HOXB13/IL17R expression ratio have been used to predict recurrence in patients with ER-positive, node negative cancer treated with tamoxifen (Paik *et al.*, 2004; Ma *et al.*, 2004) indicating that less complicated gene signatures still offer prognostic power. Indeed, several studies have been published showing that as few as three, two or even one gene (AURKA or Aurora A kinase) are significant predicators of poor prognosis leading one to increased skepticism regarding the validity of gene-signature based prognostication (Haibe-Kains *et al.*, 2012; Hallett & Hassell, 2011; Haibe-Kains *et al.*, 2008b). Indeed, no large-scale prospective study evaluating the prognostic ability of gene signatures has been conducted, though presently, two are underway (Cardoso *et al.*, 2008; Sparano & Paik, 2008).

[22] The 21-gene recurrence score has been commercialized into a prognostic test marketed as OncotypeDX by Genomic Health Inc. (Sotiriou & Piccart, 2007).

4.3 Caveats in gene signature development and microarray studies

One of the intrinsic limitations of microarray-based gene expression studies is that one requires some *a priori* knowledge of gene structure to define a complete set of transcripts. As seen above, with respect to C/EBPβ, translational or transcriptional variants can have diverse effects on biology. Indeed, RNA-seq and other third generation sequencing technologies are being employed not only to profile whole RNA, including micro RNA (Rothe *et al.*, 2011), but are also being used to map single-nucleotide polymorphisms, gene copy number variations, or even epigenetic changes due to methylation (Wong *et al.*, 2011; Tran *et al.*, 2012).

Nevertheless, array technology has matured, is nearly ubiquitous and requires some discussion. Regardless of array design, whether cDNA or oligonucleotide, the methods used for array normalization and feature intensity estimation all play a role in interpretation of RNA expression (Millenaar *et al.*, 2006; Choe *et al.*, 2005). Also, batch effects can make comparing array experiments between runs difficult if not corrected (Johnson *et al.*, 2007). Recently a number of studies have been published as part of the Microarray Quality Control Project (MAQC-II). In one of them, a systematized study of 230 breast cancers, Popovici *et al*. tested the performance of 120 classification models and found that the success of gene expression signatures for prognostication depended largely on the size the training data sets and the phenotypic classification difficulty (Popovici *et al.*, 2010). In a large study involving 36 independent analysis teams analyzing six data sets for 13 outcomes generated over 30,000 gene set classification models. Model performance varied mostly as a function of the endpoint, meaning that

classification of certain biological phenotypes (such as breast cancer, multiple myeloma) is less tractable by array than others (liver toxicity, neuroblastoma) (Shi *et al.*, 2010a). Nevertheless, MAQC-II also showed that gene signatures could be used to predict prognostic outcome with 80-90% consistency across microarray platforms if used consistently (Fan *et al.*, 2010). Furthermore, non-overlapping gene signatures sharing similar prognostic power were enriched in similar pathways rather than specific genes (Shi *et al.*, 2010b), findings previously reporter by other groups (Fan *et al.*, 2006; Yu *et al.*, 2007). However the use of traditional IHC approaches still maintains validity with a great number of physicians and researchers. A recent metastudy of over 10,000 tumors demonstrated the continued efficacy of using IHC for prognostic and therapeutic purposes (Blows *et al.*, 2010) while a gene signature meta-analysis acknowledged that both IHC and other pathological indicators (e.g. node status) had prognostic value independent of gene signatures (Wirapati *et al.*, 2008) illustrating the importance of both molecular and classical approaches to cancer evaluation.

Irrespective of technical difficulties another caveat when considering microarray studies is the variability in the quality and reporting of microarray data. In an investigation attempting to replicate 18 microarray studies published in Nature Genetics in 2005-2006, Ioannidis *et al*. could not repeat ten due to unavailability of data or incomplete methodological descriptions (Ioannidis *et al.*, 2009). The remaining eight studies were replicated only partially (Ioannidis *et al.*, 2009). A similar study analyzing 42 microarray-based profiling studies linked to clinical outcome showed that half suffered from at least one serious methodological flaw (Dupuy & Simon, 2007). In a recently

publicized case, a series of papers analyzing gene signatures predicting therapeutic sensitivity to drugs was retracted due statistical errors (Bonnefoi *et al.*, 2011; Garman *et al.*, 2011; Potti *et al.*, 2011a; Potti *et al.*, 2011b; Acharya *et al.*, 2012). These studies came under scrutiny when portions of the work could not be reproduced by independent biostatisticians (Reich, 2011).

Despite these important considerations, it should be noted that numerous journals have adopted the Minimum Information About a Microarray Experiment (MIAME) guidelines (Brazma *et al.*, 2001; Brazma, 2009) and consortia such as MAQC-II continue to advocate for the standardization of microarray data and experiments. Recent proposed guidelines for Minimum Information about a high-throughput Sequencing Experiment (MINSEQE) should further ensure reproducibility of other high-throughput data (Brazma, 2009).

5. Rationale and organization of thesis

A role for c-Src in the initiation or progression of human cancer has been documented in several studies. In particular, elevated Src kinase activity has been observed in several tumor types (Irby & Yeatman, 2000; Biscardi *et al.*, 2000). An early and defining observation of v-Src transformation is the capacity of activated Src to modify the pattern of gene expression specifically through the regulation of transcription factors such as AP-1 (Lloyd *et al.*, 1991; Suzuki *et al.*, 1994). The primary objective of this thesis was to define a set of v-Src regulated genes and processes in primary cells and to discover and characterize novel Src-regulated genes. By using recently available *Gallus gallus*

GeneChip microarray technology, we were able to undertake a systematic investigation of v-Src transformation using the prototypical primary cell systems that initially characterized Src, namely chicken embryo fibroblasts and neuroretinal cells. Furthermore, by investigating the changes of gene expression of Src-transformed CEF in response to AP-1 repression, we sought to identify genes regulated by AP-1 in conditions of Src-transformation. Characterization of one of these genes, *DAPK1*, has provided insight into a gene previously unknown to be regulated in the context of Src-transformation.

This thesis contains one published manuscript (Chapter 2) and two unpublished manuscripts (Chapters 3 and Appendix 2). All materials and methods pertaining to the specific manuscripts are contained therein. For ease of reading, references for all sections are provided in a single general references section found at the end of the thesis following the Appendices.

Chapter 2: This is a published manuscript of the work detailing the characterization of gene expression changes in CEF and CNR in response to v-Src transformation. A clustering enrichment regime was further used to define a set of 42-genes that could be used to predict poor prognostic outcome in breast and lung tumors.

Chapter 3: These are studies describing the discovery and characterization of the proapoptotic gene, *DAPK1*, previously unknown to be regulated by AP-1 in Src-transformed CEF. Analyses of the *DAPK1* promoter in the presence and absence of AP-1 repression and investigations using a C/EBPβ dominant negative allele indicate that DAPK1 expression is dependent on C/EBPβ activity and antagonized by AP-1 in Src-transformed CEF.

Chapter 4: This section contains the general discussion, future directions and perspectives.

Appendix 1: This section describes the undertaking of *in silico* discovery of novel Src-responsive units and was done as part of the focus of this thesis.

Appendix 2: This is a manuscript that describes the induction of the p20K growth arrest specific gene by CHOP repression in response to hypoxia and contact inhibition. My contribution to this work was the microarray analysis and gene expression validation that showed that the contact inhibition response in CEF resembles the hypoxia response in other systems.

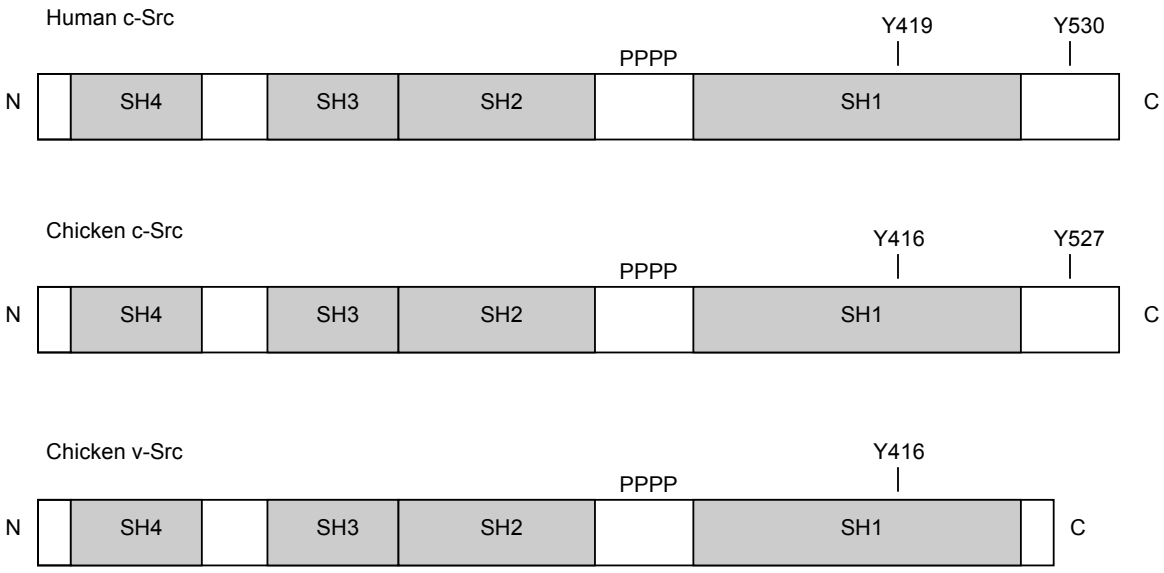


Figure 1. Domain structure of Src

The modular domain structure of Src is described in the text. Deletion of the C-terminal regulatory tyrosine in chicken v-Src leads to constitutive activation of the kinase. The SR-A RSV also contains several point mutations in the SH3 domain that are thought to interfere with the formation of the closed conformation of Src via the SH3-linker interaction (not shown). PPPP represents the linker region containing the pseudo-proline helix motif. This figure was modified from Yeatman, 2004 (Yeatman, 2004).

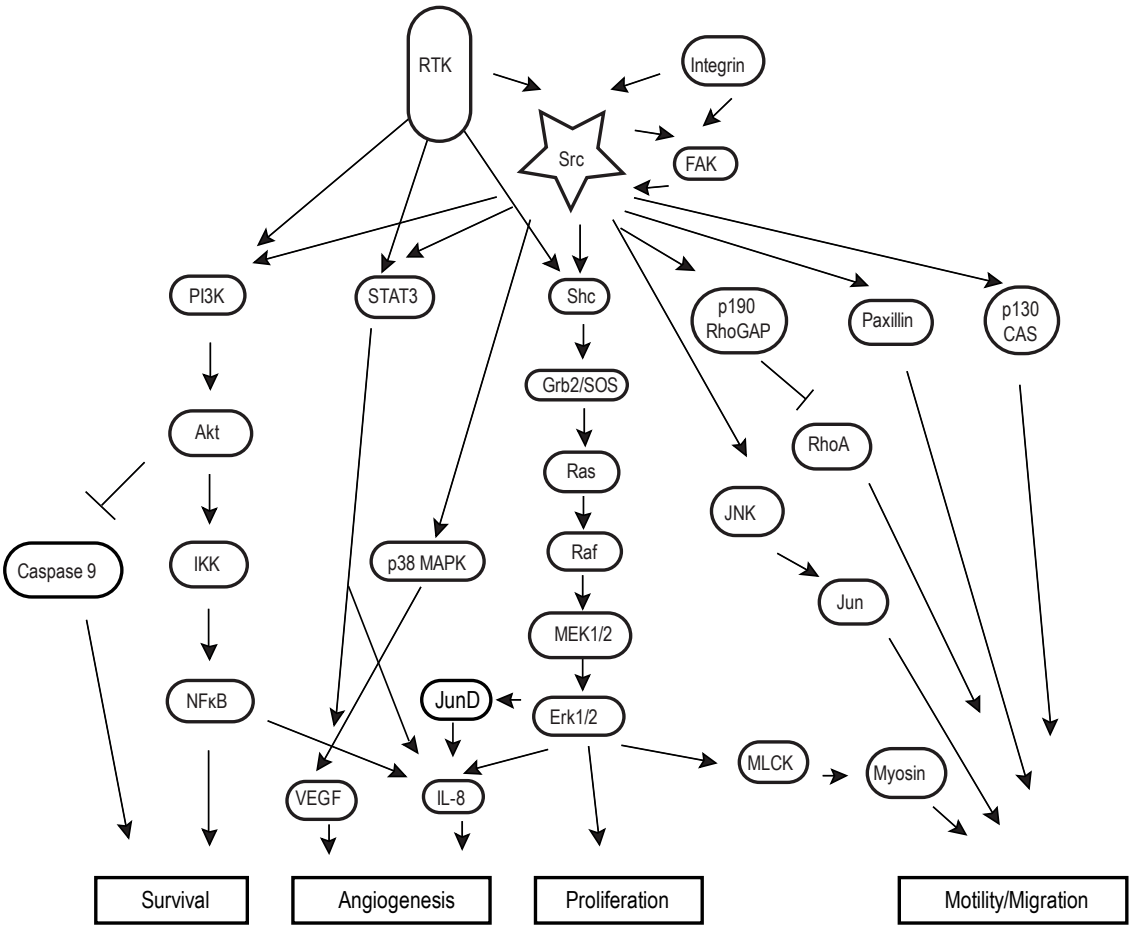


Figure 2. Src signals through numerous pathways regulating various biological processes that facilitate aggressive tumor behavior.

A schematized representation of a number of pathways and processes regulated by Src is shown. Arrows indicate activation and stems indicate repression. Figure was modified from Wheeler *et al*., 2009 (Wheeler *et al.*, 2009).

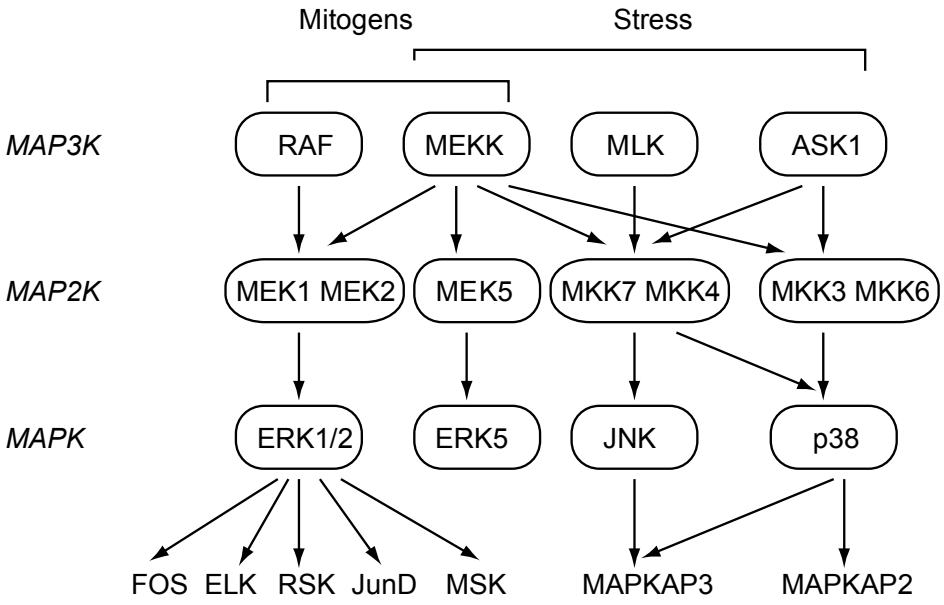


Figure 3. MAPK hierarchy

The figure describes a schematized and simplified depiction of MAPK signaling in vertebrates. MAP3K and MAP2K indicate MAP kinases kinase kinase and MAP kinase kinase respectively. Figure was modified from Yang *et al.*, 2003 (Yang *et al.*, 2003b).

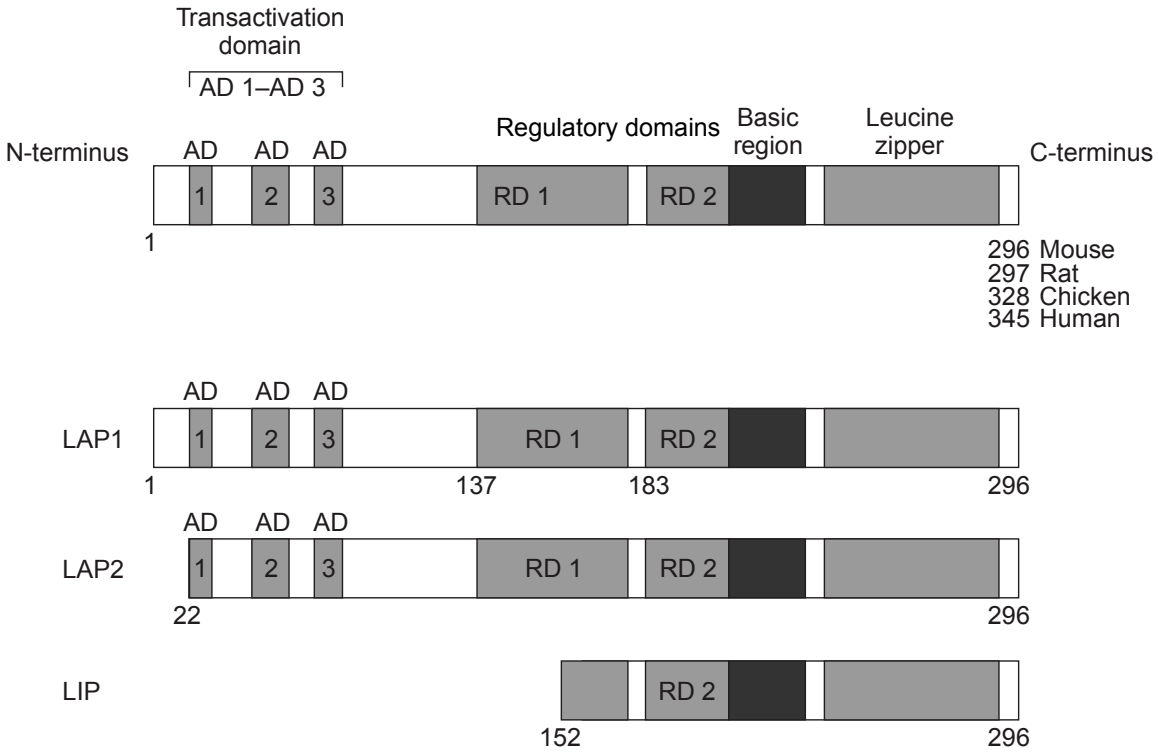
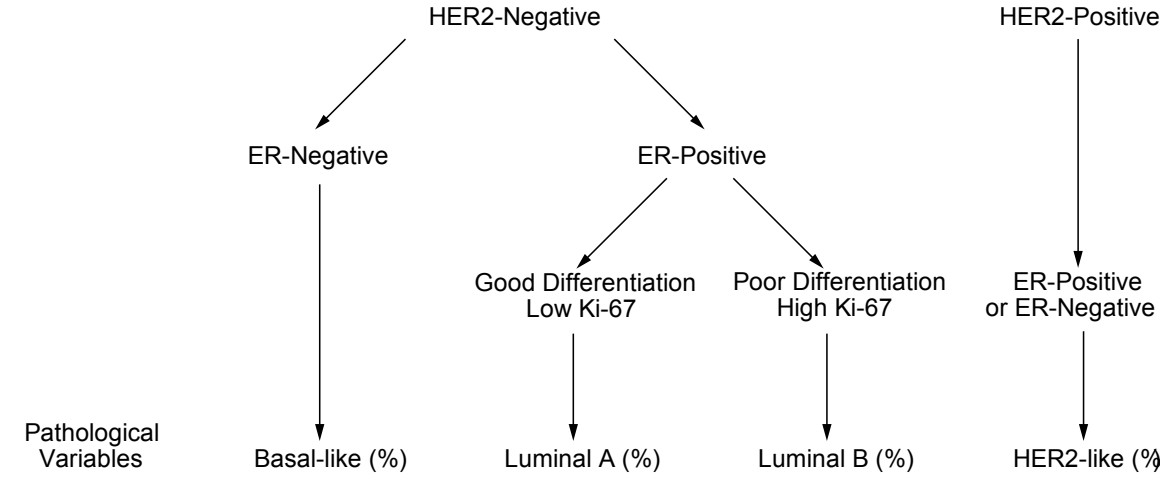


Figure 4. The domain structure of C/EBPβ and its isoforms.

Full length C/EBPβ consists of the transactivation domain (AD), the regulatory domains (RD1 and RD2) and the bZIP domain consisting of the basic domain and leucine zipper. C/EBPβ isoforms are generated by a dynamic process involving ribosomal scanning of an upstream out-of-frame ORF (see text). Figure was modified from Zahnow, 2009 (Zahnow, 2009).



Pathological Variables	Basal-like (%)	Luminal A (%)	Luminal B (%)	HER2-like (%)
HER2-positive (IHC)	10	12	20	100
ER-positive (IHC)	12	96	97	46
Grade III or greater	84	19	53	74
Tumor size >2 cm	75	53	69	74
Node-positive	40	52	65	66

Figure 5. Immunohistological classification of breast tumors

Immunohistological classification of breast carcinomas relies on HER2 and hormone receptor status. Ki-67 indicates nuclear antigen Ki-67, a marker for differentiation used in breast tumor classification. Pathology data were compiled by Sotiriou & Pusztai., 2009 (Sotiriou & Pusztai, 2009).

Chapter 2: Cellular processes of v-Src transformation revealed by gene profiling of primary cells

Context and contribution

It has long been understood that transformation by the v-Src oncogene leads to profound changes not only in cell signaling but also in gene expression (see Introduction). Over the last decade and a half, high throughput studies of oncogene-induced gene expression have focused largely on immortalized *in vitro* culture models or tumor material. Few studies have looked at primary cells with a few notable exceptions (see Introduction, section 4.1). The availability of the chicken genome sequence and the manufacture of the Affymetrix *Gallus gallus* GeneChip provided an opportunity to investigate v-Src-dependent changes in gene expression using the prototypical RSV system, chicken primary cells. In addition to using embryonic fibroblasts we solicited expertise on neuroretinal cells from Dr. Germain Gillet at the Institut de Biologie et Chimie des Protéines in Lyon, France. Together this provided the opportunity to study changes in transformation-dependent gene expression in two different primary cells derived from different germ layers. By comparing gene expression changes of a constitutively active but non-transforming allele of *Src* (NY315) with that of a fully transforming allele (SRA), we were able to identify a transformation-specific gene signature in CEF. Using thermolabile Src mutants (NY72-4) we compared the gene expression changes of CEF and CNR and subsequently identified a 42-gene signature correlated with poor prognosis in human cancers.

This article was published in BMC Cancer on February 12th, 2010. The following manuscript appears as it did for final submission with the exception of minor formatting changes and the refernces which are appended to the end of this thesis. All microarray data analyses, statistics, northern blot probe design and cloning and immunofluorescence was undertaken by the thesis author. Figure 3E, Figure 4 and Additional File 13 were contributed by Lizhen Wang, the author, and Natalie A. Rodrigues respectively. All northern blotting was conducted by Ying Wu. Benjamin D. Néel derived the CNR cells and contributed to experimental design. Germain Gillet and André Bédard coordinated the study. The manuscript was written and edited by André Bédard and the thesis author. All figures were created by the author. André Bédard conceived of the study.

Cellular processes of v-Src transformation revealed by gene profiling of primary cells – Implications for human cancer

Bart M. Maślikowski[1], Benjamin D. Néel[2], Ying Wu[1], Lizhen Wang[1], Natalie A. Rodrigues[3] Germain Gillet[2] and Pierre-André Bédard[1]*

[1]Department of Biology, McMaster University, 1280 Main street West, Hamilton, ON, Canada L8S 4K1,

[2]Institut de Biologie et Chimie des Protéines, Lyon, France,

[3]Sunnybrook Health Sciences Centre, 2075 Bayview Avenue, Toronto, ON, Canada M4N 3M5

*Corresponding author

Email addresses:

BMM: maslikbm@mcmaster.ca

BDN: b.neel@ibcp.fr

YW: wuying@mcmaster.ca

LW: wangl24@mcmaster.ca

NAR: gnatpat@yahoo.ca

GG: g.gillet@ibcp.fr

PAB: abedard@mcmaster.ca

Abstract

Background

Cell transformation by the Src tyrosine kinase is characterized by extensive changes in gene expression. In this study, we took advantage of several strains of the Rous sarcoma virus (RSV) to characterize the patterns of v-Src-dependent gene expression in two different primary cell types, namely chicken embryo fibroblasts (CEF) and chicken neuroretinal (CNR) cells. We identified a common set of v-Src regulated genes and assessed if their expression is associated with disease-free survival using several independent human tumor data sets.

Methods

CEF and CNR cells were infected with transforming, non-transforming, and temperature sensitive mutants of RSV to identify the patterns of gene expression in response to v-Src-transformation. Microarray analysis was used to measure changes in gene expression and to define a common set of v-Src regulated genes (CSR genes) in CEF and CNR cells. A clustering enrichment regime using the CSR genes and two independent breast tumor data-sets was used to identify a 42-gene aggressive tumor gene signature. The aggressive gene signature was tested for its prognostic value by conducting survival analyses on six additional tumor data sets.

Results

The analysis of CEF and CNR cells revealed that cell transformation by v-Src alters the expression of 6% of the protein coding genes of the genome. A common set of 175 v-Src regulated genes (CSR genes) was regulated in both CEF and CNR cells. Within the CSR

gene set, a group of 42 v-Src inducible genes was associated with reduced disease- and metastasis-free survival in several independent patient cohorts with breast or lung cancer. Gene classes represented within this group include DNA replication, cell cycle, the DNA damage and stress responses, and blood vessel morphogenesis.

Conclusion

By studying the v-Src-dependent changes in gene expression in two types of primary cells, we identified a set of 42 inducible genes associated with poor prognosis in breast and lung cancer. The identification of these genes provides a set of biomarkers of aggressive tumor behavior and a framework for the study of cancer cells characterized by elevated Src kinase activity.

Background

The v-Src kinase, the product of the Rous sarcoma virus (RSV), has provided a paradigm for the study of signaling pathways and mechanisms of cell transformation by receptor and non-receptor type tyrosine kinases. Its cellular counterpart, c-Src, is a member of a small family of kinases sharing a similar domain organization, overall structure and regulatory mechanism. Members of the Src family of kinases (SFK) contribute to several aspects of the activity of receptor tyrosine kinases including receptor turn-over, reorganization of the cytoskeleton and the initiation of DNA synthesis (Thomas & Brugge, 1997).

A role for c-Src in the initiation or progression of human cancer has been documented in several studies. Elevated Src kinase activity has been observed in several human cancers and in particular in breast, ovary, lung, bladder, stomach and colon

carcinomas (Irby & Yeatman, 2000). The majority of breast tumors samples (>70%) show elevated Src kinase activity that reflects increased protein levels (Biscardi *et al.*, 2000). While c-Src over-expression is not sufficient to induce cell transformation, c-Src likely cooperates with other tyrosine kinases, such as the EGF receptor, frequently over-expressed in the same tumors. An activating mutation resulting in the deletion of the c-Src C-terminal region adjacent to the negative regulatory tyrosine (Y530) has also been identified in a subset of patients with advanced colon carcinomas (Irby *et al.*, 1999b). This mutation mimics the oncogenic activation of v-Src, whose C-terminus lacks the C-terminal Src kinase (Csk) phosphorylation site.

Signaling pathways controlling cell proliferation or survival, in particular the Ras and PI3K pathways, have been the subject of intense investigation in v-Src transformed cells (Sugimoto & Erikson, 1985; Aftab *et al.*, 1997; Webb *et al.*, 2000). More recently, elevated Src kinase activity has been linked to several aspects of tumorigenesis including modification of the tumor micro-environment, vasculogenesis, metastasis and the acquisition of chemoresistance (Shah & Gallick, 2007; Zhang *et al.*, 2009). The mechanisms by which Src controls these properties of tumor cells remain largely unknown. One of the earliest and defining observations of v-Src transformation is the capacity of this oncoprotein to modify the pattern of gene expression. This was revealed by the cloning and characterization of genes aberrantly expressed in v-Src transformed cells, including genes encoding metalloproteinases and chemokines, and the trans-acting factors regulating their expression (Sugano *et al.*, 1987; Simmons *et al.*, 1989; Matrisian *et al.*, 1985; Gillet *et al.*, 1995; Bedard *et al.*, 1987a). Investigations based on gene

disruption or the use of dominant-negative mutants established the importance of transcription factors such as AP-1, members of the STAT and Ets families, and c-Myc in the proliferation and behavior of v-Src transformed cells (Wasylyk *et al.*, 1994; Turkson *et al.*, 1998; Suzuki *et al.*, 1994; Lloyd *et al.*, 1991; Johnson *et al.*, 1996; Granger-Schnarr *et al.*, 1992; Bruder *et al.*, 1992; Bromberg *et al.*, 1998).

Gene profiling studies of transformed cells or tumors characterized by elevated Src kinase activity have documented the changes in gene expression associated with this oncoprotein (Masker *et al.*, 2007; Malek *et al.*, 2002; Paz *et al.*, 2004; Liu *et al.*, 2006). However, these studies did not assess the transformation-dependence of gene expression (Masker *et al.*, 2007). Often they were performed on heterogeneous tumor specimens that limit the sensitivity of the analysis or in immortalized cell lines that may not reveal the full range of Src-dependent changes in gene expression. In this study, we employ a different strategy by comparing the expression profiles of two different primary cell types infected by the Rous sarcoma virus, namely chicken embryo fibroblast (CEF) and chicken neuroretinal cells (CNR). By using temperature sensitive or transformation deficient mutants of v-Src, we define a common set of genes regulated by v-Src. The expression of a group of 42 v-Src inducible genes of the common set was associated with reduced disease-free survival in independent cohorts of patients with breast carcinomas. High expression of this gene set was also observed in primary tumors of patients with reduced bone or lung metastasis-free survival, suggesting that the common program of v-Src transformed cells is enriched for genes associated with a more aggressive tumor phenotype. The definition of this v-Src gene signature provides a set of biomarkers for the

identification of tumors with an aggressive phenotype and the framework for the study of cellular properties conferred by high Src kinase activity in human tumors.

Methods

Cell culture and RSV strains

Chicken embryo fibroblasts (CEF) were isolated from day 10 embryos while chicken neuroretinal cells (CNR) were isolated from the retina of 7 day old embryos, as described before (Crisanti *et al.*, 1995). CNR cells do not proliferate in the absence of serum or a transforming v-Src kinase and therefore were infected with the *wt* Schmidt Ruppin-A strain (SR-A) of RSV or with the temperature-sensitive mutant *ts* NY72-4 RSV. CEF were infected with the RSV strains *wt* SR-A, *ts* NY72-4 and *td* NY315 or with RCASBP-A, a derivative of avian sarcoma viruses lacking a viral Src gene. All viruses belong to the A sub-group of ASV. Characterization of the ts NY72-4 and td NY315 RSV mutants were described before (Garber *et al.*, 1987; Cross *et al.*, 1984). CNR and CEF were cultured in high glucose Richter's modified MEM medium (HyClone #SH30601) supplemented with 5% cosmic calf serum (HyClone #SH30087.03), 5% tryptose phosphate broth, glutamine, penicillin and streptomycin. All studies were performed with actively dividing cells cultured in medium replenished the day before sample preparation to avoid starvation and acidosis of the transformed cells. Populations of *ts* NY72-4 RSV-infected CNR cells were expanded at the permissive temperature of 37.5˚C while CEF were cultured at the non-permissive temperature of 41.5˚C until transferred to the permissive temperature of 37.5˚C to induce transformation. For PI3K inhibition, CEF infected with RSV (RCASBP(A), NY315 or SRA) were incubated for 8 hours in either

100 nM wortmannin (Biomol Research Laboratories, Pennsylvania) or in 15 μM LY290042 (Biomol Research Laboratories, Pennsylvania) in complete medium. Animal use was approved by the McMaster University Animal Research Ethics Board (AUP#05-06-26) and conducted in accordance with the guidelines of the Canadian Council on Animal Care.

RNA isolation and northern blotting analysis

Total cell RNA was isolated using Trizol reagent (Invitrogen). Ten μg of total RNA was loaded per well and separated on a 1.2% agarose gel containing 3.75% formaldehyde. RNA was transferred to nylon membrane (Schleicher & Schuell) by capillary transfer, cross-linked, and probed with randomly primed radiolabeled DNA fragments as described before (Gagliardi *et al.*, 2001). Signals detected on northern blots were quantified directly by phosphor-imaging using a Storm 820 phosphorimager (Molecular Dynamics) or by scanning autoradiographs on a Umax Astra 1220U scanner. Images were quantified using ImageJ (Abramoff *et al.*, 2004) and adjusted for loading using GAPDH.

Gene profiling analyses

RNA samples were isolated using Trizol (Invitrogen). RNA quality was assessed by gel electrophoresis and examined with an Agilent 2100 Bioanalyzer. All RNA samples were first analyzed by northern blotting analysis and probed for IL8 and GAPDH expression. RNA samples with a RNA Integrity of less than 9.7 were discarded. Microarray experiments were conducted at the Toronto Centre for Applied Genomics (TCAG) at the Hospital for Sick Children (Toronto, Canada). Biotinylated cRNAs were generated at TCAG and hybridized to Affymetrix Chicken GeneChip arrays using standard Affymetrix

protocols (EukGE-WS2v4). GeneChips were scanned using the Affymetrix GeneChip Scanner 3000. Feature intensity was quantified using Command Console software and exported to CEL format.

CEL files were analysed using dChip software version 2007 (Li, 2003a). Array data were normalized against the median intensity array for each experiment using the invariant set normalization method (Li & Hung Wong, 2001). Median array intensities for the three experiments were scaled to the same value prior to normalization in order to provide comparable probe intensities for inter-experimental comparison. Expression levels were determined by the model-based expression index method using perfect-match probes only (Li & Wong, 2001; Li & Hung Wong, 2001). Two-fold changes in gene expression between experimental conditions within each experiment were determined using log-transformed expression values and statistical significance of expression was determined using one-way ANOVA (RCASBP/NY315/SR-A comparison using RCASBP(A) and NY315 as control groups) or an unpaired two-tailed t-test (NY72-4 CEF and CNR comparisons). Probe-sets whose p-values were greater than 0.05 (pairwise comparisons) or 0.01 (ANOVA) were excluded from further analysis. False discovery rates in all comparisons were estimated to be less than 3% by permutation (10,000 permutations;(Tusher *et al.*, 2001)). For clustering, redundant genes were removed by identifying probe-sets whose Entrez Gene and/or reference sequence IDs were identical. Probe-sets with the lowest p-values were retained. Gene clustering was performed using unsupervised hierarchal clustering by average Euclidean distance (Eisen *et al.*, 1998). Venn diagrams were drawn using Vennmaster v. 0.35 (Kestler *et al.*, 2005).

Experimental groups were divided into the following: CEF infected by RCASBP(A), NY315 RSV, or SR-A RSV (experimental group 1); CEF infected by ts NY72-4 RSV and grown either at the permissive or non-permissive temperature (experimental group 2); and CNR cells infected by ts NY72-4 RSV and grown either at the permissive or non-permissive temperature (experimental group 3). A minimum of three biological replicates per experimental condition was used. An additional three RCASBP(A)-infected CEF samples cultured at 37.5˚C were used to control for temperature effects (normalized within experimental group 1). Temperature-regulated genes (determined using the same criteria as for the experimental groups) were cross-referenced to all differentially expressed genes from the CEF ts NY72-4 and CNR tsNY72-4 experiments. Genes whose change in magnitude expression was greater than or equal to the same genes found to be differentially expressed in the SR-A/RCASBP(A) experiment were considered to be temperature-regulated and not attributable to v-Src-dependent transformation. This includes 13 genes from the CEF ts NY72-4 and 16 genes from the CNR ts NY72-4 experiments respectively (Additional File 1). Arrays in each experimental group were normalized within said experimental group to prevent skewing of data points from tissue-effects as determined by M-A plots for individual arrays. Array data are accessible through the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) using the accession number GSE14489.

Gene ontology and pathway analyses

Gene ontology (GO) analyses were conducted using the DAVID 2.0 bioinformatics resources (Dennis *et al.*, 2003; Hosack *et al.*, 2003) functional annotation tool using an

EASE score of 0.05 and a minimum of two genes per term. Pathway analyses were carried out using Pathway Express software available in the Onto-tools package (Draghici *et al.*, 2007). Probe-sets associated with significantly differentially expressed genes from each experimental group were converted to the orthologous probe sets from the Affymetrix human U133 Plus 2.0 array. Non-redundant orthologous probe-sets and associated fold change differences (linear-scale) were uploaded to Pathway Express for use in pathway analysis. Pathway analysis was conducted using default settings and only pathways whose corrected gamma-p-value was less than or equal to 0.05 were kept.

Tumor data analyses

Tumor expression data sets were obtained from the Gene Expression Omnibus at the National Center for Biotechnological Information. Breast tumor data from *Pawitan et al*. (Pawitan *et al.*, 2005) and *Ivshina et al*. (Ivshina *et al.*, 2006) were used as training data sets (Figure 5, panels Ai and Aii respectively). Breast tumor data from *Minn et al.* (Minn *et al.*, 2005), colon tumor data from the International Genomics Consortium Expression Project for Oncology (http://www.intgen.org/), and breast, lung and ovary tumor data from *Bild et al.* (Bild *et al.*, 2006) were used as test sets. All clinical data were obtained from the original publications. Orthologous probe-sets (Hu95av2, U133, U133 Plus 2.0 Affymetrix arrays) for the CSR gene set were obtained from NetAffyx (Affymetrix).

To ascertain if up-regulated genes in the CSR set could be used as markers of poor prognosis in human cancer, orthologous probe-sets corresponding to the up-regulated genes in the CSR set were obtained for the Affymetrix platforms used in the training data sets. Only the up-regulated orthologous CSR probe-sets were used for clustering of the

Pawitan and Ivshina training sets. Unsupervised gene and sample clustering (Pearson correlation distance metric (Eisen *et al.*, 1998)) was performed on the two training data in order to identify clusters of tumors with similar gene expression profiles. Each tumor cluster was subjected to survival analysis in order to identify tumor clusters associated with poor survival. Since the objective was to identify up-regulated CSR genes associated with poor prognosis, the genes whose expression was statistically significantly higher in the poor-prognosis tumor set versus the other three sets were chosen as candidates for the aggressive gene signature (t-test, single-tailed, $p<0.05$). The common set of 42 genes identified from the two training sets defined the aggressive gene signature.

For the test data, the sets of tumors expressing high levels of the aggressive signature were defined as the upper quartile of tumors from any given test set with the highest average gene-wise mean-centered expression of the aggressive signature set. Survival analyses were carried out using the Kaplan-Meier product limit estimator method (Kaplan, 1958) and statistical significance was evaluated using the log-rank test.

Immunofluorescence and western blotting

CEFs were grown to sub-confluence on glass slides, rinsed with PBS twice and fixed in 1% formaldehyde/PBS for ten minutes. Cells were washed three times for ten minutes in PBS and incubated with anti-CD44-FITC antibody (Abcam, cat. # ab24907) for 2 hours at room temperature and washed three times for ten minutes in PBS. Cells were visualized on a Nikon Eclipse TE2000-U inverted microscope. Western blotting was performed as described before (Papaconstantinou *et al.*, 2005) using the following antibodies: anti-GSK3-β and phospho-S9-GSK3-β (Cell Signaling #9332 and 9336S),

anti-heme oxygenase 1 (Stressgen,#SPA-896), anti-PKB (New England Biolabs #9272), anti-phospho-PKB (Cell Signaling #9271), anti-Erk1 (Santa Cruz Biotechnology #sc-94), and anti-Twist1 (Santa Cruz Biotechnology #sc-15393). Autoradiographs were scanned on a Umax Astra 1220U scanner, quantified using ImageJ and corrected for loading using Erk1 (Abramoff *et al.*, 2004).

Preparation and cloning of DNA fragments

RNA was isolated from 10^7 CEFs using 1ml of Trizol reagent (Invitrogen) and precipitated using one volume of ethanol in a final volume of 150mM NaCl. One μg of RNA was reverse transcribed (Invitrogen SuperScript RT) and treated with RNAse A (Invitrogen). Double-stranded DNA probes for northern blotting analyses were PCR amplified using Promega GoTaq and the appropriate forward/reverse primers (Table 1). DNA fragments were gel-purified and cloned into pCR2.1-TA (Invitrogen) vector via TA cloning. All cloned products were verified by sequencing at the MOBIX sequencing facility, McMaster University. CEF4/IL8 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes were generated as described previously (Gagliardi *et al.*, 2001).

Results

Identification of Transformation-Regulated genes in v-Src transformed CEF

To identify genes regulated in a transformation-dependent manner, we characterized the gene expression profiles of CEF transformed by the wt RSV strain Schmidt Ruppin – group A (SR-A) or infected with the transformation-deficient viruses NY 315 RSV or RCASBP(A). NY315 RSV is a group A virus encoding a catalytically active deletion mutant of the v-Src kinase lacking amino acids 2-14 of the SH4 domain. As a result of

this deletion, the v-Src kinase of NY315 RSV is not myristoylated, does not associate with the plasma membrane and is non-transforming (Cross *et al.*, 1984). RCASBP(A) is a group A replication competent virus of the avian sarcoma virus family lacking an oncogene (Petropoulos & Hughes, 1991). Total cellular RNA was isolated and analyzed on the Affymetrix chicken GeneChip, an array comprising 37,703 probe sets representing 19,881 UniGene clusters. A total of 3254 probe sets, corresponding to 2904 unique genes were differentially expressed by two fold or greater in cells infected by either NY315 or SR-A RSV (Additional File 2). Within regulated sequences, 2455, 1691 and 730 transcripts were differentially expressed in pair-wise comparisons between SR-A and RCASBP(A), NY315 and RCASBP(A), and SR-A and NY315 infected CEF, respectively.

Genes regulated in a transformation-dependent manner were identified by one-way ANOVA ($p \leq 0.01$) using RCASBP(A) and NY315 as control groups and defined by probe sets with differences in expression greater or equal to two-fold between any two conditions. Unsupervised hierarchal clustering shows that transformation responsive genes cluster to one of two groups, either up-regulated or down-regulated in SR-A RSV transformed CEF but not in the other two conditions (Figure 1). A total of 1095 transcripts corresponding to 418 up-regulated and 535 down-regulated genes were identified in this analysis, defining a set of genes regulated in a transformation-dependent fashion. Approximately 15% of these genes were differentially expressed by five fold or more. We refer to this set of genes as the **T**ransformation-**R**egulated genes (TR genes) of v-Src transformed CEF (Additional File 3).

Differentially expressed genes were also characterized in CEF infected with the temperature sensitive (*ts*) mutant NY72-4 RSV. A pair-wise comparison of CEF infected with NY72-4 revealed that 568 transcripts encoded by 477 unique genes (261 up- and 216 down-regulated) were differentially expressed at the permissive versus non-permissive temperatures (Figure 2 and Additional File 4). Cross-referencing of the gene lists provided by the SR-A/NY315/RCASBP(A) and NY72-4 RSV analyses revealed a set of 145 non-redundant genes (or 199 transcripts) corresponding to 84 down- and 61 up-regulated genes associated with cell transformation (Additional File 5).

To control for the effect of temperature change (cold shock), we compared the profile of RCASBP(A) infected CEF at the permissive temperature of 37.5˚C and non-permissive temperature of 41.5˚C (Additional File 1). Few genes were differentially expressed by a factor of three or more at the two different temperatures. However, some of the highly v-Src responsive genes (HMOX1, UPP1, Aquaporin 1, for instance) were also regulated by temperature change. Genes affected by temperature and *wt* v-Src, as determined by the analysis of control and SR-A RSV-transformed CEF at 41.5˚C, indicate a sub-class of genes that are both stress-responsive and v-Src-regulated.

Identification of v-Src regulated genes in chicken neuroretinal (CNR) cells

In vertebrates, the c-Src kinase is widely expressed and detected in most tissues. In addition, v-Src is capable of transforming a variety of cell types *in vitro* (Boettiger *et al.*, 1977; Tato *et al.*, 1983; Pacifici *et al.*, 1977; Menko & Boettiger, 1988; Crisanti-Combes *et al.*, 1982a; Yoshimura *et al.*, 1981; Kruger & Anderson, 1991). To compare the biological response of different cell types transformed by v-Src, we characterized the

patterns of gene expression of chicken neuroretinal (CNR) cells infected with the temperature-sensitive mutant *ts* NY72-4 RSV. Unlike CEF, neuroretinal cells do not proliferate *in vitro* unless transformed by an oncoprotein such as v-Src and cultured in serum-containing medium (Gillet *et al.*, 1993). Therefore, *ts* NY72-4 RSV infected CNR are quiescent at the non-permissive temperature of 41.5˚C but are actively dividing and transformed at the permissive temperature of 37.5˚C. Gene profiling analyses of CNR cells identified 1062 transcripts, consisting of 485 up- and 577 down-regulated RNA species, with a two-fold or greater difference in gene expression between the two temperatures (Additional File 6). A significant fraction of the v-Src regulated genes identified in *ts* NY72-4 RSV (31%) and SR-A/NY315/RCASBP(A) infected CEF (21%) overlapped with the corresponding set of genes in CNR cells (Figure 2). The total number of transcripts (probe sets) and genes differentially expressed in the three systems of v-Src transformation is provided in Table 2. Together, the total genes regulated in these three sets represent approximately 6% of the protein-coding genes in the chicken genome. When all three systems are compared, a group of 84 common genes are identified as v-Src regulated (Figure 2; Additional File 7).

A second group of 91 genes was regulated in ts NY72-4 RSV infected CEF and CNR cells but not in SR-A RSV transformed CEF (Additional File 8). Up-regulated genes in this list encode several important regulators of cell proliferation or behavior including ornithine decarboxylase (ODC), osteopontin, hyaluran synthase 2 (HAS2), cyclin A and cyclin E2. In contrast, the cyclin kinase inhibitor $p27^{Kip1}$ was down-regulated in NY72-4 RSV transformed CEF and CNR cells. Since CNR cells are

amplified and cultured at the permissive temperature for extensive periods of time, it is unlikely that the regulation of these genes represents a transient effect of ts v-Src activation. A more likely explanation for this discrepancy is that differences in the structure of the v-Src kinase encoded by NY72-4 and SR-A RSV account for this specificity in gene regulation. It is also likely that the list of genes defined above (Additional File 7) is an under-representation of the gene cohort controlled by v-Src in CEF and CNR cells. A more accurate description of this class of genes can be obtained by combining the genes described in Additional File 7 and the genes regulated uniquely in NY72-4 RSV transformed CEF and CNR cells (Additional File 8). This combined list would then consist of a total of 175 genes that we define as the **C**ommon set of v-**S**rc **R**egulated genes or **CSR** genes.

Validation and characterization of v-Src regulated genes

The expression of v-Src regulated genes was confirmed by northern blotting analyses. In this study, the expression of genes identified by gene profiling was compared to that of previously characterized markers of Src transformation including IL8/CEF-4, CD44 and Nov (Ladeda *et al.*, 2001; Jamal *et al.*, 1994; Scholz *et al.*, 1996; Sugano *et al.*, 1987; Bedard *et al.*, 1987a). For all up and down-regulated genes selected, little difference was observed between non-transformed CEF infected with the replication competent virus RCASBP(A) and CEF infected with the transformation-deficient virus NY315 RSV (Figure 3A). As reported before, the kinetics of IL8/CEF-4 RNA accumulation was biphasic and characterized by the rapid accumulation of IL8/CEF-4 (within 1 h) followed by a temporary reduction of RNA level between 4 and 8 hrs of temperature shift

(Gonneville *et al.*, 1991). Other RNA species (VIP, CD44, integrin-α–6) accumulated more slowly i.e. between 2 and 4hrs following *ts* v-Src activation and did not show the transient down-regulation observed for the IL8/CEF-4 mRNA. IL8/CEF-4 and CD44 were both induced in cells treated with cycloheximide indicating that the different kinetics of RNA accumulation do not necessarily reflect a different requirement for *de novo* protein synthesis (data not shown).

Cell heterogeneity was assessed by looking at the surface expression of CD44 by immunofluorescence (Additional File 9). A strong CD44 signal was observed in SR-A RSV transformed CEF but was absent in cells infected with RCASBP(A) and NY315 RSV. All cells expressing the ts mutant of v-Src (NY72-4 RSV) were also positive for CD44 expression at the permissive temperature. Low but detectable signals for CD44 expression were also observed in ts NY72-4 RSV-infected cells at the non-permissive temperature. This may be indicative of a certain degree of leakiness of the ts v-Src kinase, an observation accounting for the lower number of v-Src regulated genes identified in this system.

Characterization of the common set of v-Src regulated genes in CEF and CNR cells

Differences in the profiles of v-Src regulated genes in CEF and CNR cells reflect in part the proliferative and differentiation state of these cells since CNR cells do not proliferate when non-transformed and are partially committed to differentiation (Gillet *et al.*, 1993; Calothy *et al.*, 1980). However, a common program of gene expression was also evident in the profiles of v-Src transformed CEF and CNR cells. Genes such as IL8/CEF4, VIP, HMOX1, PLCPI and UPP1, were all activated in v-Src transformed CEF and CNR cells.

HMOX1 also provided an example of a gene with partial activation in CEF infected with NY315 RSV, both at the RNA and protein level (Figure 3C & E).

A comparative analysis of v-Src regulated mRNA species examined by northern blotting and microarray profiling was performed for all mRNA species included in Figure 3. This study revealed a strong correlation (Spearman ρ of 0.83, $p<0.0001$) between northern and microarray gene expression estimates. A regression coefficient of 0.91 indicates a nearly 1:1 correspondence between northern blotting and microarray gene expression values (Additional File 10, Additional File 11). Collectively the results of the immunofluorescence, northern, and western blotting analyses validated the expression of a sub-set of genes identified in the gene profiling studies and illustrated the existence of a common program of gene expression controlled by v-Src in different cell types.

Signaling pathways and biological processes regulated during v-Src transformation

Pathways potentially regulated by v-Src-induced changes in gene expression were identified by Pathway Express software (Draghici *et al.*, 2007). Pathway Express estimates whether genes differentially expressed in a given data set impact a given pathway by incorporating fold-change of gene expression, where those genes are in the hierarchy of a pathway and whether those genes are found to be statistically overrepresented in that pathway. Using this methodology, "ECM-receptor interaction", "focal adhesion" and "phosphatidylinositol signaling" were identified as pathways most significantly altered in all three data sets (corrected γ p-value ≤ 0.05; Table 3). TGFβ signaling was also identified in CEF but not CNR cells. Several pathways were also identified in a single data set and, in particular, in the large cohort of the Transformation-

Regulated gene set. Thus, "leukocyte transendothelial migration", "complement and coagulation cascades", "cell adhesion molecules", "MAPK" and "Toll-like receptor signaling" pathways were all identified in this data set (Additional File 12).

Pathway Express stresses the regulatory nature of a gene product. This is best illustrated by the genes encoding the pro-survival regulatory and catalytic sub-units of PI3K, which are part of the "focal adhesion", "PI3K" and "leukocyte transendothelial migration" pathways and are hallmarks of several cancer types (ex. "small cell lung cancer"). The up-regulation of both the catalytic and regulatory sub-units of PI3K in SR-A RSV transformed CEF implies that the pathway is strongly activated in these cells (Additional File 3). This was confirmed by looking at the phosphorylation status of PKB/Akt and GSK3β, two downstream targets of the PI3K pathway. High levels of phospho-PKB and phospho-GSK3β were detected in SR-A RSV transformed CEF but not in cells infected with RCASBP(A) or NY315 RSV, even when these cells were actively cycling (Additional File 13). The signal was reduced in cells treated with the specific inhibitor LY294002, indicating that phosphorylation of these proteins was dependent on the PI3K pathway.

Other important regulatory factors are included in the list of Transformation-Regulated genes. An example is provided by Twist1, a bHLH transcription factor promoting metastasis and capable of inducing the epithelial-to-mesenchymal transition (Additional Files 3 & 4;(Yang *et al.*, 2004)). The up-regulation of Twist1, observed as a 26 kDa doublet by western blotting, was confirmed in SR-A RSV transformed CEF (Figure 4).

The Common Set of v-Src Regulated (CSR) genes includes a subset of genes associated with reduced disease-free survival in human cancer

To test whether v-Src-induced changes in gene expression correlate with reduced disease- or metastasis-free survival in human cancers, we undertook a clustering-based enrichment regime to identify up-regulated genes of the CSR set associated with this phenotype. To this end, two independent breast carcinoma datasets with clinical information were selected and used as training sets. Mammary tumor sets were used because of the abundance of good quality array data obtained on more recent Affymetrix platforms and because a large proportion of breast carcinomas (>70%) exhibit high Src activity (Biscardi *et al.*, 2000). It was also reasoned that CSR genes were more likely to be regulated in tumors because their expression is controlled by v-Src in different cell types (i.e. CEF and CNR cells). The aggressive gene signature, defined by clustering analysis, was then tested against additional, independent tumor datasets to assess if it could be used to predict reduced disease-free survival.

Probe-sets corresponding to orthologous genes from the up-regulated CSR set and tumor samples from the two independent breast carcinoma datasets (Pawitan *et al.*, 2005; Ivshina *et al.*, 2006) were clustered to identify samples containing unique up-regulated gene clusters associated with poor disease-free survival. Survival analyses of these tumor datasets revealed tumor sample clusters associated with higher disease relapse (Figure 5A). Figure 5Ai shows that sample cluster 3 has poorest estimated mean disease-free survival (5.6 years) compared to the other three clusters (7.6, 7.8, and 6.8 years; clusters 1, 2 and 4 respectively; $P<0.001$). In Figure 5A, panel ii, cluster 4 has the poorest

estimated mean survival compared to clusters 1, 2 and 3 (7.8, 10.1, 10.7 and 8.4 years respectively; $P<0.01$). CSR genes that were up-regulated in the low-survival cluster in relation to the other tumor clusters of each tumor data set were cross-referenced to each other to generate an aggressive tumor gene signature (Table 4, see methods). This set contains 42 unique genes overlapping with an 80-87% concordance with respect to the genes found differentially up-regulated in the two tumor data training sets. This striking overlap represents over 50% of the 80 up-regulated CSR genes (Additional Files 7 & 8) indicating that the common program of v-Src gene expression is enriched for genes associated with an aggressive tumor phenotype. Functional classification of these genes showed greatest enrichment for gene ontology biological processes (GOBP) such as "Cell Cycle" ("M-phase", "Mitosis", "Cell Division"), "DNA Metabolic Process" ("Response to DNA damage stimulus"), "Response to Stress", "Cell Proliferation" and "DNA Replication". (Additional File 14)

To assess whether the aggressive tumor gene signature could be used to predict poor disease-free survival, six independent tumor datasets were interrogated to determine if high expression of the 42 genes defining this signature was associated with a poor outcome. By comparing the top 25% of tumors expressing the highest levels of the aggressive gene signature against the bottom 75%, four data sets out of six showed that patients with tumors characterized by high expression of the 42 gene cohort have reduced disease-free or metastasis-free survival (Figure 5B). Reduced relapse-free survival was seen in the breast and lung carcinoma datasets while reduced lung and bone metastasis-free survival was observed in two other breast tumor datasets.

Analysis of tumors expressing the highest levels of the aggressive signature genes in the breast tumor set from *Minn et al.,* (Minn *et al.*, 2005) indicated that the patients from whom the tumors were derived had reduced disease-free survival with respect to lung metastasis (mean estimated survival 5.4 years versus 10.1 years, $P<0.001$; Figure 5Bi). Similarly, using bone metastasis data from the same study, the high aggressive signature expressing tumors were associated with reduced metastasis-free survival in patients (mean estimated survival 6.4 years versus 9.3 years, $P<0.05$; Figure 5Bii). Analysis of a second breast tumor data set (Bild *et al.*, 2006) also shows that patients whose tumors express high levels of the aggressive signature genes have a reduced disease-free survival (mean estimated survival 71 months versus 106 months, $P<0.02$; Figure 5Biii). Lung tumor data from the same study indicates a similar trend where patients with tumors expressing high levels of the aggressive signature have reduced disease-free survival (mean estimated survival 29 months versus 54 months, $P<0.001$, Figure 5Biv). Analysis of ovarian tumor data from the same study however did not yield any statistically significant difference (data not shown). Since survival data was not available for the colon carcinoma dataset, we asked whether high expression of the aggressive tumor gene signature could identify patients with distal metastases. Analysis of the relative proportions of metastasis in patients exhibiting high expression of the aggressive signature to those that did not, failed to show any statistically significant difference (data not shown). Taken together, these data show that a subset of the v-Src-regulated CSR genes correlates with poor prognosis in patients with breast tumors, exhibiting some specificity for metastasis to lung and bone.

Discussion

Definition of a gene signature for v-Src transformation of primary cells

Previous gene profiling studies of v-Src transformed cells have relied principally on the use of immortalized rodent cell lines that may not reflect the full spectrum of biological processes altered during v-Src transformation (Paz *et al.*, 2004; Malek *et al.*, 2002). In this study, we used transformation-deficient (NY315 RSV) and temperature sensitive mutants of the Rous sarcoma virus (NY72-4 RSV) to identify gene products regulated in a transformation-dependent manner in primary cells. Further, all experiments were performed in conditions promoting asynchronous cell division and limiting acidosis. A separate study of RSV transformed CEF was reported previously (Masker *et al.*, 2007). However, most of the genes identified in our studies (80%) were not included in the gene set described by these investigators. Moreover, the results of this study differ from ours in some important aspects. First, Masker and co-workers did not assess the transformation-dependence of differentially expressed genes since their analysis was restricted to a pair-wise comparison of SR-A RSV transformed CEF and CEF infected with a replication competent virus devoid of any oncogene. Second, several established markers of v-Src transformation such as IL8/CEF-4 and CD44 (Sugano *et al.*, 1987; Bedard *et al.*, 1987a; Ladeda *et al.*, 2001), were poorly regulated in their experimental conditions and did not pass the significance criteria of the analysis. Third, Masker and co-workers identified some transcription factors such as c-Jun and c-Myc, as being up-regulated by v-Src while we did not. We previously established that early immediate genes such as c-Myc are activated by v-Src in quiescent cells but are not differentially

expressed when actively dividing cells are compared (Bedard *et al.*, 1987a). Therefore, some of these differences may reflect the experimental conditions of the studies and, in particular, the conditions of cell culture.

A study performed with ts NY72-4 v-Src identified a limited number of v-Src regulated genes in NIH 3T3 fibroblasts (Liu *et al.*, 2006). Key transcriptional regulators identified by these authors, such as Hmga2 (high mobility group AT-hook2) and Id4 (inhibitor of DNA binding 4), are also included in the list of v-Src-regulated genes in CNR cells (Additional File 6). In addition, Liu and co-workers described the regulation of important regulators of the cell cycle and angiogenesis by SSeCKS, a metastasis-suppressor gene down-regulated by v-Src (Liu *et al.*, 2006). Interestingly, tumor suppressor genes such as DKK3 and gamma FBPα, the avian homolog of HIC-1 (hypermethylated in cancer 1), were repressed markedly by v-Src in CEF. Thus, the abrupt down-regulation of tumor-suppressor genes may be required for v-Src-dependent cell transformation (Figure 3 and Additional File 3; (Liu *et al.*, 2006)).

The common set of v-Src regulated genes (CSR genes) is enriched for genes associated with an aggressive tumor phenotype

Since the c-Src kinase is expressed ubiquitously in most vertebrate tissues, we reasoned that genes regulated by v-Src in multiple cell types would better represent targets of this kinase in tumorigenesis. Using CEF and CNR cells, we thus described a common program of gene expression consisting of 175 genes regulated by v-Src in both cell types. This program, referred to as the **C**ommon set of v-**S**rc **R**egulated genes or **CSR** genes, was then used to interrogate several independent tumor data sets with corresponding

clinical information. Beginning with data from two independent cohorts of breast carcinomas as training sets, we performed hierarchal clustering analyses to identify a group of v-Src inducible genes highly expressed in tumors associated with reduced disease-free survival (Table 4). Significantly, the majority (80-87%) of the 42 genes included in this group were identified in both independent tumor data sets. Since the CSR gene cohort consists of a total of 80 up-regulated genes, the 42 genes identified in this analysis suggest that CSR genes are highly enriched for genes associated with a more aggressive tumor phenotype. To confirm this finding, we then interrogated several additional tumor data sets including some used previously by other investigators (Bild *et al.*, 2006; Minn *et al.*, 2005). This analysis confirmed the expression of the v-Src inducible gene set in primary tumors of patients with reduced disease-free or metastasis-free survival in four of six tumor data sets, including breast and lung carcinomas (Figure 5). In contrast, no correlation was observed for ovarian and colon carcinomas suggesting tissue specificity of the v-Src aggressive tumor gene signature. A recent report by Zhang and co-investigators shed some light on the role of Src in bone metastasis of breast cancer cells (Zhang *et al.*, 2009). Knockdown of c-Src in the highly metastatic human BoM-1833 cell line impaired the proliferation of these cells in the bone micro-environment but had no effect on lymph node metastasis in a mouse model. Moreover, these authors provided evidence that Src enhanced the survival of the metastatic cancer cell line by mediating the activation of PKB/Akt in response to CXCR4 activation (Zhang *et al.*, 2009).

Prognostic gene signatures for breast cancer have been described previously by several groups (Yu *et al.*, 2004) (Sotiriou *et al.*, 2006; Haibe-Kains *et al.*, 2008a; Wang *et al.*, 2005; van 't Veer *et al.*, 2002). A comparative analysis of the 70-gene, 76-gene and the Gene expression Grade Index (GGI) signatures revealed that they have similar prognostic ability despite limited overlap in gene identity (Haibe-Kains *et al.*, 2008a). Recently, *Yu et al.* showed that a common set of biological processes is enriched among these gene signatures even when defined by different genes. For instance, "Cell Cycle" is enriched in the 70-gene, 76-gene and the 97-gene GGI signatures even though cyclin E2 (CCNE2) is the only gene of this pathway common to all three signatures (Sotiriou *et al.*, 2006; van 't Veer *et al.*, 2002; Wang *et al.*, 2005; Yu *et al.*, 2007). Interestingly, CCNE2, the only gene found in more than two signatures (Yu *et al.*, 2007) was also identified in our aggressive gene signature along with EXO1 and KIF11, found in two other studies (Yu *et al.*, 2004; Sotiriou *et al.*, 2006)(Table 4). Functional classification of our 42-gene aggressive signature shows that three of the 16 enriched GOBP terms ("Cell Cycle", "Mitosis" and "DNA replication") are identical to three of the 36 core GOBP terms described by *Yu et al*. Twelve remaining terms from our aggressive signature map to parent terms of one or more of the 36 core terms while two, "blood vessel morphogenesis" and "cell proliferation", are unique to our gene set (Additional File 14). This similarity in enriched processes shared with other breast cancer signatures reinforces the hypothesis that there is a common aggressive tumor "pathway-signature" (Yu *et al.*, 2007).

Biological processes defined by the CSR gene signature – DNA damage and the stress response

Several genes of the DNA damage and stress responses are included in the 42 genes of the "aggressive tumor signature" DNA damage resulting from the production of reactive oxygen species (ROS) or DNA replication stress has been observed in cells transformed by several oncogenes (Vafa *et al.*, 2002; Lee *et al.*, 1999; Bartkova *et al.*, 2006; Di Micco *et al.*, 2006; Mallette *et al.*, 2007). However, evidence of DNA damage, as indicated by the levels of γH2AX, is presently lacking in v-Src transformed CEF (our unpublished results). Therefore, the pathways responsible for the production of ROS or replicative stress may be attenuated in these cells. Src-transformed cells are known to be more resistant to conditions of oxidative stress and the induction of genes such as HMOX1 or UPP1 enhance cell survival in conditions of hypoxia or glucose depletion i.e. in conditions promoting the production of ROS (Deichman *et al.*, 1996; Kocanova *et al.*, 2007; Zuckerbraun & Billiar, 2003; Choi *et al.*, 2006; Choi *et al.*, 2008). Therefore, genes of the aggressive tumor signature may limit the effects of oncogenic stress and provide a survival advantage to the cell.

Several genes identified in this study were also regulated in response to temperature change (cold shock). Since temperature change had a modest effect on the activation of these genes with induction levels of 3-fold or less (Additional File 1), and since all v-Src regulated genes affected by temperature change were also identified in the RCASBP/NY315/SR-A RSV analysis at 41.5˚C, it is unlikely that these genes represent a class of genes regulated uniquely as a result of temperature change. Genes like HMOX1,

Aquaporin 1 and the metallothioneins MT2A and 3 are induced in response to a wide variety of stress conditions and are widely regarded as general stress response genes. Since HMOX1, Aquaporin 1 and UPP1 are activated by temperature change and are included in the Common Set of v-Src Regulated (Additional Files 7 & 8), they may be part of a general and previously unrecognized stress-response program controlled by v-Src.

Biological processes affected by v-Src transformation: Motility and Invasiveness

The large cohort of Transformation-Regulated genes (TR genes; Additional File 3) includes several genes that may contribute to a more aggressive tumor phenotype. Src-transformed CEFs are highly motile and characterized by structures mediating cell migration called podosomes (Sohara *et al.*, 2001; Platek *et al.*, 2004). Like the related invadopodia described in other cancer cells, the podosomes are actin-rich structures closely associated with adhesion molecules and ECM-degrading enzymes such as the matrix metalloproteinases MMP-1, MMP-2 or MMP-9 (Gimona *et al.*, 2008). In CEF, v-Src transformation stimulated the expression of four critical regulators of actin polymerization and podosome formation, namely N-WASP, cortactin, gelsolin and the p41 subunit of the actin related protein complex 2/3 (Arp 2/3 subunit 1b-p41; Additional File 3 (Tehrani *et al.*, 2006; Kempiak *et al.*, 2005; Yamaguchi *et al.*, 2005; Webb *et al.*, 2006)). Two of these factors (Arp 2/3 subunit 1b-p41 and cortactin) were also activated in CNR cells indicating that v-Src controls the expression of these genes in diverse cell types (Additional File 6). The gene encoding the p41 subunit of the Arp2/3 complex is frequently amplified in pancreatic cancer and is a regulator of the motility and invasion of

pancreatic cell lines (Laurila *et al.*, 2009). In contrast, caldesmon and transgelin were repressed by v-Src in CEF. Transgelin is a multi-functional protein with roles independent of podosome formation. In particular, transgelin is a negative regulator of MMP-9 expression and a suspected tumor suppressor (Assinder *et al.*, 2009; Nair *et al.*, 2006). Independent studies have shown that caldesmon, an actin filament cross-linker, antagonizes the action of the Arp 2/3 complex and is a negative regulator of podosome formation and invasion in transformed cells (Morita *et al.*, 2007; Yoshio *et al.*, 2007; Eves *et al.*, 2006). Therefore, v-Src transformation is characterized by changes in gene expression promoting the dynamic remodeling of the actin cytoskeleton and the assembly of podosomes. It is also significant that wt v-Src induces the expression of the p110δ catalytic subunit, a PI3K isoform required for cell migration in macrophages and breast cancer cells (TR genes; Additional File 3; (Vanhaesebroeck *et al.*, 1999; Sawyer *et al.*, 2003)).

Biological processes affected by v-Src transformation: Epithelial-to-mesenchymal transition and drug resistance.

Several studies have linked the expression of v-Src and other Src family kinases to resistance of a variety of chemotherapeutic agents including cisplatin, geftinib, paclitaxel, oxaliplatin and tamoxifen (Duxbury *et al.*, 2004; Griffiths *et al.*, 2004; Chen *et al.*, 2005b; Hiscox *et al.*, 2006; Masumoto *et al.*, 1999). Since chemoresistance is often associated with increased cell motility and invasiveness, it has been suggested that v-Src controls these activities by inducing the epithelial-to-mesenchymal transition (EMT) in tumor cells (Shah & Gallick, 2007). In support of this notion, Sham and co-workers reported recently

that the up-regulation of the basic-helix-loop-helix factor Twist1 by NF-kB increases chemoresistance of PC3 prostate cancer cells treated with daunorubicin or cisplatin (Pham *et al.*, 2007). In a separate report, Cheng *et al*. identified Twist1 and its target Akt2/PKBβ as factors contributing to the metastatic potential of highly invasive breast carcinoma cell lines. In the same study, these investigators showed that Twist1 and Akt2/PKBβ determine the resistance of these cells to paclitaxel (Cheng *et al.*, 2007). Twist1 is a potent inducer of the EMT and a member of the Transformation-Regulated gene cohort identified in this study (Figure 4 and Additional File 3). Since forced expression of Twist1 is sufficient to induce the EMT in mammary epithelial cells, the regulation of this factor provides a mechanism by which v-Src may induce the EMT and enhance chemoresistance (Yang *et al.*, 2004). Twist1 is not the only marker of the EMT identified in this study since N-cadherin mRNA expression was also up-regulated in SR-A transformed CEF (TR genes; Additional File 3).

Conclusion

Elevated Src kinase activity has been described in several unrelated human tumors and in cells derived from these tumors (Irby & Yeatman, 2000). In human cancers, high Src activity correlates with progression to a more malignant phenotype and the increased metastatic potential of tumor cells (Irby *et al.*, 1999b; Talamonti *et al.*, 1993; Termuhlen *et al.*, 1993). In this study, we define a signature of 42 v-Src inducible genes whose expression is associated with reduced disease-free survival in breast and lung cancer patients from several independent studies. In one dataset, this 42 gene signature was also associated with reduced bone and lung metastasis-free survival. Several genes of the

larger cohort of Transformation-Regulated Genes (TR genes) have also been associated with features of aggressive tumors such as invasiveness and chemoresistance. These genes provide a set of biomarkers and candidate therapeutic targets for the treatment of patients with tumors characterized by more aggressive behavior. The functional characterization of these genes represents a roadmap for the study of tumor cells characterized by elevated Src kinase activity.

Competing Interests

The authors declare that they have no competing interests.

Authors' contributions

BMM performed microarray and data analyses, statistics, CEF cell culture, immunofluorescence, immuno-blotting, northern blot probe design and cloning, and contributed to experimental design. BDN derived and cultured the CNR cells and participated in experimental design. YW cultured CEF and performed northern blotting analyses. LW and NAR participated in CEF cell culture, treatment and western blotting analysis. GG participated in experimental design and coordination. PAB conceived and coordinated the study, derived the CEF, and contributed to experimental design. BMM and PAB wrote the manuscript. All authors read and approved the manuscript.

Acknowledgments

BDN is the recipient of scholarships from the Ministère de la Recherche, the Association pour la Recherche sur le Cancer (France) and the European Council of doctorate candidates and young researchers (Eurodoc). This work was made possible by grants from the Association pour la Recherche, the Ligue Nationale contre le Cancer and the Région Rhône Alpes (France) to GG, and the Canadian Institutes of Health Research (MOP#10272) to PAB.

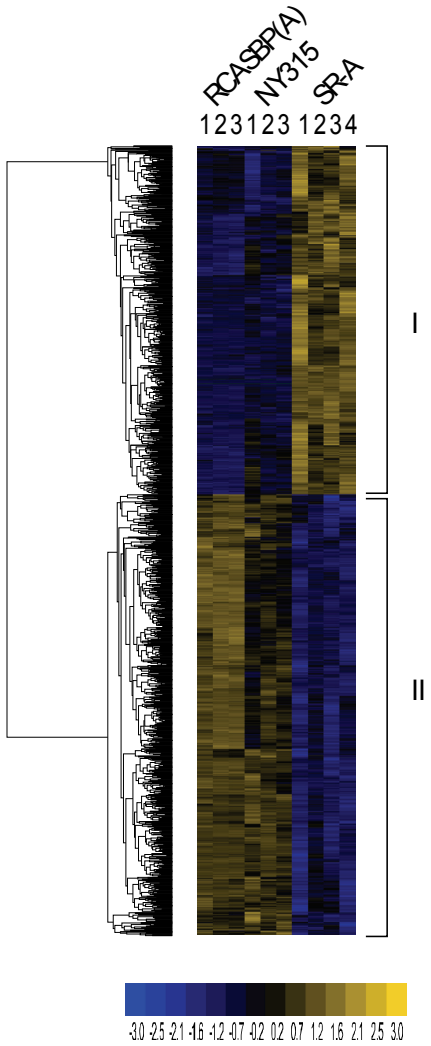


Figure 1. Transformation regulated (TR) genes belong to two clusters of differentially expressed genes in SR-A RSV transformed CEF.

Unsupervised hierarchal clustering was performed on the Transformation-Regulated (TR) gene set revealing transformation responsive genes clustering into one of two approximately equal sized clusters. Class I includes genes up-regulated during transformation representing approximately 44% of the TR genes while class II comprises the down-regulated genes (56% TR genes). The color scale indicates standard deviations from mean centered gene expression values.

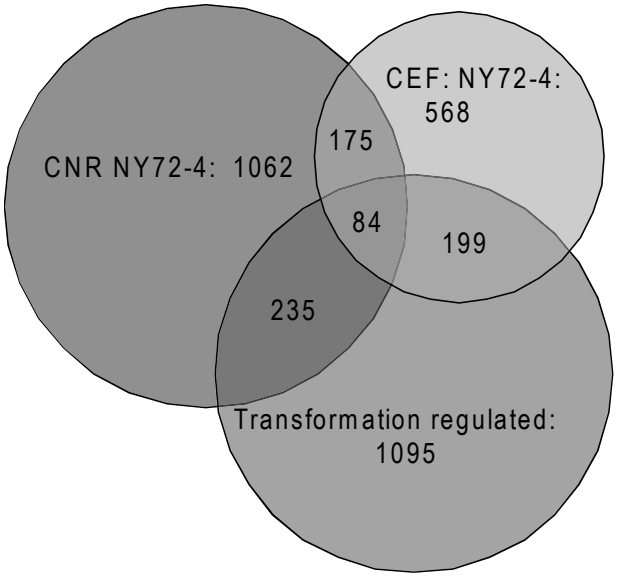


Figure 2. Euler representation of genes differentially expressed between control and v-Src transformed cells.

Comparison of gene sets differentially expressed by two-fold between CEF or CNR cells infected with the temperature sensitive mutant NY72-4 RSV, or the set of Transformation-Regulated (TR) genes defined in Additional File 3. Numbers indicate total probe sets.

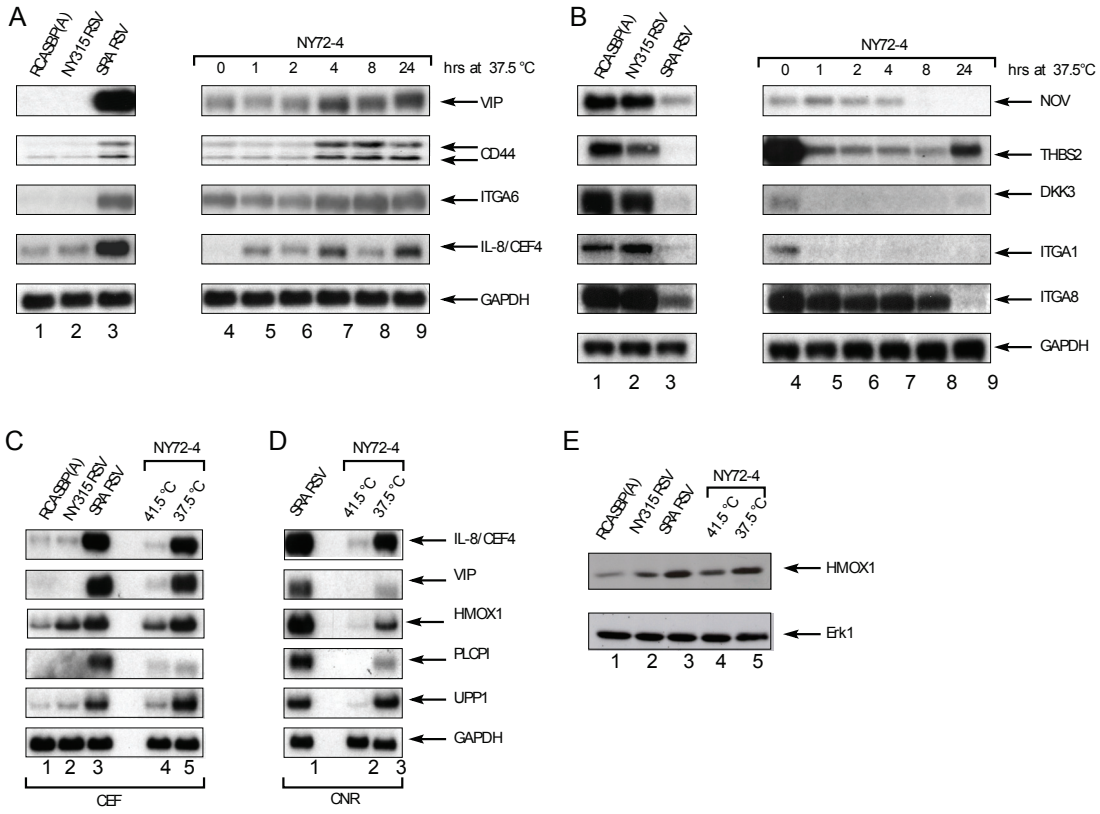


Figure 3. Validation of gene profiling results by northern and western blotting analyses.

Steady state transcript levels for a selected set of v-Src regulated genes were determined by northern blotting analyses (A-D). CEF infected with RCASBP(A), NY315 or SR-A RSV were maintained at 41.5˚C for the duration of the analysis while NY72-4 RSV infected cells were cultured at the permissive and non-permissive temperatures of 37.5˚C and 41.5˚C, respectively, for the indicated period of time. A-B) CEF infected with NY 72-4 RSV were grown at the non-permissive temperature of 41.5˚C and transferred to 37.5˚C for the indicated period. C-D) CNR cells infected with NY 72-4 RSV were grown at the permissive temperature of 37.5˚C and either maintained at this temperature or transferred to 41.5˚C for a 24 hr period before RNA isolation. CNR cells transformed by SR-A RSV were kept at 41.5˚C. RNA loading was assessed by probing for GAPDH. E) Western blotting analysis of heme oxygenase 1 (HMOX1) in CEF. Protein samples were prepared from CEF infected RCASBP(A), NY315 or SR-A RSV at 41.5˚C. CEF infected with NY 72-4 RSV were either kept at the non-permissive temperature of 41.5˚C or transferred to 37.5˚C for 24 hrs before lysis. Erk1 was used as a control to assess protein loading.

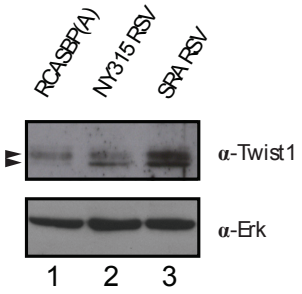


Figure 4. Up-regulation of Twist1 in v-Src-transformed CEF.

Twist1 protein levels are up-regulated in v-Src transformed CEF. Two Twist1 immunoreactive protein species of 26 kDa of molecular weight are detected in normal and v-Src transformed CEF.

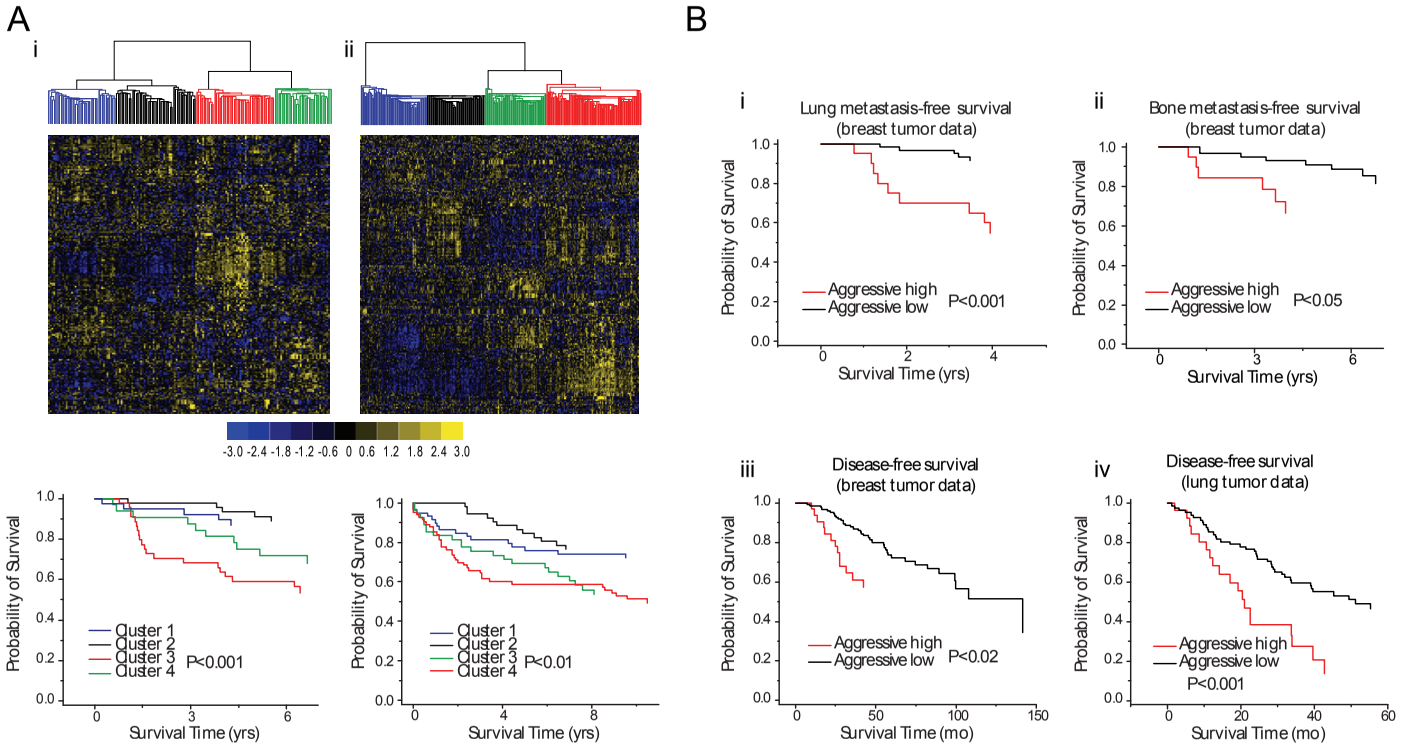


Figure 5. A subset of CSR genes predicts poor prognosis in human tumors.

A) Hierarchal clustering of two breast tumor datasets with respect to up-regulated CSR genes reveals tumor sample clusters associated with low disease-free survival (cluster 3 in panel i and cluster 4 in panel ii). The color scale beneath the heat maps indicates standard deviations from mean centered gene expression values. Colors of survival curves correspond to the colored clusters indicated in the heat maps above each survival plot. B) A common set of up-regulated genes associated with low-survival clusters in panel A, termed the aggressive tumor gene signature (Table 4), correlates with poor prognosis in several patient cohorts. Tumor clusters expressing high levels of the aggressive signature genes (aggressive high) correlate with lower lung and bone metastasis-free survival in patients with breast tumors (i and ii), as well as with lower disease-free survival in separate cohorts of patients with breast (iii) or lung tumors (iv).

Table 1. Primer sequences used for the amplification of probe DNA.

Gene	Forward primer	Reverse primer
CD44	TTACTCCGTACTCACATATGCC	CGTCACATGCTCCTGTTCG
DKK3	AAAACCCAGCATACACACTGC	CAGACTTCACACCTGCTTGG
HMOX1	CTGCCCTGGAGAAAGACTTG	AAGCTCTGCCTTTGGCTGTA
ITGA1	CTCTTCTCTACATTACGACG	ATTTTCTTCTTCAGTGGC
ITGA6	GTAATGGCAAATGGCTG	GAACGCTGGAAGAACC
ITGA8	TGGAAAGAGGGAAGAGC	AAGAAGATTGGTGGAAGG
NOV	ATGAAGTGCTCCTGGGAGG	GACATGGGATCTAATGGCTGG
PLCPI	CTCCTCAGAACCACTGCACA	TTCAAGTGTATTTTATTCTCCTGCAT
THBS2	GGGTTATTCGCCACCAAGG	TAGACCTAATCGTCCACCAGC
UPP1	TATGAAGGACAGGGCAGGTT	TTTCAAACGTCACAGCAAGC
VIP	TAGAAAACGAGTTAGCTCCCAGGA	AGAGTTTGCTAGGTGTCCTTCAGA

Table 2. Summary of probe sets found differentially regulated in all three systems of v-Src regulation.

	Total probe-sets			Unique transcripts		
Analysis	up	down	total	up	down	Total
Transformation regulated	483	612	1095	418	535	953
CEF NY72-4	319	249	568	261	216	477
CNR NY72-4	485	577	1062	444	503	947

Number of transcripts differentially expressed in the Transformation-Regulated dataset (RCASBP(A), NY315, SR-A RSV analysis) and the ts NY72-4 RSV infected CEF and CNR cells is shown. Numbers under the *total probe-sets* heading indicate the total number of Affymetrix probe-sets hybridizing to differentially expressed transcripts, whereas *unique transcripts* do not include redundant probe-sets whose Entrez Gene and/or reference sequence ID were identical.

Table 3. Pathway Express output summary.

		#Input genes in pathway			Corrected γ p-value		
Pathway Name	# genes in pathway	TR	CEF NY72-4	CNR NY72-4	TR	CEF NY72-4	CNR NY72-4
ECM-receptor interaction	87	19	10	14	1.83E-11	3.37E-06	3.14E-07
Focal adhesion	195	26	14	19	6.87E-11	1.44E-06	3.47E-06
Phosphatidylinositol signaling system	77	3	1	3	7.71E-10	4.80E-11	1.71E-03
TGF-beta signaling pathway	84	10	7	N.D.	2.90E-06	2.12E-04	N.S.
Regulation of actin cytoskeleton	208	19	N.D.	11	1.27E-05	N.S.	5.03E-03
Small cell lung cancer	86	12	6	8	2.56E-05	9.19E-03	1.26E-02
Complement and coagulation cascades	69	4	2	N.D.	1.31E-03	2.15E-02	N.S.
Epithelial cell signaling in Helicobacter pylori infection	67	3	1	N.D.	2.80E-03	1.09E-05	N.S.
Leukocyte transendothelial migration	116	7	2	7	3.19E-03	1.73E-02	2.75E-02
Type II diabetes mellitus	44	4	1	N.D.	2.60E-02	2.85E-02	N.S.

Common pathways found to be dysregulated in the Transformation-Regulated (TR), CEF NY72-4 and CNR NY72-4 gene sets are shown. The *number of genes in the pathway* refers to the number of genes in the associated KEGG pathway (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/; (Kanehisa & Goto, 2000)). *Input genes* refer to the number of differentially expressed genes that were found in that pathway. Corrected γ p-value is a measure of significance as calculated by Pathway Express. N.D. and N.S. indicate *not determined* and *not significant* (corrected γ p-value > 0.05) respectively.

Table 4. v-Src aggressive tumor gene signature.

Gene symbol	Gene name
ATAD3A	ATPase family, AAA domain containing 3A
C13orf3	chromosome 13 open reading frame 3
CCNA2	cyclin A2
CCNE2	cyclin E2
CEP55	centrosomal protein 55kDa
CSTA	cystatin A (stefin A)
E2F8	E2F transcription factor 8
EAF2	ELL associated factor 2
EXO1	exonuclease 1
GAR1	GAR1 ribonucleoprotein homolog (yeast)
HELLS	helicase, lymphoid-specific
HMOX1	heme oxygenase (decycling) 1
HSP90AB1	heat shock protein 90kDa alpha (cytosolic), class B member 1
IL8	interleukin 8
ITGA4	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)
KIF11	kinesin family member 11
KIF2A	kinesin heavy chain member 2A
LBR	lamin B receptor
MPP1	membrane protein, palmitoylated 1, 55kDa
NASP	nuclear autoantigenic sperm protein (histone-binding)
NOC2L	nucleolar complex associated 2 homolog (S. cerevisiae)
NOP14	NOP14 nucleolar protein homolog (yeast)
NPM3	nucleophosmin/nucleoplasmin, 3
ODC1	ornithine decarboxylase 1
PDCD6	programmed cell death 6
PLAU	plasminogen activator, urokinase
RIOK3	RIO kinase 3 (yeast)
RRM1	ribonucleotide reductase M1
RRM2	ribonucleotide reductase M2 polypeptide
SHC4	SHC (Src homology 2 domain containing) family, member 4
SLC2A14	solute carrier family 2 (facilitated glucose transporter), member 14
SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3
SLC36A4	solute carrier family 36 (proton/amino acid symporter), member 4
SOCS1	suppressor of cytokine signaling 1
TRIP13	thyroid hormone receptor interactor 13
TTC35	tetratricopeptide repeat domain 35
UHRF1	ubiquitin-like with PHD and ring finger domains 1

UPP1	uridine phosphorylase 1
USP1	ubiquitin specific peptidase 1
USP18	ubiquitin specific peptidase 18
VRK1	vaccinia related kinase 1
ZDHHC21	zinc finger, DHHC-type containing 21

Aggressive gene signature used as a predictor of poor prognosis as determined by hierarchal clustering enrichment regime (see text, Figure 5).

Table 4 continued.

Additional files

The following two links can be used to access additional files:
http://www.biomedcentral.com/1471-2407/10/41
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2837010/?tool=pubmed

Additional File 1
File name: Additional File 1.csv
File format: comma separated value
Temperature regulated genes

Additional File 2
File name: Additional File 2.csv
File format: comma separated value
Pairwise comparisons of CEF infected with RCASBP(A), NY315, or SRA RSV

Additional File 3
File name: Additional File 3.csv
File format: comma separated value
Transformation-Regulated genes in CEF

Additional File 4
File name: Additional File 4.csv
File format: comma separated value
Genes regulated by ts NY72-4 infected CEF

Additional File 5
File name: Additional File 5.csv
File format: comma separated value
v-Src regulated genes identified in SR-A and ts NY72-4 RSV transformed CEF

Additional file 6
File name: Additional File 6.csv
File format: comma separated value
Genes regulated by ts NY72-4 infected CNR

Additional File 7
File name: Additional File 7.doc
File format: Microsoft Word document
Genes commonly regulated in all three v-Src transformed cell systems

Additional File 8
File name: Additional File 8.doc

File format: Microsoft Word document
Genes uniquely regulated in NY72-4 RSV transformed CEF and CNR

Additional File 9
File name: Additional File 9.pdf
File format: portable document format
Expression of CD44 in normal and transformed CEF.
Surface expression of CD44 was examined by immunofluorescence in CEF infected with RCASBP(A), NY315 or SR-A RSV. NY72-4 infected CEF were either grown at the non-permissive (41.5°C) or the permissive temperature (37.5°C) for 24 hours prior to fixing and staining for CD44.

Additional File 10
File name: Additional File 10.pdf
File format: portable document format
Comparison of expression data of v-Src regulated genes as determined by gene profiling and northern blotting analyses.
Gene expression by northern blotting analysis (Figure 3) was quantified and analyzed against microarray data to confirm a correlation in gene expression as measured by the two methods. Analysis of correlation of $\log_2$-transformed gene expression ratios ($\log_2$(experimental/baseline)) indicates a strong correlation (Spearman ρ of 0.83; $p<0.0001$) between northern blot and microarray gene expression estimates. This ρ value is higher than typically observed in array validations (Morey *et al.*, 2006), and consistent with northern blotting as a superior method of gene expression validation (Ding *et al.*, 2007). A slope of 0.91 for the regression line indicates a nearly 1:1 ratio between $\log_2$ expression ratios of northern and microarray data.

Additional File 11
File name: Additional File 11.doc
File format: Microsoft Word document
Comparison of gene expression data quantified from northern blots (Figure 3) compared to values obtained from microarray analysis.

Additional File 12
File name: Additional File 12.doc
File format: Microsoft Word document
Full Pathway Express output summary
Common pathways found to be dysregulated in the Transformation-Regulated (TR), CEF NY72-4 and CNR NY72-4 gene sets are shown. The *number of genes in the pathway* refers to the number of genes in the associated KEGG pathway. *Input genes* refer to the number of differentially expressed genes that were found in that pathway. Corrected γ p-value is a measure of significance as calculated by Pathway Express. N.D. and N.S. indicate *not determined* and *not significant* (corrected γ p-value > 0.05) respectively.

Additional File 13
File name: Additional File 13.pdf
File format: portable document format
Activation of the PI3K/PKB-Akt pathway in v-Src-transformed CEF.
The activation of PKB-Akt in RCASBP(A), NY315 and SR-A RSV infected CEF was investigated by western blotting analysis. Antibodies for PKB-Akt and the Ser-473 phosphorylated form of PKB-Akt were used to determine the expression and activation of PKB-Akt, respectively. The level of phospho-PKB-Akt was examined in cells treated with 1% DMSO (D; diluent) or the PI3K inhibitors LY290042 (LY) and wortmannin (W). PKB-Akt was hyper-phosphorylated in v-Src transformed CEF but phospho-PKB-Akt levels decreased upon treatment with the PI3K inhibitors. The activation of PKB-Akt coincided with increased Ser-9 phosphorylation of GSK3-β.

Additional File 14
File name: Additional File 14.doc
File format: Microsoft Word document
GO biological process terms most enriched in the aggressive gene signature.

Chapter 3: JunD/AP-1 antagonizes the induction of *DAPK1* by C/EBPβ to promote the survival of v-Src transformed cells

Context and contribution

In a previous study published by our lab in which this author was a contributor (Wang *et al.*, 2011), we demonstrated that blocking different AP-1 factors in Src-transformed CEF induced different phenotypes. In particular, expression of the Jun dominant negative allele TAM67 or the JunD shRNA induced significant apoptosis. In order to understand the gene expression changes that led to apoptosis in CEF we undertook a microarray study to assess transcriptional changes in normal and transformed CEF expressing TAM67 or the JunD shRNA.

The discovery of the activation of the proapoptotic gene *death-associated protein kinase 1* (*DAPK1)* in transformed CEF expressing either TAM67 or the JunD shRNA revealed a plausible method by which AP-1 repression inhibited survival. We therefore pursued the hypothesis that repression of *DAPK1* expression by AP-1 bypassed an oncogene checkpoint that would otherwise lead to cell death. Since *DAPK1* expression was previously shown to be regulated by IFNγ in a C/EBPβ-dependent manner (Gade *et al.*, 2008), this led us to the investigation of the role of C/EBPβ in the activation of the *DAPK1* gene in response to AP-1 repression.

All microarray and data analyses in this manuscript were carried out by the thesis author. Reporter assay experiments, qPCR, chromatin immunoprecipitation experiments and cloning were also conducted by the author with the exception of cloning of the shRNA constructs which was done by former technician Shi Yan. Colony formation

assays were conducted by former PhD student Lizhen Wang and technician Ying Wu. Quantification of colony formation assays and ImageJ automation was done by the author. Immunoblots in Figure 2B and 3D were contributed by Lizhen Wang and Figure 4B was contributed by former MSc student Ben Fielding. All figures were made by the author. The manuscript was written by the author and edited by Pierre-André Bédard.

JunD/AP-1 antagonizes the induction of *DAPK1* by C/EBPβ to promote the survival of v-Src transformed cells

Bart M. Maślikowski, Lizhen Wang, Ying Wu, Ben Fielding and Pierre-André Bédard

Department of Biology, McMaster University, Hamilton, Ontario, L8S 1K4, Canada

Running Title: Regulation of DAPK1 by C/EBPβ and AP-1/JunD

Key words: DAPK1, C/EBPβ, AP-1, JunD, Src, apoptosis, transformation

* To whom correspondence should be addressed: Phone: (905) 525-9140 ext. 23149, Fax: (905) 522-6066, e-mail: abedard@mcmaster.ca

Abstract

The activation of the AP-1 family of transcription factors is a well-characterized feature of cell transformation by tyrosine kinases. Previously, we reported that blocking AP-1 activity using the c-Jun dominant-negative mutant TAM67 induced both senescence and apoptosis in v-Src-transformed chicken embryonic fibroblasts (CEF), whereas, inhibition of JunD by shRNA specifically induced apoptosis. To investigate the role of AP-1 in Src-mediated transformation, we undertook a microarray study to characterize the transcriptomes of v-Src-transformed CEF infected with either TAM67 or the JunD shRNA. Our study revealed a cluster of 18 probe-sets up-regulated in v-Src-transformed CEF over-expressing either TAM67 or the JunD shRNA but not up-regulated in normal CEF or CEF transformed only by v-Src. Four of these probe-sets correspond to genes involved in the interferon pathway. One gene in particular, *death-associated protein kinase 1* (*DAPK1*), is a C/EBPβ-regulated mediator of apoptosis in IFN-γ-induced cell death. Here we show that inhibition of DAPK1 abrogates cell-death in v-Src-transformed cells expressing the JunD shRNA. Further, we show that expression of DAPK1 is dependent on C/EBPβ and is enhanced by the repression of AP-1 activity. Chromatin immunoprecipitation data indicated that C/EBPβ is recruited to the DAPK1 promoter but JunD is not. We conclude that JunD promotes survival by indirectly antagonizing C/EBPβ-dependent expression of DAPK1.

Introduction

The Src non-receptor tyrosine kinase has served as the prototypical kinase model for signalling in vertebrates. Understanding the fundamental basis for Src signalling has provided insight into both the normal function of kinase signalling in the cell as well as contributing to the understanding of human disease. In particular, high Src activity has been linked to poor prognosis and metastasis in breast and colon cancers (Biscardi *et al.*, ; Irby & Yeatman, 2000). Given Src's role in human disease, *v-Src* continues to serve as a model system for understanding intracellular signalling and has provided a system for studying transformation both *in vitro* and in animal models. It is well established that Src-dependent transformation induces profound changes in gene expression (Dehbi & Bedard, 1992). A previous gene expression-profiling study conducted by our group showed that v-Src induces expression changes in over two thousand genes in two different primary cell types. Over-expression of a core subset of these genes was found to be correlated with poor prognosis in breast and lung cancer (Maslikowski *et al.*, 2010).

Despite the importance of signal transduction cascades mediated by Src, these changes in gene expression are ultimately mediated through the activation of transcription factors acting on promoter/enhancer regions. Indeed, the role of Ets, Stat3 and AP-1 on transformation has been documented through the inhibitory effect that their dominant negative alleles exert on v-Src or RasV12-dependent transformation (Bruder *et al.*, 1992; Granger-Schnarr *et al.*, 1992; Lloyd *et al.*, 1991; Suzuki *et al.*, 1994; Turkson *et al.*, 1998; Wasylyk *et al.*, 1994) Inhibition of AP-1 activity in immortalized *jun-/-* mouse embryo fibroblasts (MEFs) renders cells resistant to transformation by activated Ras. Ectopic

expression of c-Jun restores transformation of these MEFs *in vitro* and is capable of generating tumors in nude mice (Johnson *et al.*, 1996). Similarly, over-expression of JunB and JunD partially restores the transformation and tumor generating potential in *jun-/-* MEFs (Johnson *et al.*, 1996). Knock-out *c-jun* and *junD* MEFs also proliferate slowly and are prone to early senescence (Wisdom *et al.*, 1999; Weitzman *et al.*, 2000). *junD-/-* MEFs, furthermore, are sensitized to apoptosis when treated with TNFα (Weitzman *et al.*, 2000). Taken together, these data illustrate the importance of the AP-1 family, particularly *jun* members, in transformation and survival.

Recently, our group demonstrated the pleiotropic action of AP-1 by inhibiting AP-1 activity through the expression of short hairpin RNAs (shRNAs) targeting *c-Jun JunD* and *Fra-2* or by repressing AP-1 via the c-Jun dominant negative allele TAM67. In normal chicken embryo fibroblasts (CEFs) TAM67 induces senescence, however, when transformed by v-Src the same cells exhibit a pleiotropy where three distinct phenotypes are visible. In addition to senescence, a proportion of cells undergo adipogenesis while another portion of cells undergo apoptosis (Wang *et al.*, 2011a). Since TAM67 can dimerize with Jun and Fos members, (Grondin *et al.*, 2007; Hsu *et al.*, 1994; Ozanne *et al.*, 2007) these heteromeric interactions may alter the activity of these proteins as well as c-Jun. Presumably, through a stochastic process, cells give rise to different phenotypes through differential inactivation of different AP-1 members. Resection of these distinct cell fates by shRNA showed that c-Jun, Fra-2 and JunD mediate distinct fates by antagonizing senescence, adipogenesis and apoptosis respectively (Wang *et al.*, 2011a).

Therefore, by comparing the transcription profiles of TAM67-infected versus JunD shRNA-infected cells, in both transformed and untransformed backgrounds we sought to identify genes regulating apoptosis in v-Src-transformed CEF with inhibited AP-1 activity. Our array analysis identified a list of eighteen candidate genes involved in apoptosis. Four genes in this list are members of the interferon (IFN) pathway. Among these, the *death-associated protein kinase 1* (*DAPK1*) gene product was initially identified as a C/EBPβ-dependent proapoptotic protein in IFN-γ induced cell death ((Gade *et al.*, 2008) and reviewed in (Raveh & Kimchi, 2001)). DAPK1 is an activator of the Arf/p53 pathway, and an established tumour suppressor (Raveh *et al.*, 2001; Raveh & Kimchi, 2001). Furthermore, our lab has shown that inhibition of p53 by shRNA restores colony formation in v-Src-transformed JunD shRNA CEF (Wang *et al.*, 2011a) suggesting that JunD-inhibited apoptosis may be acting through DAPK1 in a p53-dependent manner. In this study we show that inhibition of DAPK1 restores survival in v-Src-transformed CEF with inhibited AP-1 activity. Furthermore, DAPK1 expression is dependent on C/EBPβ and is antagonized by JunD/AP-1 expression.

Materials and Method

Cell culture

Early passages (n<10) of CEFs were cultured at 41.5ºC in Dulbecco's Modified Eagle Medium with 5% heat-inactivated (at 57ºC for 30 minutes) Cosmic Calf serum or 5% new-born bovine serum (BioMedia, Cansera, Rexdale, Ontario), 5% tryptose phosphate broth, 1% L-glutamine, and 1% penicillin/streptomycin solution (GIBCO BRL). CEFs

were transfected with B-type RSV viral vectors expressing shRNAs (JunD, C/EBPB, DAPK1, JunD-DAPK1 double shRNA, JunD-C/EBPβ double shRNA, GFP control) or the c-Jun TAM67 mutant using the calcium phosphate method. CEFs were superinfected with the temperature sensitive A-type RSV NY72-4 one passage following transfection and cultured for three passages or until the apoptosis phenotype was observed in AP-1 inhibited CEF at the permissive temperature. Infected CEFs were cultured at the non-permissive temperature of 41.5ºC until such time when temperature shift was performed. Temperature shift was performed for six to eight hours at 37.5ºC.

Soft agar assays and colony formation assays

The assay was performed in 60mm dishes with a lower layer of 0.5% low-melting-point agar in 1X DMEM medium containing 5% cosmic calf serum, 4% chicken serum, 5% tryptose phosphate broth, and 1% penicillin/ streptomycin. 10^5 cells were resuspended in the same medium with 0.35% low-melting-point agar and then were over-laid on the lower agar. The dishes were then incubated in a 37˚C incubator containing 5% CO_2 for 5 days or until colonies were visible. Images of colonies were documented at 10X magnification using an inverted microscope (Magnification 20X). Colony formation assays were quantified using ImageJ software (Abramoff *et al.*, 2004).

Generation of retroviral vectors for shRNA expression

Generation of the single and double shRNA RCASBP(B)-shRNA-ΔU6 retroviral vectors used in this study were described previously (Wang *et al.*, 2011a). We selected several target sequences for C/EBPβ and DAPK1. These target sequences were subcloned into the modified microRNA operon on the transfer plasmid pRFPRNAi(-) and later

subcloned into the RCAS vectors following the original supplier's instructions (ARK Genomics; (Das *et al.*, 2006)). All constructs were designated using the number of the first nucleotide of the targeted sequence. For example, construct containing nt99-120 of *c/ebpβ* is termed RCASBP(B)-shRNA-ΔU6-*C/EBPβ*99. For simplicity, shRNA constructs are written as RCAS(B)-*JunD* shRNA, RCAS(B)-*C/EBPβ* shRNA etc. The control virus RCASBP(B)-shRNA-ΔU6-*EGFP* and RCASBP(B)-shRNA-ΔU6-*JunD* (or simply RCAS(B)-GFP RNAi and RCAS(B)-JunD shRNA) were described previously (Wang *et al.*, 2011a). Nucleotides corresponding to 298-319 of the *DAPK1* coding region were used for silencing. Suppression of gene expression was ascertained by immunoblotting for the corresponding proteins.

Gene profiling analyses

RNA samples were isolated using Trizol (Invitrogen) as described previously (Maslikowski *et al.*, 2010). All RNA samples were first analyzed by northern blotting analysis and probed for IL8 and GAPDH expression to assess integrity. RNA quality was assessed by gel electrophoresis and examined by Bioanalyzer (Agilent). RNA samples with a RNA Integrity Index of less than 9.7 were discarded. Microarray experiments were conducted at the Centre for Functional Genomics (CFG) at McMaster University, Hamilton, Canada. Biotinylated cRNAs were generated at CFG and hybridized to Affymetrix Chicken GeneChip arrays using standard Affymetrix protocols (EukGE-WS2v4). GeneChips were scanned using the Affymetrix GeneChip Scanner 3000. Feature intensity was quantified using Command Console software and exported to CEL format. All experimental groups consisted of three biological/technical replicates except for

NY72-4/RCASBP(B)-shRNA-ΔU6-*JunD* permissive (NY72-4/RCAS(B) *JunD* shRNA P) which was conducted in sextuplicate. These samples undergo apoptosis rapidly upon transformation and show a greater intragenic variance in gene expression than the other samples. Therefore the number of replicates was increased in order to increase statistical power of the experiment.

CEL files were analysed using Affymetrix Expression Console software v1.1. Array data were normalized and probe intensities were estimated using the Affymetrix probe logarithmic intensity error (PLIER) algorithm. Two-fold changes in gene expression between experimental conditions within each experiment were determined using $\log_2$-transformed expression values and statistical significance of expression was determined using a Bonferroni-corrected two-tailed t-test (α=0.05). Gene clustering was performed using unsupervised hierarchal clustering by average Euclidean distance (Eisen *et al.*, 1998). Mean-centered gene expression values and clustering was carried out using PLIER-calculated values in dChip version 2007 (Li, 2003b). Venn diagrams were drawn using Vennmaster v.0.35 (Kestler *et al.*, 2005). Pathway perturbation analysis was carried out using Pathway Express software (Draghici *et al.*, 2007) as described previously (Maslikowski *et al.*, 2010) except that orthologous human Entrez gene annotation was used instead of probe IDs. Annotations were retrieved from the Affymetrix NetAffx service (http://www.affymetrix.com/analysis/index.affx).

Antibodies and immunoblotting

Immunoblotting was performed as described before (Papaconstantinou *et al.*, 2005; Wang *et al.*, 2011a) using the following antibodies: anti-Erk1 (Santa Cruz Biotechnology #sc-

94), anti-JunD (Santa Cruz Biotechnology #sc-74), anti-DAPK1 (#sc-8163), anti-MYL12A (Santa Cruz Biotechnology # sc-9449), anti-MYL9 phospho-Ser19 (cross-reacts with phospho-Ser19 on MYL12A; Santa Cruz Biotechnology, # sc-293109). Anti-chicken C/EBPβ was generated in house and is described previously (Gagliardi *et al.*, 2001). Autoradiographs were scanned on a Umax Astra 1220U scanner, quantified using ImageJ (Abramoff *et al.*, 2004) and corrected for loading using Erk, or Erk and MYL12A (Figure 2E, Suppl. 8). For ChIP, rabbit IgG and anti-JunD were obtained from Santa Cruz Biotechnology (sc-2027 and sc-74). Anti-C/EBPβ anti-serum used in ChIP was a gift from Achim Leutz (Katz *et al.*, 1993).

Real time PCR

For expression validation, DNase I treated RNA samples were reverse-transcribed using the ProtoScript cDNA synthesis kit (New England Biolabs). PCR amplification and quantification was performed using PerfeCTa SYBR Green FastMix low ROX (Quanta Biosciences) on a Stratagene MX3000P Real-time PCR instrument. Probe amplification was quantified using the ΔΔCt method (Livak & Schmittgen, 2001) using GAPDH as the reference gene for array validation assays or input DNA for ChIP assays. The following oligonucleotides were used to amplify the targets indicated:

GAPDH
F:GTCGGAGTCAACGGATTTGGCCG
R:ATGGCCACCACTTGGACTTTGCC

DAPK1
F:TCAACCCAACAAGCATGGAACACCT
R:CGGGATCCACGCTTTAGAAGCAACT

DAPK1 promoter
F:CCCTCAGGGCTGAGCAGTGCA
R:GGTGGCTGGATCATATGTGAGGAGC

DAPK1 intron
F:GTCCCTAGGCCCCAGCTGCC
R:CGGAGGGGCAGTGCTGAGCT

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) was described previously (Wang *et al.*, 2011a) and followed the method and reagents described in the EZ-ChIP protocol (Upstate Biotechnology-Millipore). Chromatin was prepared from CEF infected with NY72-4 and RCAS(B) GFP RNAi or NY72-4 and RCAS(B) TAM67 at both permissive and restrictive temperatures. Chromatin corresponding 500 μg of protein was immunoprecipitated using one μg normal rabbit IgG, anti-C/EBPβ antibody or anti-JunD antibody. Purified chromatin was amplified by real-time PCR as described above. One percent of the input from each sample was used to normalize PCR amplification. All PCR experiments were performed using a minimum of three replicates.

Transient reporter assays

pGLuc-derived reporter constructs and RSV-βgal control plasmid (described previously (Dehbi *et al.*, 1992)) were co-transfected into NY72-4-infected CEF along with RCASB-GFP RNAi, RCASB-TAM67, or RCASB-JunD shRNA as described in the results except for the experiment summarized in Figure 8B. For this experiment pGLuc-359 and RSV-βgal were transfected into NY72-4-infected CEF along with RCASB-GFP RNAi and RCASB-TAM67 or RCASB-Δ184 and RCASB-TAM67. NY72-4-infected CEFs were seeded onto six-well plates a day prior to transfection. For two six-well plates one 100mm plate of nearly confluent CEF was split 1:12. CEF were transfected by the DEAE-dextran method for four to six hours and shocked using 10% DMSO-PBS shock for 2 minutes.

Medium was replaced the following day. The next day half the cells were moved to permissive temperature. After six-eight hours, CEF were lysed in 70 µl 250mM Tris (pH 6.8) and 1% NP-40. Lysate was centrifuged to remove debris. 30 µl of lysate was used for βgal normalization. Five to ten µl of lysate was assayed for *Gaussia* luciferase using the *Gaussia* luciferase assay kit (New England Biolabs) as per the manufacturer's recommendations. Luminescence was quantified on a Berthold Lumat 9501 luminometer. For the JunD shRNA reporter assays (Figure 8A), NY72-4-infected CEF were transfected, fed the following day, and split 1:2 two days following feeding. Since DEAE-dextran inhibits growth of CEF for a few days following transfection, the cells were not overgrown nor contact inhibited. Half the cells were immediately shifted to permissive temperature to induce v-Src-transformation and harvested the next day. All reporter assays were conducted using a minimum of six replicates.

Cloning

We employed the *Gaussia* luciferase system since previous experiments using several firefly luciferase vectors revealed that they could be induced by AP-1 activity even in the absence of a promoter (our unpublished data). pGluc-TATA was derived by cloning the human β-globin TATA box into the pGluc-Basic reporter plasmid (New England Biolabs). The TATA box was cloned into the pGluc-Basic BamH1 site by oligonucleotide cloning. This generated a BamHI/NdeI fragment that could be used to ascertain directionality of the clone. *DAPK1* promoter fragments were cloned into pGluc-TATA using directional cloning. The full length DAPK1 promoter was PCR-amplified from chicken genomic DNA and cloned into pCR2.1 (Invitrogen) using TA cloning and into

pGluc-TATA as an Ecor1/BamH1 fragment. The deletion mutants were PCR amplified from the pCR2.1 construct and subcloned into pGluc-TATA. Constructs -134,-83, and -46 had an MfeI site on the 5' primer instead of EcoR1. MfeI generates cohesive compatible ends that can be ligated to EcoR1 cut DNA. Ligation of MfeI/EcoR1 cohesive ends destroys both sites generating a useful diagnostic marker for cloning small DNA fragments. Binding site mutants were constructed by site-directed mutagenesis. Each oligonucleotide pair was designed to mutagenize at least two critical sites in the binding motif and to introduce a diagnostic restriction site for clone screening. PCR cloning of the full-length and truncation mutants was carried out using GoGreen Taq (Promega). Site directed mutagenesis was done using Pfu Turbo (Stratagene). All clones were sequenced at the MOBIX sequencing facility at McMaster University. The following oligonucleotides were used to generate the constructs:

Construct	Direction	Sequence	Restriction sites
pGLuc-TATA	F	GATCGGATCCGAGTATAAAAGGTGAGGT AGGATCAGTTGCTCCTCACATAT	EcoR1
	R	GATCATATGTGAGGAGCAACTGATCCTA CCTCACCTTTTATACTCGGATCC	NdeI
pGluc-DAPK1-359	F	AAGCAGCA TGATGGAACT GCACC	EcoR1
	R	TATAGCGGATCCGTTCCTTTAATCTGCACT	BamH1
pGluc-DAPK1-146	F	TGTGCTGAA TTCTTAACCT CTGATGAGCA	EcoR1
	R	TATAGCGGATCCGTTCCTTTAATCTGCACT	BamH1
pGluc-DAPK1-134	F	CCT CTGATGAGCA ATTGTCTTTC TGAAAATC	MfeI
	R	GATTTTCAGAAAGACAATTGCTCATCAGAGG	BamH1
pGluc-DAPK1-83	F	GGGAGCTGA CAATTGCTCA GTGACCTGAC	MfeI
	R	GTCAGGTCACTGAGCAATTGTCAGCTCCC	BamH1
pGluc-DAPK1-46	F	CCCTCAGGGC TGAGGAATTC AGATTAAAGG	MfeI
	R	CCTTTAATCTCCATTGCTCAGCCCTGAGGG	BamH1
pGluc-DAPK1 µ146	F	CCTCTGATGAGCTCTCCTCTTTCTGAAAATCACAAAGC	SacI
	R	ATTTTCAGAAAGAGGAGAGCTCATCAGAGG TTAAGAGTTC	SacI
pGluc-DAPK1 µ 134	F	CAATCCTCTAGATGAAAATCACAAAGCTCA GATGGGAGC	XbaI
	R	AGCTTTGTGATTTTCATCTAGAGGATTGCTC ATCAGAGGTTAAG	XbaI

pGluc-DAPK1 μ 83	F	GTGACCTGATCACATGCACTCCCTCAGGGCTG	BclI
	R	GGGAGTGCATGTGATCAGGTCACTGAGCAGCTTTC	BclI

Results

Micorarray analysis of AP-1 inhibition in v-Src-transformed CEF

To investigate the role of AP-1 in Src-mediated transformation, we undertook a microarray study to characterize the transcriptomes of v-Src-transformed CEF infected with the c-Jun dominant negative mutant TAM67 or a JunD shRNA. It was reasoned that by comparing the transcriptomes of transformed CEF repressing AP-1 activity using either the TAM67 dominant negative or the JunD shRNA, we could identify a gene or genes involved in affecting survival in v-Src transformed cells. CEF infected with the temperature sensitive RSV NY72-4 virus were co-infected with the TAM67-containing virus, the JunD shRNA-containing virus or the RCAS(B) GFP shRNA control virus. Following array normalization and probe-set intensity estimation, two multiple pair-wise analyses were conducted to identify genes of interest. The first set was a multiple pair wise analysis of gene differentially expressed by two-fold or more between CEF infected with NY72-4/RCASB at permissive and restrictive temperatures, CEF infected with NY72-4/JunD shRNA at permissive and restrictive temperatures and between CEF infected with NY72-4/JunD shRNA and NY72-4/RCASB both at permissive temperature. Similarly, the second set was an analysis of differential gene expression between CEF infected with NY72-4/RCASB at permissive and restrictive temperatures, CEF infected with NY72-4/TAM67 at permissive and restrictive temperatures and between CEF infected with NY72-4/TAM67 and NY72-4/RCASB both at permissive temperature (see

Suppl. 1 for all significantly differentially expressed genes for both analysis sets and Suppl. 2 and 3 for a summary of the number of probe-sets corresponding to differentially expressed genes in each comparison). A total of 2468 probe-sets corresponding to 1539 unique differentially expressed genes passed the significance criteria.

In order to identify potential gene candidates involved in the control of apoptosis in v-Src transformed cells, we considered the probe-sets corresponding to genes that were differentially expressed both in transformed CEF expressing either TAM67 or the JunD shRNA in relation to normal transformed CEF. Pathway perturbation analysis of orthologous genes in these two sets (NY72-4 TAM67 permissive/NY72-4 RCASB permissive and NY72-4 JunD shRNA permissive/NY72-4 RCASB permissive; or simply TAM67 P/RCASB P and JunD shRNA P/RCASB P) and the set of genes differentially expressed between normal and transformed CEF (NY72-4 RCASB permissive/ NY72-4 RCASB non-permissive; or simply RCASB P/RCASB NP) shows 32 dysregulated pathways. Thirteen pathways are shared between two or more sets and eight are shared by all three (phosphatidylinositol signaling system, focal adhesion, ECM-receptor interaction, TGF-β signaling pathway, pathways in cancer, natural killer cell mediated cytotoxicity, MAPK signaling pathway, and calcium signaling pathway). Five of the thirteen pathways are shared by TAM67 P/RCASB P and JunD shRNA P/RCASB P but not by RCASB P/ RCASB NP (cytokine-cytokine receptor interaction, regulation of actin cytoskeleton, Jak-STAT signaling pathway, GnRH signaling pathway, and basal cell carcinoma; Suppl. 4 & 5). Although there are a number of pathways unique to each gene set, there are no uniquely shared pathways between either TAM67 P/RCAS P and RCAS

P/RCAS NP, or JunD shRNA P/RCAS P and RCAS P/RCAS NP. Put differently, there are no exclusively shared pathways between either AP-1 repressed data set and the Src-transformed data set whereas there are five pathways exclusively perturbed by both AP-1 repressed sets (Suppl. 4 & 5). Moreover the number of genes present in each pathway in the AP-1 repressed comparisons is approximately double to the number of pathway genes for the RCAS P/RCAS NP set (Suppl. 5). Therefore, the similarity of pathways dysregulated by the two AP-1 repressed sets, and the number of genes present in each pathway suggests a shared biochemistry that underlies the phenotypic similarity shared by TAM67 and the JunD shRNA expressing CEF as compared to normal versus transformed CEF.

Pooling those probe sets from TAM67 P/RCAS P and JunD shRNA P/RCAS P that are up-regulated in relation to NY72-4 RCASB P, we performed unsupervised hierarchal clustering to identify gene clusters that were up-regulated in transformed CEF with repressed AP-1 activity but not in non-transformed or normal transformed CEF (Suppl. 6). Using this process, we identified a cluster of 18 probe-sets corresponding to eleven unique annotated genes that are up-regulated in transformed CEF with repressed AP-1 activity, but not in normal or transformed CEF (Figure 1, Table 1). Pathway perturbation analysis indicates that four of the top eight most dysregulated pathways associated with these genes are those involved in cancer (ErB signalling, pathways involved in cancer, Jak-STAT signalling, and bladder cancer; see Suppl. 7). Four genes in this list are members of the interferon (IFN) pathway (*DAPK1, IFIT5, OASL, SERPINB2*; presently the KEGG pathway “cytokine-cytokine receptor interaction”, which subsumes

IFN signalling, does not include the genes). DAPK1 is a C/EBPβ-dependent proapoptotic protein that mediates apoptosis in response to IFN-γ signalling (Gade *et al.*, 2008). Previously, we showed that C/EBPβ can antagonize AP-1 signalling, attenuating AP-1 activity and promoting adipogenesis (Gagliardi *et al.*, 2003; Wang *et al.*, 2011a). Since the role of DAPK1 is recognized in C/EBPβ-dependent apoptosis and C/EBPβ activity is stimulated in response to AP-1 inhibition (Wang *et al.*, 2011a), we chose to investigate the anti-proliferative role of DAPK1 in v-Src-transformed CEF.

Validation of *DAPK1* activation

To assess the induction of *DAPK1* in response to AP-1 inhibition in transformed CEF, we conducted qPCR using the same conditions as were used for the microarray. Validation of expression by qPCR shows that the *DAPK1* expression increase two to four fold over control conditions and shows a similar profile of expression as compared to microarray (Figure 2A). Immunoblotting shows a two-fold induction of DAPK1 in v-Src transformed CEF expressing the *JunD* shRNA (Figure 2B, lane 4; quantified using ImageJ, data not shown). Similar two to four fold increases in DAPK1 expression were reported in response to IFNγ-stimulation and p53 activation elsewhere (Gade *et al.*, 2008; Martoriati *et al.*, 2005).

Although inhibition of AP-1 increases *DAPK1* transcript and protein levels in v-Src-transformed CEF, Src can attenuate DAPK1 activity by phosphorylating DAPK1 and thus it is possible that while DAPK1 levels are increased in response to AP-1 inhibition, its activity is not. One of the substrates of DAPK1 is the regulatory subunit of myosin light chain 2 (MYL12A, formerly MLC2B). DAPK1 regulates the formation of stress-

fibres by phosphorylating Ser19 on MYL12A (Kuo *et al.*, 2003). Initially we attempted to perform a kinase assay using a GST-MYL12A fusion protein as per Kuo *et al*. (Kuo *et al.*, 2003), however, we were unsuccessful. The protocol published by these authors and others relies on using immunoprecipitates from cells ectopically expressing DAPK1. Since we are using endogenous protein, it is possible that the signal obtained by precipitating endogenous DAPK1 is too weak. We therefore blotted directly for Ser19 on MYL12A to determine if DAPK1-like activity changed during v-Src transformation and inhibition of AP-1. In Figure 2C lane 8 shows that phosphorylation of MYL12A is decreased upon transformation of CEF. This decrease is not seen in transformed CEF expressing either TAM67 or JunD shRNA (Figure 2C, lanes 10 and 12) suggesting that Ser19-MYL12A kinase activity increases upon inhibition of AP-1 by TAM67 and the JunD shRNA.

To examine the possibility that MYL12A is being differentially phosphorylated by another kinase, we examined the array data to asses if other known myosin kinases changed their expression in response to v-Src transformation and AP-1 inhibition. Previously, we observed that myosin light chain kinase (MYLK) expression decreases in v-Src transformed CEF (Maslikowski *et al.*, 2010). Since MYLK phosphorylates MYL12A at Thr18 and Ser19 (Ikebe & Hartshorne, 1985) it is possible that the change in MYL12A phosphorylation is attributable to a change in MYLK levels. Only one probe set corresponding to *MYLK* (Gga.4091.1.S2_a_at) shows a statistically significant decrease in expression between NY72-4 RCAS(B) non-permissive and permissive conditions. Examination of this probe-set shows no correlation between *MYLK* expression and

MYL12A phosphorylation (Spearman's ρ=0.486, p=0.356; Suppl. 1, Suppl. 8). Moreover, the fluctuation of MYLK levels over an order of magnitude in the various conditions examined suggests that MYL12A phosphorylation at Ser19 is unrelated to MYLK expression. Other MYLK family members, MYLK2, MYLK3 and MYLK4, are not found to be differentially expressed in our array analysis. Regulatory subunits of the myosin light chain phosphatase (MYLP) complex (PPP1R12A, PPP1R12B, PPP1R16A, PPP1R12C and PPP1R16B) and the catalytic subunit (PPP1C; reviewed in (Grassie *et al.*, 2011)) were likewise not found to be dysregulated in our array analysis. While further investigation is needed, taken together, these data suggest that the increase in MYL12A phosphorylation in v-Src-transformed CEF with repressed AP-1 activity results from a change in DAPK1 expression and/or activity and not as a result of changes in MYLK or MYLP expression.

Repression of *DAPK1* by shRNA restores transformation in v-Src-transformed CEF with inhibited AP-1 activity

Previous work by our lab showed that inhibition of JunD activity by shRNA increased apoptosis in v-Src-transformed CEF approximately twelve-fold and abrogated colony formation in soft agar (Wang *et al.*, 2011a). Co-inhibition of JunD with p53 restored colony formation (Wang *et al.*, 2011a). To assess if DAPK1 functions in this pathway, a double knock-down construct was used to assay colony formation in transformed CEF. Double inhibition of JunD and DAPK1 restored colony formation in transformed cells (Figure 3A-C). Similarly, inhibition of C/EBPβ restored the ability of v-Src-transformed CEF expressing the JunD shRNA to form colonies (Figure 4) suggesting that C/EBPβ is

required for the activation of the *DAPK1* gene. Interestingly, double-inhibition of JunD and DAPK1 produced a greater number and size of colonies when compared to control alone suggesting that DAPK1 restricts colony formation even in control v-Src-transformed CEF (see Figure 3B-C).

Activation of *DAPK1* expression is dependent on C/EBPβ and is antagonized by AP-1

In mammals, IFN-γ-mediated apoptosis is dependent on C/EBPβ whose activity is potentiated by ERK1/2 (Gade *et al.*, 2008). Analysis of the murine *dapk1* gene shows that activation of the promoter is mediated through a proximal cyclic AMP response element (CRE) and a distal C/EBPβ-binding site (Gade *et al.*, 2008). Recent work by Zhu and colleagues (Hu *et al.*, 2009) showed that ERK2 represses expression of IFN-γ-induced genes by binding to a modified C/EBPβ binding site known as a GATE element; originally characterized in the context of IFN-γ signalling (Roy *et al.*, 2000). Analysis of the upstream regulatory region of the chicken *DAPK1* gene identified a putative TATA box and initiator element upstream of an ~800bp GC-rich putative 5' untranslated region. We have also identified two putative C/EBP binding sites, along with one CRE, and a potential GATE element in the proximal promoter region (Figure 5, see Suppl. 9 for full sequence). In addition, we identified a potential C/EBPβ binding site known as a CARE element. CARE elements are comprised of two-half sites that bind to C/EBPβ and ATF4 in response to stress and amino acid deprivation (reviewed in (Kilberg *et al.*, 2009))

To assess if inhibition of AP-1 de-represses DAPK1 transcription, we performed a series of *in vitro* reporter assays to delineate the function of these putative binding sites. 5' deletion analysis of the upstream regulatory region led to the identification of critical

elements of the DAPK1 promoter (Figure 6). Since infection with the JunD shRNA takes several passages to repress the target protein and since transformed JunD shRNA cells tend to undergo apoptosis in great number, promoter mapping experiments were conducted using the less lethal RSV-TAM67 CEF co-infected with NY72-4.

In Figure 6, deletion mapping shows that removal of the CARE element attenuated reporter activity in CEF but did not eliminate induction upon transformation in TAM67 infected cells. Deletion of the 5' C/EBP site markedly reduced inducibility of the promoter as seen in constructs -146 and -83. Deletion of region including the CRE abolished any residual promoter activity.

To ascertain the role of individual sites in the promoter, we created point mutation constructs for the distal C/EBP binding site, the GATE element and the CRE. Mutation of either the distal C/EBP site or the CRE (constructs μ146, μ83) decreased reporter activity whereas mutation of the GATE element had no effect (Figure 7). Both μ146 and μ83 retained inducibility under conditions of AP-1 repression. The mutant CRE retained approximately four times more activity than the mutated C/EBP site consistent with the deletion data and suggesting that the distal C/EBP site is a more powerful activator of *DAPK1* transcription. Taken together, these data show that the CRE and the distal C/EBP site are the primary sites required for the activation of the *DAPK1*. Moreover, the combined effect of these sites is greater than additive implying that there is a synergism between these sites and that neither site on its own is sufficient for activation of *DAPK1*. Although the function of the CARE element was not assessed directly, deletion of the CARE element and the promoter region upstream of the distal C/EBP site attenuated

reporter activity, but did not inhibit inducibility by TAM67. This suggests that the CARE element, or some other element upstream of the distal C/EBP, acts to potentiate basal *DAPK1* promoter activity.

Attempts to assess promoter activity using the JunD shRNA proved challenging. For transient *in vitro* reporter assays the length of time required to establish knock-down exceeds the duration during which the reporter is active. In contrast, transfection of stably-infected JunD shRNA CEF stresses the cells which are compromised by JunD inhibition leading to little or no reporter activity. It was therefore necessary to modify the existing protocol to allow for detection of reporter activity without losing all the cells (see Materials and Method). Using this method we were able to show that inhibition of JunD in transformed CEF induces reporter activity using the full-length the *DAPK1* promoter (Figure 8A). Because of the large amount of apoptosis, reporter activity was approximately an order of magnitude lower (before normalization) in the full length reporter using JunD shRNA than it was for TAM67. Nevertheless, these results indicate that insofar as the regulation of the DAPK1 promoter is concerned, TAM67 and the JunD shRNA are functionally similar.

To ascertain the role of C/EBPβ in the regulation of the DAPK1 promoter we undertook a reporter assay experiment where C/EBPβ was inhibited by the Δ184 C/EBPβ dominant negative mutant. In CEF infected with NY72-4, TAM67 and Δ184, reporter activity was attenuated when compared to CEF infected with NY72-4, TAM67 and a control virus (Figure 8B). Furthermore, there was no reporter activation in CEF infected with NY72-4, TAM67 and Δ184 at the permissive condition when compared to the non-

permissive. These data suggest that C/EBPβ is required for the activation of the DAPK1 promoter in response to AP-1/JunD inhibition.

Although the role of C/EBPβ appears to be the result of direct binding to the *DAPK1* promoter, the case for AP-1/JunD is less clear. Chromatin immunoprecipitation experiments were carried-out in order to determine if C/EBPβ or JunD associates directly with the DAPK1 promoter. AP-1 factors are known to interact with CREs (Nagamoto-Combs *et al.*, 1997; Andersson *et al.*, 2001) and JunD has been shown to repress CRE-mediated transcription through promoter binding (Guberman *et al.*, 2003). JunD therefore may be repressing *DAPK1* through direct promoter binding. Alternatively JunD could repress *DAPK1* indirectly, perhaps through competition for co-activators such as p300/CBP. AP-1 is known to repress glucocorticoid signalling by just such a mechanism (reviewed in (Webster & Cidlowski, 1999)).

CEF were infected with NY72-4 and an RCASB control virus or with NY72-4 and TAM67, grown at either permissive or non-permissive temperature and lysed. Chromatin was immunoprecipitated from the lysates using an anti-C/EBPβ or anti-JunD antibody, or rabbit IgG control serum. The JunD antibody was previously used to show interaction of JunD with the IL8 promoter in response to v-Src activation (Wang *et al.*, 2011a). Analysis of the qPCR data shows that C/EBPβ binds to the *DAPK1* promoter in response to AP-1 inhibition in transformed CEF (Figure 9A). In contrast, the signal using anti-JunD does not exceed the background level seen in the IgG control suggesting that JunD does not bind to the *DAPK1* promoter under any condition. Amplification of the *DAPK1* intron was an order of magnitude weaker than the promoter indicating that the

binding of C/EBPβ was not the result of contamination or non-specific immunoprecipitation conditions. These data show that C/EBPβ binds directly to the *DAPK1* promoter in v-Src-transformed cells under conditions of AP-1 repression. No evidence of JunD interaction with the promoter was observed suggesting that the effect of AP-1/JunD on *DAPK1* expression is indirect.

Discussion

In order to identify potential genes involved in the inhibition of survival in AP-1-repressed v-Src-transformed cells, we carried out an expression profile analysis on v-Src transformed primary CEFs with and without AP-1 inhibition. By using a c-Jun dominant negative allele and a *JunD* shRNA to repress AP-1 activity, we could better identify common targets that repress survival in v-Src-transformation. Analysis of the six conditions showed a total of 1539 unique dysregulated genes. Pathway perturbation analysis of the comparison sets used for clustering showed a dysregulation of thirteen pathways involved in cancer signaling, immunity or cell adhesion. Five of these were uniquely shared between the AP-1 repressed comparison sets (Suppl. 4-5) illustrating the similarity of pathway regulation between JunD shRNA and TAM67. Clustering analysis revealed a set of eleven unique genes up-regulated in response to AP-1-inhibition in v-Src-transformed CEF but not in normal transformed or untransformed cells. Four of these genes are involved in the IFNγ response. In particular, *DAPK1* activation by IFNγ is dependent on C/EBPβ (Li *et al.*, 2007). Consistent with this, down-regulation of AP-1 enhances C/EBPβ activity (Gagliardi *et al.*, 2003; Wang *et al.*, 2011a). DAPK1 has been

shown to be indispensable in cell death mediated by IFNγ, TGFβ, TNFα, DNA damage, and oxidative stress (reviewed in (Bialik & Kimchi, 2006)). DAPK1 plays a role both in early tumour development and in metastasis, and is known to play a role in autophagy (Bialik & Kimchi, 2006; Inbal *et al.*, 1997). Promoter hypermethylation on *DAPK1* has been observed in numerous tumours (Harden *et al.*, 2003; Gonzalez-Gomez *et al.*, 2003; Narayan *et al.*, 2003) making DAPK1 and its regulation a topic of interest amongst clinicians and basic scientists alike.

By repressing the expression of DAPK1 we were able to rescue the colony formation phenotype in AP-1-inhibited v-Src transformed CEF. Furthermore, this could be recapitulated by the repression of C/EBPβ suggesting that DAPK1 and C/EBPβ function in the same pathway in CEF. Interestingly, repression of both *DAPK1* and *JunD* led to more numerous and larger colony formation in v-Src-transformed CEF as compared to normal v-Src-transformed control cells suggesting that DAPK1 restricts v-Src transformation even in the absence of AP-1 inhibition. DAPK1 activity is known to be attenuated by the phosphorylation of Tyr491 by Src (Wang *et al.*, 2007) indicating a synergistic mechanism of DAPK1 regulation; both via Src-dependent transcriptional repression by AP-1 and by direct Src-phosphorylation.

Promoter analyses and chromatin immunoprecipitation indicated that DAPK1 expression is C/EBPβ-dependent and its inducibility is mediated through a C/EBPβ-binding element and a CRE. Interestingly, this reflects the structure of the murine *dapk1* promoter, which too is regulated by a proximal CRE and a distal C/EBPβ-binding site. The conservation of this regulatory mechanism across distant taxa emphasizes the

important role that DAPK1 plays in regulating apoptosis. Although the inducibility of the *DAPK1* promoter was dependent on C/EBPβ activity (Figure 8B), some promoter activity was still retained suggesting that other trans-acting factors are involved. C/EBPβ is known to dimerize with the stress responsive factor ATF4 and bind to CREs as an ATF4/ C/EBPβ heterodimer on other genes (Vallejo *et al.*, 1993; Park *et al.*, 1993). Cellular transformation induces oxidative stress and DAPK1 has been shown to act in the oxidative stress response pathway mediated by PKD (Eisenberg-Lerner & Kimchi, 2007). It is therefore possible that ATF4 cooperates with C/EBPβ in the regulation of DAPK1. Binding of JunD was not observed in the ChIP assays suggesting that the role of AP-1 in repression of *DAPK1* is indirect. It was previously shown that over-expression of C/EBPβ antagonized AP-1 activity and decreased AP-1 levels, while repression of C/EBPβ using a dominant negative mutant showed the inverse (Gagliardi *et al.*, 2003). More recently, we showed that the inhibition of AP-1 leads to an increase of C/EBPβ activity and *vice versa* (Wang *et al.*, 2011a). While AP-1 does not interact directly with the *DAPK1* promoter, we hypothesize that AP-1 and C/EBPβ compete for common co-activators such as p300. Similar inhibition of gene expression by co-activator sequestration by AP-1 has been observed previously (Webster & Cidlowski, 1999). This could be addressed by a series of immunoprecipitation experiments designed to assess if C/EBPβ or AP-1 preferentially competes for p300. Additionally if this hypothesis holds true, *DAPK1* repression during AP-1 activation should be relieved by the over expression of p300.

The regulation of DAPK1 by gene expression is confounded by a significant amount of cross talk between the different pathways regulating DAPK1, and the

pathways regulated by DAPK1 itself. Phosphorylation by ERK on S735 (Chen *et al.*, 2005a) up-regulates DAPK1 activity while paradoxically, RSK, a target of ERK, decreases DAPK1 activity by phosphorylating S289 on DAPK1. ERK also activates C/EBPβ by phosphorylating T188 in the trans-activating domain thereby activating the *DAPK1* locus (Gade *et al.*, 2008). However, DAPK1 can sequester ERK in the cytosol thereby attenuating ERK activity and hence DAPK1 though a negative feedback loop. Src, which activates the MAPK pathway and therefore ERK, inhibits DAPK1 by directly phosphorylating Y491 on DAPK1 while the LAR phosphatase reverses this during periods of low Src activity (Wang *et al.*, 2007). While DAPK1 activates the JNK-mediated oxidative stress pathway via PKD activation, JNK activates JunD (Eisenberg-Lerner & Kimchi, 2007), which suppresses *DAPK1* expression as presented here. Similarly, DAPK1 has been shown to up-regulate p53 through p14Arf (Raveh *et al.*, 2001) even though *DAPK1* itself is a target of p53 activity (Martoriati *et al.*, 2005). The various transcriptional and post-transcriptional regulatory mechanisms offer numerous positive and negative feedback loops to control DAPK1 activity presumably to allow the cell to respond to pro- and anti-apoptotic stimuli under various contexts. As such, a straight forward assessment of DAPK1 activation is not trivial. While we show that inhibition of AP-1 de-represses MYL12A phosphorylation in transformed CEF, and while we show that MYLK expression and the expression of other myosin light chain kinases and phosphatases does not correlate with the reduced phosphorylation of MYL12A, we cannot rule out that differential regulation of these enzymes through Ca^{++}/calmodulin and PKA-dependent pathways is not occurring. Previous groups have used the

phosphorylation status of S308 on DAPK1 to gauge whether the kinase is in an active conformation (Shohat *et al.*, 2001). However, since Src and RSK can both inactivate DAPK1 by phosphorylation on different residues, and since calmodulin is required to stabilize DAPK1 into an active conformation, S308 may be insufficient to address the activity of DAPK1.

Regardless, a modest two-to four fold increase in DAPK1 expression is sufficient to suppress growth in v-Src-transformed CEF. Similar modest increases in DAPK1 expression were reported before in response to IFNγ and p53–induced apoptosis (Gade *et al.*, 2008; Martoriati *et al.*, 2005). v-Src transformation leads to both oxidative stress and a loss of adhesion. Since DAPK1 plays a role in oxidative stress and anoikis (Eisenberg-Lerner & Kimchi, 2007; Wang *et al.*, 2002), it is possible that even modest increases of DAPK1 activity are sufficient to sensitize cells to apoptosis under conditions of transformation induced stress. Repression of DAPK1 by AP-1 during v-Src-transformation appears to be the cell's attempt to by-pass this oncogenic checkpoint.

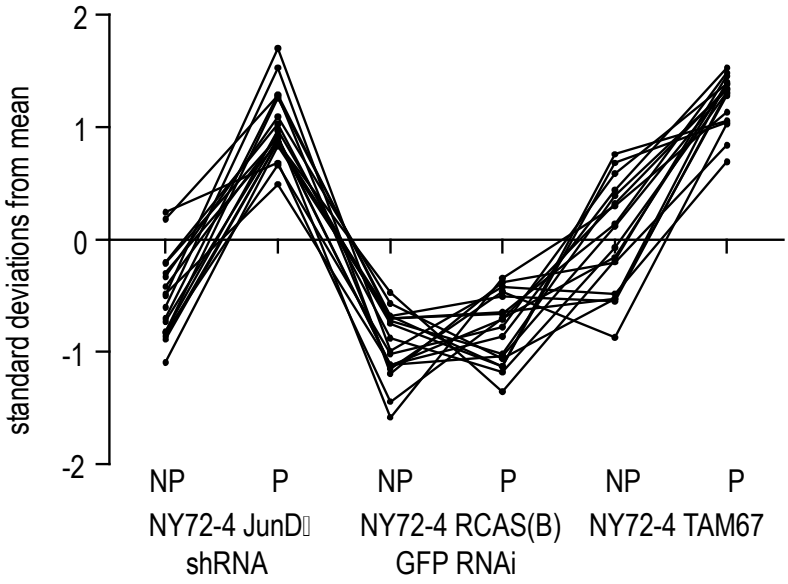


Figure 1. Line plot indicating gene-wise mean-centred expression values for genes found to be up-regulated in NY72-4/JunD and NY72-4/TAM67 infected CEF grown at permissive temperature in relation to control cells (NY72-4 and RCAS(B) GFP-RNAi co-infected CEF).

Table 1. Mean log2 gene expression values for genes found to be up-regulated in NY72-4/JunD knock-down and NY72-4/TAM67 infected CEF grown at permissive temperature in relation to control cells (NY72-4 and RCAS(B) GFP-RNAi co-infected CEF). NP and P indicate non-permissive and permissive respectively.

					NY72-4 JunD KD		NY72-4 RCAS(B)		NY72-4 TAM67	
Probe Set	Gene Symbol	Gene Title	Entrez Gene ID	Representative Public ID	NP	P	NP	P	NP	P
Gga.10034.2.S1_a_at	SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	420896	BU419559	9.5	12.49	8.84	9.61	9.54	11.12
Gga.10034.2.S1_s_at	SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	420896	BU419559	10.15	12.92	9.37	10.43	10.67	12.03
Gga.13110.1.S1_at	---	---	---	BU388661	5.75	6.91	4.36	5.07	4.65	6.64
Gga.1442.1.S1_at	---	---	---	BU334089	7.76	9.18	6.61	6.7	8.17	9.73
Gga.18046.1.S1_at	CTSK/L†	cathepsin K/L	---	BU437111	5.46	6.34	5.33	4.67	5.55	6.78
Gga.18379.1.S1_at	---	---	---	BU458521	6.75	8.02	5.64	6.82	7.43	8.22
Gga.389.1.S1_at	HBEGF	heparin-binding EGF-like growth factor	395654	NM_204849.1	7.52	9.49	7.27	7.58	9.4	10.45
Gga.4502.1.S1_at	SFRS5	splicing factor, arginine/serine-rich 5	423258	BU443510	9.23	9.58	7.87	8.47	8.9	10.14
Gga.536.1.S1_a_at	OASL	2'-5'-oligoadenylate synthetase-like	395908	NM_205041.1	7.75	10.64	7.96	8.2	8.14	10.71
Gga.6121.1.S1_at	---	---	---	BU480628	8.95	10.14	8.95	8.98	9.06	10.14
Gga.6815.1.A1_at	IL6*	interleukin 6	395337	BX273308	6.67	7.42	6.26	6.08	6.75	7.59
Gga.9179.1.S1_at	DAPK1	death-associated protein kinase 1	431670	CF257373	7.3	8.04	6.82	7.01	8	8.83
GgaAffx.20888.1.S1_at	CBLB	Cas-Br-M (murine) ecotropic retroviral transforming sequence b	418406	CR523727.1	9.51	10.99	9.16	9.53	10.46	11.39
GgaAffx.21915.1.S1_at	IFIT5	interferon-induced protein with tetratricopeptide repeats 5	423790	ENSGALT00000010311.1	6.66	9.1	7.16	7.21	8.22	9.93
GgaAffx.23472.1.S1_at	CHN2	chimerin (chimaerin) 2	428437	ENSGALT00000018199.1	9.39	10.49	9.48	9.29	10.19	10.79
GgaAffx.24929.1.S1_at	---	---	---	ENSGALT00000026308.1	8.99	9.69	8.88	8.64	8.9	9.84
GgaAffx.9237.1.S1_s_at	PPP1R3C	protein phosphatase 1, regulatory (inhibitor) subunit 3C	425328	ENSGALT00000000817.1	7.96	9.34	8.12	7.77	9.25	9.49
GgaAffx.9237.2.S1_at	PPP1R3C	protein phosphatase 1, regulatory (inhibitor) subunit 3C	425328	ENSGALT00000010788.1	8.64	9.94	8.69	8.41	9.73	9.99

*Based on sequence homology to chicken *IL6* † Based on sequence homology to vertebrate *CTSK* and *CTSL*

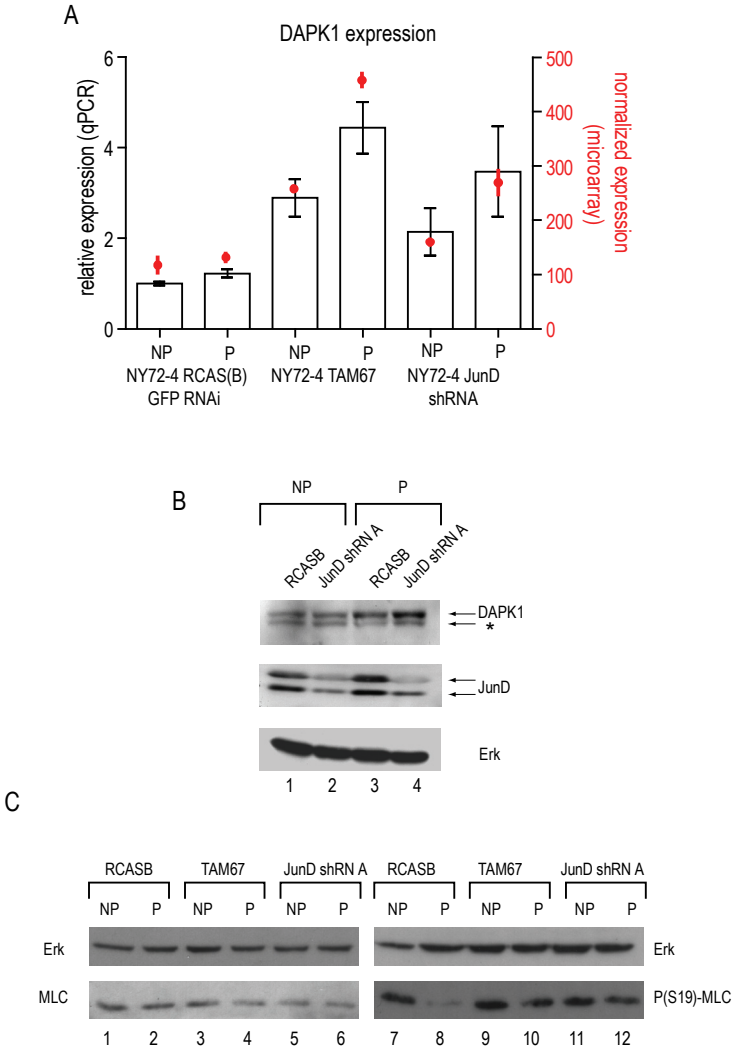


Figure 2. Validation of *DAPK1* expression and activity.

A. qPCR expression data comparing expression of *DAPK1* by qPCR to microarray data. Red points indicate mean expression of *DAPK1* on the microarray while the bar graph shows mean expression data as determined by qPCR. Error bars indicate standard error of mean. **B.** Immunoblotting validation of DAPK1 induction by v-Src in conditions of JunD inhibition by shRNA. The star indicates the position of a product of degradation of DAPK1 generated during sample preparation.**C.** Immunoblotting shows a decrease in the phosphorylation of S19 on MYL12A (MLC) in transformed CEF (lane 8), an indication of reduced DAPK1 activity. Repression of AP-1 by TAM67 or JunD alleviates this repression in transformed CEF (lanes 10, 12). NP and P indicates non-permissive and permissive respectively.

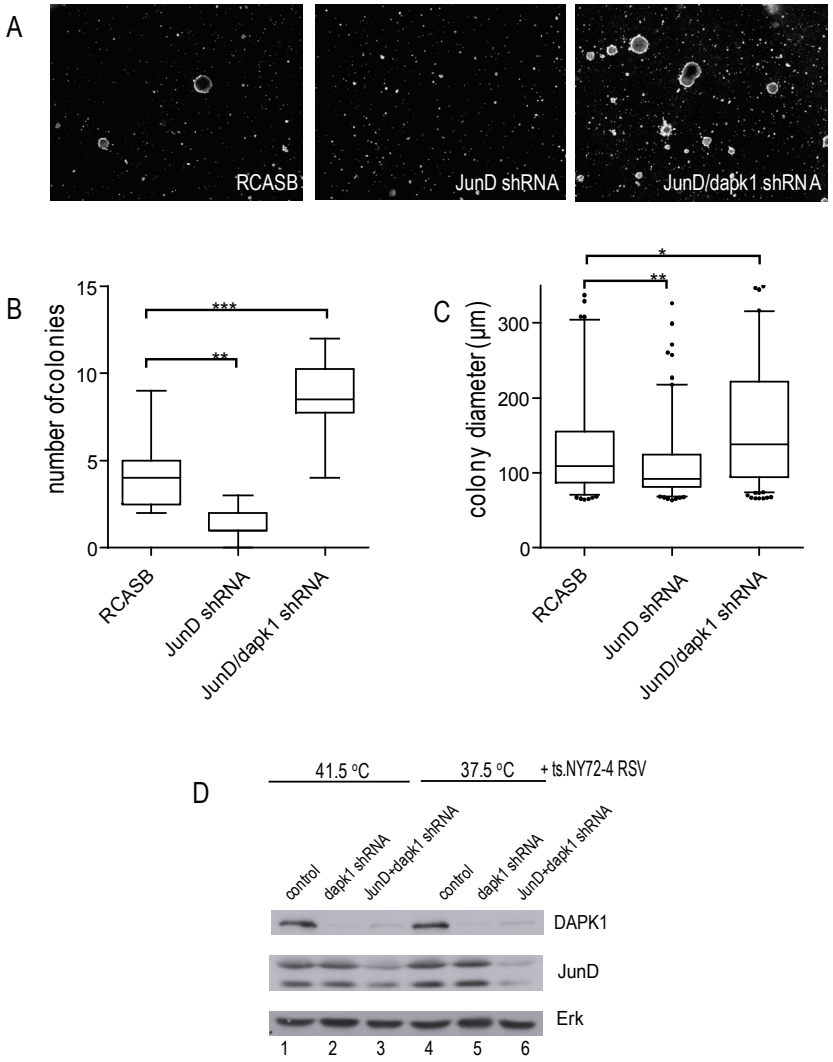


Figure 3. v-Src-transformed JunD/DAPK1 double knock-down CEF form larger and more numerous colonies in soft agar.

A. Representative fields of colony formation assay are shown. All CEFs are v-Src-transformed and co-infected with viruses indicated. **B.** Number of colonies per field greater than 50 µm in each condition is shown. Mean number of JunD/DAPK1 double-knock-down CEF colonies is greater than RCASBRNAi control, and the JunD single knock-down CEF (one-way ANOVA followed by Tukey post-hoc test, $p<0.05$). Mean number of colonies per field for RCASB, JunD, and JunD/DAPK1 are 4.22, 1.40 and 8.70, respectively. **C.** Mean colony diameter of JunD/DAPK1 knock-down cells is greater than RCASBRNAi control, and the JunD single knock-down CEF (Kruskal-Wallis test followed by Dunn's post-hoc test, $p<0.05$). Outlier data greater than 350 µm are not shown. Mean colony sizes for RCASB, JunD, Fra2 and JunD/DAPK1 are 138.6 µm, 113.7 µm and 163.5 µm, respectively. Whiskers in both plots indicate 5^{th} -95^{th} percentiles. Levels of significance in pair-wise tests are indicated in brackets above box-plots. *, ** and *** indicate p values less than 0.05, 0.01 and 0.001 respectively. Representative fields from colony formation assays quantified in B and C are shown at 2X magnification. Colony formation assays were quantified using ImageJ (Abramoff *et al.*, 2004). All features in each field greater than 50µm were counted. Feret's diameter was calculated and used for statistics. A minimum of 9 fields per condition were used. Distribution normality was ascertained using the Shapiro-Wilk's test followed by the appropriate analysis of variance (Kruskal-Wallis or ANOVA). **D**. Immunoblotting validation shows DAPK1 and JunD levels are knocked-down using single or double shRNA constructs as indicated.

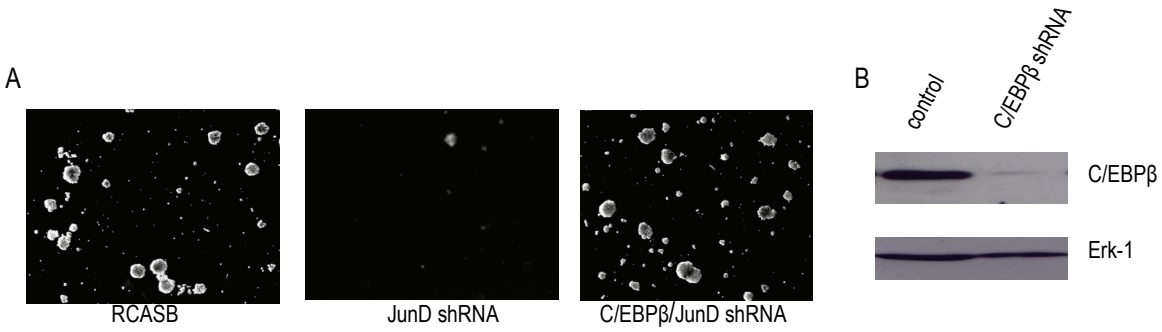


Figure 4. The co-inhibition of C/EBPβ restores the capacity of v-Src transformed CEF to grow in soft agar in conditions of JunD repression.

A. Colony formation assays show that repression of C/EBPβ can restore the transformation phenotype in JunD shRNA v-Src-infected CEF. **B.** Immunoblotting validation of C/EBPβ knock-down.

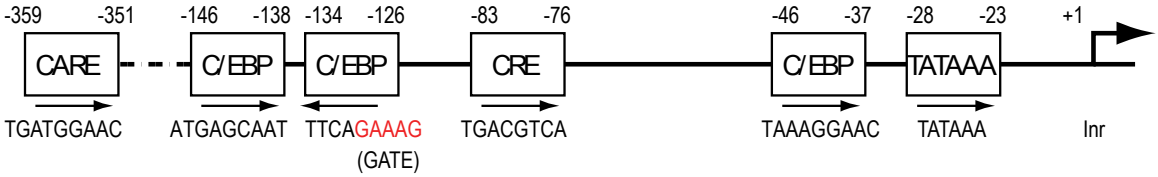


Figure 5. Structure of the upstream regulatory region of chicken *DAPK1*.

The positions of the transcription start site (+1), TATAAA box, C/EBP binding sites, cAMP response element (CRE) and CARE element are indicated. A GATE element (in red) is embedded within the -134 C/EBP binding site. Arrows indicate orientation of the element.

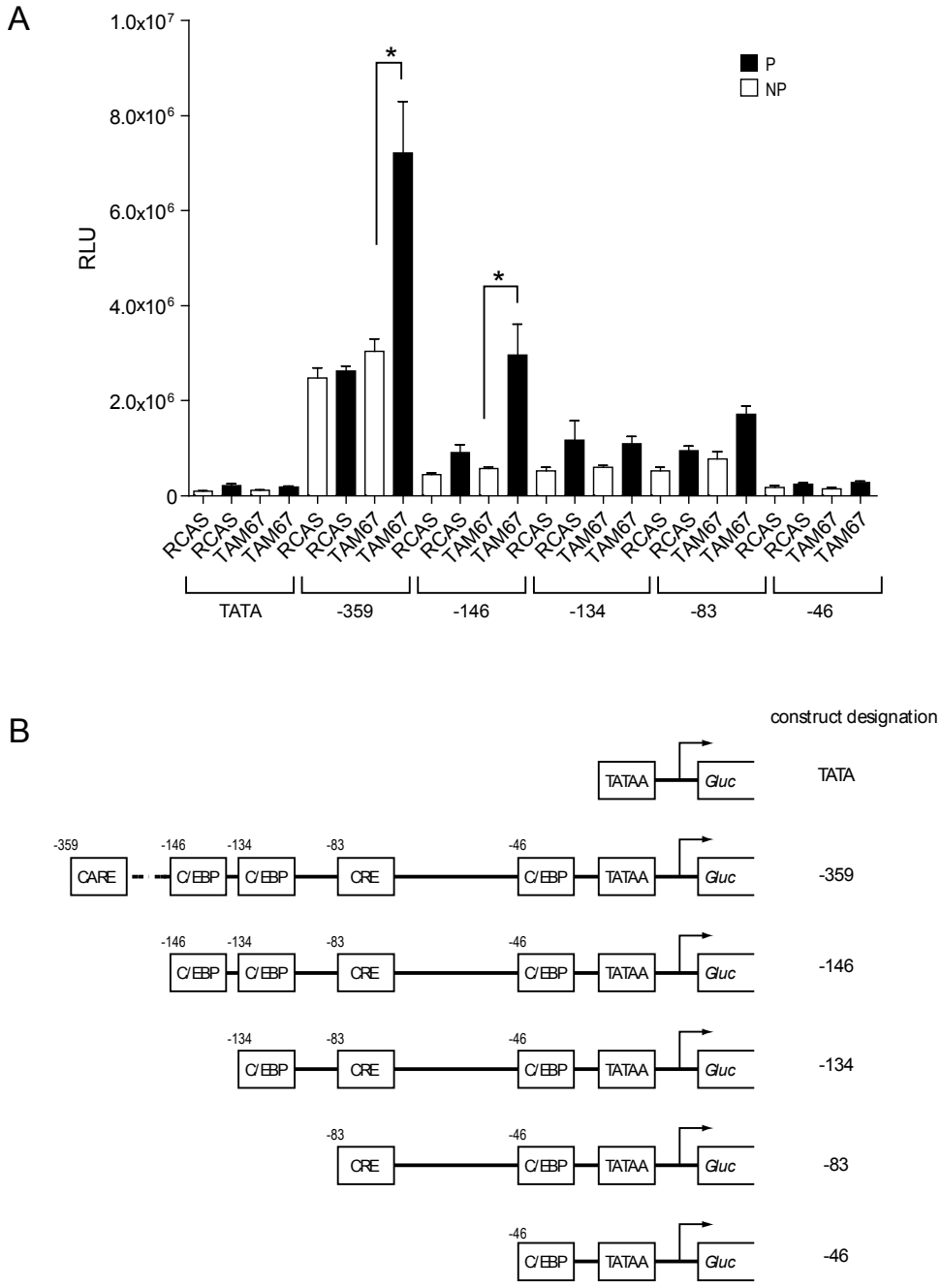


Figure 6. Deletion mapping of the *DAPK1* upstream regulatory region

Panel A shows Quantification of *Gaussia* luciferase activity in ts72-4-infected CEF using reporter constructs indicated. Cells were co-infected with RCAS control virus or TAM67 as shown. Asterisks indicate statistical significance as determined by multiple pairwise t-tests (Bonferroni-corrected, $p<0.05$). Six replicates per condition were used. Error bars indicate standard error of mean. Panel B shows schematic representations of the deletion constructs used in panel A. NP and P refers to non-permissive and permissive temperatures.

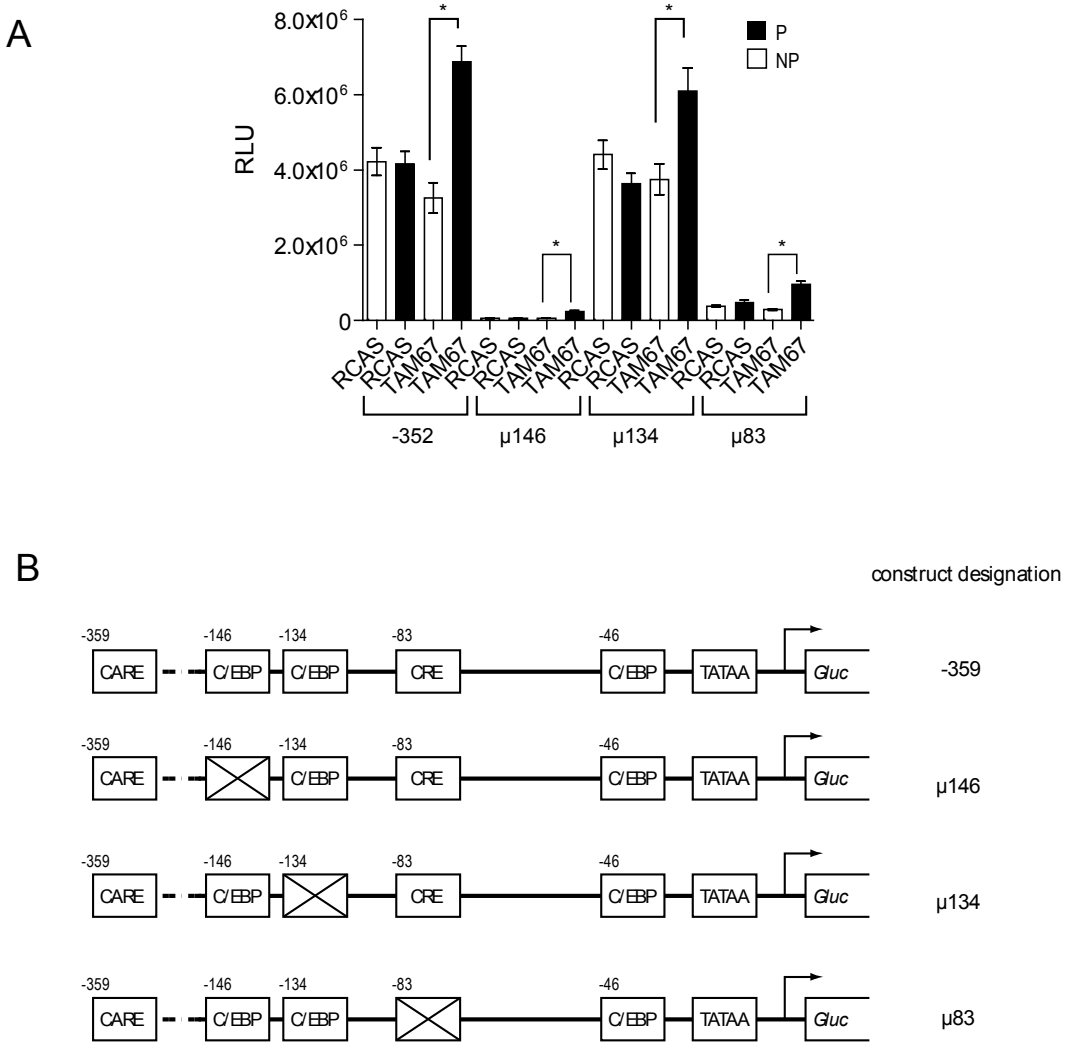


Figure 7. Mutation mapping of the *DAPK1* upstream regulatory region

Panel A shows quantification of *Gaussia* luciferase activity in ts72-4-infected CEF using reporter constructs indicated. Cells were co-infected with RCAS control virus or TAM67 as shown. Asterisks indicate statistical significance as determined by multiple pairwise t-tests (Bonferroni-corrected, $p<0.05$). Error bars indicate standard error of mean. Six replicates per condition were used. Panel B shows schematic representations of the mutation constructs used in panel A. NP and P refers to non-permissive and permissive temperatures.

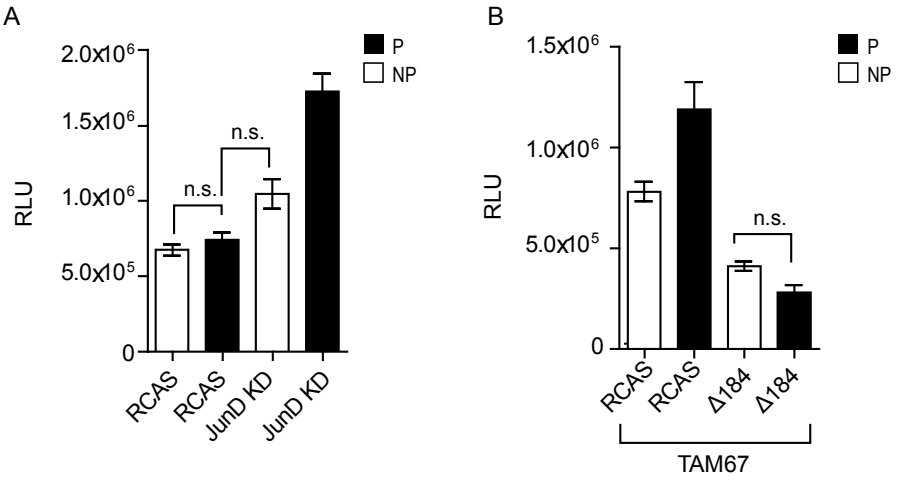


Figure 8. The full length *DAPK1* upstream regulatory region is regulated by JunD and C/EBPβ when measured using *in vitro* reporter assays

A. Repression of JunD by JunD shRNA activates the full length *DAPK1* promoter in v-Src-transformed CEF. NY72-4 infected cells were co-infected with RCAS(B) control virus or JunD shRNA (JunD KD) as shown. **B.** Repression of C/EBPβ by the Δ184 dominant negative mutant of C/EBPβ abrogates activity of the full length *DAPK1* promoter in transformed and untransformed CEF. NY72-4 infected cells were co-infected with RCAS(B) control virus and TAM67 or Δ184 and TAM67 as shown. Error bars indicate standard error of mean. Differences between means are significant unless indicated by n.s. (multiple pairwise t-tests, Bonferroni-corrected, $p<0.05$). A minimum of six replicates per condition were used. NP and P refer to non-permissive and permissive temperatures.

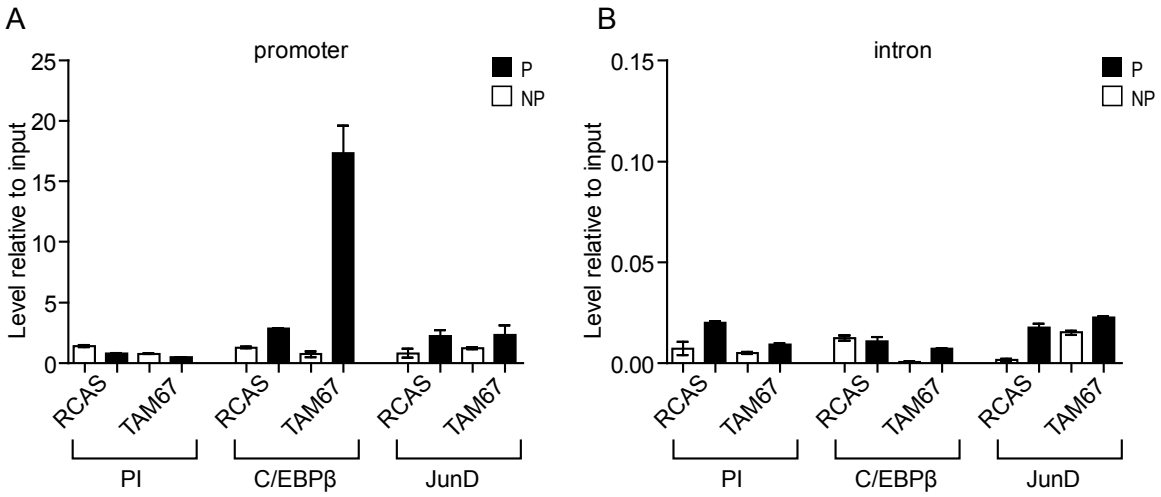
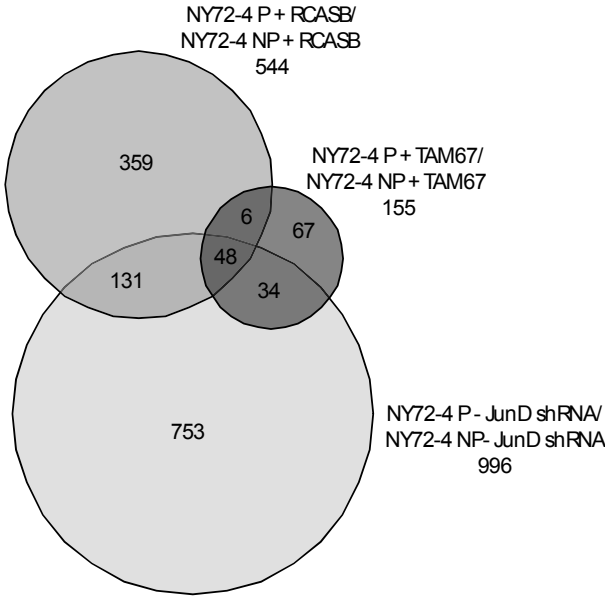


Figure 9. Chromatin immunoprecipitation indicates that C/EBPβ is recruited to the *DAPK1* promoter under conditions of AP-1 inhibition.

NY72-4 infected CEF were co-infected with either RCAS(B) control virus or TAM67 as indicated. Cells were expanded and then grown at either permissive (P) or non-permissive (NP) conditions for eight hours before being lysed for immunoprecipitation. **A.** C/EBPβ immunoprecipitated *DAPK1* promoter region is PCR amplified under conditions of AP-1 repression in v-Src-transformed CEF. Only background signal is observed in samples immunoprecipitated using rabbit IgG (PI) or anti-JunD antibody. **B.** A distal intron sequence is not amplified when chromatin precipitated using any of the antibodies indicated. Error bars indicate standard error of mean.

Supplementary Data 1. Number of probe-sets corresponding to genes found to be differentially expressed by two-fold or more in the comparisons studied. See electronic resource.



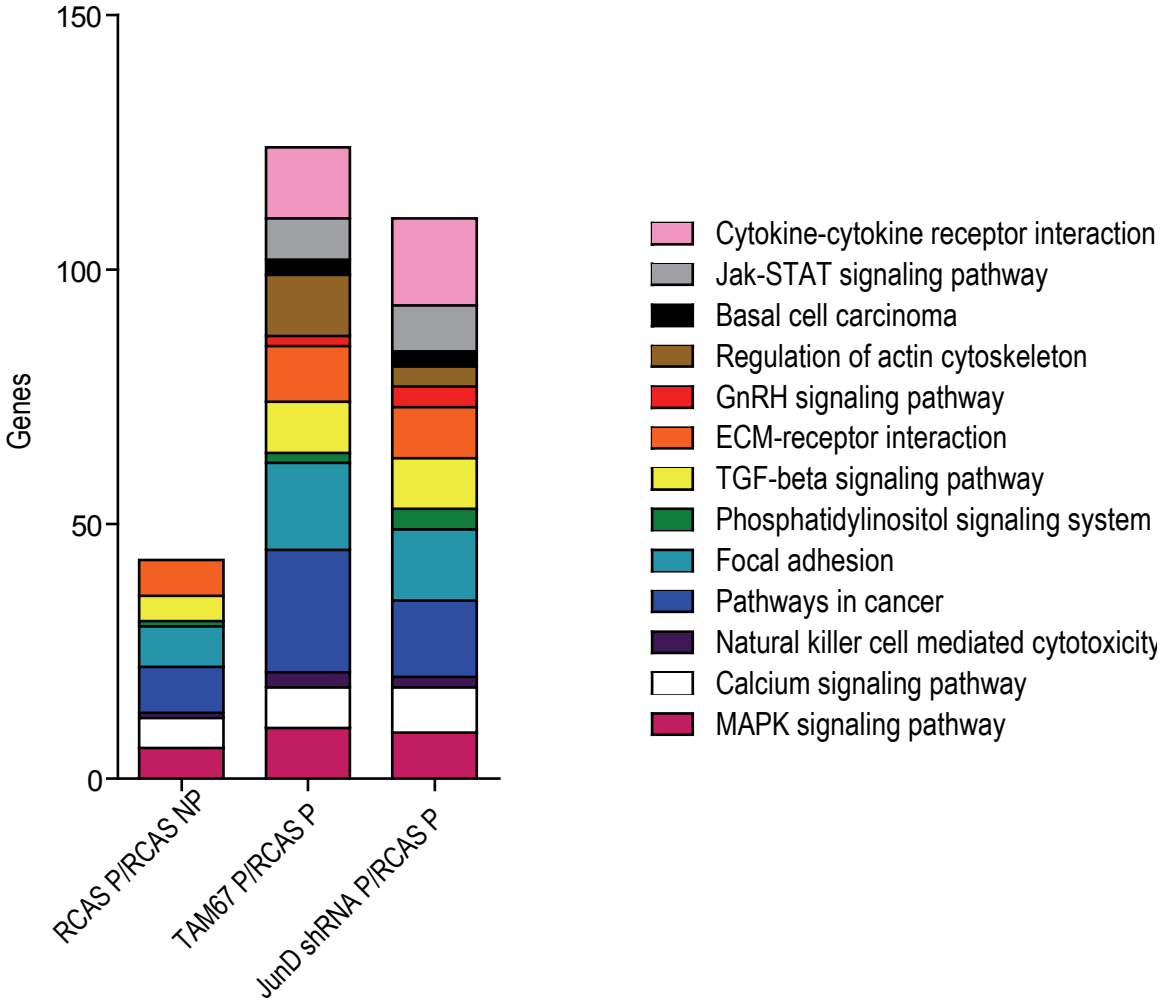
Supplementary Data 2. Euler representation of genes differentially expressed between control and AP-1 inhibited CEF under transformed and non-transformed conditions.

Each set indicates number probe-sets corresponding to genes differentially expressed at a two-fold or greater level and found to be statistically significant in the comparison indicated (multiple pairwise t-tests Bonferroni-corrected, $p<0.05$).

Supplementary Data 3. Summary of differentially expressed transcripts as determined by microarray analysis.

	Comparison	Probe-sets		
		up-regulated	down-regulated	total
1	NY72-4 P - RCASB control/NY72-4 NP - RCASB control	176	368	544
2	NY72-4 P - TAM67/NY72-4 NP - TAM67	83	72	155
3	NY72-4 P - TAM67/NY72-4 P – RCASB control	514	593	1107
4	NY72-4 P - JunD(shRNA)/NY72-4 NP- JunD(shRNA)	716	280	996
5	NY72-4 P - JunD(shRNA)/NY72-4 P - RCASB control	510	157	667

Number of transcripts differentially expressed in the various pair-wise comparisons is shown. Significance cut-off was defined as a Bonferroni-corrected t-test satisfying $P \leq 0.05$ and a minimum two-fold change in expression in each comparison. Numbers under the probe-sets heading indicate the total number of Affymetrix probe-sets hybridizing to differentially expressed transcripts. NP and P indicate CEF cells grown at non-permissive and permissive temperatures respectively.



Supplementary Data 4. Number and distribution of genes found in perturbed pathways in NY72-4 P RCASB /NY72-4 NP RCASB, NY72-4 P TAM67/NY72-4 P RCASB and NY72-4 P JunD shRNA/NY72-4 P RCASB gene comparison sets (referred to here as RCASB P/ RCASB NP, TAM67 P/ RCASB P and JunD shRNA P/ RCASB P respectively).

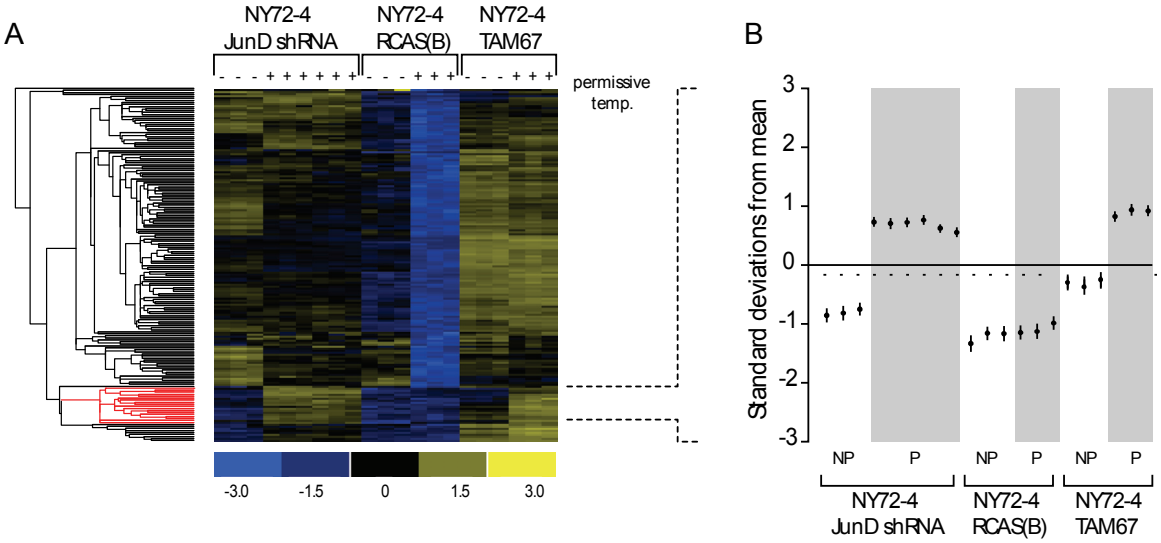
Pathway perturbation was determined by Pathway Express. Only pathways that were significantly dysregulated (corrected γ p-value <0.05) in two or more gene sets are shown.

Supplementary Data 5. Common pathways found to be dysregulated in NY72-4 P RCASB control/NY72-4 NP RCASB, NY72-4 P TAM67/NY72-4 P RCASB and NY72-4 P JunD shRNA/NY72-4 P RCASB gene comparison sets

Gene sets are referred to herein as RCASB P/ RCASB NP, TAM67 P/ RCASB P and JunD shRNA P/ RCASB P respectively.

		#Input Genes in Pathway			corrected γ p-value		
Pathways	#Genes in Pathway	RCASB P/ RCASB NP	TAM67 P/ RCASB P	JunD shRNA P/ RCASB P	RCASB P/ RCASB NP	TAM67 P/ RCASB P	JunD shRNA P/ RCASB P
Phosphatidylinositol signaling system	76	1	2	4	2.16E-13	1.79E-02	4.64E-06
Focal adhesion	203	8	17	14	5.53E-03	1.50E-05	5.49E-06
ECM-receptor interaction	84	7	11	10	9.74E-05	2.11E-05	1.51E-06
TGF-beta signaling pathway	87	5	10	10	1.66E-03	3.54E-05	2.04E-06
Pathways in cancer	330	9	24	15	8.00E-03	1.74E-05	1.99E-04
Natural killer cell mediated cytotoxicity	135	1	3	2	6.96E-06	1.36E-02	1.74E-03
MAPK signaling pathway	272	6	10	9	2.09E-02	1.24E-02	1.45E-02
Calcium signaling pathway	182	6	8	9	2.92E-02	2.50E-02	2.47E-03
Cytokine-cytokine receptor interaction	263	N.S.	14	17	N.S.	1.58E-02	2.73E-06
Regulation of actin cytoskeleton	217	N.S.	12	4	N.S.	1.21E-03	2.45E-02
Jak-STAT signaling pathway	155	N.S.	8	9	N.S.	3.24E-02	1.59E-03
GnRH signaling pathway	103	N.S.	2	4	N.S.	3.10E-03	5.00E-02
Basal cell carcinoma	55	N.S.	3	3	N.S.	4.99E-02	5.14E-03
Vibrio cholerae infection	62	N.S.	N.S.	4	N.S.	N.S.	1.22E-08
Proteasome	48	N.S.	N.D.	2	N.S.	N.D.	3.19E-05
Adherens junction	78	1	N.S.	N.S.	2.05E-04	N.S.	N.S.
p53 signaling pathway	69	N.D.	8	N.S.	N.D.	1.38E-03	N.S.
Melanoma	71	1	N.S.	N.D.	1.43E-03	N.S.	N.D.
Homologous recombination	28	N.S.	5	N.S.	N.S.	2.77E-03	N.S.
Tight junction	135	N.D.	5	N.S.	N.D.	3.26E-03	N.S.
Gap junction	96	N.S.	N.S.	4	N.S.	N.S.	6.30E-03
Type II diabetes mellitus	45	N.S.	3	N.S.	N.S.	9.67E-03	N.S.
Ubiquitin mediated proteolysis	138	N.S.	10	N.S.	N.S.	1.11E-02	N.S.
Fc epsilon RI signaling pathway	78	1	N.S.	N.S.	1.64E-02	N.S.	N.S.
Wnt signaling pathway	152	N.S.	N.S.	6	N.S.	N.S.	2.05E-02
T cell receptor signaling pathway	108	2	N.S.	N.S.	2.32E-02	N.S.	N.S.
Epithelial cell signaling in Helicobacter pylori infection	68	1	N.S.	N.S.	2.36E-02	N.S.	N.S.
Complement and coagulation cascades	69	N.S.	3	N.S.	N.S.	3.02E-02	N.S.
Long-term depression	75	N.D.	1	N.S.	N.D.	3.24E-02	N.S.
Antigen processing and presentation	89	N.S.	N.S.	4	N.S.	N.S.	3.33E-02
Toll-like receptor signaling pathway	102	4	N.S.	N.S.	4.31E-02	N.S.	N.S.
Cell adhesion molecules (CAMs)	134	N.S.	7	N.S.	N.S.	4.98E-02	N.S.

The number of genes in the pathway refers to the number of genes in the associated KEGG pathway (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/; (Ogata *et al.*, 1999)). Input genes refer to the number of differentially expressed genes that were found in that pathway. Corrected γ p-value is a measure of significance as calculated by Pathway Express. N.D. and N.S. indicate not determined and not significant respectively.



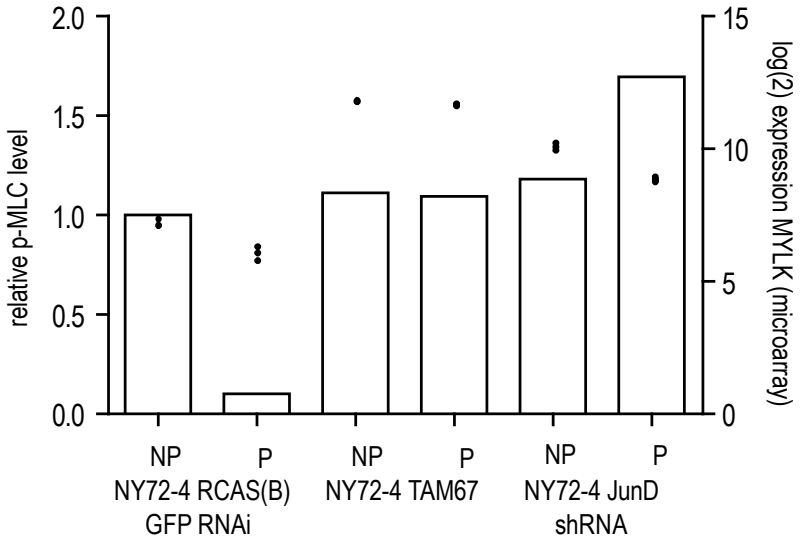
Supplementary Data 6. A cluster of 18 genes that are up-regulated in JunD shRNA and TAM67-infected cells at permissive temperature but not at the restrictive temperature, nor in either of the RCAS(B) GFP-RNAi controls

Panel A depicts unsupervised clustering of genes up-regulated in NY72-4/JunD and NY72-4/TAM67 infected CEF grown at permissive temperature in relation to control cells (NY72-4 and RCAS(B) GFP-RNAi co-infected CEF) grown at permissive temperature. Examination of clusters reveals a group of 18 probe-sets that are up-regulated in JunD shRNA and TAM67-infected cells at permissive temperature but not at the restrictive temperature, nor in either of the RCAS(B) GFP-RNAi controls (indicated in red on the dendrogram). Scale on heat map indicates standardized expression values (-3 to 3 standard deviations). "+" indicates permissive temperature. Panel B shows gene-wise mean-centred expression values for genes found in the cluster. Each point indicates the mean standardized gene expression in a replicate array for the condition indicated at the bottom. Error bars show the standard error of mean of standardized expression. NP and P indicate non-permissive and permissive respectively.

Supplementary Data 7. Common pathways found to be dysregulated in the AP-1 repression gene cluster

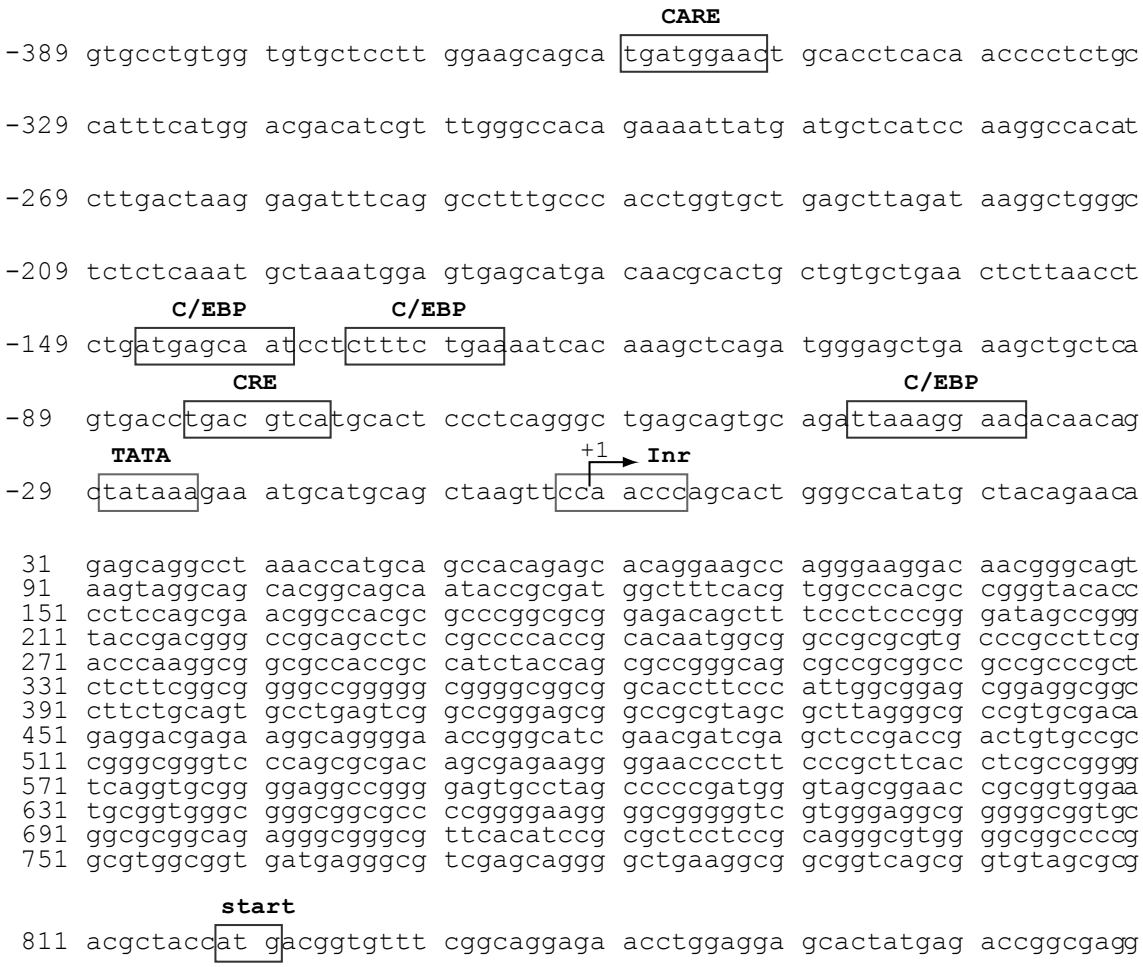
Pathway Name	#Genes in Pathway	#Input Genes in Pathway	corrected γ p-value
ErbB signaling pathway	87	2	3.08E-04
GnRH signaling pathway	103	1	5.67E-04
Pathways in cancer	330	3	9.00E-04
Jak-STAT signaling pathway	155	2	3.78E-03
Insulin signaling pathway	138	2	5.15E-03
Epithelial cell signaling in Helicobacter pylori infection	68	1	7.80E-03
Bladder cancer	42	1	1.87E-02
T cell receptor signaling pathway	108	1	4.29E-02

The number of genes in the pathway refers to the number of genes in the associated KEGG pathway (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/; (Ogata *et al.*, 1999)). Input genes refer to the number of differentially expressed genes that were found in that pathway. Corrected γ p-value is a measure of significance as calculated by Pathway Express. Pathways with p-values greater than 0.05 are not shown.



Supplementary Data 8. Relative levels of p-MLC and expression of MYLK are not correlated.

Bars show relative level of MYL12A phosphorylation at Ser19. Scatter-plot shows mean expression of MYLK. Note that MYLK level varies over fifteen fold between the lowest and highest levels of expression (NY72-4 RCAS(B) permissive and NY72-4 TAM67 non-permissive). Each point on the scatter plot represents a single replicate measurement of MYLK expression by microarray in the condition indicated. NP and P indicate non-permissive and permissive respectively.



Supplementary Data 9. The *DAPK1* upstream regulatory region includes several putative cis-regulatory elements

Nucleotide numbering is based on the putative transcriptional start at +1.

Chapter 4: Discussion

1.1 The Src-transformation signature in primary chicken cells

When this study was initiated the chief goal was to identify targets of Src-induced gene expression in primary chicken cells. By using two different primary cell types, (CEF and CNR) and four virus strains, we were able to identify gene expression signatures corresponding to transformation, and to identify similarities and differences among different v-*Src* alleles and primary cell types. Src-transformed CEF are characterized by accelerated growth, a lack of contact inhibition and high motility. CNR cells are post-mitotic and cannot be expanded *in vitro* in the absence of a transforming oncogene such as *v-Src* or *v-Myc* (Pessac, 1987). In the absence of a transforming agent, CNR eventually differentiate into neurons, photoreceptors, retinal glial cells (Müller cells), pigmented epithelial cells and lens epithelial cells (Pessac, 1987). It is perhaps unsurprising that Src-transformed CNR are enriched for genes regulating cell cycle and mitosis. Pathway enrichment analysis indicated that from the three systems studies, only the CNR set was significantly enriched for cell cycle pathway perturbation (Maslikowski *et al*. 2010; Additional File 6 & 12). This is not unexpected since transformation in CEF has normally been carried out under conditions of exponential growth in order to differentiate genes regulated by transformation and not by proliferation *per se*. A number of genes encoding proteins promoting cell cycle progression or mitosis such as cyclins, minichromosome maintenance complex factors, proliferating cell nuclear antigen, E2F1 and polo-like-kinase 1 were up-regulated in transformed CNR (*ANAPC4, BUB1, BUB1B, CCNA2,*

CCNB2, CCNE2, CDC20, CDC45L, , E2F1, MCM2, MCM3, MCM5, MCM6, ORC4L, PCNA, and *PLK1*). Conversely, the CDK inhibitor p27 and the antiproliferative gene TGFβ3 were down-regulated (*CDKN1B* and *TGFB3*). In both CEF and CNR, Src-transformation results in refractile morphology and increased motility suggestive of extensive rearrangements to the cytoskeleton and cell adhesion. Indeed pathway analyses indicated perturbations to pathways controlling ECM-receptor interaction, focal adhesion, PI3K signaling and the actin cytoskeleton (Maslikowski *et al*., 2010; Table 3). Moreover a number of genes involved in the stress response were noted in all systems studied possibly due to increased production of reactive oxygen species (see Discussion in Maslikowski *et al.*, 2010). High Src activity has been linked to poor prognosis and metastasis in breast and colon cancers (Biscardi *et al.*, 2000; Irby & Yeatman, 2000; Irby *et al.*, 1999a) and successful metastasis requires both intra- and extravasation of tumorigenic cells. As such, enrichment for genes involved in leukocyte transendothelial migration (Maslikowski *et al*., 2010; Table 3) is consistent with an invasive phenotype induced by Src-transformation.

A notable feature of our study is the relative dissimilarity of the genes that were differentially expressed between our three experimental groups (TR set, CNR NY72-4 set and CEF NY72-4 set) (Maslikowski *et al.*, 2010; Figure 2). However, there were a number of biological processes that were commonly dysregulated among all three systems. In one way, this observation demonstrates a feature noted by groups studying prognostic tumor signatures, namely that different gene signatures demonstrate a concordance with respect to pathways or processes, but not always specific genes (Yu *et*

al., 2007; Thomassen *et al.*, 2007; Wirapati *et al.*, 2008; Haibe-Kains *et al.*, 2008a). A similar observation was made by Marius Sudol's group when comparing Src activation in CEF and murine 3T3 fibroblasts. Src transformation was shown to activate the urokinase plasminogen activator (PLAU) gene in CEF but not in murine 3T3 cells. Instead, 3T3 cells showed activation of downstream genes such as *mmp1b* (Sudol, 2011). The differences observed between SR-A-transformed and NY72-4 transformed CEF are similarly striking. The differences would suggest that thermolabile and constitutively transforming v-*Src* alleles are qualitatively different and that ts strains are not merely attenuated versions of a constitutively transforming RSV, an observation consistent with previous studies (Stoker *et al.*, 1986; Garber *et al.*, 1987; Hamaguchi *et al.*, 1988).

A more recent analysis of the data revealed additional aspects of Src-transformation in primary cells pertaining to the role of Src in differentiation in CEF and CNR. Moreover it provides insight into the 42-gene aggressive tumor signature and the rationale for the study. These will be discussed below.

1.2 Src promotes a state of de-differentiation in CEF

One of the groups of processes regulated by Src is epithelial to mesenchymal transition (EMT) (Shah & Gallick, 2007). Fibroblasts are not epithelial cells and do not technically undergo EMT, however, Src-transformation in CEF regulates a number of processes that phenotypically resemble some aspects of EMT. EMT is characterized by cells with poor differentiation, high motility, invasiveness, reduced adhesion, and chemoresistance (Qin *et al.*, 2012). Indeed, Src-transformed CEF and CNR exhibit a repression of markers of

differentiation (see below) and work in our lab has shown that v-Src-transformed CEF are more motile than their non-transformed counterparts and are more resistant to the chemotherapeutic drugs etoposide and thapsigragin (see Appendix 1, Figure 8 and (Gregory, 2009; Liang, 2010)). EMT is further characterized by a number of changes to molecular markers, most notably the cadherin switch (Yilmaz & Christofori, 2009). In both CEF and CNR infected with either the ts or wt RSV-Src, the *CDH2* (N-cadherin) gene was up-regulated while in CEF, E-cadherin was down-regulated at the protein level[23] (*Maslikowski et al.*, 2010, Additional Files 2 & 4; Appendix 1, Figure 13). Importantly, the EMT phenotype is associated with cancer invasion and stemness, and an instrumental driver of this transformation is the transcription factor Twist and the related factors Snail (*SNAI1*) and Slug (*SNAI2*) (Qin *et al.*, 2012). Overexpression of Twist alone is sufficient to induce EMT (Yang *et al.*, 2004) and *Twist* is up-regulated in CEF infected with either SR-A or NY72-4 (Maslikowski *et al.*, 2010, Figure 4, Additional Files 2 & 4). EMT is normally a temporary de-differentiated state induced during periods of cellular migration during embryogenesis, however, its induction during tumorigenesis promotes metastasis (Yang *et al.*, 2004). While Src can induce a de-differentiated EMT-like state in CEF, our study also showed that Src antagonizes differentiation in CEF and CNR by several pathways other than EMT.

Though Src is known to promote or regulate differentiation in a number of cell types, including myeloid and lymphoid cells and osteoclasts (Horne *et al.*, 2005; Johnson,

[23] The up-regulation of *CDH2* in CNR infected with NY72-4 was significantly different but was only increased by 1.87 fold and therefore did not meet the threshold criterion for analysis. E-cadherin levels were not determined for CNR.

2008; Salmond *et al.*, 2009), a number of early seminal studies showed that Src represses differentiation of a number of mesodermally-derived cell types including chondroblasts and myoblasts (Pacifici *et al.*, 1977; Boettiger *et al.*, 1977; Fiszman, 1978; Boettiger *et al.*, 1983; West & Boettiger, 1983). Consistent with this, examination of the genes down-regulated in CEF transformed by SR-A or NY72-4 shows enrichment for genes involved in differentiating myoblastic and chondroblastic lineages. In SR-A infected CEF, genes coding for a number of structural muscle proteins such as cardiac and skeletal muscle actin α1 and cardiac muscle myosin heavy chain α6 (*ACTC1*, *ACTA1* and *MYH6* respectively) are repressed. The gene coding for connective tissue growth factor (*CTGF*) is down-regulated in NY72-4 infected CEF while a number of chondrocyte or connective tissue specific collagen genes (*COL2A1*, *COL11A1* and *COL3A1*) are found down-regulated in both NY72-4 and SR-A infected CEF.

Although our study was not aimed specifically at addressing differentiation, other work by colleagues in our lab uncovered a role for AP-1 in the trans-differentiation of fibroblasts into adipocytes. Indeed, a more recent study by our lab showed that repression of Fra2 in v-Src transformed CEF promotes adipogenesis indicating that Fra2 blocks differentiation under conditions of Src-transformation. Similarly, inhibition of AP-1 by the *Jun* dominant-negative allele *TAM67* converts a proportion of CEF into adipocytes presumably by the sequestration and inhibition of Fra2 (Wang *et al.*, 2011a). In CEF transformed by ts Src, PPARγ, a master regulator of adipogenesis, is repressed two and half fold (Maslikowski et al., 2010; Additional File 4). The expression profile of transformed CEF expressing TAM67 but not the *JunD* shRNA shows a two fold increase

in expression of PPARγ compared to normal transformed CEF (raw p= 0.023 and 0.53 respectively)[24] consistent with the notion that Fra2 inhibits adipogenesis in Src-transformed CEF by repressing *PPARγ*. Indeed, a previous study by Fu *et al.* demonstrated that overexpression of AP-1 factors inhibited activation of the PPARγ promoter in cultured human hepatocytes and rat aortic smooth muscle cells (Fu *et al.*, 2003). Interestingly, C/EBPα expression is also increased over two fold in transformed CEF expressing TAM67 but not the *JunD* shRNA when compared to normal transformed CEF (Chapter 3, Supplementary Data 1). Recently we showed that C/EBPβ activity was stimulated by the repression of AP-1 by TAM67 (Wang *et al.*, 2011a). Indeed, both adipogenic transcription factors *PPARγ* and *C/EBPα* lay downstream of C/EBPβ and require C/EBPβ activation for induction (Farmer, 2005). Together, these data would suggest that adipogenic conversion of Src-transformed CEF by TAM67 works through a dual mechanism of derepression of PPARγ by inhibition of AP-1 (specifically Fra2) and the activation of PPARγ and C/EBPα by C/EBPβ (Figure 1). Increased expression of PPARγ and C/EBPα then generates an autoregulatory loop that further sustains PPARγ and C/EBPα levels resulting in exit from cell cycle and adipogenic conversion. The observation that *Fra2* shRNA is a less potent activator of adipogenesis than TAM67 (Wang *et al.*, 2011a) is consistent with this model. Moreover, previous work in our lab has shown that overexpression of C/EBPβ induces adipogenesis in CEF (Kim *et al.*, 1999b)

[24] The p-values for this study were corrected using the Bonferroni method. The Bonferroni-corrected p-value for PPARγ is 0.069 in transformed CEF expressing TAM67 compared to normal transformed CEF. It should be noted that Bonferroni correction is considered a conservative method of correcting for multiple testing and may increase the possibility of type II error (Bland & Altman, 1995).

1.3 Src promotes a state of de-differentiation in CNR

Avian neuroretinal cells in culture express some features of differentiated cells even when proliferating and transformed by v-Src. v-Src transformed CNR and QNR (quail neuroretinal) cells can form synapses and express elevated levels of glutamic acid decarboxylase, the enzyme mediating the synthesis of the neurotransmitter GABA (Pessac, 1987; Crisanti *et al.*, 1985; Crisanti-Combes *et al.*, 1982b). A role for Src in neurite outgrowth has also been described in pheochromocytoma-derived PC12 cells (Alema *et al.*, 1985; Thomas *et al.*, 1991) and embryonic neural tissues in the chick have elevated tyrosine kinase activity mediated by the expression of a splicing variant of c-Src (Martinez *et al.*, 1987; Brugge *et al.*, 1985). However, expression of a ts *v-Src* allele has also been shown to reversibly block differentiation of QNR and lens epithelial cells into lentoid bodies and to block expression of α and δ-crystallin lens proteins (Simonneau *et al.*, 1986; Menko & Boettiger, 1988) suggesting a role for Src in maintaining a de-differentiated state in neuroretinal cultures.

Although in our study cells were not maintained in culture for the extended periods of time required for neuroretinal cell differentiation (Calothy *et al.*, 1980), genes for several kinesin motor proteins, including the neuron-specific *KIF5C* gene, were activated in v-Src transformed CNR cells suggesting that some of the features of differentiation were established in our experimental conditions. Nevertheless, a number of down-regulated genes involved in the positive regulation of axon guidance were identified by PathwayEpress in our study (Maslikowski et al., 2010; Additional File 6 &

12) including genes coding for ephrin receptors, Fak, class 3 semaphorins and the Rac1 effector Pak1 (*EFNA5, EPHA3, EPHA7, FAK, PAK1, SEMA3C* and *SEMA3D;* (Giger *et al.*, 2010; Chacon & Fazzari, 2011; Schmid *et al.*, 2004)). Moreover, several markers of lens differentiation such as the gene for αB-crystallin (*CRYAB*) and *Pax6* were found to be down-regulated more than five fold (Maslikowski *et al.*; Additional File 6). Overexpression of *Pax6*, a master regulatory gene, in vertebrate ectoderm and insect imaginal discs is capable of inducing ectopic eye development (Halder *et al.*, 1995; Chow *et al.*, 1999). *Pax6* mouse knock-outs result in the ablation of eye development while wild-type levels of *Pax6* expression are required for the formation of the retinal pigmented epithelium from the neuroretina, suggesting roles for Pax6 both in eye morphogenesis and melanogenesis (Grindley *et al.*, 1995; Baumer *et al.*, 2003) and reviewed in (Kozmik, 2008; Kozmik, 2005)). Indeed, Pathway Express data of the CNR set show enrichment of five down-regulated genes; CREB2L3, endothelin1, endothelin receptor type B, frizzled 1 and PLC beta 2, in the melanogenesis pathway (Maslikowski *et al.*, 2010; Additional Files 6 & 12). These genes are members of the Wnt, cAMP/PKA, and endothelin receptor signaling pathways known to regulate melanogenesis, which is required not only for development of the retinal pigmented epithelium but also the retina itself (Yanfeng *et al.*, 2003; Sturm *et al.*, 2001; Abdel-Malek *et al.*, 2008; Jeffery, 1998; Strauss, 2005). Consistent with this, early studies showed that differentiated melanosomes transformed by ts alleles of v-Src reversibly lose their epithelial morphology, melanin production and melanosome assembly (Boettiger *et al.*, 1977).

Pax6 is also required for the activation of various crystallin protein genes in vertebrates and in other phyla (Kozmik, 2005). In chicken, Pax6 was shown to act cooperatively with USF-1 and CREB to activate the αA-crystallin gene in primary lens epithelial cells but not in CEF (Cvekl *et al.*, 1994). Conversely, EMSA analysis of nuclear extracts from CEF but not from lens cells showed the presence of the AP-1 proteins JunD and Fra2 bound to the inactive αA-crystallin gene promoter suggesting that AP-1 acts as a negative regulator of αA-crystallin expression (Cvekl *et al.*, 1994). It should be noted that JunD and Fra2 are the dominant forms of AP-1 in v-Src transformed CEF and that Fra2 functions in part to suppress differentiation in v-Src-transformed CEF (Wang *et al.*, 2011a). Together, these data would indicate that Src functions through multiple parallel pathways to repress differentiation in CNR cells. Further, these data may show that the repression of myoblast and chondroblast markers, along with suppression of adipogenesis in CEF, and the suppression of melanogenesis and lens differentiation in CNR, is a result of a generalized program of differentiation suppression by v-Src in primary avian cells.

1.4 A 42-gene set derived from primary avian cell predicts poor prognosis in human cancer

In this thesis we defined a set of 175 CSR (Common set of v-Src Regulated) genes as the convergent set of all genes regulated in CEF and CNR by v-Src. Using the 80 up-regulated CSR genes we employed hypothesis-driven (bottom-up) approach aimed at identifying possible prognostic markers in breast cancer. By interrogating two independent tumor data sets we identified a set of 42 genes found to be up-regulated in

the most aggressive tumors. Validation of this gene set against six independent tumor data sets showed that tumor samples expressing a high level of the aggressive gene set relative to low expressing tumor samples were associated with poor prognosis or high recurrence of metastases in breast and lung tumors but not in colon or ovarian tumors (Maslikowski *et al.,* 2010; Figure 5). The study was designed to characterize the global gene expression pattern in Src-transformed avian primary cells and was not focused on developing prognostic gene expression markers for human disease. As such, the analysis is non-canonical and includes some caveats to which attention should be drawn. First it should be noted that the 42-gene signature is an aggregate signature and does not assign preferential weighting to individual genes and therefore prognostic ability of each gene was not evaluated. Second, no objective gene expression threshold was employed in evaluating the prognostic ability of the 42-gene set but only a statistical measure, namely the upper quartile of high expressing samples was compared against the remaining data. Consistent with this, lowering the threshold by comparing the bottom and top halves of the survival data reduces the power to discriminate between a poor and good prognosis as determined by receiver operator characteristic analysis (Figure 2). Despite this, the 42-gene signature, as employed above, performs similarly well to the five gene signatures tested in the meta-analyses by Yu *et al.* (average AUC[25] of 0.67 and 0.70 for ER+ and ER- tumors respectively versus a range of 0.665-0.725 for the tumor sets we tested). However, since relative measures of expression were used in our study, this approach, as

[25] AUC indicates area under the curve. In this case, the interpretation for AUC is that it represents the probability that a randomly selected individual with a "poor" survival outcome will be classified as having a "poor" outcome versus a randomly selected individual with a "good" outcome.

is, could not be used for prognostication on a case by case basis but would require a pooled database of expression data or an objective quantitative measure of gene expression. Moreover, inter-experimental and platform variability would make it difficult to compare relative expression of different tumor samples unless processed simultaneously.

Nevertheless, this study is a proof of concept that even highly divergent vertebrate *in vitro* biological models can be used to make testable predictions about complex disease states like cancer. Indeed, functional analysis of the aggressive signature shows that all but two of the sixteen of the GO categories (see Chapter 2, Additional File 14) to which the genes belong, overlap with the core GO categories identified by Yu *et al*. as the defining poor-prognosis pathway (Yu *et al.*, 2007). The five signatures tested by Yu *et al.* were well-validated signatures including the GGI signature, and two signatures since commercialized into diagnostic tests (Mammaprint and OncoType DX) (Yu *et al.*, 2007; Sotiriou & Piccart, 2007). Other studies testing the validity of multiple gene signatures have similarly concluded that successful prognostic signatures are enriched for genes involved in proliferation, cell cycle progression and also differentiation (Thomassen *et al.*, 2007; Wirapati *et al.*, 2008; Haibe-Kains *et al.*, 2008a). Of the three genes from the 42-gene signature (*CCNE2, EXO1* and *KIF11*) that were found in two or more studies analysed by Yu *et al.,* all three genes are involved in proliferation or cell cycle progression. Interestingly, despite no attempt to classify tumors by IHC criteria, the 42-gene signature may be able to discriminate between different tumor types. The top three hits of a gene signature comparison analysis using GeneSigDB (Culhane *et al.*, 2012)

show that the 42-gene list is enriched for genes discriminating luminal/basal/apocrine breast tumors as well as ER and p53 status ((Farmer *et al.*, 2005; van 't Veer *et al.*, 2002; Miller *et al.*, 2005); $p<10^{4}$; 21, 18 and 18 genes respectively).

1.5 Appropriate biological material and correct assumptions are essential for accurate expression profile analysis

Finally, our study underscores the importance of basing gene expression profiling analyses on proper biological starting material and protocol. Comparison of our study with a similar one conducted by Masker *et al*. (Masker *et al.*, 2007) showed little overlap between gene signatures despite using the same RSV strains and cell type. Inspection of their data shows expression of genes not known to be normally activated in v-Src transformed CEF such as the immediate early genes *Myc* and *Jun* (see Introduction). Their expression profile also lacks induction of known markers of Src-transformation such as IL8 and CD44 (Bedard *et al.*, 1987a; Jamal *et al.*, 1994) possibly suggesting different culture conditions or microarray post-processing conditions.

In a high profile study from Duke University, Bild *et al.* identified gene signatures associated with several oncogenes including Src (Bild *et al.*, 2006). Gene signature comparisons showed that their gene set for activated Ras is more enriched for our 42-gene set than their Src set (GeneSigDB; Benjamini-Hochberg corrected $p<10^{-4}$ and $p>0.05$ respectively). Unlike overexpression of activated Ras, overexpression of WT Src is not transforming. Therefore, since Bild *et al.* did not employ an activated *Src* allele our signature may be more similar to an activated *Ras* profile because Ras signals

downstream of activated Src. Interestingly, follow-up work based on this study demonstrated that the Bild *et al.* Src signature performed poorly in assessing dasatanib sensitivity when compared to two other gene indices (see Introduction; (Moulder *et al.*, 2010)) further suggesting that the study by Bild *et al.* relied on a false premise, namely that overexpression of a wt *Src* allele is an appropriate model for cancer.

One of the central rationales for conducting our gene expression study in primary cells was that immortalized cell lines display some characteristics of transformation, namely indefinite mitotic potential, increased proliferation, aneuploidy and in some cases, a lack of contact inhibition. Indeed, some cultured fibroblast lines exhibit spontaneous transformation at high densities, a property affected by culture conditions and serum concentrations (Matthews, 1993). Therefore, one could reason that some immortalized cells are either partially transformed or primed for transformation. A recent gene expression profile study of DF-1 cells provided some insight into the validity of this rationale. DF-1 cells are spontaneously immortalized fibroblasts derived from endogenous virus-free CEF. DF-1 cells exhibit a more fusiform morphology when compared to CEF and grow more rapidly (Kong *et al.*, 2011). Kong *et al.*, recently conducted a gene profile study comparing normal low passage CEF to DF-1 cells (Kong *et al.*, 2011). Among the 904 annotated differentially expressed genes, they found several up-regulated oncogenes (*Src, APCDD1, Twist2*) and several down-regulated tumor suppressors including *CDKN2B* ($p15^{INK4B}$). Perhaps most interestingly, 18% of the dysregulated genes are also found in our transformation-regulated (TR) gene set (Fisher's exact test, p=0.023). Among these, 11 genes from the 42-gene aggressive gene signature

were found up regulated in the Kong *et al.* study (*CEP55, E2F8, HELLS, ITGA4, KIF11, NASP, NOC2L, ODC1, SOCS1, TTC35*, and *VRK1;* Fisher's exact test, $p<10^{-4}$). Taken together, these data would suggest that DF-1 cells are, if not transformed, then well on the way to becoming so. Any experimental undertaking to assess the changes in gene expression in relation to Src-transformation using this cell line would have underestimated the changes in gene expression, or confounded interpretation of the data.

2.1 AP-1 promotes survival in CEF by antagonizing C/EBPβ-dependent activation of *DAPK1*

Prolonged activation of certain oncogenes leads to premature growth arrest, a phenomenon known as oncogene-induced senescence (OIS) (Mallette & Ferbeyre, 2007). MEFs transformed by activated Ras have elevated levels p53, $p16^{INK4A}$ and $p21^{Waf1}$ and undergo OIS unless transformed by a cooperating oncogene or accompanied by the inactivation of p53 (Serrano *et al.*, 1997). Transformation of CEF by v-Src is sufficient to bypass OIS but inhibition of AP-1 reduced proliferation via three distinct processes: senescence, apoptosis and transdifferentiation (Wang *et al.*, 2011a). The most dramatic phenotype, that of apoptosis, was induced by both the Jun dominant negative allele *TAM67* and by *JunD* down-regulation by shRNA. To identify potential genes regulating this process, we compared expression profiles of v-Src-transformed and non-transformed CEF expressing either TAM67 or *JunD* shRNA. We identified a co-regulated cluster of 18 probe-sets. Four of the thirteen annotated genes belonged to the interferon pathway, including the pro-apoptotic gene *DAPK1* (Deiss *et al.*, 1995). Previous work by our lab

showed that inhibition of JunD activity by shRNA increases apoptosis in v-Src-transformed CEF approximately twelve-fold and abrogates colony formation in soft agar (Wang *et al.*, 2011a). Inhibition of DAPK1 by shRNA rescued colony formation in transformed CEF expressing the *JunD* shRNA. Similarly shRNA repression of an upstream factor of DAPK1 (C/EBPβ) and a downstream factor (p53), (Raveh *et al.*, 2001; Gade *et al.*, 2008) rescued colony formation (Chapter 3, Figures 3 & 4; Wang *et al.*, 2011). Molecular resection of the DAPK1 promoter revealed two activating elements consisting of a proximal CRE and a distal C/EBP-binding site. Analyses by previous groups showed that the murine DAPK1 promoter was similarly arranged suggesting evolutionary conservation between avians and mammals in the regulation of DAPK1 expression. Down-regulation of C/EBPβ using a dominant negative allele was shown to attenuate DAPK1 promoter activity while inhibition of AP-1 by TAM67 or *JunD* shRNA activated DAPK1 promoter activity in conditions of Src-transformation. Interestingly, ChIP experiments showed recruitment of C/EBPβ during conditions of transformation and AP-1 inhibition, however no direct interaction was observed for JunD under normal, transformed or AP-1 repressed conditions suggesting an indirect repression of DAPK1 activity by AP-1 (Chapter 3, Figure 9). One hypothesis for this is that AP-1 and C/EBPβ compete for coactivators such as p300/CBP, a phenomenon described previously for both factors (Webster & Cidlowski, 1999; Manna *et al.*, 2009). Indeed, previous studies by our lab showed that overexpression of AP-1 inhibits CAAT-box activity while TAM67 activates it and, conversely, overexpression of C/EBPβ represses TRE activity while expression of the C/EBPβ dominant negative mutant activates it (Gagliardi *et al.*, 2003;

Wang *et al.*, 2011a). Since neither C/EBPβ nor AP-1 bind to the cognate binding sites of the other, these data are consistent with the coactivator competition hypothesis. Nevertheless, this has not been formerly shown and will require further analysis.

2.2 A novel gene interaction network regulating survival in CEF

A second outstanding question that remains is how C/EBPβ and AP-1 are regulated in the context of DAPK1 signaling. Interestingly, all three factors converge on extracellular regulated kinase (ERK). Src transformation is known to activate ERK2, resulting in the phosphorylation and activation of Fra2 (Murakami *et al.*, 1997), while work by our lab and others has shown that both the Jun and JunD TAD are activated by ERK (Vinciguerra *et al.*, 2004) (data not shown). Activation of the *DAPK1* gene in response to IFNγ was shown to be dependent on ERK1/2 activation of C/EBPβ (Gade *et al.*, 2008) and Hu and colleagues have recently demonstrated that ERK2 can function as a transcriptional repressor in IFNγ-signaling by binding variant C/EBPβ sites known as GATEs (gamma activated transcription element) (Hu *et al.*, 2009). Although work in our lab has shown that ERK2 can repress activation of the growth arrest associated gene *p20K* under conditions of normal cell division (Athar, 2011), there is presently no evidence that it does so in the case of *DAPK1*. Indeed, mutation or deletion of the putative GATE in the *DAPK1* promoter should have potentiated reporter activity in Src-transformed CEF but did not (Chapter 3, Figures 6 & 7). Interestingly, DAPK1 itself has been shown to preferentially bind to ERK2, leading to its activation and subsequent cytoplasmic sequestration of ERK in a bidirectional signaling mechanism described by Chen and

colleagues (Chen *et al.*, 2005a). I therefore propose the following model as illustrated in Figure 3. Activation of Src activates ERK1/2 which stimulates survival by activating AP-1. Simultaneously ERK1/2 activates C/EBPβ; however, coactivator competition prevents full activation of C/EBPβ-dependent genes, i.e. *DAPK1*. In Src-transformed CEF expressing TAM67 or the *JunD* shRNA, full activation of C/EBPβ occurs, allowing full induction of *DAPK1* ultimately resulting in apoptosis. How this is achieved in the v-Src-CEF system is presently unknown, but according to Chen *et al.*, DAPK1 is activated by ERK and simultaneously prevents ERK translocation. This results in a bipartite switch in equilibrium from pro-survival, to pro-apoptosis. By preventing nuclear translocation, ERK cannot phosphorylate its pro-survival targets (Chen *et al.*, 2005a), simultaneously, activated DAPK1 induces p53-dependent anoikis by suppressing integrin signaling (Wang *et al.*, 2002), or directly by up-regulating the $p19^{ARF}$-p53 pathway (Raveh *et al.*, 2001). Whether this occurs in CEF remains to be investigated.

2.3 Future directions in DAPK1 research

2.3.1 Coactivator competition hypothesis

There are two predictions that can be made to test this hypothesis. First, if coactivators are limiting, overexpression of p300/CBP should alleviate DAPK1 repression by AP-1/JunD. Second, the amount of p300/CBP associated with C/EBPβ should increase upon repression of AP-1 and *vice versa*. The first prediction can be investigated by immunoblotting or qPCR for DAPK1 in conditions of p300/CBP overexpression, or in the case of excessive lethality, DAPK1 promoter reporter assays could be conducted as a

proxy measure of *DAPK1* activation. The second prediction could be addressed by co-immunoprecipitation of p300/CBP-C/EBPβ under conditions of AP-1/JunD repression in transformed CEF.

2.3.2 ERK-dependent signaling model

To explore this model, ERK-dependent activation of C/EBPβ can be investigated by the use of MEK inhibitors or by RNAi targeting of ERK1 and ERK2 in conditions of AP-1/JunD inhibition in transformed CEF. If suppression of C/EBPβ activity is observed in AP-1-repressed cells, attempts to rescue the apoptosis phenotype could be conducted by the expression of C/EBPβ containing a phospho-mimetic ERK phosphorylation site (T188D in mouse, T220D in chicken). ERK-DAPK1 co-localization could further be addressed by fluorescence microscopy in the presence and absence or MEK inhibitors or ERK1/2 RNAi in conditions of AP-1/JunD.

2.3.3 Activation of *DAPK1* CRE by ATF4

Another question that remains concerns the regulation of *DAPK1* by the CRE. Mutational analyses of the chicken *DAPK1* promoter showed that the proximal CRE is required for full activation of *DAPK1* under conditions of AP-1 inhibition in transformed CEF (Chapter 3, Figures 6 & 7). What is not known is whether C/EBPβ acts solely or in concert with other factors. Indeed, overexpression of C/EBPβ in transformed CEF does not induce apoptosis (data not shown), suggesting that C/EBPβ cooperates with one or more factors to activate *DAPK1*. This observation also suggests that C/EBPβ requires the cooperativity of a factor induced under conditions of AP-1/JunD inactivation. One

possible factor is ATF4. C/EBPβ is known to dimerize with ATF4 to activate a number of gene promoters including somatostatin, IL1B, enkaphalin and phosphoenolpyruvate carboxykinase (Vallejo *et al.*, 1993; Vinson *et al.*, 1993; Tsukada *et al.*, 1994). In addition to binding canonical CREs, (Podust *et al.*, 2001), C/EBPβ-ATF4 heterodimers have been shown to bind C/EBP-CRE composite sites known as CAREs (C/EBP-ATF response elements) in order to induce transcription of genes in response to amino acid starvation and ER-stress (the so-called amino acid response and unfolded protein response, or AAR and UPR respectively; reviewed in (Kilberg *et al.*, 2009; Tsukada *et al.*, 2011)) Translational induction of ATF4 occurs under conditions of amino acid depletion and endoplasmic reticulum-stress (ER-stress) resulting from overactive metabolism and redox imbalance (Hetz, 2012; Clarke *et al.*, 2012). Previous studies in MEFs and transgenic mice have shown that both Jun and JunD protect against oxidative stress by lowering intracellular H_2O_2 (Gerald *et al.*, 2004; Laurent *et al.*, 2008; Meixner *et al.*, 2010). It is possible therefore that AP-1/JunD repression in rapidly-dividing transformed CEF may lead to stress-induced activation of ATF4. Induction of ATF4 along with full activation of C/EBPβ could then lead to full activation of *DAPK1*.

In order to address this question, one would have to assess ATF4 proteins levels in transformed CEF with and without AP-1 repression via immunoblotting. Second, one could investigate if ATF4 interacts with the *DAPK1* CRE under conditions of AP-1 repression in transformed CEF by using ChIP. To address if ATF4 is activating *DAPK1* via the CRE site, reporter assays could be carried out on the promoter deletion/mutation constructs. To see if AP-1/JunD repression changes the equilibrium of C/EBPβ-ATF4 to

C/EBPβ-C/EBPβ dimers, immunoprecipitation experiments could be carried-out in conditions of AP-1 repression in transformed CEF to see if C/EBPβ-ATF4 dimers form preferentially compared to transformed CEF with normal AP-1 activity. Finally, to assess the biological relevance of ATF 4 in the induction of *DAPK1*, one could repress ATF4 by shRNA to see if *DAPK1* expression and the apoptosis phenotype are rescued in the JunD shRNA background in transformed CEF.

2.3.4 DAPK1 and oxidative stress

DAPK1 has been characterized in a number of stress induced contexts (Michie *et al.*, 2010). Several studies have shown DAPK1 to play a role in inducing apoptosis or autophagy under conditions of oxidative stress (Eisenberg-Lerner & Kimchi, 2007; Eisenberg-Lerner & Kimchi, 2012) while a recent study has also shown that DAPK1 can induce the active conformation of the enzyme pyruvate kinase M2 (PKM2) to mitigate the Warburg effect (Mor *et al.*, 2012). Src on the other hand has been shown to protect cells under conditions of oxidative stress by activating NFκB (Storz *et al.*, 2004a; Storz *et al.*, 2004b). Conversely, JunD deficient MEFs were shown to be sensitive to oxidative stress and exhibit higher H_2O_2 levels leading to the activation of the HIF1α-inducible pro-angiogenic factor VEGF-A. (Gerald *et al.*, 2004; Lamb *et al.*, 2003b). Indeed, our array studies show an increase of the VEGF-A in transformed cells lacking JunD (Chapter 3, Additional File 1). Interestingly, both DAPK1 and Src have been shown to signal through the serine/threonine kinase PKD. Src and DAPK1 regulate responses to oxidative stress via the phosphorylation of different PKD residues resulting in the activation of different downstream pathways (Eisenberg-Lerner & Kimchi, 2007; Eisenberg-Lerner & Kimchi,

2012; Storz *et al.*, 2004a; Storz *et al.*, 2004b). Gene profiling experiments on contact inhibited CEF have shown the activation of a number of hypoxia markers. Follow-up experiments by our group have shown the C/EBPβ-dependent induction of several hypoxia responsive genes under conditions of low O_2 suggesting that C/EBPβ plays a role in oxygen sensing (See Appendix 2). Since previous studies have observed that reactive oxygen species (ROS) play a role in oxygen sensing (Brunelle *et al.*, 2005), it is possible that C/EBPβ may function in ROS sensing as well. Furthermore, under conditions of rapid cell growth, as is the case during oncogenic transformation, cells in culture frequently undergo chronic or intermittent hypoxia due to rapid consumption of O_2 from the surrounding medium (C. Nurse & S. Brown, pers. comm.). Although a speculative line of inquiry, the investigation of C/EBPβ-dependent DAPK1 signaling under conditions of oxidative stress, hypoxia or glucose limitation could be of great interest given the recent focus in metabolic aspects of tumorigenesis over the last decade (Mor *et al.*, 2012).

3 Perspective on *in silico* promoter discovery

One of the objectives throughout this thesis was the discovery and characterization of novel Src-responsive genes. One aspect of this was the *de novo* characterization of Src-responsive genes using *in silico* approaches. To assist the process, a number of premises were defined to guide the investigation. First, we defined an SRU as having a minimum of four binding sites consisting of an AP-1, an NFkB and two C/EBPβ sites. Second, we defined the SRU as spanning a relatively short region of DNA, namely 120 bp. Third, we

believed that by using an integrative approach aimed at correlating Src-responsive gene expression to SRU position and/or probability score, we could quickly define novel Src-responsive genes. The complexity of the endeavor proved challenging and the underlying assumptions must be questioned. Indeed, as demonstrated in the case of the *K60* and *ITGA8* pSRUs tested, the mere presence of all four requisite binding sites (AP-1, NF-kB and two C/EBPβ) (Gagliardi *et al.*, 2001) was not sufficient to activate transcription in response to Src activation. The one pSRU that was inducible was very weak and likely non-functional *in vivo*. Moreover, the gene to which it was proximal (*PIK3CD*) may not be a *bona fide* Src-inducible gene (see Appendix 1).

Since both AP-1 and NFκB members are known to synergize with each other and C/EBPβ (Ozanne *et al.*, 2007) via direct interaction, it is likely that the distances and arrangement between the sites, i.e. the organization of transcription factors along the DNA helix, is important for Src-induced activation. Underlying assumptions about what precisely constitutes an SRU, such as the number of binding sites, distance between sites and perhaps even types of sites may have to be revised. Certainly the relative distance and position of sites has to be considered. Structural data on the binding of the NFκB proteins RelA and p50 to the HIV LTR promoter shows that NFκB dimers bind in opposing orientation on the DNA strand (Stroud *et al.*, 2009). The cores of the two binding sites are spaced 15 nucleotides apart indicating 90^{o} orientation of these sites. Another group showed that C/EBPβ interacts directly with c-Myb on the *tom1* promoter via DNA loop formation (Tahirov *et al.*). The same group has structural data indicating that C/EBPβ can dimerize tandemly by loop formation as well (Protein Database ID: 1GTW). Mutational

data from our lab suggest that C/EBPβ works cooperatively with AP-1 and NFκB in the activation of *IL8* implying that relative positioning of the sites may indeed be important for the SRU (Gagliardi *et al.*, 2001; Bojovic *et al.*, 1996). Although there has been success with *in silico* discovery of functional transcription binding sites (Silvestri *et al.*, 2008), other groups have reported that *de novo in silico* discovery has high false positive rates and have indicated that other empirical data are required for better binding site discovery (Warnatz *et al.*, 2010). For example, recent developments in protein-binding microarray technology allow for a high-throughput methodology for defining consensus biding sites (Bulyk, 2007). This is only one piece of the puzzle however. Although promoter strength is correlated with proximity to the transcriptional start site, both enhancer and insulator regions can act over megabases (Splinter & de Laat, 2011). Furthermore, DNA binding sites must be in regions of DNA that are accessible and not heterochromatized. Regardless, some key piece of information is lacking in our approach and would be better served by a greater understanding of promoter structure. Furthermore, the integration of multiple data types including methylation and DNAse I profiles, and ChIP-chip data would provide a firmer empirical foundation for the characterization of novel Src-responsive units (Splinter & de Laat, 2011).

4 Concluding Remark

The primary objective of this PhD thesis was to define a set of v-Src regulated genes and processes in primary fibroblasts and to discover novel Src-regulated genes by gene expression profiling and *in silico* analyses. The work described in Chapter 2 defined a set

of gene expression profiles and processes for two different primary avian cell types and four different RSV strains (Maslikowski *et al.*, 2010). It furthermore defined a set 42 genes associated with poor prognosis in breast and lung cancer and supported our assumption that primary cell lines are more suitable for cancer gene profiling than their immortalized counterparts (Maslikowski *et al.*, 2010; Discussion). The work in Chapter 3 defined a set of co-regulated genes between two different transformed CEF lines exhibiting AP-1 repression. Furthermore, we showed that one of these genes, *DAPK1* is a novel regulator of apoptosis in v-Src-transformed CEF. In collaboration with others I have addressed the role of different AP-1 factors in v-Src-transformation (Wang *et al.*, 2011a) and have showed via gene expression profiling that CEF under conditions of contact inhibition exhibit a gene profile similar to hypoxia (Appendix 2). Although the SRU study did not discover novel SRU-containing genes, I was however able to demonstrate that some of the underlying assumptions about pSRU discovery are likely flawed.

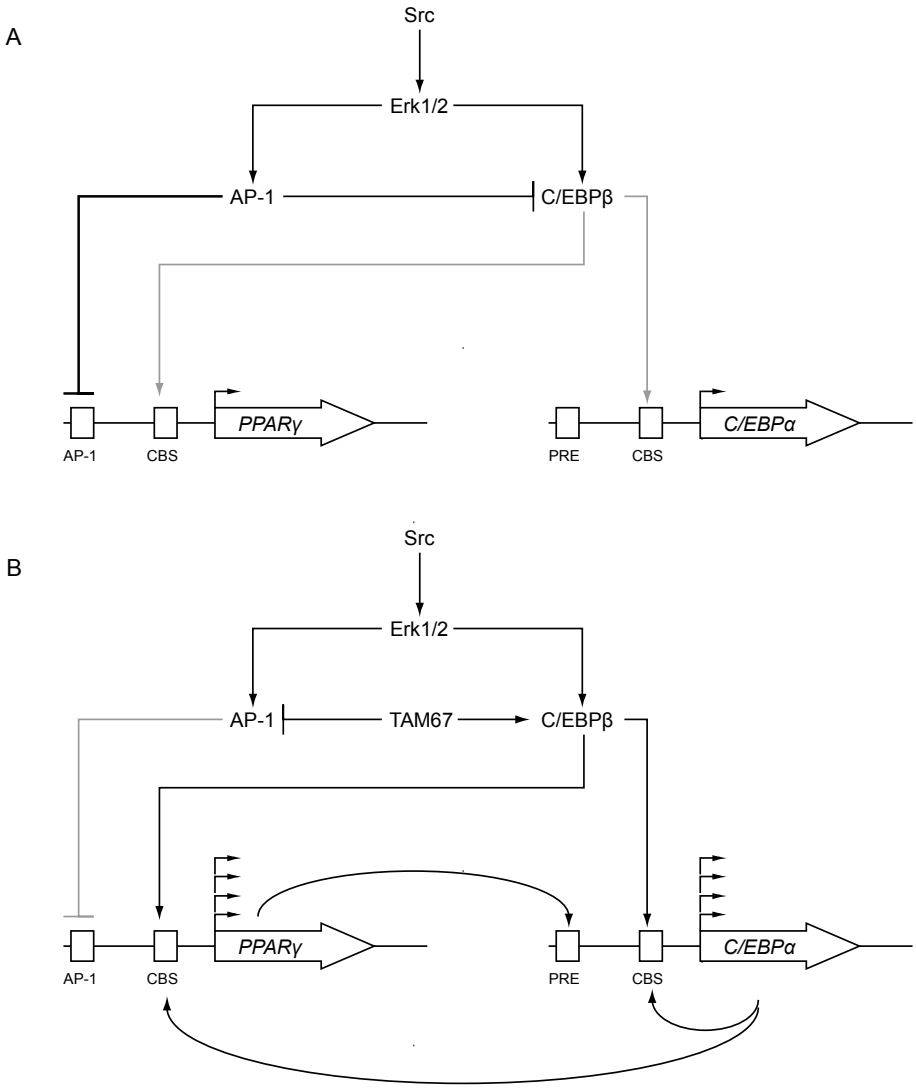


Figure 1. Model of adipogenesis in v-Src transformed CEF expressing the Fra2 shRNA.
In normal Src-transformed CEF, PPARγ expression is repressed directly by AP-1 and by the inhibition of C/EBPβ. Inhibition of AP-1 by TAM67 or the *Fra2* shRNA results in a de-repression of both PPARγ expression and C/EBPβ activity. Increased C/EBPβ activity results in a positive feedback loop between C/EBPα and PPARγ resulting in adipogenic conversion. Arrows indicate activation while stems indicate inhibition. Grey stems or arrows indicate an attenuated response. CBS and PRE stand for C/EBP binding site and PPARγ response element.

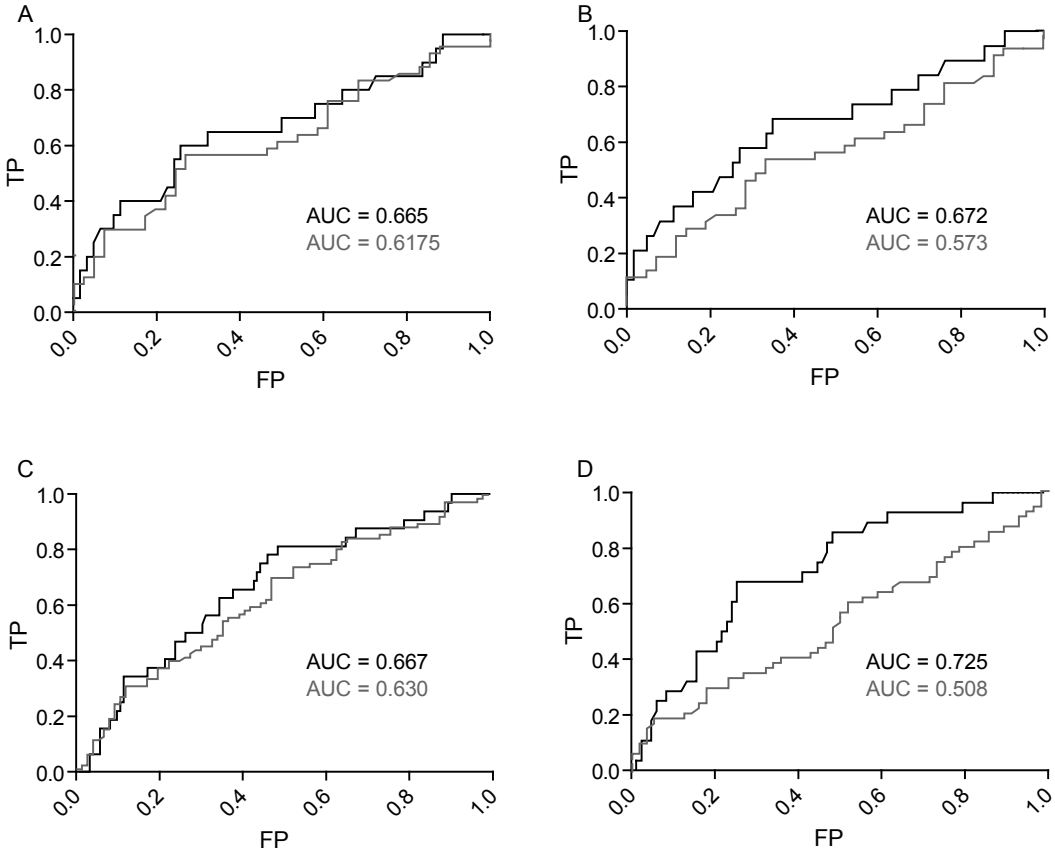


Figure 2. Receiver operator characteristic (ROC) analysis shows a reduction in ability to discern between poor and good prognoses when 42-gene signature threshold is reduced.

Data in panels A-D correspond to panels B *i-iv* in Maslikowski *et al.*, Figure 5. Black (upper) curves indicate ROC analysis performed using the upper quartile threshold while grey (lower) curves indicate ROC using the median threshold. Differences in AUCs were only significant for panels B and D ($P<0.01$). Statistical significance was calculated using the method by Hanley and McNeil (Hanley & McNeil, 1983). TP and FP indicate true positive and false positive respectively.

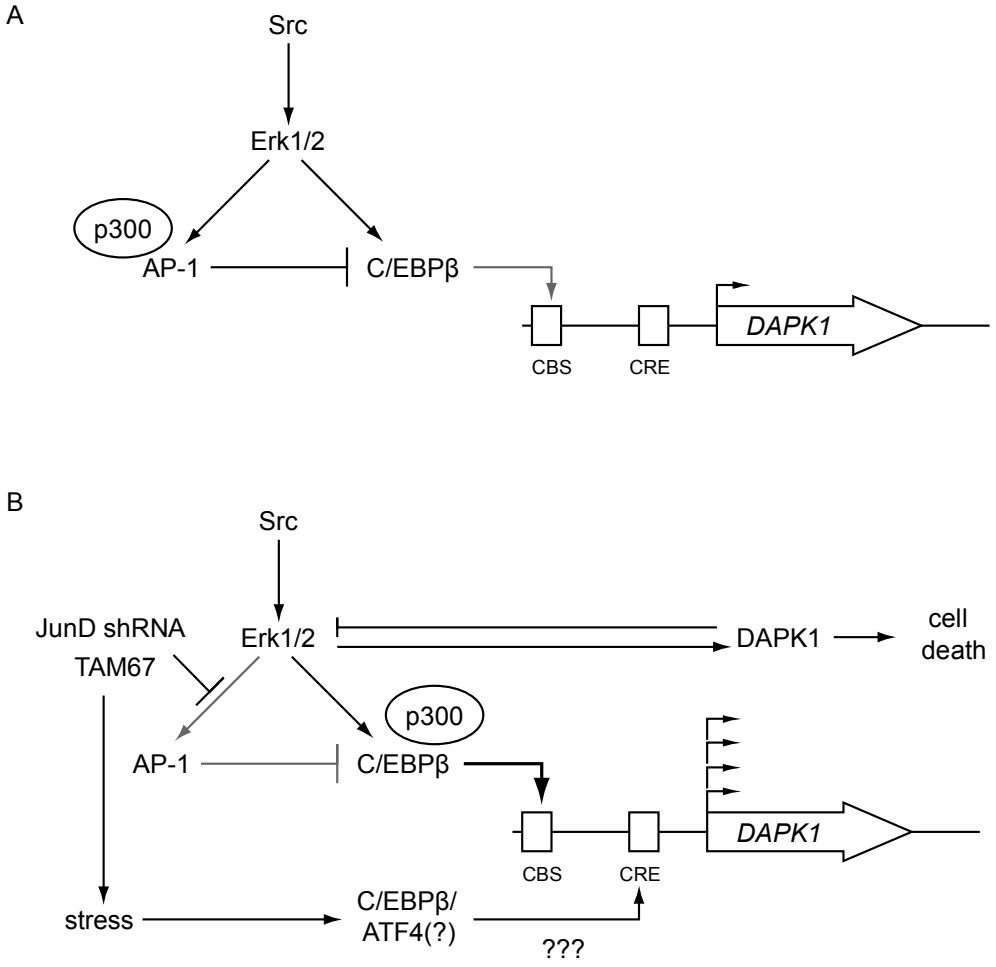


Figure 3. Model of *DAPK1* regulation in v-Src transformed CEF.

Schematic representation of the model of regulation of DAPK1 in v-Src transformed CEF under normal transforming conditions (A) or under conditions of AP-1/JunD inactivation (B). Arrows indicate activation while stems indicate inhibition. ATF4-dependent activation of *DAPK1* via the CRE, as indicated with question marks, is hypothetical and warrants further investigation (described in Discussion, section 2.3.3). Grey stems or arrows indicate an attenuated response. CBS and CRE stand for C/EBP binding site and cyclic AMP response element.

Appendix 1: Identification and assessment of putative Src-responsive units in *Gallus gallus*

Context and contribution

Consistent with the objective to discover novel Src-regulated genes, one approach used was to employ genomic sequence data to identify novel Src-responsive transcriptional units. The publication of the draft chicken genome allowed for the systematic analysis of the chicken genome for regulatory elements. This appendix describes the work undertaken toward discovering and validating novel Src-responsive genes using *in silico* discovery.

All experiments and data analyses in this appendix were carried out by the author of this thesis with the exception of the cloning of the shRNA constructs which was undertaken by technician Shi Yan and the motility assay which was conducted by former undergraduate thesis student Caitlin Gregory (Figure 8). The protocol for the motility assay was devised by the author.

1. Introduction

v-Src has served as a model system for understanding intracellular signalling and has provided system for studying transformation both *in vitro* and in animal models. The involvement of Src in human cancer in the context of cell signalling has been well documented. Our group has concluded microarray-based gene profiling studies that have shown that up to 6% of the protein-coding chicken genes is differentially expressed at a two-fold level in Src-transformed chicken embryonic fibroblasts (CEF) indicating that substantive changes at the transcriptional level result from Src-transformation. Our analyses indicated that genes related to cell adhesion and communication, motility, invasion, proliferation and metabolism were dysregulated. Studies in chicken neuroretinal (CNR) cells indicated that Src-induced changes in gene expression are cell type-dependent and that v-Src induces changes in gene expression that both antagonize and promote differentiation. Moreover, my analyses also showed that despite the diversity of Src-induced gene expression in different cellular and viral backgrounds, similar regulatory pathways linked to survival and invasion were activated in our different models.

Despite the importance of signal transduction cascades mediated by Src, downstream components, such as the activator protein-1 (AP-1) group of transcription factors are necessary determinants of transformation. Previous groups have shown that immortalized MEFs derived from *jun-/-* mice are v-Src and activated Ras transformation resistant and primary MEFs derived from *junD-/-* mice senesce after 2-3 passages *in vitro*

(Johnson *et al.*, 1996; Weitzman *et al.*, 2000). Dominant negative mutants of Jun/AP-1, Ets and Stat3 are known to abrogate Src-mediated transformation (Granger-Schnarr *et al.*, 1992; Johnson *et al.*, 1996; Lloyd *et al.*, 1991; Suzuki *et al.*, 1994; Turkson *et al.*, 1998). Our group has shown that both v-Src dependent activation of AP-1 and NFκB are essential for cell survival (Cabannes *et al.*, 1997). More recently, we have shown that virally mediated RNAi knock-down of *JunD*, *Fra2* and *Jun* expression antagonizes transformation in cells over-expressing constitutively active v-Src (Wang *et al.*, 2011a). Previous studies of the prototypical Src-responsive gene IL8 have shown that four regulatory elements are required for maximal transcriptional activation. This Src-responsive unit (SRU) is composed of a TPA responsive element (TRE or AP-1 binding site), an NFκB binding site, and two C/EBPβ binding sites flanking the TRE and NFκB sites (Dehbi *et al.*, 1992; Gagliardi *et al.*, 2001) (Figure 1). Mutation of any of these sites abrogates Src-dependent IL8 gene expression in CEF while overexpression of their cognate factors (AP-1, NFκB, C/EBPβ) activates the IL8 promoter (Dehbi *et al.*, 1992; Cabannes *et al.*, 1997; Gagliardi *et al.*, 2001). Repression of AP-1 and C/EBPβ in turn, attenuates IL8 promoter activation (Gagliardi *et al.*, 2001; Wang *et al.*, 2011a). Together, these data indicate that Src-dependent IL8 expression necessitates a functional SRU. Therefore, it was hypothesized that using genomic information coupled with gene expression profiling one could characterize novel Src-responsive genes through the discovery of putative SRUs (pSRUs) associated with differentially expressed genes in Src-transformed CEF. This appendix describes the discovery and experimental characterization of novel pSRUs and the genes thought to be regulated by them.

2. Materials and Methods

Computational analyses of pSRUs using SiteSearch

Software developed by the Wrana/Attisano labs at the University of Toronto was used to identify potential SRUs in the *Gallus gallus* genome. Search keys corresponding to SRUs comprising IUPAC consensus sequences were used to identify potential SRUs within a 20kb distance of the transcriptional start site (TSS) of all annotated *Gallus gallus* genes. The parameters for the search required that one NFκB site, two C/EBPβ sites and one AP-1(TRE) site were to be located within a 120bp window. The following IUPAC nucleotide sequences were used for the identification of potential SRUs: NFκB: GGGRNNYYC (Kucharczak *et al.*, 2003), AP-1 (TRE): KNVVTCA (determined by TransFac data compilation), C/EBPβ: TKDNGMAAK (Bédard unpublished). The frequency matrix for the AP-1 site was generated by WCONSENSUS (Hertz & Stormo, 1999) using non-redundant vertebrate sequences from the TransFac database. The IUPAC consensus sequence was then derived by Maslikowski and Bedard. Phylogenetic comparison was conducted by cross-referencing chicken SRU hits to hits from *Homo sapiens*, *Pan troglodytes*, *Canis familiaris*, and *Rattus norvegicus*. SRU searches were cross-referenced to the microarray data by LocusLink ID. Correlation analysis of SRU data was carried out using OpenStat v1.4 (Miller, 2006). Briefly, expression data for the SR-A/NY315 dataset (all significant differentially expressed genes) were cross-referenced to their putative SRU by their most proximal location to the TSS.

Computational analyses of pSRUs using matrix-scan

Non-redundant vertebrate sequences corresponding to binding sites for NFκB, AP-1 (TRE) and C/EBPβ were obtained from the TransFac database (Version 7) and converted to frequency matrices using WCONSENSUS (Hertz *et al.*, 1990). Genomic sequences corresponding to 10kb upstream and 5kb downstream of the TSS of genes in the transformation-regulated (TR) set (Maslikowski *et al.*, Additional File 3) were retrieved using the Regulatory Sequence Analysis Tool (RSAT) sequence retrieval tool (Turatsinze *et al.*, 2008). Coding regions of upstream genes or downstream exons were masked to minimize the discovery of false-positives. Sequences were scanned using the matrix-scan employing default cut-offs with a CRER (cis-regulatory enriched region) window size of 30-120bp. The background model was derived from the input sequence using a Markov order of 1. All CRER hits were cross-referenced to the TR-gene set expression values using Ensembl gene IDs. Correlation analysis of SRU data was carried out using OpenStat v1.4 (Miller, 2006) or Prism GraphPad.

Statistics and graphics

Expression/SRU-distance and expression/CRER_sig data were analysed for correlation using Spearman's rank correlation. Significance was determined by approximation to the Student's t-distribution (Sokal & Rohlf, 1995). All other statistical analyses are indicated within the figure legends. Gene schematics were created using GenePallette v1.2 (Rebeiz & Posakony, 2004). pSRU data were visualized using the RSAT feature map tool (Turatsinze *et al.*, 2008). Sequence logos were generated using WebLogo (Crooks *et al.*, 2004).

Cell culture and RSV strains

All cell culture was performed as described previously in Chapters 2 & 3, Materials and Methods sections. The A-type RCAS(A), NY315, SRA and the *ts* NY72-4 RSV strains were used as described previously in Chapter 2, Materials and Methods section. For the shRNA experiments, the LA90 strain of RSV was used to transform cells. This viral strain belongs to the B sub-group of ASV/ALV and can be used to co-infect CEF with an A-type virus. Similar to NY72-4, LA90 encodes a temperature sensitive mutant of *v-Src*, which is transforming at the permissive temperature of 37.5°C and non-transforming at the non-permissive temperature of 41.5°C.

Transient reporter assays, shRNA vectors and transfection

Transfection was carried out by the DEAE-dextran method as described in Chapter 2 Materials and Methods section. Chloromphenicol acetyltransferase (CAT) and *Gaussia* luciferase reporter assays were carried out as described previously (Wang *et al.*, 2011a) and as in Chapter 3, Materials and Methods section. Generation of the shRNA RCASBP(A)-shRNA-ΔU6 retroviral vector used in this study was described previously (Wang *et al.*, 2011a). The *PIK3CD* shRNAi hairpins used for this experiment target the following sequences:

1041: 5'- TTTCACGGCAATGAGATGCTG-3',

1992: 5' - TTGAGGTTTGGCTTGATCCTG-3'

K60, ITGA8, *IL8* and *PIK3CD* SRUs were PCR amplified from genomic chicken DNA and cloned into either pJFCAT-TATA or pGLuc-TATA using the primers and restriction sites listed below. pJFCAT-TATA and pGLuc-TATA were described previously in (Dehbi *et al.*, 1992) and in Chapter 3, Materials and Methods section.

Construct	Direction	Sequence	Restriction sites
pJFCAT-IL8	F	AAACTGCAGTCAGCTAGTCTATTTCACAACA	PstI
	R	AAAAGATCTCTAGCATGTAAATTGCGTTAGA	BglII
pJFCTA-K60	F	AAACTGCAGTATAGGCAGGATTTCTAAAGAC	PstI
	R	AAAAGATCTTTCTCCCCAGCTTTCCCCAACT	BglII
pJFCTA-ITGA8	F	AAACTGCAGAAAAGCTTGCATTAGGCAATTA	PstI
	R	AAAAGATCTTTACTGGTCTTATTTCAAAAGG	BglII
pGluc-IL8	F	AAAGAATTCAGCTAGTCTATTTCACAACA	EcoR1
	R	AAAAAGCTTCTAGCATGTAAATTGCGTTAGA	HinDIII
pGluc-PIK3CD	F	GACTATAGATCTTTGCTTTTTGAAAGTTGAACAAGTAA	BglII
	R	TGCCGAGCTCCTGACATTTCATCAACTGACTCAA	SacI
GAPDH qPCR	F	GTCGGAGTCAACGGATTTGGCCG	N/A
	R	ATGGCCACCACTTGGACTTTGCC	N/A
IL8 qPCR	F	GCTCTGTCGCAAGGTAGGACGCT	N/A
	R	GCCGCTTGGCGTCAGCTTCAC	N/A
PIK3CD qPCR	F	TCTGATGGCTGCACGGGGCA	N/A
	R	TCGCTGGAAAGCATCAGGTGAGCTA	N/A

Antibodies and immunoblotting

Actively growing CEF were washed twice in phosphate-buffered saline (PBS) and lysed immediately in sodium dodecyl sulfate (SDS) lysis buffer supplemented with 10mM $NaVO_4$, 10mM NaF and 1X protease inhibitor cocktail (Roche Biosciences mini cat.#11836170001). Samples were immediately denatured by boiling for five minutes. Proteins were separated by standard SDS-PAGE and transferred to nitrocellulose (Whatman Protran) by standard electroblotting procedures. Membranes were blotted with the following anti-bodies: anti-PIK3CD (Novus Biologicals, cat#NB600-1062; 1:1500);

anti-Erk1 (Santa Cruz, cat#SC-94; 1:10000); anti-E-cadherin (BD Transduction Labs, cat# 610182; 1:3000).

Cell proliferation assay

Cell proliferation assay was performed to assess the effect of each RCASBP(A) shRNAi vector on cell survival. Cells were seeded in 24 well dishes at a density of 10^4 cells per well. Four replicates for each experimental group were counted by Coulter counter (Beckman-Coulter) every day for eight days following transfection.

TUNEL and senescence-associated β-galactosidase assays

Apoptosis in CEF was assessed by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Cover slips were seeded with cells from each experimental group in 60mm dishes. Cells were washed twice in 1x PBS, and fixed for 15 minutes in 10% formaldehyde in PBS. Cells were permeabilized with 0.01% Triton X-100 in PBS, washed and then labeled as per manufacturer's directions (Roche Biosciences cat#12156792910). Cells were also stained with diamidino-2-phenylindole (DAPI), to facilitate counting and to discriminate pyknotic nuclei. Nuclei and TUNEL stain was visualized under fluorescence microscopy and quantified using ImageJ (Abramoff *et al.*, 2004).

Senescence β-galactosidase staining was carried out according to the manufacturer's instructions (Cell Signaling Technology, cat#9860). Briefly, cells from each experimental group were seeded into 35mm dishes and allowed to proliferate overnight. Cells were fixed, washed with 1x PBS and then incubated in the staining solution at 37.5°C overnight or until the staining was discernible. All components other

than PBS were supplied by the manufacturer. Cells were labeled with DAPI and visualized by phase contrast and fluorescence microscopy and quantified using ImageJ (Abramoff *et al.*, 2004).

Motility assay

Cells were assayed for motility using a modified Boyden chamber assay. Cell culture inserts containing a porous polyethylene terepthalate (PET) membranes (BD Falcon, cat#353097) were inserted into a 24 well culture plate and seeded with 10^4 cells resuspended in Richter's modified MEM medium with 2% cosmic calf serum. A serum concentration gradient was achieved by adding medium with 10% serum to the lower chamber. Following seeding, plates were incubated at the appropriate temperature for eight hours. Cells that failed to migrate through the membrane were removed by mechanical dissociation using a sterile swab. Remaining cells were fixed with ice-cold methanol for 10 minutes. Inserts were excised, cells were stained with DAPI, visualized under fluorescence microscopy and quantified using ImageJ (Abramoff *et al.*, 2004).

3. Results and Discussion

3.1 Identification of novel pSRUs from genomic and expression data using SiteSearch

Several gene profiling studies have attempted to delineate Src-dependent changes in gene expression using various systems. Many of these studies used tumour-derived samples or immortalized cell lines and as such do not necessarily reflect the subset of genes

expressed purely as a result of Src-dependent transformation (Malek *et al.*, 2002; Irby *et al.*, 2005; Bild *et al.*, 2006).

By using embryonically derived primary chicken embryonic fibroblasts (CEFs), we were able to circumvent issues relating to immortalized cell lines. For our experiments, a transcription profile obtained from myristylation-deficient, constitutively kinase-active non-transforming mutant RSV (NY315)-infected CEFs was compared to the wildtype v-Src (SR-A)-transformed CEF profile and RCASA (replication competent avian leukemia virus long terminal repeat splice acceptor virus) (Hughes *et al.*, 1987) infected cells. By comparing the gene expression profiles of NY315 and v-Src infected cells, it was possible to differentiate between transcriptional differences that account for cellular transformation and not merely Src-dependent protein phosphorylation (Maslikowski *et al.*, 2010).

In order to narrow down targets directly regulated by Src transformation and to identify novel putative Src Responsive Units (pSRUs), we have employed a computational approach directed at identifying novel pSRUs by scanning the chicken genome to identify potential regulatory units composed of a TRE, NFκB binding site and two C/EBPβ binding sites. This software, developed by our collaborators, the Attisano and Wrana labs at the University of Toronto, employs a string-based search algorithm designed to find exact matches of IUPAC-defined sequences, or combinations of sequences within a prescribed distance of annotated transcriptional start sites (TSSs). Their approach has so far allowed them to identify a number of genes responsive to TGF-β signalling (Silvestri *et al.*, 2008). Although the program is suboptimal for the

identification of *bona fide* target sequences without a large number of false positives, in combination with gene expression data, the method has allowed us to winnow out potential Src-unresponsive genes to create a database of potential SRUs.

Data indicated that there are 156 unique targets common to both lists, indicating possible novel Src-responsive genes (Table 1). Interestingly, these data have also indicated that there may be SRUs acting in a negative regulatory fashion, providing an unanticipated avenue that will warrant further investigation. Data also indicate that these pSRUs are located both up and downstream of the TSS of their presumably cognate genes (Figure 3).

Table 1. Summary of GeneChip and pSRU data from SiteSearch

Experiment	Genes (2 fold diff.)	% genes [a]	*G.g.* SRU	% *G.g.* SRU [b]	vertebrate SRU	% vertebrate [c] SRU
NY315/RCASA	1406	5.40%	282	6.00%	1	2.30%
SR-A/NY315	709	2.70%	156	3.30%	3	6.80%
SR-A/RCASA	2222	8.50%	451	9.60%	8	18.20%
Total genes	26109 [d]	100.00%	4710	100.00%	44	100.00%

a Expressed as the percentage of genes significantly differentially expressed at a two-fold level in relation to the total number of non-redundant probe-sets on the chicken GeneChip
b Expressed as the percentage of putative chicken SRUs cross-referenced to the expression data in relation to the total number of putative SRU
c Expressed as the percentage of putative vertebrate SRUs cross-referenced to the expression data in relation to the total number of putative SRU
d Total number of non-redundant probe-sets representing genes on the *G.gallus* GeneChip

In an effort to characterize the putative SRUs for possible trends, an investigation of the relationship between the difference in expression of a target gene and the orientation and distance of its SRU from the TSS was undertaken. Taken as a whole, there is no

correlation between differences in expression and the distance of a pSRU from the TSS. Similarly, when looking at upstream pSRUs, there is no relationship between the magnitude of differentially expressed genes and the upstream location of the SRU. However, there is a negative correlation between differences in gene expression and pSRU distance from the TSS in genes whose pSRUs are downstream of the TSS (Spearman rank correlation test, $\rho = -0.172$, $P = 0.0199$; Figure 4) in the SR-A/NY315 dataset demonstrating that pSRUs further downstream of the TSS correlate with a repression of expression of its respective gene. Given that there are no known examples of SRUs that regulate transcription through repression, it is reasonable to hypothesize that down-regulation of gene expression could be mediated through non-coding RNA such as anti-sense or micro RNA, transcription start sites may be variously located in introns, exons, and UTRs of coding genes (Mattick & Makunin, 2006).

3.2 *K60* and *ITGA8* pSRUs are not Src-responsive transcriptional units

Two initial candidates representing both a positively and negatively regulated pSRU-containing genes were chosen for validation based on the sequence of their pSRUs and by their interesting ontologies. *K60*, a chemokine gene located several kilobases upstream of *IL8*, has no known mammalian homolog, shares 72% amino acid sequence identity with chicken *IL8*, and may represent an *IL8* paralog under transcriptional control similar to *IL8*. *ITGA8* is the only repressed gene common to all the vertebrate SRU searches and has a putative SRU roughly five and a half kilobases downstream of the TSS in intron 7 (Figure 3). Both *K60* and *ITGA8* show pSRU sequences similar to the canonical *IL8* SRU

(Figure 1). *K60* and the *ITGA8* pSRUs were cloned into the JFCAT-TATA plasmid along with the *IL8* SRU and assayed using the chloramphenical acetyltransferase (CAT) assay. It was found that the *K60* and *ITGA8* promoters were unresponsive to Src-activation (Figure 5). Furthermore, there was no detectable activity in either the non-induced or the induced condition, indicating that these regulatory units were non-functional.

3.3 Identification of novel pSRUs from genomic and expression data using position-specific scoring matrices

The previous strategy for identifying pSRUs was based on an approach where *in silico* discovery of pSRUs was cross-referenced to Src-responsive genes identified in our array experiments. Initially, SiteSearch provided the pSRU data. However, the string-based algorithm used by SiteSearch is known to be prone to high rates of false-discovery, does not allow for preferential scoring of putative targets (Stormo, 2000), nor does it allow for the background correction of nucleic acid composition of the genome (Bulyk, 2003; Turatsinze *et al.*, 2008). Analysis of two pSRUs proximal to the *K60* and *ITGA8* genes identified by SiteSearch did not show Src-inducible reporter activity. In contrast to SiteSearch, a position-specific scoring matrix (PSSM) approach employing a probabilistic measure allows for ranking of putative cis-regulatory units and can be used to assess the probability of finding a specific site in a given genomic background and therefore may be better at discriminating between real and false binding sites (Turatsinze *et al.*, 2008).

Using the Regulatory Sequence Analysis Tools (RSAT) portal (Thomas-Chollier *et al.*, 2011), sequences corresponding to transformation-regulated (TR) genes identified

in our microarray study were retrieved. Although eukaryotic enhancer elements are frequently located far from the genes they regulated, proximal promoter regions (within 5kb of the transcriptional start of a gene) account for the majority of *cis*-regulatory elements in eukaryotic genomes (Margulies *et al.*, 2003). For the purpose of this study, analysis was restricted to the first 10 kb upstream, and 5kb downstream of annotated transcriptional start sites (TSS). Frequency matrices for AP-1, NFkB and C/EBPβ were derived from the TRANSFAC public database (Figure 2). Using a window size of 120bp, matrix-scan was used to find overrepresented clusters of cis-regulatory enriched regions (CRERs) in addition to individual putative binding sites.

Matrix-scan identified 5862 CRERs proximal to 476 out of 623 annotated TR genes (76%) (Additional File 1.). Genes of interest previously identified by SiteSearch (*ITGA8, K60, HMOX1, AQ1, MMP2, PTGS2, DKK3 PLAU,* and *FLT1*) were identified as having proximal putative CRERs. Indeed, 85% (162) of TR genes identified by SiteSearch were also identified by matrix scan. Since matrix scan identified 76% of TR genes as being proximal to CRERs, this overlap is not surprising and likely does not represent a biologically significant result. Consistent with this, reanalysis of the putative *ITGA8* and *K60* SRUs using matrix-scan did not reveal sequences corresponding to the pSRUs identified by SiteSearch (Figure 6). These data would suggest that the negative results obtained in the CAT-reporter assay reflected non-regulatory DNA sequences and not SRUs. Further analysis of matrix-scan data did not show any correlation between magnitude of gene expression, CRER position or CRER significance score (Figure 7).

However, preliminary visual analysis of the orientation of several candidate genes did reveal potential targets of interest, such as *PIK3CD*.

3.4 The *PIK3CD* pSRU is a weakly responsive enhancer element but *PIK3CD* is not a Src responsive gene

The PI3K/PKB pathway has long been understood as an important pathway for survival and motility (see Chapter 1). In v-Src transformed CEF, cells characterized by high motility and resistance to apoptosis, these pathways display marked activation (Maslikowski *et al.* 2010, Additional file 13). PI3Ks comprise a family of heteromeric lipid kinases consisting of a smaller 85kDa regulatory subunit and a 110kDa catalytic subunit. The different isoforms of the p110 catalytic subunit appear to have different functions in cell proliferation, survival, actin rearrangement and migration (Vanhaesebroeck *et al.*, 1999). *PIK3CD* codes for the p110δ catalytic subunit of PI3K. Over-expression of PIK3CD has been shown to constitutively activate the PKB pathway (Kang *et al.*, 2006) and PIK3CD has been shown to be required for chemotaxis (Sawyer *et al.*, 2003) in some cancer cell lines and for migration in white blood cells (Vanhaesebroeck *et al.*, 1999). *PIK3CD* was identified as a transformation regulated gene in the array studies and as a gene that has a proximal SRU-like CRER approximately 3kb upstream of its TSS (Figure 1). PIK3CD is upregulated in numerous cancers and is associated with poor clinical outcome (Martinez *et al.*, 2003; Lee *et al.*, 2004; Ruano *et al.*, 2008). Furthermore, as an enzyme, PIK3CD is a potential target for small molecule drug design, making it of clinical interest.

Experiments were carried-out to assess the role of *PIK3CD* in cell motility and v-Src transformation. Results from a Boyden chamber motility assay show that v-Src transformed cells migrated faster in response to serum (Figure 8). Furthermore, silencing of *PIK3CD* using two different shRNA constructs appeared to reduced motility of CEF even when transformed by v-Src (Figure 8). Since the PI3K/PKB pathway is known to play a role on apoptosis, the role of *PIK3CD* in this process was investigated in normal CEF using TUNEL. Knock-down using both constructs exhibited a modest increase in apoptosis compared to normal CEF infected with RCAS(BP)shRNA vector (referred to herein as RCAS unless otherwise indicated), with a three fold increase using construct #1992 compared to control cells (Figure 9). Since the RAS/PI3K pathway is known to play a role in oncogene-induced senescence (Bardeesy & Sharpless, 2006; Courtois-Cox *et al.*, 2006), the role of PIK3CD in senescence of normal CEF was investigated. Cells infected with *PIK3CD* shRNA constructs, or the parental control virus, were assayed for senescence-associated β-galactosidase (SAβG) activity. *PIK3CD* knock-down CEF exhibited a modest increase in senescence in cycling cells, with construct 1992 showing a greater increase (Figure 10). Similarly, proliferation assays indicated a reduced rate of growth over an eight day period when comparing *PIK3CD* knock-down CEF to control infected cells (Figure 11).

To assess whether *PIK3CD* is a bona fide target of transcription activation via Src-transformation, the *PIK3CD* pSRU was tested using a *Gaussia* luciferase reporter assay optimized previously (data not shown). Reporter assays using a TATA-only reporter or a *PIK3CD* pSRU coupled to a TATA box showed that the *PIK3CD* pSRU is

inducible about three fold in CEF infected with the *ts*72-4 RSV virus at the permissive (transforming) condition (Figure 12). However, basal level of expression of the *PIK3CD* pSRU is approximately equal to basal expression of the TATA box alone indicating that this is likely not a real cis-regulatory element. In contrast, basal level of expression for the *IL8* SRU is one to two orders of magnitude greater than TATA alone, depending on the reporter assay used (Figure 5; data not shown).

Validation immunoblots aimed at assessing the knock down efficiency of the *PIK3CD* constructs revealed that both constructs 1041 and 1992 knocked down PIK3CD protein levels approximately five to seven fold (Gregory, 2009). However PIK3CD levels in Src-transformed cells were also decreased three to ten fold when compared to untransformed CEF (Figure 13). Since the availability of good quality antibodies is a perennial problem when using avian cells, qPCR was employed in order to confirm the observations made by immunoblotting. *PIK3CD* RNA levels showed a similar pattern of expression to that in the immunoblots (Figure 14).

The above experiments show that knock-down of *PIK3CD* affects motility, apoptosis and senescence in CEF, however the expression validation data contradict the array findings and undermine the rational for knocking-down *PIK3CD* initially. Since both transformed cells and knock-down cells demonstrate decreased levels of PIK3CD but exhibit different phenotypes, it is unclear what role, if any, PIK3CD plays in the context of v-Src transformed CEF. Little is known about the regulation and expression of PIK3CD transcript variants. Both the human and murine orthologs of *PIK3CD* code for nine and thirteen variants respectively, most of which are protein coding (Ensembl gene

IDs ENSG00000171608 and ENSMUSG00000039936). Although the chicken PIK3CD transcripts have not been characterized, it seems reasonable that avian *PIK3CD* codes for several transcript and protein variants. Indeed, a minimum of three anti-PIK3CD cross-reacting bands were detected by immunoblotting suggesting multiple protein isoforms. Furthermore, the relative abundance of these bands differs depending on which *Src* allele is expressed (compare LA90 and SRA, Figure 13) suggesting differing modes of regulation at the transcript and/or protein level. Since the PI3K family of proteins are regulated substantively at the post translational level (reviewed in (Vanhaesebroeck *et al.*, 2010)), it is likely that a combination of regulatory mechanisms contributes to the PIK3CD knock-down motility phenotype.

It should also be noted that the differential expression of *PIK3CD* was detected by only one out of three probe sets on the chicken microarray (Maslikowski, *et al.*, 2010, Additioanl File 2). Moreover, there was no difference in expression of *PIK3CD* in CEF or in CNR transformed by ts72-4 suggesting that *PIK3CD* may not in fact be a universally Src-inducible gene. Indeed, protein and transcript level validation would suggest that this is the case (Figures 13 & 14). Interestingly, microarray analysis of *PIK3CD* levels in response to TAM67 expression shows a repression of *PIK3CD* in both transformed and untransformed CEF (Figure 15). However, since TAM67 is known to inhibit Fos, ATF, and Maf families in addition to Jun, repression of PIK3CD by TAM67 may not be a result of AP-1 inhibition. Indeed, repression of JunD by shRNA did not reveal any changes in PIK3CD levels, in either Src-transformed or untransformed cells (Figure 15). Since JunD

is the dominant AP-1 member in Src-transformed CEF, this observation would suggest that PIK3CD is not an AP-1 responsive gene.

4. Summary

By using two computational approaches, one string-based (SiteSearch) and one probability matrix-based (matrix-scan), this study identified several thousand putative Src-responsive transcriptional units proximal to Src-responsive genes in the chicken genome. Analysis of three of these pSRUs indicated that two were non-functional (proximal to *K60* and *ITGA8*) while a third (*PIK3CD*) was weakly inducible in Src-transformed CEF. The absence of significant overlap in the sites that were identified by the two programs and the generally poor performance of both programs in identifying SRUs suggest an inherent limitation to the *de novo* discovery of transcription factor binding sites in vertebrates even when defined by an experimentally validated consensus sequence or frequency matrix. Functional analysis of *PIK3CD* by RNA interference in normal and transformed CEF showed that PIK3CD plays a role in promoting survival and motility. However, induction of *PIK3CD* in response to Src-transformation could not be validated. Indeed, in Src-transformed CEF, PIK3CD expression was decreased at both the transcript and protein levels confounding the interpretation of what role PIK3CD plays in the afore mentioned biological phenomena. Together these data would suggest that *PIK3CD* is not a positively regulated Src-responsive gene and does not possess a *bona fide* SRU. Nevertheless repression of *PIK3CD* in CEF does appear to affect motility, growth and survival and therefore warrants future investigation. Characterization of

individual transcript and protein variants in particular would provide insight into the regulation of PIK3CD levels and what role they play in motility and survival.

IL8
TAATACGTTATATTATTTGCAGCAGAATAATTCATCATCAGCTAGTCTATTTCACAACAGTCATG**TGACTCA**
TTTTTACTACATCTGGGAAATTCCTCTAACGCAATTTACATGCTAGCATAGCTCTGCACACGGGCACAAAAC
TGTATAAAAGCACAATGGGTCTCCCTGTTCAGACATCAGCAATCCTCTGACAGGAGAGATCACAGCTCCACA
AAACCTCAGCTCAGAAAACAAGCCAAACACTCCTAACCATGAACGGCAAGCTTGGAGCTGTCCTGGCC

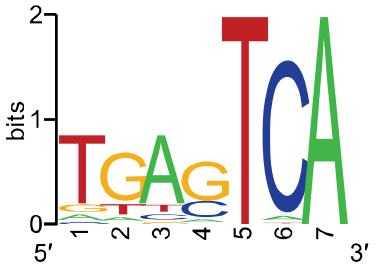
K60
TCTGGATCTAAAACTTCAGGCATATAGGCAGGATTTCTAAAGACAATGGTGAGGAACTGCTGAGGGGAGT**GA**
GGTCAAAGCAGTGCTGGAGGATCACCAGAGGAAAGAAATCCCCCAT**TGATCAC**AGCTGTATAAGGAAATGTG
AAGTTCCAGGTCACTCACCAGTTGGGGGAAAGCTGGGGAGAAGCACCTAGGAAATAATCTGAGGAACTCATTT
CTTTCACTCAAGCACTTTCCTACCTTTTGCTATGACATAGCAAAACAGACGAGGAAGCCTTCACTAGT

ITGA8
GAAGGATTGAAAGATGAAAACAAGCTTTTAAAAGCTTGCATTAGGCAATTAGAAAACAAAATTACATCTCT
GATAGGGAGAACTGCCTTACTTCCAAATGGCACTTCACCTCTGAAATTAAGATCCATATCCTTAGAAGAAG
AAAAAGAC**TTGGGTCA**GAAGGATTCATCAATCCAGGTTGAATCTAAGAACAAAAAGAAAGCCAAGCAGACT
AAAATCCAACTAGAGGATCTGGGAACCT**C**GGATCCTTTTGAAATAAGACCAGTAATAACTCAAAAAATGCA

PIK3CD
GGTTTGCTTTTTGAAAGTTGAACAAGTAATTTCCCTACCCAACTGGCAGGGCAAAGTGTGGCTGTGGGTGCT
GAATCCTCAGCACTTTTCCCCAGTGCGGCCTTT**TGAGTCA**GTTGATGAAATGTCAGGT

Figure 1. SRU sequence for *IL8* and pSRU sequences for K60, ITGA8 and *PIK3CD*.
Highlighted, underlined and bold sequences show C/EBPβ, NFκB and AP-1 biding sites respectively. Note the relative arrangement of sites in the *IL8* and *PIK3CD* sequences.

```
AC   AP-1
XX
P0       A      C      G      T
01       4      2      5     36
02       4      2     33      8
03      36      4      2      5
04       3     13     29      2
05       0      0      0     47
06       2     44      0      1
07      47      0      0      0
XX
//
```




```
AC   NFKB
XX
P0       A      C      G      T
01       0      0     40      0
02       0      0     40      0
03       1      0     39      0
04      27      0     13      0
05      21     13      1      5
06       8      1      3     28
07       1      2      2     35
08       2     18      0     20
09       3     36      0      1
10       0     38      0      2
XX
//
```


```
AC   C/EBPB
XX
P0       A      C      G      T
04       1      0      0     20
05       1      0      7     13
06       7      0      8      6
07       5      2      5      9
08       0      0     21      0
09       8      9      0      4
10      20      1      0      0
11      21      0      0      0
12       3      1      6     11
XX
//
```

Figure 2. Frequency matrices for AP-1, NFκB and C/EBPβ.

Left panel shows frequency matrices representing consensus binding sequences for transcription factors indicated. Right panel shows sequence logo representations of the frequency matrices.

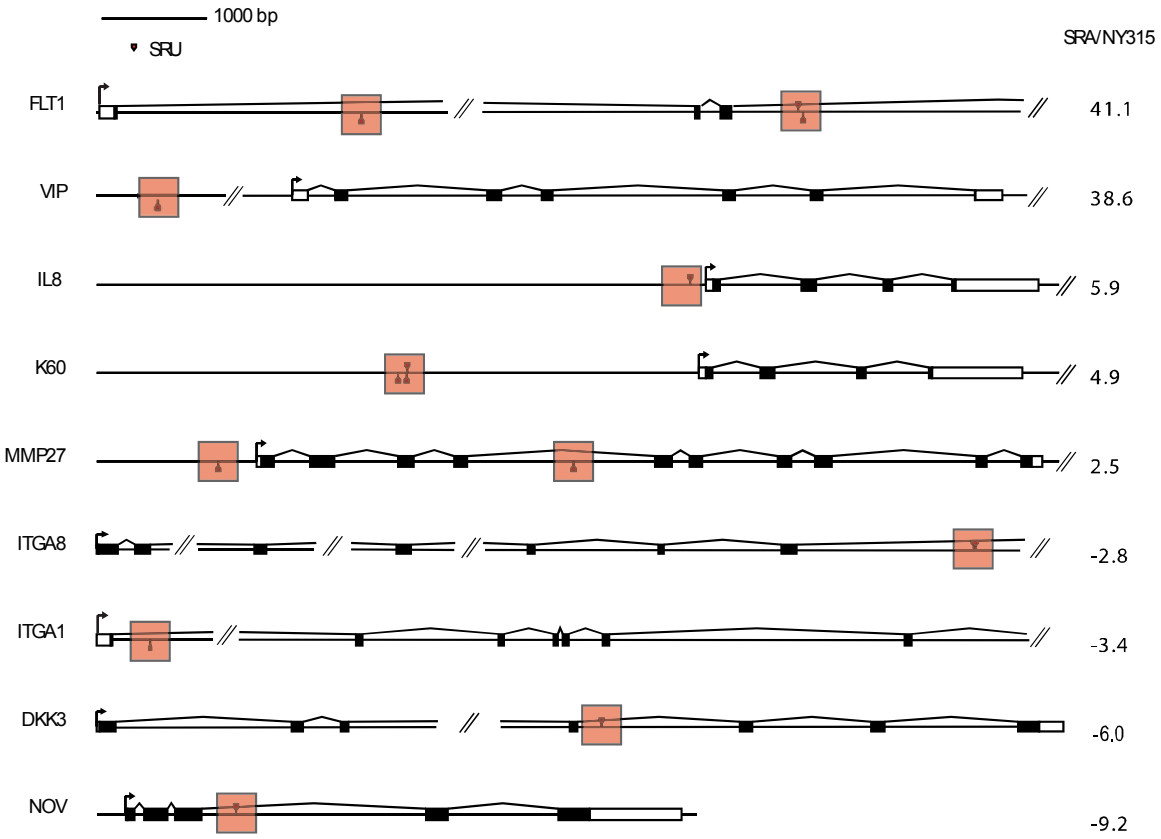


Figure 3. Distribution of putative pSRUs in genes differentially expressed between SRA and NY315 infected CEF

Red flags indicate location of pSRUs. Numbers indicate fold change gene expression between SRA and NY315- infected cells.

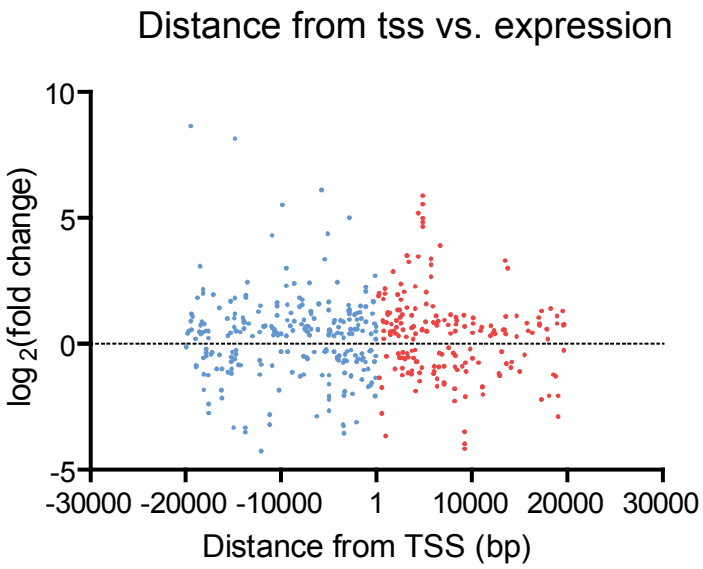


Figure 4. Scatter plot of pSRU distance from TSS versus expression of Src-responsive genes

Expression is shown as the $\log_2$(fold change) of SRA expression over NY315. Red series shows pSRUs downstream of the TSS. These data showed a negative correlation between differences in gene expression and pSRU distance from the TSS in genes whose pSRUs are downstream of the TSS.

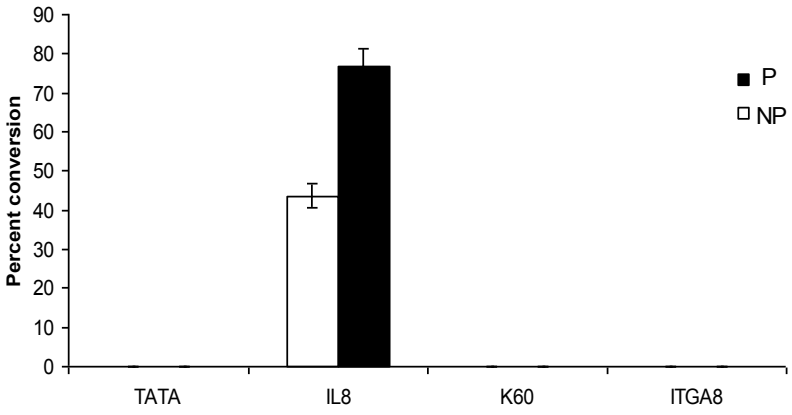


Figure 5. CAT reporter assay showing activity of different SRUs in response to Src activity.
CEF infected with NY72-4 were transfected with pJFCAT-TATA, pJFCAT-IL8, pJFCAT-K60 or pJFCAT-ITGA8 and RSV-βgal and incubated at permissive (37^0C) or non-permissive (42^0C) temperature following transfection. Cells were lysed and assayed for CAT and βgal activity 48 hours following transfection. Percentage conversion indicates the total percentage of chloroamphenicol acetylated. CAT activity was normalized to transfection efficiency by assaying βgal activity. All transfections were performed in triplicate; error bars indicate standard deviation.

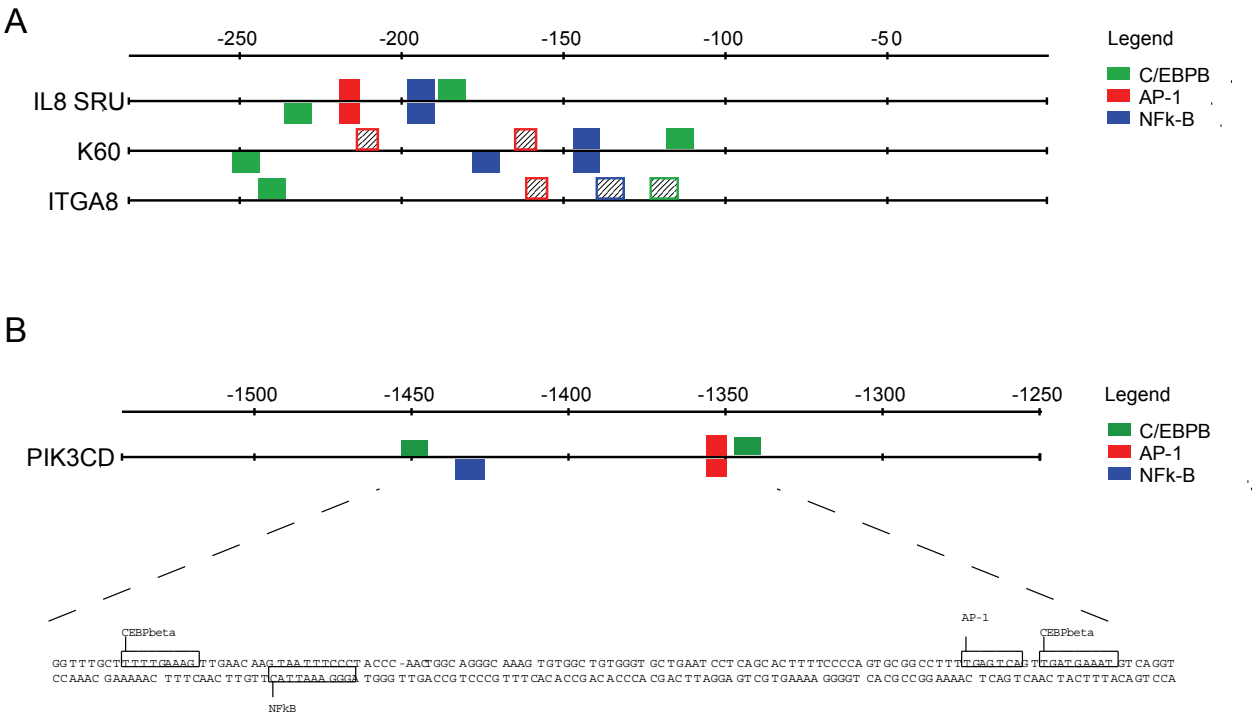


Figure 6. Schematic representations of SRUs.

A. Two putative SRUs, one located upstream of the *K60* gene and the other in an intron of *ITGA8* are not functional by reporter assay. Both putative SRUs display scattered organization of individual sites compared to the *IL8* SRU. Moreover, some predicted sites detected by SiteSearch were not detected by matrix-scan as indicated by hatched boxes. Numerical values on the scale bar indicate scale in nucleotides. B. A putative SRU for the *PIK3CD* gene (PI3'K p110δ) identified by matrix-scan indicates clustering of binding sites similar to the *IL8* SRU (panel A). Scale bar indicates actual distance from the *PIK3CD* transcriptional start.

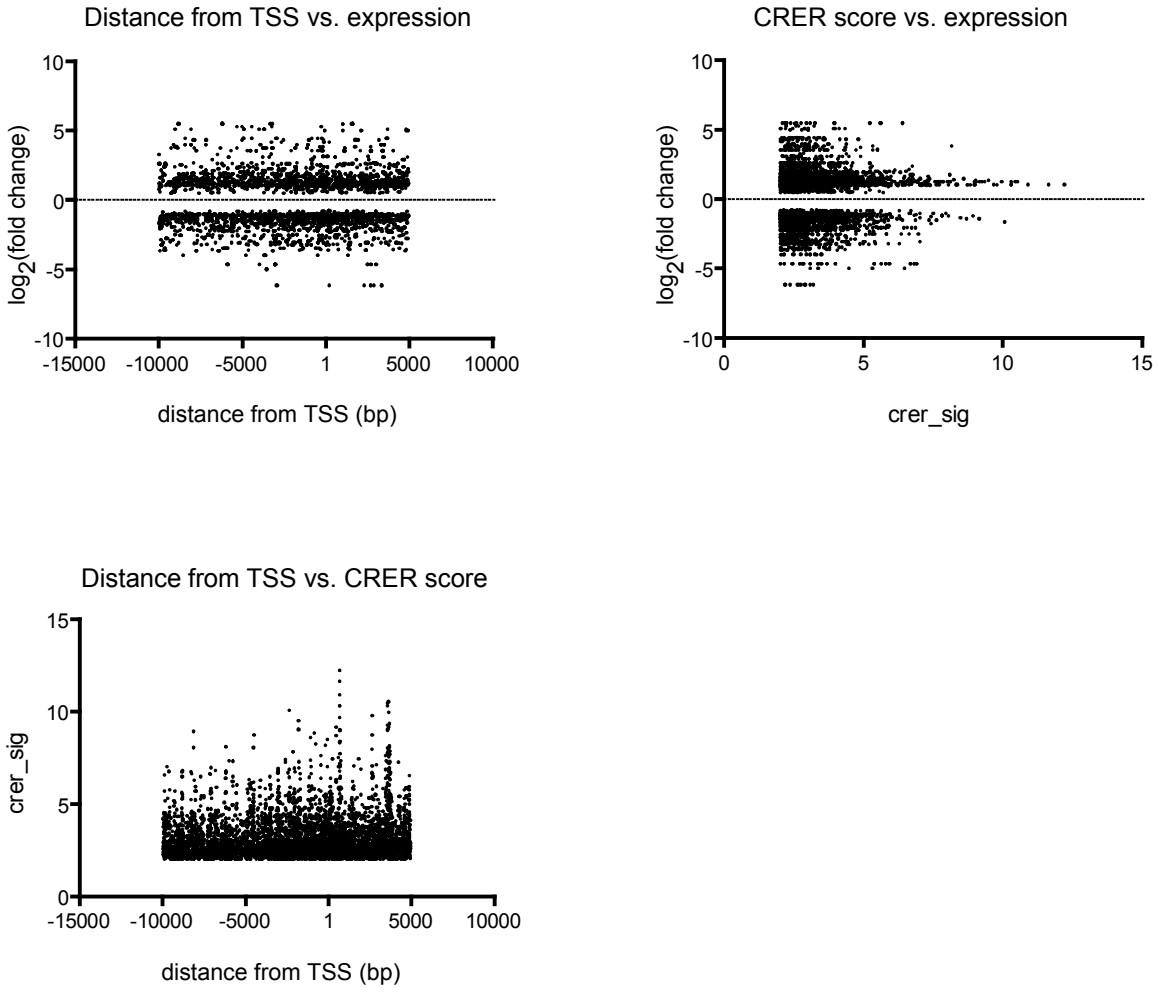


Figure 7. Pairwise comparisons of CRER distances from TSS, TR-gene set expression values and CRER scores.

crer_sig is the CRER score defined as the –log of the estimate probability of finding a CRER per given sequence. A crer_sig of 3 indicates an estimated probability of finding one CRER per 1000 nucleotides.

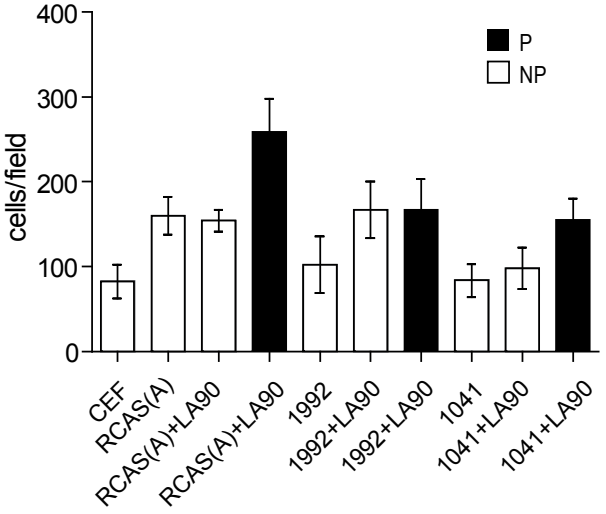


Figure 8. Knock-down of *PIK3CD* retards migration of CEF in a Boyden chamber assay

10^4 CEF were seeded per chamber and allowed to traverse a 2-10% serum gradient over an eight hour period. Lower membrane surfaces were stained with DAPI and quantified using ImageJ (Abramoff *et al.*, 2004). Eight fields per well were counted. Number of cells per field was significantly different between RCAS(A)+LA90 P and 1992+LA90 P and between RCAS(A)+LA90 P and 1041+LA90 P (one way ANOVA, with Tukey post-hoc test; p,0.01). LA90 is a type B temperature sensitive RSV. 1041 and 1992 refer to two different *PIK3CD* shRNA constructs. RCAS(A) is a control virus carrying the a GFP shRNA. NP and P refer to non-permissive and permissive temperatures. Error bars indicate standard deviation.

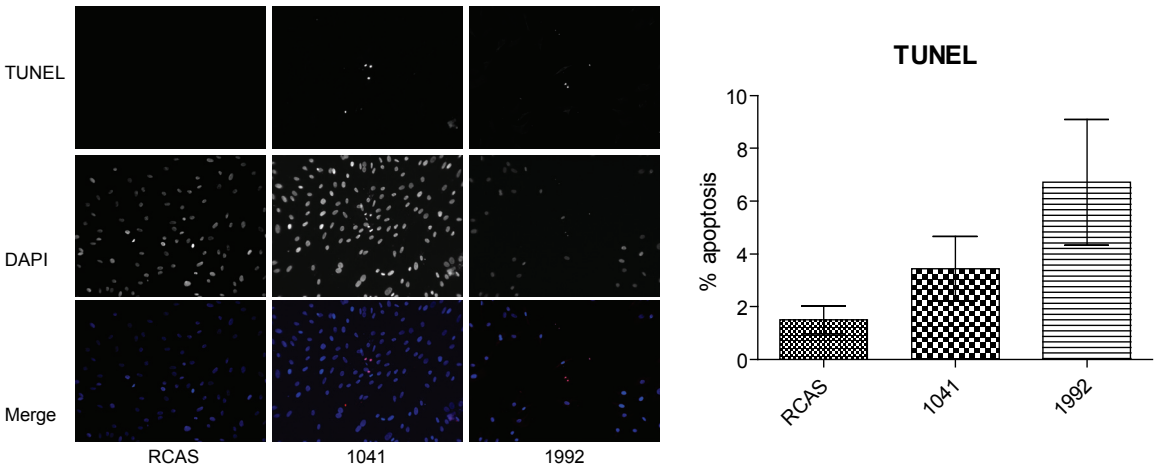


Figure 9. Percentage of cells undergoing apoptosis is greater in cells with the 1992 *PIK3CD* knock-down than in normal CEF infected with control virus (RCAS)

($p<0.05$, t-test, 2-tailed, Bonferroni-corrected). A minimum of six fields per condition were counted. Counting and quantification was done using ImageJ (Abramoff *et al.*, 2004). 1041 and 1992 refer to two different *PIK3CD* shRNA constructs. Error bars indicate standard error of mean.

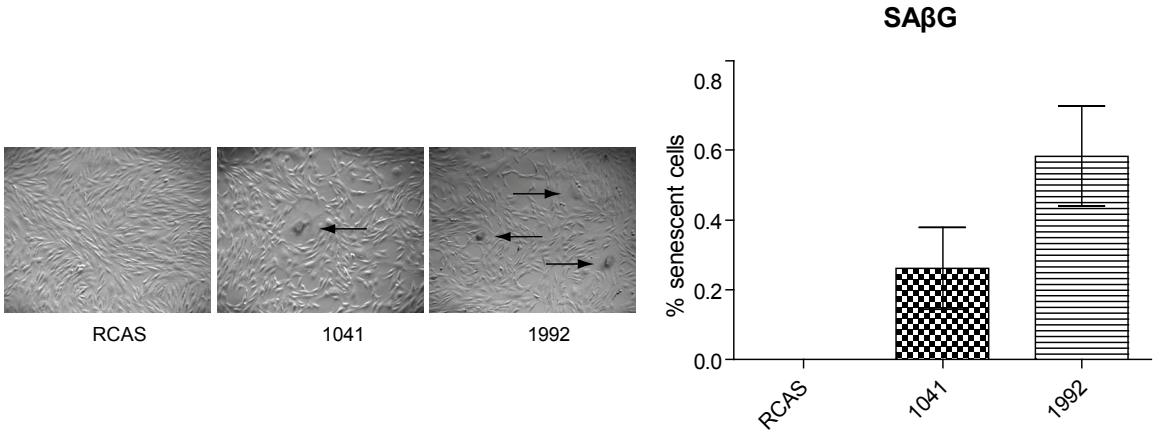


Figure 10. Percentage of senescent CEF is greater in cells with the *PIK3CD* knock-down than in normal CEF infected with control virus (RCAS)

($p<0.01$, t-test, 2-tailed, Bonferroni-corrected). 1041 and 1992 refer to two different *PIK3CD* shRNA constructs. Arrows indicate positive SAβG stain. A minimum of eight fields per condition were counted. Counting and quantification was done using ImageJ (Abramoff *et al.*, 2004). Error bars indicate standard error of mean.

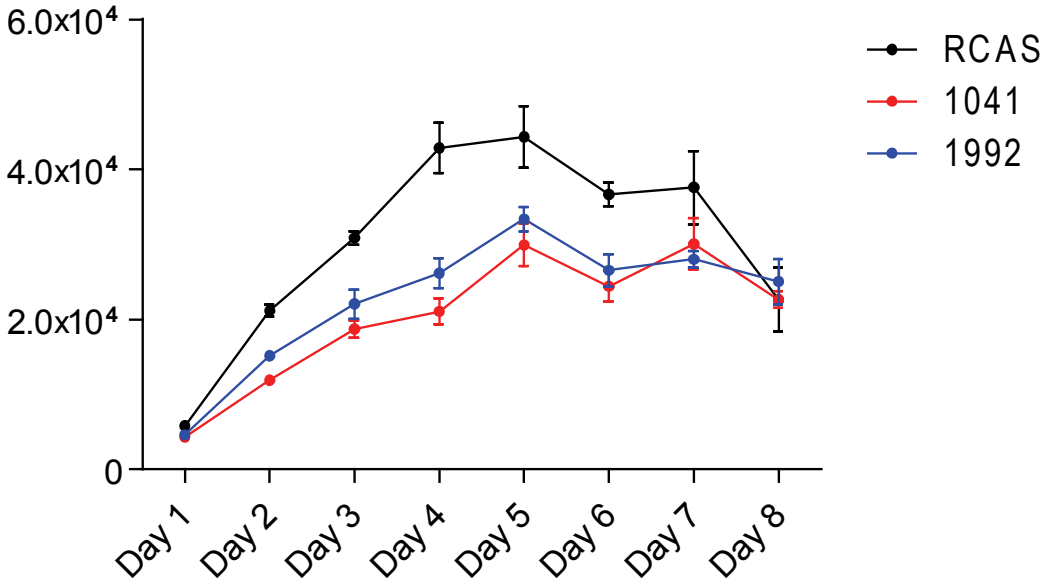


Figure 11. Growth rates for CEF infected with *PIK3CD* shRNA are decreased compared to CEF infected with control virus (RCAS).

10^4 CEF were seeded per well in a 24-well plate. Cells were counted everyday for eight days using a coulter counter. Each condition was repeated in four replicates. 1041 and 1992 refer to two different *PIK3CD* shRNA constructs. Error bars indicate standard error of mean.

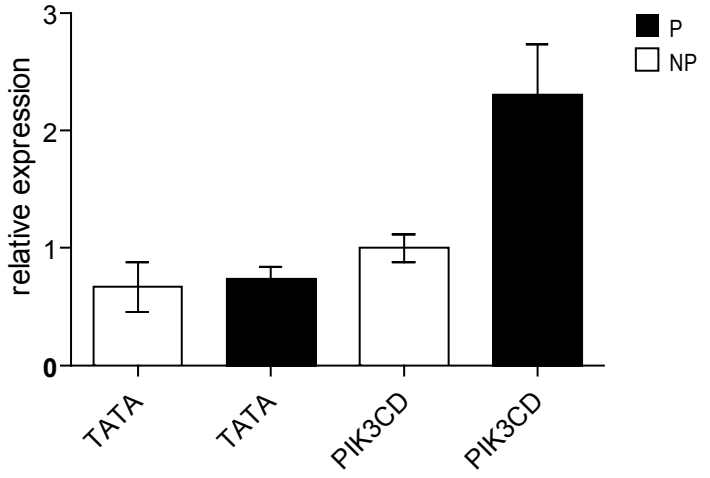


Figure 12. The *PIK3CD* putative SRU is a weak Src-responsive cis-regulatory element as determined by *Gaussia* luciferase reporter assay.

v-Src activates the *PIK3CD* pSRU approximately two-fold compared to ts72-4 infected CEF at the restrictive temperature (t-test, two tailed, $p<0.05$). NP and P refer to non-permissive and permissive temperatures. Error bars indicate standard error of mean. TATA refers to reporter constructs containing a TATA box only whereas PIK3CD refers to reporter constructs containing a TATA box and the *PIK3CD* pSRU (see Figure 1).

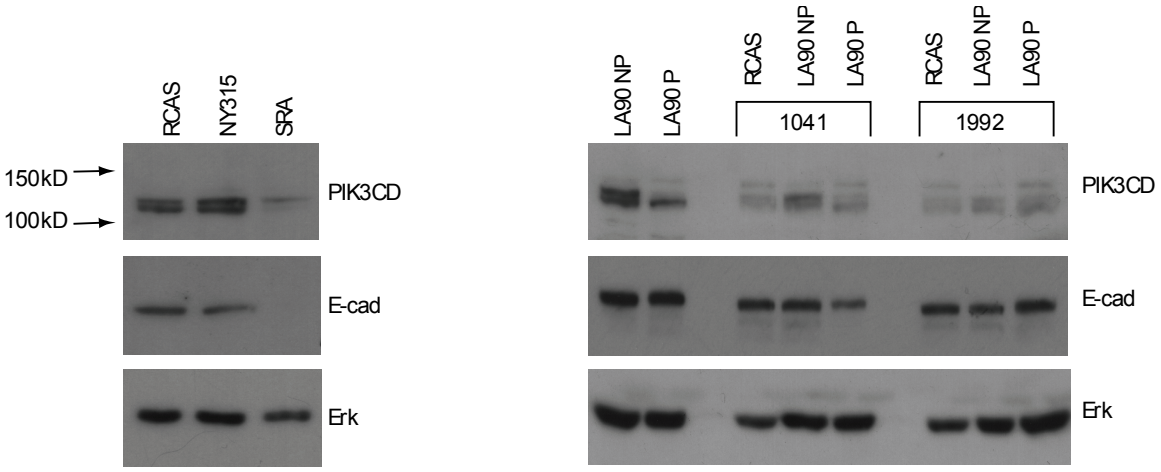


Figure 13. *PIK3CD* shRNA constructs knock down PIK3CD expression but PIK3CD is also reduced in transformed CEF.

RCAS and NY315 refer to non-transforming control virus whereas SRA refers to the highly transforming Schmidt-Ruppin A RSV. LA90 is a type B temperature sensitive RSV. 1041 and 1992 refer to two different *PIK3CD* shRNA constructs. NP and P refer to non-permissive and permissive temperatures. E-cad refers to the E-cadherin protein. Erk was used as a loading control.

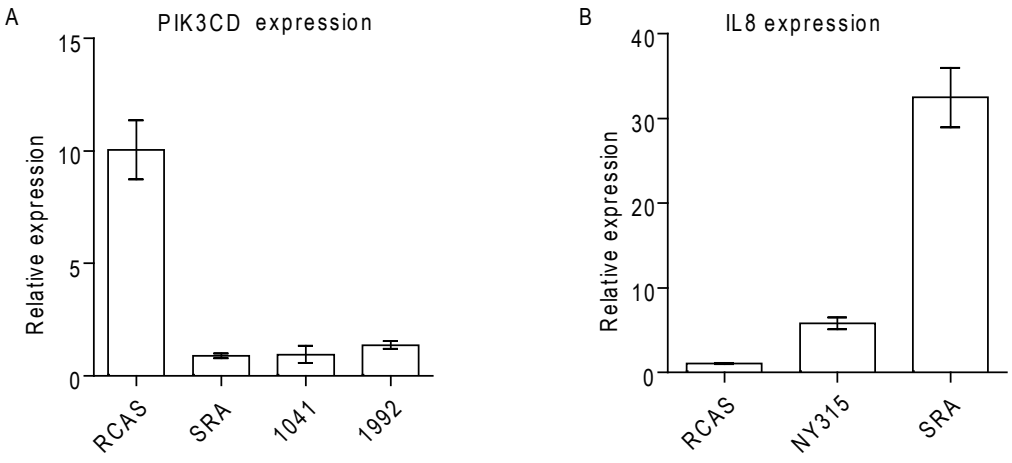


Figure 14. *PIK3CD* expression is decreased in transformed CEF

A. qPCR experiment showing that *PIK3CD* expression is decreased in transformed CEF and in CEF expressing the *PIK3CD* shRNA. **B.** qPCR experiment showing that the v-Src-responsive gene *il8* is activated in transformed CEF. RCAS and NY315 refer to non-transforming control virus whereas SRA refers to the highly transforming Schmidt-Ruppin A RSV. 1041 and 1992 refer to two different *PIK3CD* shRNA constructs. Error bars indicate standard error of mean.

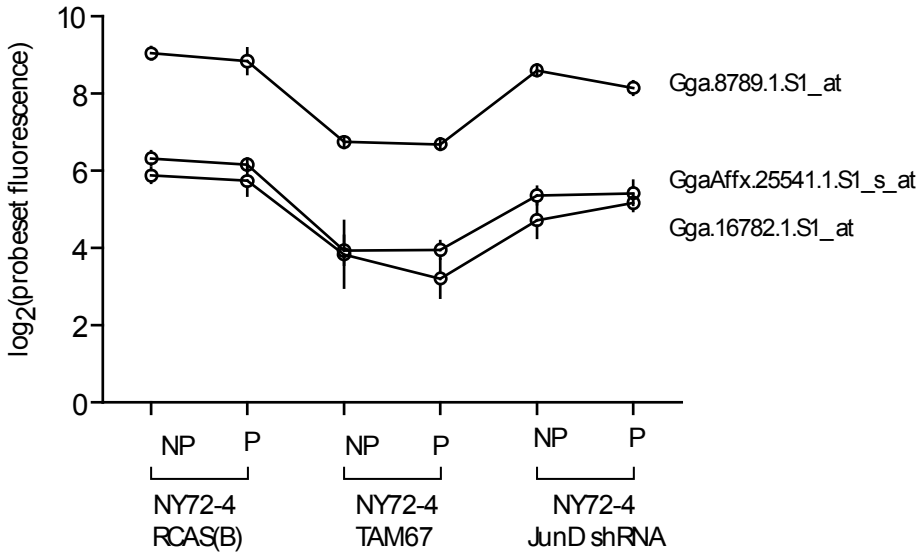


Figure 15. Microarray analysis shows that PIK3CD transcript levels are not increased in CEF transformed by the ts72-4 RSV.

TAM67 attenuates PIK3CD expression at both permissive and non-permissive temperatures but JunD shRNA has no effect. PIK3CD Probeset designations are indicated at the right of the graph. RCAS(B) is a control virus carrying the a GFP shRNA. NP and P refer to non-permissive and permissive temperatures. Error bars indicate standard deviation.

Appendix 2: CHOP repression mediates the induction of the p20K growth arrest specific gene in response to contact inhibition and hypoxia.

Context and contribution

Previous experiments in our lab showed that p20K expression was dependent on C/EBPβ activity and negatively correlated with CHOP expression. p20K expression was repressed by abrogating C/EBPβ activity and could be enhanced by CHOP repression. In search of novel growth arrest specific genes, we undertook a microarray study of serum-starved, contact inhibited and serum-starved/contact inhibited CEF. Based on the up-regulation of known HIF1α responsive genes (CA9, CA12, ENO2) and on other known hypoxic markers it was determined that the contact-inhibition response resembled hypoxia. qPCR expression analysis of several candidate genes revealed that some of these were regulated by hypoxia and that their expression could be repressed by the a C/EBPβ dominant negative allele indicating that genes previously unknown to be regulated by hypoxia were in fact regulated by this stimulus. Moreover, the hypoxia response was C/EBPβ-dependent.

The microarray analyses were carried out by the author of this thesis along with the design of the qPCR experiments to validate expression candidates. The qPCR experiments were carried out by exchange student Flore-Anne Poujade under the author's supervision. Results from the expression profiling and qPCR experiments led directly to the characterization of the hypoxia-response of the p20K promoter by former MSc student Ben Fielding. Additional cell biology, RNA isolation and CHOP analysis was

conducted by former MSc student Romita Ghosh. Cloning of CHOP and initial characterization of the QRU were carried out by the other contributors. The manuscript was written by Pierre-André Bédard with the exception of certain portions of materials and methods written by Romita Ghosh, Ben Fielding and the author of this thesis. Additional text in the results was also contributed by the thesis author. The manuscript was edited by Pierre-André Bédard and the author of this thesis.

CHOP repression mediates the induction of the p20K growth arrest specific gene in response to contact inhibition and hypoxia.

Ben Fielding[1†] Romita Ghosh[1†], Bart M. Maslikowski[1], Tetsuaki Miyake[2‡] , Flore-Anne Poujade[1], Shylan Yan[1], Scott Maynard[2¶], Mark Gagliardi[2§], Ying Wu[1] and P-A. Bédard[1*]

[1]Department of Biology, McMaster University, Hamilton, Ontario, L8S 1K4, Canada
[2]Department of Biology, York University, Toronto, Ontario, M3J 1P3

Running Title: Regulation of CHOP-C/EBPβ by hypoxia

Key words: Growth arrest specific genes, p20K lipocalin, hypoxia, CHOP, C/EBPβ

† These authors have contributed equally to this work

Present addresses:[¶] Institute of Cellular and Molecular Medicine, Center for Healthy Ageing, University of Copenhagen, Blegdamsvej 3B, DK-2200, Copenhagen, Denmark; [§] McEwen Centre for Regenerative Medicine, Toronto Medical Discovery Tower - MaRS Centre, Toronto, ON, M5G 1L7, Canada; [‡] Best Institute, 102 College Street, Toronto, ON, M5G 1L6.

* To whom correspondence should be addressed: Phone: (905) 525-9140 ext. 23149, Fax: (905) 522-6066, e-mail: abedard@mcmaster.ca

Summary

The p20K lipocalin gene is expressed in chicken embryo fibroblasts (CEF) entering G_0 as a result of contact inhibition. This expression is mediated at the transcriptional level by the interaction of C/EBPβ with two elements of a 48bp region of the promoter designated the Quiescence Responsive Unit or QRU. The signals mediating the activation of p20K and the factors cooperating with C/EBPβ in this process are presently unknown. In this report, we describe the association of CHOP (C/EBP HOmologous Protein) with C/EBPβ in actively dividing CEF. Experiments based on forced expression or down-regulation of CHOP by RNA interference revealed that CHOP inhibits the expression of p20K in cycling cells and in response to endoplasmic reticulum (ER) stress. Contact inhibition caused the repression of CHOP and the induction of p20K. Gene profiling analyses revealed that several genes induced by cell density are also activated by hypoxia. Western blotting and promoter analyses indicated that p20K is also strongly inducible by low oxygen concentrations (2% O_2 for 24 hrs) in cycling cells and that this induction is mediated by the QRU and C/EBPβ. As described for contact inhibited CEF, CHOP was repressed in response to hypoxia and forced expression of CHOP inhibited the induction of p20K in these conditions. These results suggest that hypoxia provides the signal for the induction of the p20K gas gene at contact inhibition, a process dependent on the repression of CHOP.

Introduction

Cells exiting the cell cycle enter a state of quiescence known as the G_0 phase. The entry into G_0 is poorly characterized but involves the activation of a group of genes referred to as "growth arrest-specific" or gas genes (Schneider *et al.*, 1988). The role of gas genes is largely undefined but may contribute directly to growth arrest (Goldstein *et al.*, 1991; Del Sal *et al.*, 1992), to a greater capacity of the cell to survive oxidative stress (Kops *et al.*, 2002), to the synthesis of the extra-cellular matrix (Coppock *et al.*, 1993; Mauviel *et al.*, 1995; Casado *et al.*, 1996), the modulation of lipid metabolism (Bedard *et al.*, 1989; Bohmer *et al.*, 1988; Provost *et al.*, 1991; Iyer *et al.*, 1999) and the preparation for re-entry into the cell cycle (Lih *et al.*, 1996; Sang *et al.*, 2008). The regulatory mechanisms of quiescence-specific gene expression are also poorly understood. Some of the gas genes are regulated at the post-transcriptional level while others depend on transcriptional activation for expression in response to contact inhibition or serum starvation (Fornace *et al.*, 1989; Krauss *et al.*, 1990; Krauss & Weinstein, 1991; Mao *et al.*, 1993; Mauviel *et al.*, 1995; Smith & Steitz, 1998).

We previously characterized the activation of the p20K lipocalin gene by contact inhibition. In chicken embryo fibroblasts (CEF), p20K is activated predominantly at confluence and, to a lesser extent, by serum/medium starvation. We identified a 48 bp region of the promoter, termed the "Quiescence-Responsive Unit" or QRU, required for the activation of this gene at contact inhibition (Mao *et al.*, 1993). C/EBPβ binds to two elements of the QRU and, when over-expressed, is capable of inducing the expression of p20K in cycling cells (Kim *et al.*, 1999c).

C/EBPβ is activated in response to several stimuli and plays an important role in biological processes unrelated to growth arrest. For instance, we reported that the activity of C/EBPβ is induced in CEF transformed by the Rous sarcoma virus (RSV) i.e. in conditions where p20K is not expressed (Gagliardi *et al.*, 2001). We showed that mitogenic stimulation reprograms the cell to direct the activity of C/EBPβ toward expression of genes of the G_0/G_1 transition. AP-1, a factor controlling the expression of IL8 and cyclin D1, inhibits the expression of p20K and thus plays a major role in this process (Gagliardi *et al.*, 2003). Cells over-expressing c-Jun, JunD or Fra-2, the main components of AP-1 in cycling CEF, do not express p20K, are unable to enter G_0 and undergo apoptosis at high cell density. Normal CEF entering G_0 down-regulate the activity of AP-1 by a number of mechanisms that include the repression of c-Jun, JunD and Fra-2. The expression of a dominant negative mutant of C/EBPβ blocks the expression of p20K and dramatically enhances the activity and expression of AP-1 proteins in CEF. Therefore, AP-1 and C/EBPβ play opposing roles in the expression of gas genes and the control of CEF proliferation (Gagliardi *et al.*, 2003).

The signals underlying the induction of p20K and the factors (if any) cooperating with C/EBPβ in this process are unknown. In this report, we describe the regulation of p20K and novel growth arrest specific genes by hypoxia. Promoter analyses and the over-expression of a dominant negative mutant of C/EBPβ revealed that the induction of p20K by low oxygen concentrations was mediated through the QRU in a C/EBPβ-dependent manner and, thus, did not involve HIF, the hypoxia-inducible factor. Cloning of the C/EBPβ inhibitor CHOP (C/EBP HOmologous Protein) and co-

immunoprecipitation experiments indicated that CHOP is associated with C/EBPβ in cycling CEF but is repressed by contact inhibition or hypoxia. This promotes the formation of C/EBPβ homodimers and the expression of gas genes, including p20K. These results suggest that hypoxia provides the signal for the induction of gas genes at contact inhibition, a process mediated by the repression of CHOP.

Materials and Methods

Cell culture.

Early passages (n<10) of CEFs were cultured at 41.5ºC in Richter-improved minimal medium containing insulin and zinc (I^+ medium, Irvine Scientific, Santa Ana, CA) with 5% heat-inactivated (at 57ºC for 30 minutes) Cosmic Calf serum or 5% new-born bovine serum (BioMedia, Cansera, Rexdale, Ontario), and 5% tryptose phosphate broth, and 1% L-glutamine, penicillin, and streptomycin solution (GIBCO BRL) ("complete medium"). CEFs were also starved in medium without the serum ("serum-free medium) after being washed twice with the serum-free medium. Hypoxia was induced by culturing CEF in 2% O_2, or by treatment with 1 mM dimethyloxalylglycine (DMOG) for the indicated period of time.

Proliferation assays.

The cells were collected from the plate by trypsinization (EDTA Trypsin (GIBCO BRL)). 200μL aliquots of the cell suspension in 10mL of diluent were counted in a Coulter counter (lower limit was 10μm) for three times. The averages of triplicated samples (each

counted three times) were used for proliferation curve generations. The error bars represent the standard deviations.

Cloning and plasmid construction.

The chicken CHOP clone was isolated from the quiescence specific λ cDNA library using as a probe an EST insert encoding a partial CHOP cDNA (clone pat.pk0050.f8f, chicken EST project, University Delaware). The positive cDNA was inserted in the *EcoRI* site of the Cla12 adaptor plasmid and sub-cloned into the *ClaI* site of the RCASBP vector (Hughes, 2004; Hughes *et al.*, 1987).

Construction of retroviral vectors for CHOP knockdown by shRNA.

The 22 nucleotide target sequences of the CHOP gene were chosen using the design tool at www.genscript.com/ssl-bin/app/rnai. The 5' base of the sense strand was altered in all cases so that it mismatched the guide strand base to mimic the structure found in endogenous miRNA30. Hairpins for the first miRNA cloning site were generated by PCR using 10ng of each gene-specific oligonucleotide A and B together with 100ng of two generic flanking oligonucleotides C and D in a 50ul reaction using ProofStart polymerase (Qiagen; see Table 1). PCR conditions were 5min at 95° followed by 25 cycles of 30s at 94°, 30s at 55° and 45s at 72° using GeneAmp PCR system 2700 (Applied Biosystems). PCR products were purified by PCR purification kit (GE Healthcare), digested with *Nhe*I and *Mlu*I and subcloned into pRFPRNAiC(U6-). The miRNA expression cassettes for CHOP were then subcloned from pRFPRNAiC(U6-)-CHOP into a modified RCASARNAi vector (ARK-Genomics; (Wang *et al.*, 2011a)) as a *Not*I-*Cla*I fragment .

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

A	GAGAGGTGCTGCTGAGCGACAGCTGAGTGCACACAACGAGTAGTGAAGCCA CAGATGTA
B	ATTCACCACCACTAGGCAGCAGCTGAGTGCACACAACGAGTACATCTGTGGC TTCACT
C	**GGCGGGGCTAGCTGGAGAAGATGCCTTCCGGAGAGGTGCTGCTGAGCG**
D	GGGTGGACGCGTAAGAGGGGAAGAAAGCTTCTAACCCCGCTATTCACCACC ACTAGGCA

Northern blotting analysis.

Total RNA was isolated by the high salt-urea precipitation method (Bedard *et al.*, 1987a). Briefly, the cells were washed with ice-cold 1×PBS for three times and then collected with lysis buffer (6M Urea, 3M LiCl, and 10mM sodium vanadate). The Cells were lysed by sonication three times for 15sec intervals on ice, and the RNA was precipitated overnight on ice. The pellet was centrifuged, resuspended in TES solution (5mM EDTA, 10mM Tris-HCl pH8.0, and 0.2% SDS) and extracted with phenol/chloroform. The RNA was ethanol precipitated, collected by centrifugation and dissolved in DEPC treated ddH_2O. The concentration and quality of extracted RNA were determined by optical density reading of 260/280nm and gel analysis (see below).

7.5μg of the total RNA were run on 1% agarose-3%formaldehyde, 1×MOPS (5mM NaAcetate, 1mM EDTA pH8.0, and 20mM MOPS adjusted to pH7.0 with NaOH) gel for electrophoretic separation. The RNAs were transferred onto Nytran membrane (Schleicher and Schuell) by blotting. The blot was air-dried briefly, and the RNAs were fixed onto the membrane by baking in a vacuum oven at 80ºC for 90min. The RNAs were pre-hybridized with heat-denatured purified salmon sperm DNA (200μgm/ml) in the hybridization solution (50% deionized formamide, 5×Denhardt's, 5×SSC, 5mM EDTA,

and 0.1% SDS) at 42ºC for at least 2 hrs. Finally, the RNAs were hybridised with heat-denatured ^{32}P-labeled cDNA probe in the hybridisation solution with gentle shaking at 42ºC overnight. The blot was washed twice with 2×SSC, 5mM EDTA pH8.0, and 0.1% SDS at room temperature for 15min each and then washed with 0.1×SSC, 5mM EDTA pH8.0, and 0.1% SDS at 55ºC for 20min. Film (Kodak X-OMAT) was exposed to the blot for a few days.

qRT-PCR.

DNase I treated RNA samples were reverse transcribed using commercially available reagents (ProtoScript cDNA synthesis kit, New England Biolabs) and quantitative amplification was performed using commercially available reagents (PerfeCTa SYBR Green FastMix low ROX (Quanta Biosciences), a real-time PCR instrument from Stratagene (MX3000P) and the following primers:

CAIX Carbonic Anhydrase IX
F : **TAGGTTGGGCCAAGGGAGAACCC** (5 > 27)
R : **CGGCAATGTTGAACCCGGGCA** (110 > 90)

PRKCH Protein Kinase C η
F : **CGCTTGCAGCGAAGTCGAGGA** (6 > 26)
R : **ACGCTGGACAGTGCTGGGAC** (134 > 115)

SULT1E1 sulfotransferase family 1E, estrogen-preferring, member 1
F : **ACGGACTGGCCGTTCTGGCT** (485 > 504)
R : **CGGGGACATGGACCATCTGATCCTT** (601 > 577)

P20K p20K Lipocalin
F : **GCCCAGCCAGGAGGAATGCA** (519 > 538)
R : **AGCAGCCTCGAGCTTTGGCA** (618 > 599)

Gene profiling analyses.

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

GST-Fusion protein purification and antibody production.

CHOP-pGEX-2T vector was transformed into BL21 *E.coli* strain. The production of GST-CHOP fusion protein was induced by the addition of IPTG (100mM) for 2 hours. The bacteria were collected by centrifugation and washed with ice-cold PBS. The bacteria were lysed by sonication three times for 20 seconds on ice in I% Triton X-100 containing PBS (PBS-T). Cell debris was removed by brief centrifugation. The GST-fusion protein was purified according to the instructions provided by the supplier and used to generate rabbit polyclonal antibodies, as described before (Kim *et al.*, 1999c).

Western blotting analysis.

Total proteins were isolated from whole cells. The cells were washed twice with ice-cold 1×PBS, scraped and collected in 1×PBS. The cells were lysed by for 5min by boiling in SDS sample buffer (2.3% SDS, 5% β-mercaptoethanol, 10% glycerol, and 62.5mM Tris-HCl adjust to pH 6.8 with 0.5mM phenylmethylsulfonyl fluoride and 0.03% each of Antipain, Aprotinin and Leupeptin). After determining protein concentration by the Bradford assay, 30μg total proteins were loaded onto 12% SDS-PAGE. After electrophoresis, the proteins were transferred onto the nitro-cellulose membrane (Schleicher and Schuell, BA85) by blotting. First, the membrane was blocked with 5% skim-milk powder in TBS-T (5M Tris-HCl and 0.15M NaCl adjust to pH7.4 with0.1% Tween-20) at room temperature for 2.5hrs. For apoA-I protein detection, 5% BSA TBS-T was used thereafter instead of the 5% milk TBS-T. The membrane was incubated with the primary antibody indicated in the 5% milk TBS-T at 4ºC overnight with gentle agitation. (The primary antibodies used in this study are listed below.)

Chicken p20K (601Y)	Dilution 1:2000; Previously described (Bedard *et al.*, 1987b)
Chicken CHOP	Dilution 1:1500; This study
Chicken C/EBPβ	Dilution 1:1500; Previously described (Gagliardi *et al.*, 2001)
ERK-1 (SC-94)	Dilution 1:2000; Santa Cruz Biotechn. (Santa Cruz, CA)
HIF1α (ab2185)	Dilution 1:500; Abcam

After removing excess primary antibody by washing in TBS-T a few times, the blot was incubated with secondary anti-rabbit, anti-mouse, or anti-goat IgG antibody conjugated

with horseradish peroxidase at room temperature in 5% milk TBS-T. Chemiluminescent signals generated by the enzyme of the secondary antibody in the immuno-complex were detected according to the protocol provided by the supplier (ECL, GE Healthcare).

Co-immunoprecipitation experiments.

Protein lysates were prepared by incubating cells in 1 ml/100mm plate of TNE buffer (50 mM Tris pH7.6, 150 mM NaCl, 1% Nonidet P-40, 10mM NaF, 2 mM EDTA pH8, 1 mM EGTA, pH8.5, complete protease inhibitor cocktail tablet (Roche Diagnostics #11 836 170 001)) for 20 min on ice. To remove cell debris, the lysates were centrifuged in the cold for 5 min at maximum speed in a microfuge. Antibodies for C/EBPβ (2 μl), HIF1α (4 μl) or corresponding pre-immune sera (C/EBPβ) or normal rabbit IgG (Santa Cruz #2027) were added to equal amounts of proteins (500 μg) and incubated overnight at 4˚C on a rotator. The next day blocked Protein G Sepharose beads (GE Healthcare 17-0618-01) were added to each IP and incubated for 1 hr at 4˚C on a rotator. The beads were pelleted, washed several times with 1 ml of TNE, suspended in 60 μl of SDS sample buffer and heated for 3 min at 100˚C to elute proteins. Proteins were then analyzed by polyacrylamide gel electrophoresis and western blotting analysis.

Results

Cloning and characterization of chicken CHOP. The sequence of the quiescence-responsive unit (QRU) of the p20K promoter was submitted to Signal Scan in order to identify binding sites for known transcription factors (Prestige, 1991). This analysis

revealed that the C/EBPβ element located at the 5' end of the QRU matches the consensus for the CHOP transcription factor (Fig.1A). CHOP, also known as Gadd153 and C/EBPζ, is a divergent member of the C/EBP family that can only bind DNA when dimerized with other members of the family, such as C/EBPβ (Sok *et al.*, 1999; Ron & Habener, 1992). C/EBP factors generally bind to the core sequence TGCAAT while the C/EBP/CHOP heterodimer shows preference for an extended version of this element (Ubeda *et al.*, 1996). This extended binding site resembles closely to the C/EBP element of domain A of the QRU [Fig.1A; (Kim *et al.*, 1999c; Mao *et al.*, 1993)].

To determine if p20K is a target of CHOP, we first searched for cDNAs with homology to CHOP in the collection of chicken EST clones of the University of Delaware. One such clone, pat.pk0050.f8.f, showed partial sequence similarity to mammalian CHOP. Using this EST clone as a probe, we screened a quiescence-specific cDNA library and isolated a full-length cDNA encoding a protein with similarity to mammalian CHOP. This clone encodes a protein of 156 amino acids in length that is 31% identical to human CHOP (Fig.1B). As expected, the highest degree of similarity was found at the C-terminus, in the region corresponding to the basic domain and leucine zipper of CHOP. However, human and murine CHOP are 88% identical at the amino acid level suggesting that our clone encodes a new member of the C/EBP family or that CHOP has diverged rapidly during evolution. Two features, however, suggest that our clone encodes avian CHOP. First, all mammalian homologs of CHOP contains a small open reading frame in the 5' untranslated region of their mRNA coding for a conserved peptide of 36 amino acids in length (Jousse *et al.*, 2001). As shown in Fig.1C, our

chicken cDNA includes a similar ORF coding for a 34 amino acid peptide with 33% sequence identity with the 5' ORFs of mammalian CHOP. Secondly, the α–helical structure typical of the DNA binding domain of mammalian CHOP is perfectly conserved in the protein encoded by our clone. Mammalian CHOP proteins are unable to form homodimers because two of the leucine residues of the dimerization domain are located on the adjacent side of the α-helix (Supplem. Fig.1). The same is true for the putative avian CHOP leucine zipper. By comparison, chicken C/EBPβ contains 5 leucine residues perfectly aligned along the axis of the α-helix. Moreover, the charged amino acids adjoining the leucine residues and favoring the interaction with C/EBPβ are also highly conserved in avian CHOP (Ciarapica *et al.*, 2003). Therefore, the structure of the DNA binding and dimerization domain strongly suggests that our clone encodes the chicken homolog of CHOP.

CHOP is a negative regulator of p20K expression in growth-arrested CEF.

To determine if CHOP contributes to the induction of p20K at high cell density, CEF were infected with a retrovirus expressing a shRNA for avian CHOP and p20K expression was examined in cycling and contact inhibited CEF maintained at confluence for an increasing number of days (Fig.2). In mammalian cells, these conditions of confluence cause starvation, ER stress and the induction of CHOP (Fornace *et al.*, 1989). In contrast, p20K is poorly induced by starvation and repressed by severe ER stress (Mao *et al.*, 1993). This is shown in Fig.2A. Interestingly, the down-regulation of CHOP enhanced the expression of p20K in confluent CEF and prolonged its expression in

conditions of starvation. Therefore, the accumulation of CHOP antagonized the expression of p20K in density-arrested CEF.

Control CEF and CEF expressing the shRNA for CHOP were also seeded at high density and then transferred to serum-free medium for increasing periods of time. In both cases, cell numbers began to decline upon prolonged starvation but CEF expressing the CHOP shRNA remained viable for a longer period (Fig.2B). Therefore, not only was CHOP dispensable for CEF survival but, in fact, it reduced the survival of control CEF in the absence of serum. Thus, as described in other species, avian CHOP functioned as a mediator of ER stress, limiting the expression of p20K and the survival of serum-starved CEF.

Control of growth arrest specific gene expression by hypoxia.

Gene profiling was performed to identify genes regulated by contact inhibition in CEF. A summary of this analysis is provided in Table 2. In this study, we compared the pattern of gene expression of cycling, contact inhibited, serum starved and sub-confluent, and serum starved confluent CEF (Fig.3). While there was some overlap in the patterns of genes regulated by different conditions of growth arrest, it is also clear that several genes, such as p20K, were activated primarily by contact inhibition while others, such as CHOP, were only induced in conditions of serum starvation (Fig.3A and data not shown). Interestingly, four genes dysregulated by contact inhibition (*ERO1L, FAM13A, NDRG1* and *CDC25*) were also found in the twenty-eight gene list of core hypoxia genes found commonly dysregulated in a study of hypoxia response in four epithelial cell types (Chi *et al.*, 2006). Another ten functional/structural homologues to eight of the core hypoxia

genes were also dysregulated (*FAM13A, LDHB, LOXL1, LOXL3, P4HA1, P4HA3, SLC2A5, CYP1A1, E2F5* and *E2F8*). Strikingly, all these genes are dysregulated in the same manner, i.e. their direction of change is the same for hypoxia versus normoxia as it is for contact inhibited CEF compared to cycling CEF. Some of the genes induced most markedly at contact inhibition are also well characterized targets of HIF1α and the response to hypoxia (Wenger *et al.*, 2005). This is the case for carbonic anhydrase IX and XII, as well as enolase 2 (Table 3). In fact, the expression of the mRNA for HIF1α, a key factor of the response to hypoxia, was also elevated in contact inhibited but not serum-starved CEF. Interestingly, CHOP mRNA was expressed, albeit at low levels, in cycling CEF and was down-regulated when cells reached confluence (Fig.3A and Table 2).

Gene validation was performed by quantitative RT-PCR (qRT-PCR) for p20K, carbonic anhydrase IX and genes for two signaling molecules, PKCη (PRKCH) and sulfotransferase 1E1 (SULT1E1) previously not known to be regulated by contact inhibition or hypoxia. This was done in control CEF and CEF expressing a dominant negative mutant of C/EBPβ designated Δ184-C/EBPβ, in conditions of normoxia or hypoxia (24 hrs in 2% O_2). As shown in Fig.4, all four genes were induced by both hypoxia and contact inhibition, although differences were evident in the degree of induction of these genes in these two conditions. For instance, p20K was strongly induced by contact inhibition and hypoxia while carbonic anhydrase IX (CAIX) was primarily activated by hypoxia. These genes were also distinguishable by the response to the dominant negative mutant Δ184-C/EBPβ. Indeed, the expression of PKCη,

sulfotransferase 1E1 and p20K was abolished by the dominant negative mutant while the induction of carbonic anhydrase IX was partially reduced in hypoxia and stimulated in contact inhibited CEF. Collectively, these results suggest that C/EBPβ functions as a global regulator of gas gene expression in contact inhibited CEF.

To confirm that the expression of p20K is regulated by hypoxia, cycling or density-arrested CEF were maintained in 2% O_2 for 24 hrs (hypoxia) and compared to cells in conditions of normoxia. As shown in Fig.5A, p20K was strongly induced by hypoxia even when CEF were actively dividing (cycling); this induction was further enhanced in density-arrested CEF. In this experiment, the expression of HIF1α was stimulated modestly by either hypoxia or contact inhibition. Cycling CEF were also treated with dimethyloxalylglycine (DMOG), a hypoxia mimetic and potent inhibitor of proline hydroxylases. Again, a marked induction of p20K was observed in these conditions but not in the presence of Δ184-C/EBPβ (Fig.5B).

Dual effects of N-acetyl-L-cysteine in the induction of p20K by contact inhibition and hypoxia.

Reactive oxygen species (ROS), produced by the mitochondrial complex III, play an important role in the response to hypoxia (Semenza, 2011; Chandel *et al.*, 2000). To determine if ROS are involved in the induction of p20K by contact inhibition, confluent CEF were treated with 5 or 8 mM N-acetyl-L-cysteine (NAC) for 4 or 24 hrs and p20K expression was monitored by western blotting analysis. The addition of NAC reduced modestly the expression of p20K at the 4 hr time point but had no effect at 24 hrs. In fact, there was a small but statistically significant increase in p20K expression at the later time

point (Fig.6). This dual effect of NAC was even more apparent when p20K expression was induced by hypoxia (Fig.7), suggesting that NAC functions through different mechanisms early and late after addition to CEF cultures.

P20K induction is dependent on the QRU and C/EBPβ in conditions of hypoxia.

Two potential HIF-response elements (HRE) are located at position -415 and -458 of the 5' flanking region of the p20K gene [Fig.8A; (Kimura *et al.*, 2001)]. To identify the region of the p20K promoter responsible for activation by hypoxia, 5' deletion constructs of the promoter region fused to the CAT reporter gene were transfected in cycling CEF and analyzed in conditions of normoxia and hypoxia (2% O_2 for 24hrs) by transient expression assays (Fig.8B-C). The p20K promoter was strongly inducible by hypoxia. This induction was observed with all 5'deletion constructs but that lacking the QRU (-169 to -217 region), indicating that the same region responsible for activation in contact inhibited CEF is also required for the induction by hypoxia. This result is consistent with the qRT-PCR data, indicating that the induction of the p20K mRNA by contact inhibition and hypoxia is abolished by the expression of the dominant negative mutant Δ184-C/EBPβ (Fig.4). In support of this notion, western blotting analysis confirmed that Δ184-C/EBPβ abolishes the induction of p20K by contact inhibition and hypoxia (Fig.8D). These results suggest that C/EBPβ is a novel hypoxia-responsive factor.

The down-regulation of CHOP mediates the induction of p20K by hypoxia.

Since CHOP mRNA expression is inhibited by contact inhibition (Fig.3A), we performed a western blotting analysis of CHOP in response to increasing periods of moderate hypoxia (2% O_2). This analysis revealed that the induction of p20K did not correlate with

peak accumulation of HIF1α, in agreement with the notion that p20K is not regulated by this factor in low oxygen concentrations (Fig.9). In contrast, p20K expression followed closely the delayed down-regulation of CHOP in response to hypoxia.

Two experiments were performed to look at proteins interacting with C/EBPβ in response to hypoxia. Since HIF1α has been reported to function as a co-activator of C/EBPα in leukemic cells (Jiang *et al.*, 2005; Janardhan, 2008), we first looked at a potential interaction between this factor and C/EBPβ by co-immunoprecipitation/western blotting analysis (IP-western). Multiple forms of C/EBPβ, corresponding to the transcriptional activators LAP and LAP*, as well as the transcriptional inhibitor LIP (Descombes *et al.*, 1990), were detected in total CEF lysates (Fig.10A). However, none of the C/EBPβ isoforms was detected in immunoprecipitates of HIF1α. Similar experiments were performed with CHOP. A stable interaction of CHOP with C/EBPβ was detected by this approach (Fig.10B). However, the amount of CHOP associated with C/EBPβ decreased in response to hypoxia, consistent with the repression of CHOP in these conditions (Fig.10B, lanes 1-2). To determine if CHOP functions as an inhibitor of p20K induction by hypoxia, CEF were infected with a retrovirus encoding CHOP or a control virus devoid of any cDNA insert, and p20K expression was examined in conditions of normoxia and hypoxia. P20K accumulated markedly in control CEF subjected to 2%O_2 for a 24hr period (Fig.11). However, this induction was nearly abolished in cells over-expressing CHOP. Collectively, these results indicate that the repression of CHOP mediates the induction of p20K by hypoxia. The repression of

CHOP would promote the formation of the potent C/EBPβ homodimer, the induction of p20K and possibly other gas genes regulated by this factor in quiescent CEF.

Discussion

Regulation of growth arrest specific gene expression by hypoxia.

The p20K lipocalin is induced rapidly in response to contact inhibition but poorly when sub-confluent cells are serum-starved, indicating that not all conditions of growth arrest promote the expression of this gene. Gene profiling analyses confirmed that different sets of genes are induced in response to contact inhibition and serum starvation (Tables 3-5). Since most of the previous studies performed on growth arrest-specific genes relied on serum starvation to induce quiescence, it is not surprising that few of the contact inhibition-specific genes identified in our studies have been described before (Coppock *et al.*, 1993; Iyer *et al.*, 1999; Fornace *et al.*, 1989; Schneider *et al.*, 1988).

One of the distinguishing features of the gene signature of contact inhibited CEF is the presence of genes induced by hypoxia, including carbonic anhydrase IX, carbonic anhydrase XII and enolase 2 (Table 3). This result prompted the analysis of p20K in response to oxygen limitations. Western blotting analysis confirmed that p20K expression is induced in CEF cultured in 2% O_2 or treated with chemicals capable of inducing the response to hypoxia, such as the specific prolyl hydroxylase inhibitor DMOG (Fig.5). Carbonic anhydrases are regulators of intracellular pH, providing protection against reactive oxygen species (ROS). Since lipocalins function in part as lipid scavengers, the induction of p20K may represent an adaptive response to the

presence of ROS and peroxidated lipids. A second lipid transport protein, FABP4, is also part of the genes strongly activated by contact inhibition, suggesting that processes involved in lipid metabolism are particularly affected in these conditions of growth arrest. Whether or not the induction of p20K and FABP4 promotes the survival of contact inhibited CEF remains to be investigated.

Contact inhibition and wound healing share a common gene signature.

Novel gas genes regulated by hypoxia have been uncovered in the study of contact inhibited CEF (Table 3, Fig.4). Two of the genes most strongly activated by contact inhibition and hypoxia, namely PRKCH encoding PKCη and SULT1E1 encoding sulfotransferase 1E1, have also been implicated in wound healing. In particular, mice with a disruption of the PRKCH gene have impaired wound healing and display increased skin carcinogenesis (Chida *et al.*, 2003). PKCη is unique among PKC members because it is strongly activated by 3'sulfo-cholesterol, a product of sulfotransferase 1E1 (Chida *et al.*, 1995; Ikuta *et al.*, 1994). Wound healing is a multi-stage process consisting of an inflammatory phase, a connective and epithelial tissue regeneration phase promoting wound closure, and a scar-remodeling phase responsible for the formation of an epidermal barrier (Werner *et al.*, 2007). Angiogenic factors play a supportive role in these processes by inducing the formation of blood vessels providing nutrients to newly regenerated tissues. In this respect, it is tempting to speculate that the strong activation of angiopoietin-like 5 and prokinecitin 2, two of the genes most dramatically activated at contact inhibition and potent regulators of angiogenesis, reflect a role in the early phase of wound healing (Monnier & Samson, 2010). While it has been speculated that contact

inhibition of fibroblasts provides an *in vitro* model to study wound healing, little information exists to support a link between these two processes. In this respect, our gene signature of contact inhibited CEF opens new avenues of research to address this question.

Block to differentiation, enhanced survival and other C/EBPβ-dependent processes underlying reversible growth arrest.

C/EBPβ, sulfotransferase 1E1 and PKCη all play a role in the differentiation of keratinocytes, a key process of wound healing (Kashiwagi *et al.*, 2002). Significantly, C/EBPβ-deficient keratinocytes have defects in growth arrest, survival and differentiation, indicating that C/EBPβ controls several aspects of the physiology of these cells (Zhu *et al.*, 1999; Zhu *et al.*, 2002). Likewise, fibroblasts undergo a process of differentiation during wound healing. In these conditions, fibroblasts differentiate into myofibroblasts to acquire contractile activity and promote wound closure (Hinz *et al.*, 2007). Less is known about the role of C/EBPβ in the differentiation of myofibroblasts. This process is dependent on a complex series of paracrine interactions between fibroblasts and keratinocytes and, thus, is unlikely to take place in our *in vitro* system (Werner *et al.*, 2007). In agreement with this notion, smooth muscle actin, a key modulator of contractile activity in myofibroblasts, is not expressed in cycling or contact inhibited CEF [our unpublished results; (Hinz *et al.*, 2001)]. Whether or not the absence of a myofibroblast gene signature simply reflects the absence of appropriate cues or is also an active process of reversible growth arrest remains to be determined. Differentiation of myofibroblasts depends on chromatin modification and global genome

reprogramming (Mann *et al.*, 2007). In this respect, it is tempting to speculate that the marked induction of the chromodomain containing M-phase phospho-protein 8 (Mpp8) contributes to the maintenance of a state of reversible growth arrest in contact inhibited CEF. Mpp8 is expressed in numerous human carcinoma cells where it plays an important role in the maintenance of mesenchymal properties in these cells (Kokura *et al.*, 2010).

The sulfo-transferase 1E1 and PKCη axis is likely to be important for multiple aspects of the physiology of fibroblasts. In particular, PKCη has been shown to promote cell survival by inhibiting the JNK pathway (Hara *et al.*, 2011; Rotem-Dai *et al.*, 2009). Therefore, the role of this axis may be to enhance the survival of contact inhibited CEF in the absence of receptor tyrosine kinase and PI3K signaling.

H_2S gene signature of contact inhibited CEF.

Treatment of contact inhibited CEF with N-acetyl-L-cysteine (NAC) revealed a dual effect of this reducing agent. In the early phase, NAC had a modest inhibitory effect while it enhanced the expression of p20K at the later time points in both contact inhibited and hypoxic CEF (Fig.6-7). NAC antagonizes the action of ROS produced by the mitochondrial complex III in hypoxia but also generates L-cysteine upon prolonged incubation (Whiteman *et al.*, 2011). L-cysteine is then transformed into H_2S, a potent mediator of the hypoxic response (Li *et al.*, 2011; Mustafa *et al.*, 2009). Under physiological conditions, the production of H_2S is catalyzed by two main enzymatic systems, namely cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (Wang). It is significant that the expression of CBS is stimulated by contact inhibition (Table 3). Thus, in addition to sulfotransferase 1E1, CBS provides a second example of

an enzyme involved in sulfur metabolism in growth arrested CEF. Whether or not H_2S leads to the sulfhydration of proteins and regulation of numerous enzymatic systems in contact inhibited CEF remains to be investigated (Mustafa *et al.*, 2009).

C/EBPβ acts as a "switch" factor in the control of reversible growth arrest.

C/EBPβ often fulfills paradoxical roles in cell proliferation and differentiation, cell survival and cell death. This pleiotropic action is dependent on a series of post-translational modifications and the association with other members of the C/EBP family or with unrelated transcription factors (Sebastian & Johnson, 2006). We showed previously that C/EBPβ cooperates with AP-1 and NF-κB in the activation of IL8 by v-Src and that AP-1 blocks the C/EBPβ-dependent induction of p20K (Gagliardi *et al.*, 2003; Gagliardi *et al.*, 2001). More recently, we showed that the inhibition of JunD/AP-1 in v-Src transformed CEF results in a high incidence of apoptosis, a process that can be abolished by the concomitant down-regulation of C/EBPβ in these cells [(Wang *et al.*, 2011a); our unpublished results]. In this study, we uncovered a role for CHOP in the control of p20K by contact inhibition (Fig.2&11). Since the expression of a dominant negative mutant of C/EBPβ blocks apoptosis in response to prolonged starvation (Maynard, 2011), C/EBPβ acts as "switch" factor controlling the proliferation and survival of CEF. As depicted in Fig.12, this pleiotropic action depends largely on the interaction of C/EBPβ with factors activated in different physiological contexts.

ACKNOWLEDGMENTS

We thank Dr. Colin Nurse and Dr. Stephen Brown for the discussion of gene regulation by hypoxia and the use of their microscopy facility. This work was made possible by a grant from the Natural Sciences and Engineering Research Council of Canada to P.-A.B.

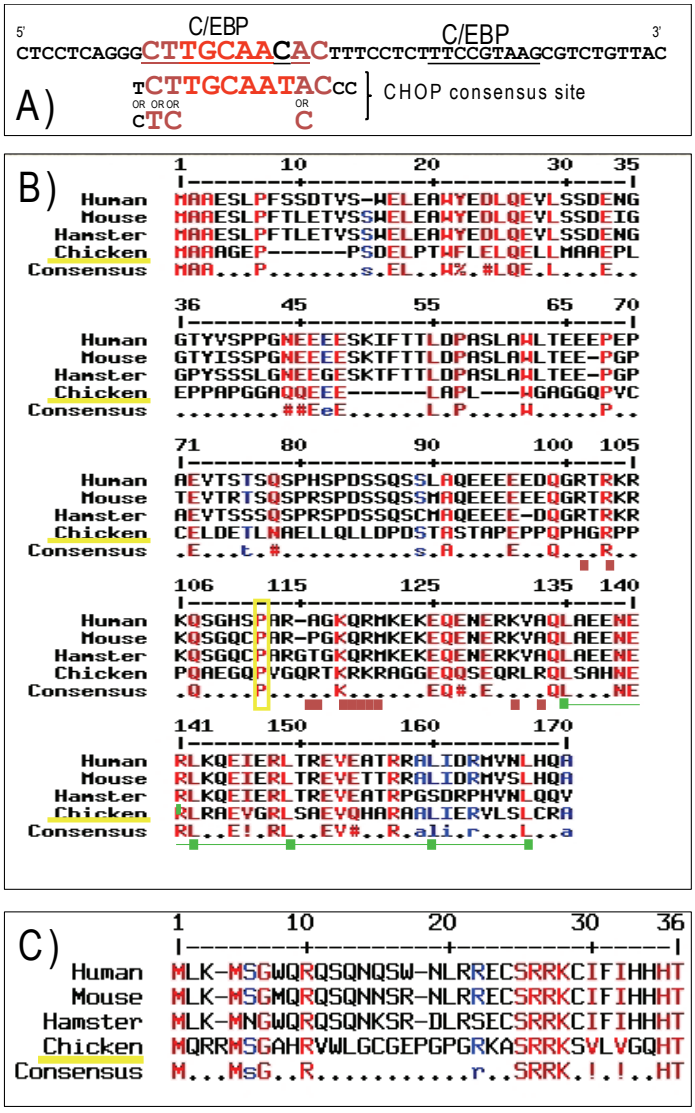


Figure 1. Characterization of chicken CHOP

A) Potential CHOP/C/EBPβ binding site of the p20K QRU - The 48bp QRU of the p20K promoter is shown. Two C/EBP binding sites are located within the QRU (underlined). The 5' distal C/EBP site matches closely the CHOP consensus binding site sequence (Ubeda *et al.*, 1996). Red letters are nucleotides protected by the C/EBP dimer (Sok *et al.*, 1999). **B)** Amino Acid sequence analysis of chicken CHOP**.** Avian and mammalian CHOP display a 31% amino acid sequence identity. The C-terminus end of chicken CHOP has a heptad repeated pattern of leucines, a hallmark of leucine zipper transcription factors. Upstream of the leucine zipper, chicken CHOP includes a basic region for DNA binding. The proline residue located within the bZIP region, typical of CHOP factors, is indicated by the yellow box. **C)** Amino Acid sequence analysis of uORF in the 5'UTR of chicken CHOP. The length of the 5' ORF of chicken CHOP is very similar (36 VS 34 amino acids) to that of the consensus sequence. Conserved amino acids (colored), defining the consensus, are also present in the chicken 5' ORF (12/36).

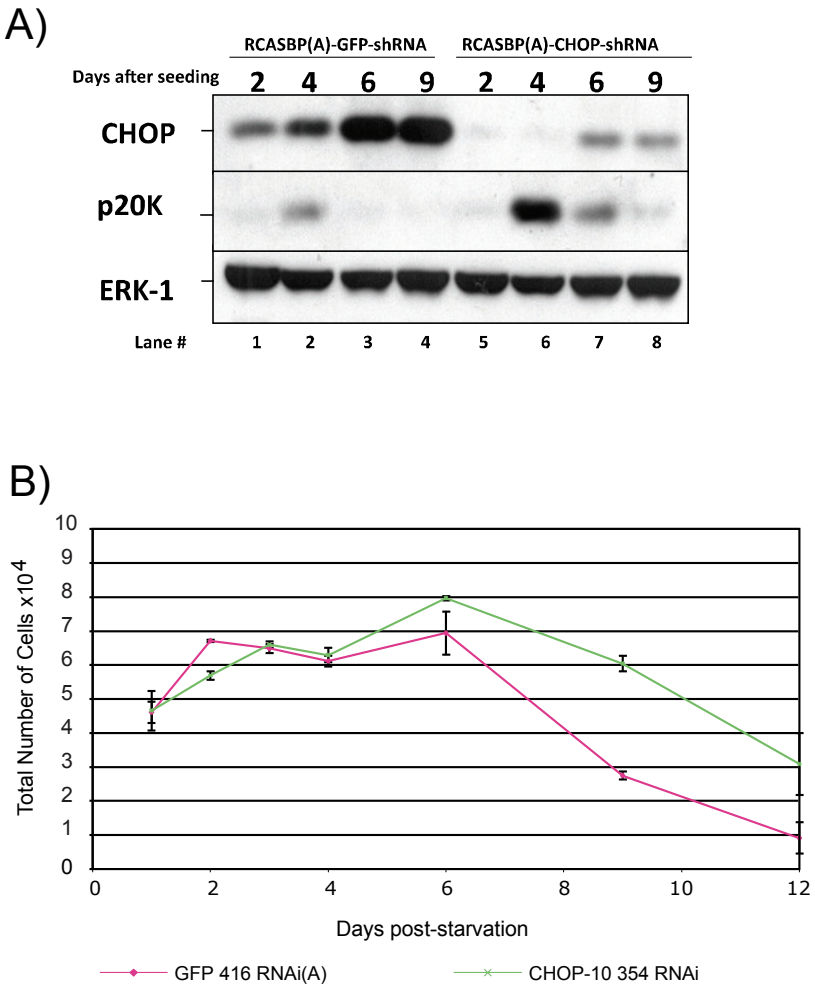


Figure 2. Effect of CHOP inhibition on growth of CEF

A) p20K expression in conditions of CHOP inhibition by shRNA. CEFs were infected with a RCASBP control or CHOP shRNA expressing virus (CHOP-ShRNA-354). CEFs were seeded onto 100mm plates and harvested on the indicated day. Whole cell lysates were prepared and subjected to western blotting analysis with the indicated anti-sera. Equal protein loading (80ug) was monitored by the level of ERK-1. The inhibition of CHOP enhanced p20K expression in contact inhibited CEF and prolonged its expression in response to ER (endoplasmic reticulum) stress. Confluence was reached at day 3 after seeding. **B) Proliferation and survival of CEF in conditions of CHOP down-regulation by shRNA**. Equal numbers of CEF were seeded onto 24-well dishes at day 0 and grown in complete medium. At day 1, CEFs were transferred to serum-free medium. Cell numbers were determined in a Coulter counter. Each time point represents the average of four separate samples; error bars represent the standard deviation. Down-regulation of CHOP improved CEF survival in conditions of serum/nutrient depletion.

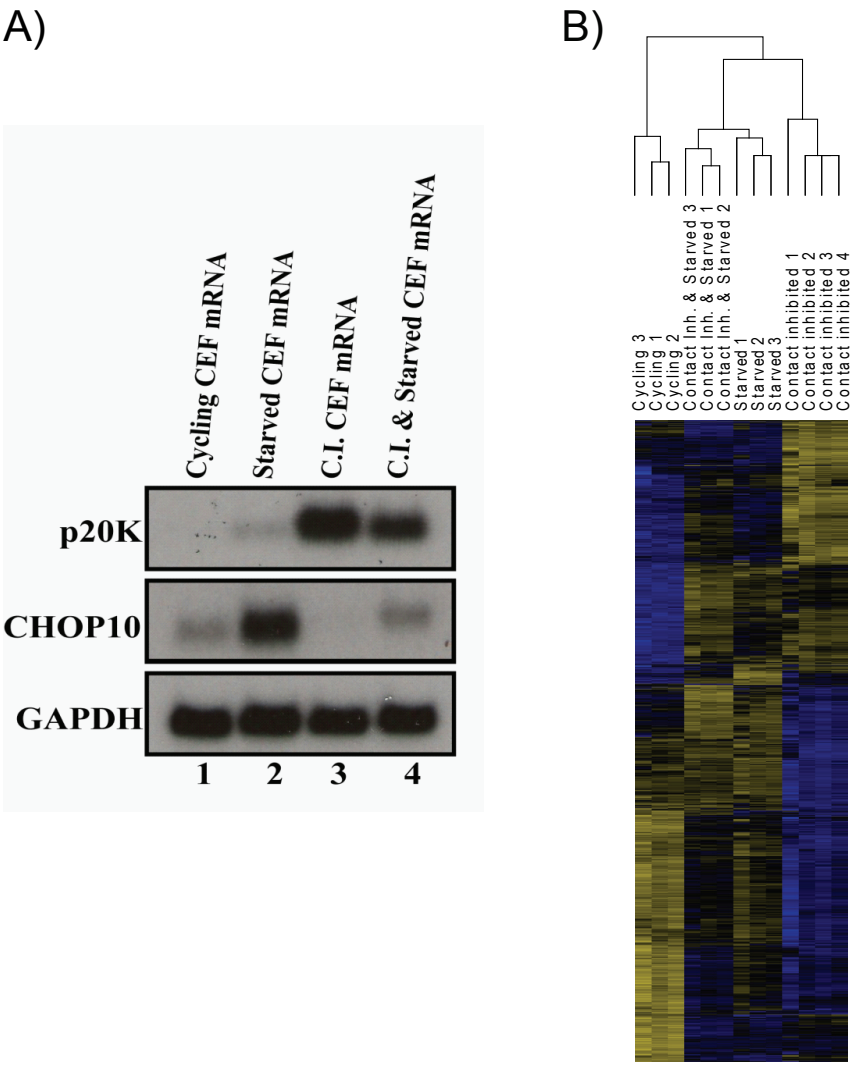


Figure 3. Different conditions of growth arrest regulate different sets of genes.

A) Northern blotting analyses of genes regulated by different programs of growth arrest: contact inhibition and serum-starvation. p20K is a marker for contact inhibition and CHOP is a marker for starvation. **B)** Unsupervised hierarchal clustering demonstrating a minimum of two-fold change of gene expression between at least two conditions is indicated. Statistical significance of differences of expression was determined by two-way ANOVA using contact inhibition and serum starvation as factors ($\alpha = 0.05$). Two-fold or greater changes in gene expression were determined by unpaired t-test on all pair-wise comparisons between experimental conditions (Bonferroni-corrected $\alpha = 0.05$). Yellow indicates genes that are upregulated and blue represents genes that are repressed.

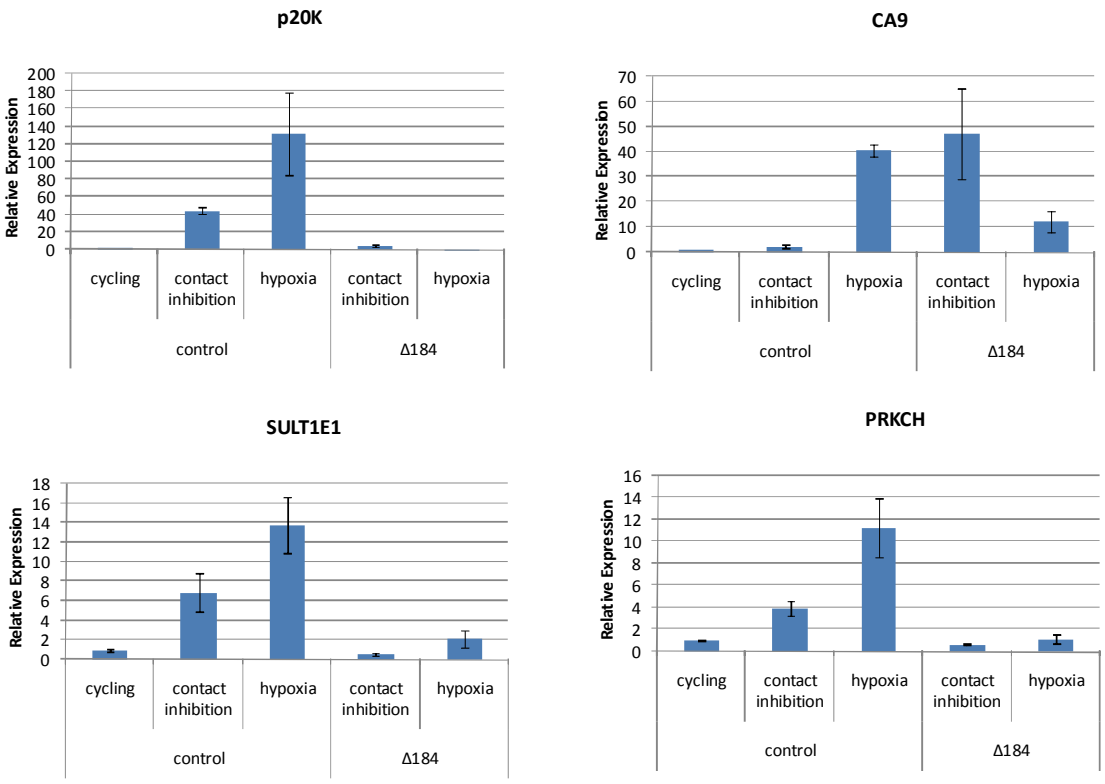


Figure 4. Validation of growth arrest specific genes by quantitative RT-PCR.

RNA was extracted from CEF infected with a control virus lacking an insert (RCASBP or WT) or a virus expressing a dominant negative mutant of C/EBPβ designated Δ184-C/EBPβ (Δ184). Expression at contact inhibition refers to the fold activation between contact inhibited CEF (sample) and cycling CEF (control). Expression in hypoxia refers to the fold activation between cycling CEF cultured in 2%O_2 for 24 hrs (sample) and cycling CEF in normoxia (control). This is shown for **A)** p20K, **B)** CAIX (encoding carbonic anhydrase IX), **C)** PRKCH (encoding Protein Kinase C eta) and **D)** SULT1E1 (encoding sulfotransferase 1E1). Error bars represent the standard deviation of triplicate samples.

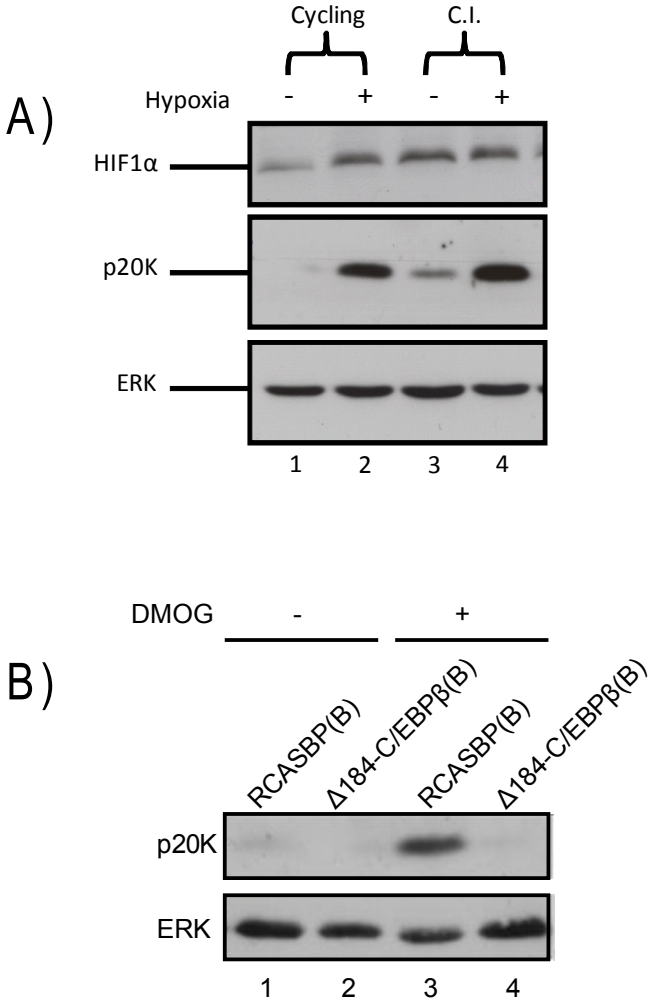


Figure 5. Activation of p20K by hypoxia.

A) Western blotting analysis of p20K and HIF1α expression in actively dividing CEF (cycling) and contact inhibited CEF (C.I.) in conditions of normoxia (-) or hypoxia (+; 24 hrs in 2% O_2). ERK-1 was used as a control for protein loading. B) Induction of p20K expression by the proline hydroxylase inhibitor dimethyloxalylglycine (24 hrs in 1 mM DMOG) in control CEF (RCASBP(B)) or CEF expressing a dominant negative mutant of C/EBPβ (Δ184-C/EBPβ). The dominant negative mutant blocks the induction of p20K by DMOG.

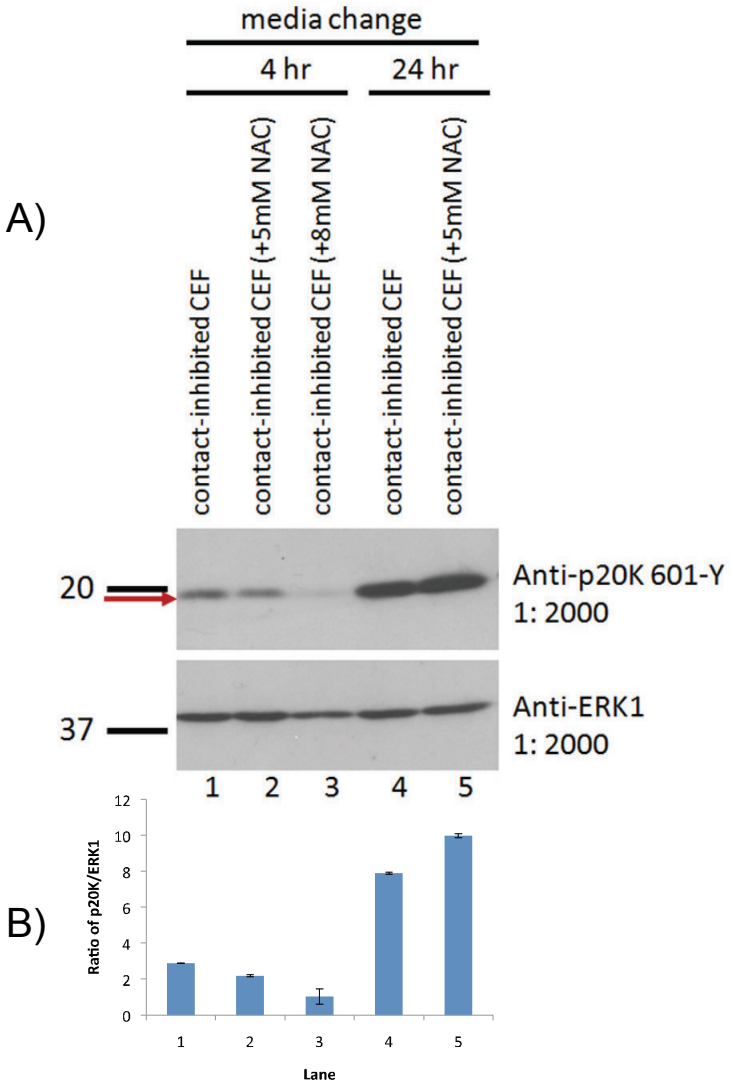


Figure 6. Effect of N-acetyl-L-cysteine (NAC) on the expression of p20K at contact inhibition

A) Effect of N-acetyl-L-cysteine (NAC) on the expression of p20K at contact inhibition (western blotting analysis). Media change was performed to increase the cell number and extent of contact inhibition 24 hrs before sample preparation. NAC was added for 4 or 24 hrs before sample preparation. **B)** Quantitation of p20K expression, as determined in A), after correction for protein loading using ERK-1 as a control. NAC reduced the expression at 4 hr but led to a superinduction of p20K at the 24 hr time point.

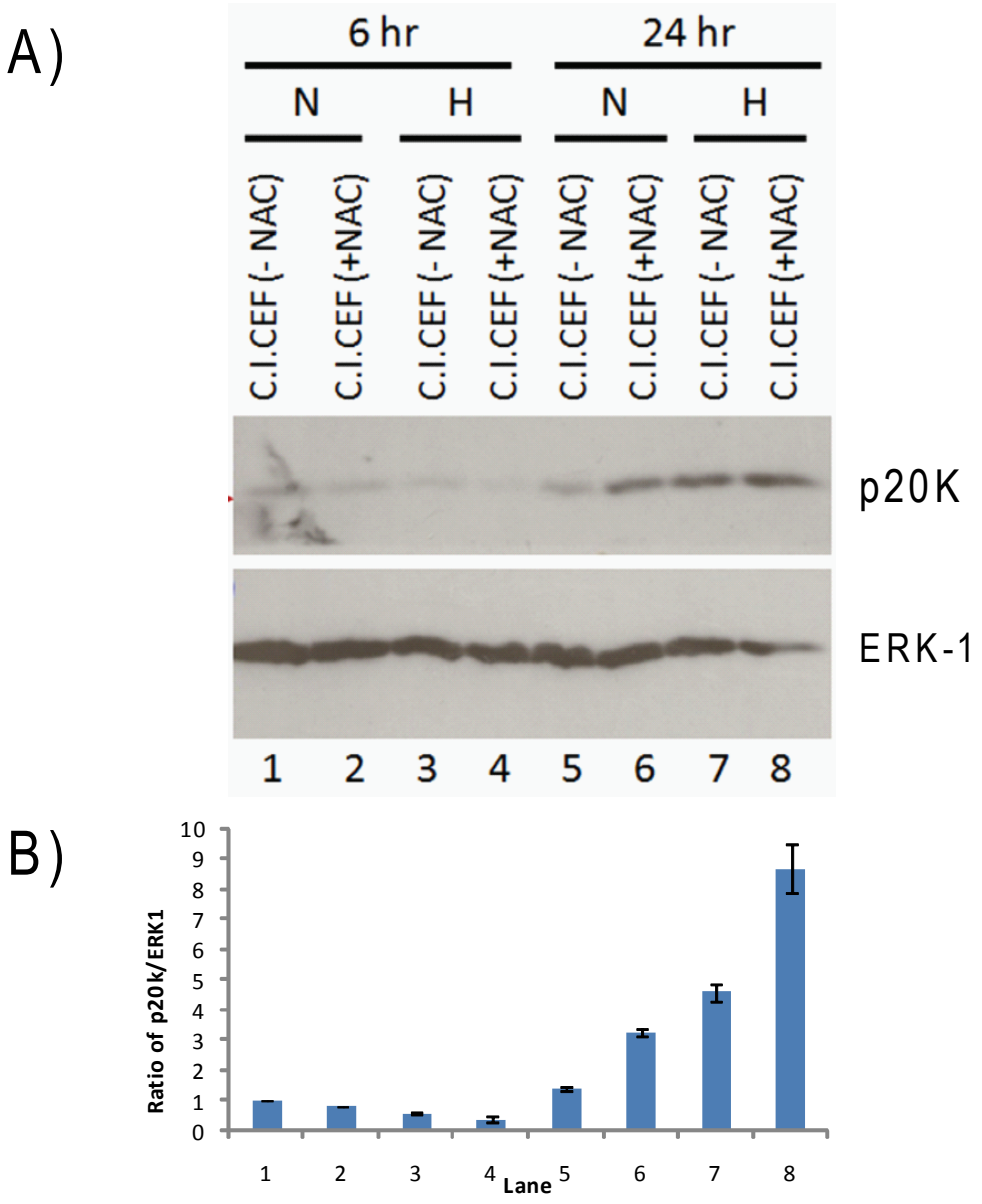


Figure 7. Superinduction of p20K expression by hypoxia and treatment with N-acetyl-L-cysteine (NAC). A) Western blotting analysis of p20K expression in contact inhibited CEF treated with 8 mM NAC for 6 or 24 hrs in conditions of normoxia or hypoxia (24 hrs in 2% O2). **B)** Quantitation of p20K expression, as determined in A), after correction for protein loading using ERK-1 as a control. NAC treatment caused a superinduction of p20K at the 24 hr time point.

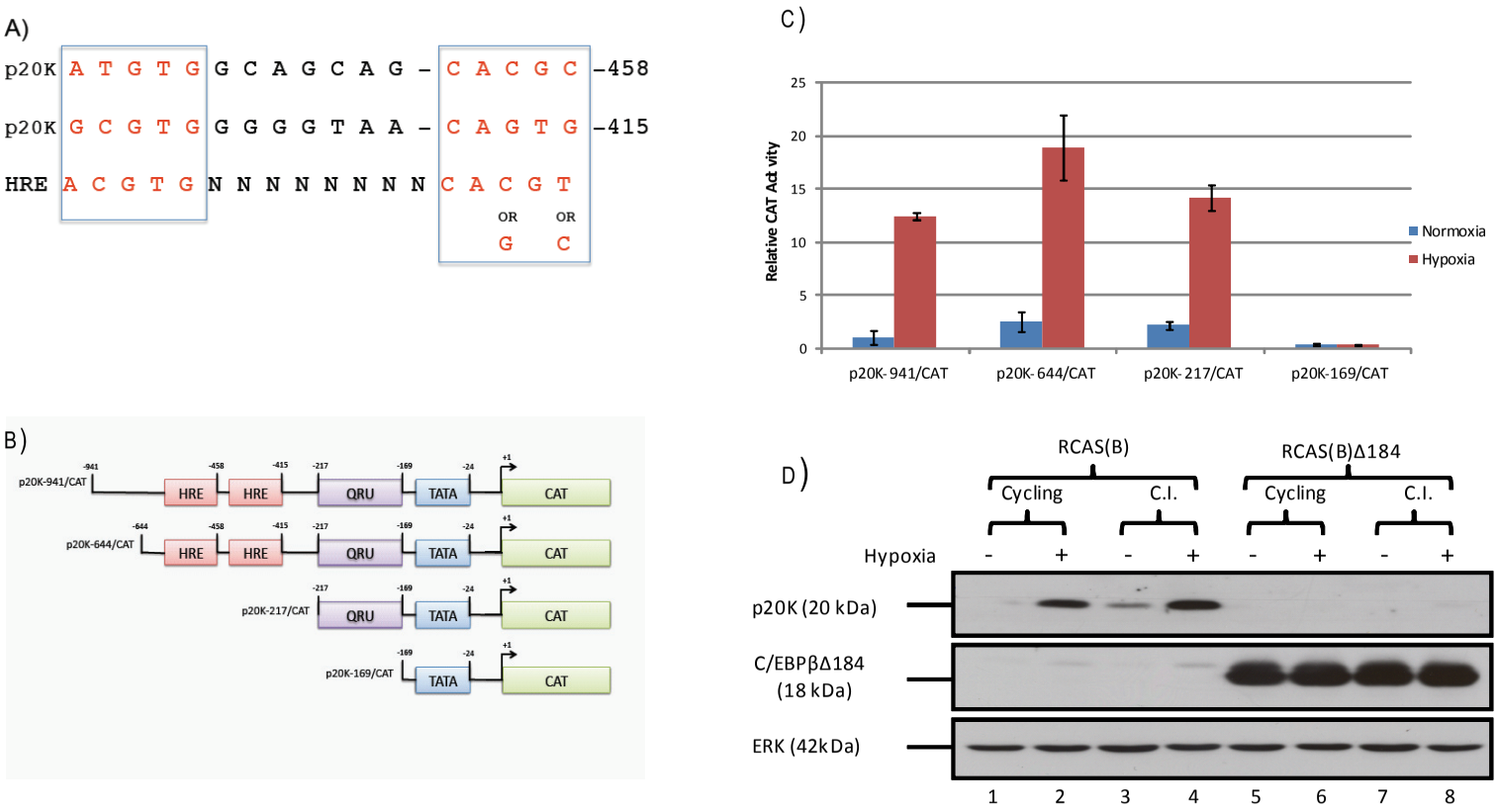


Figure 8. The QRU mediates the induction of the p20K promoter by hypoxia.

A) Sequence of two candidate HIF-response elements (HRE) located at position -415 and -458 of the p20K gene. The consensus sequence of the HRE is also shown (Kimura *et al.*, 2001). **B)** Schematic representation of 5'deletion constructs of the p20K promoter fused to the CAT reporter gene. **C)** Results of transient expression assays of p20K reporter constructs analyzed in cycling CEF in conditions of normoxia or hypoxia (24 hrs in 2% O2). The QRU is required for activation of the p20K promoter. **D)** Western blotting analysis of p20K expression in actively dividing (cycling) and contact inhibited (C.I.) CEF in conditions of normoxia or hypoxia (24 hrs in 2% O2). Results are shown for CEF infected with a control virus lacking an insert RCASBP(B) or expressing a dominant negative mutant of C/EBPβ designated Δ184-C/EBPβ (Δ184). The dominant negative mutant blocks the induction of p20K by contact inhibition and hypoxia.

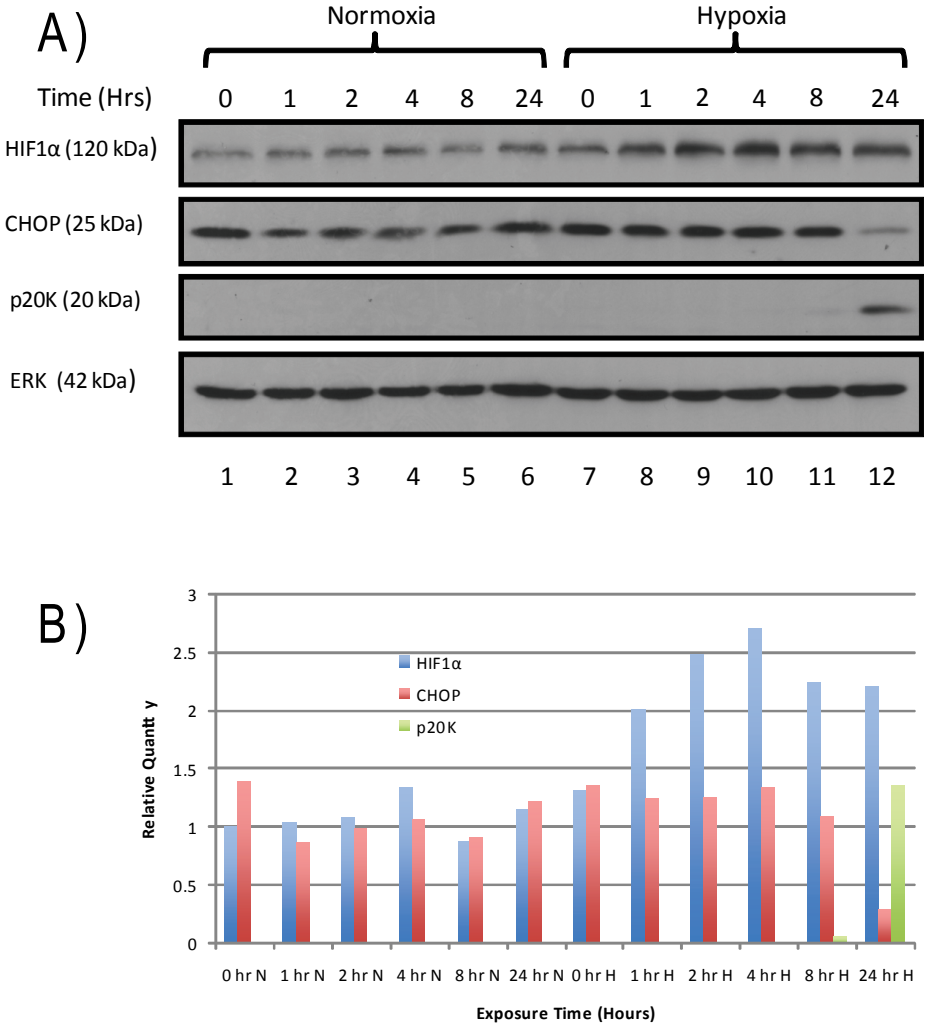


Figure 9. Kinetics of p20K, CHOP and HIF1α expression in cycling CEF cultured in conditions of normoxia or hypoxia for the indicated time (2% O2).

A) Western blotting analysis indicates that p20K induction occurs after the peak expression of HIF1α but correlates with the down-regulation of CHOP in conditions of hypoxia. **B)** Quantitative representation of results shown in A) after correction for protein loading using ERK-1 as a control.

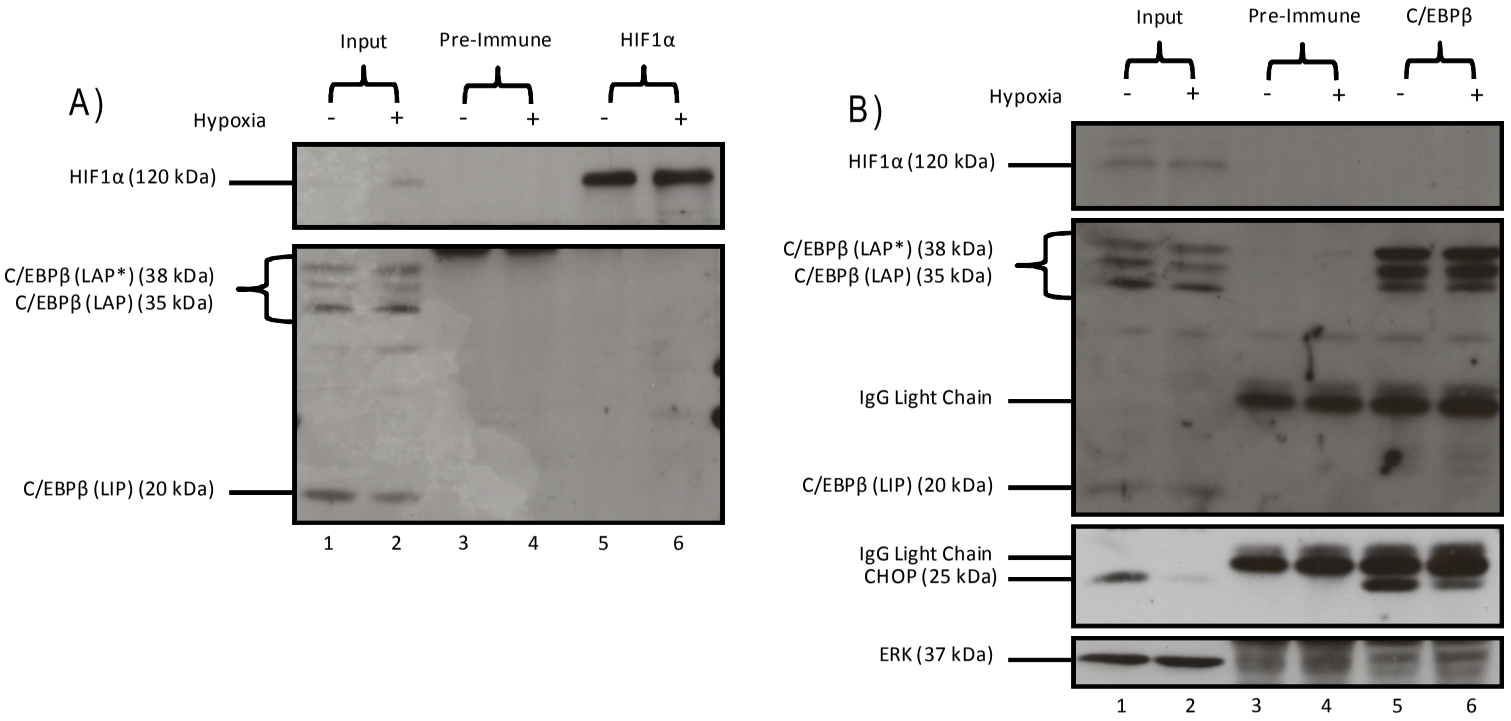


Figure 10. Co-immunoprecipitation analysis of C/EBPβ, CHOP and HIF1α.

A) HIF1α was immunoprecipitated and the interaction with C/EBPβ was examined by western blotting. The expression of HIF1α and C/EBPβ was also determined in total protein lysates of CEF in conditions of normoxia or hypoxia (24 hrs in 2% O2; lanes 1-2). Multiple forms of C/EBPβ, corresponding to LAP, LAP* and LIP (Descombes *et al.*, 1990), are detected in total cell lysates but not in the HIF1α immunoprecipitate (lanes 5-6). **B)** C/EBPβ was immunoprecipitated and the interaction with HIF1α and CHOP was examined by western blotting. The expression of HIF1α and C/EBPβ was also determined in total protein lysates of CEF in conditions of normoxia or hypoxia (24 hrs in 2% O2; lanes 1-2). CHOP but not HIF1α was detected in the C/EBPβ immunoprecipitates. Hypoxia caused a decrease in CHOP levels and in the amount of CHOP associated with C/EBPβ. Immunoprecipitations were also performed with the corresponding pre-immune serum, as a control.

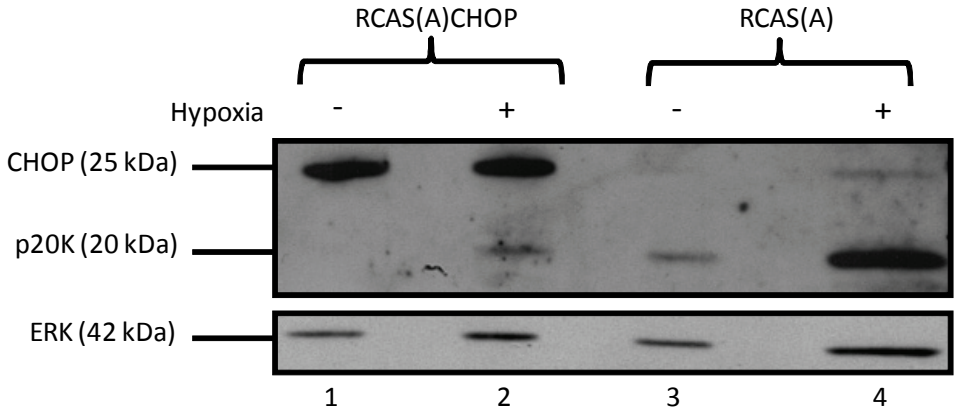


Figure 11. Inhibition of p20K by forced expression of CHOP.

CEF were infected with a virus expressing CHOP or the corresponding control virus lacking an insert (RCASBP(A)). The expression of p20K was examined for infected CEF in conditions of normoxia or hypoxia (24 hrs in 2% O2) by western blotting analysis. The expression of p20K was inhibited by the over-expression of CHOP.

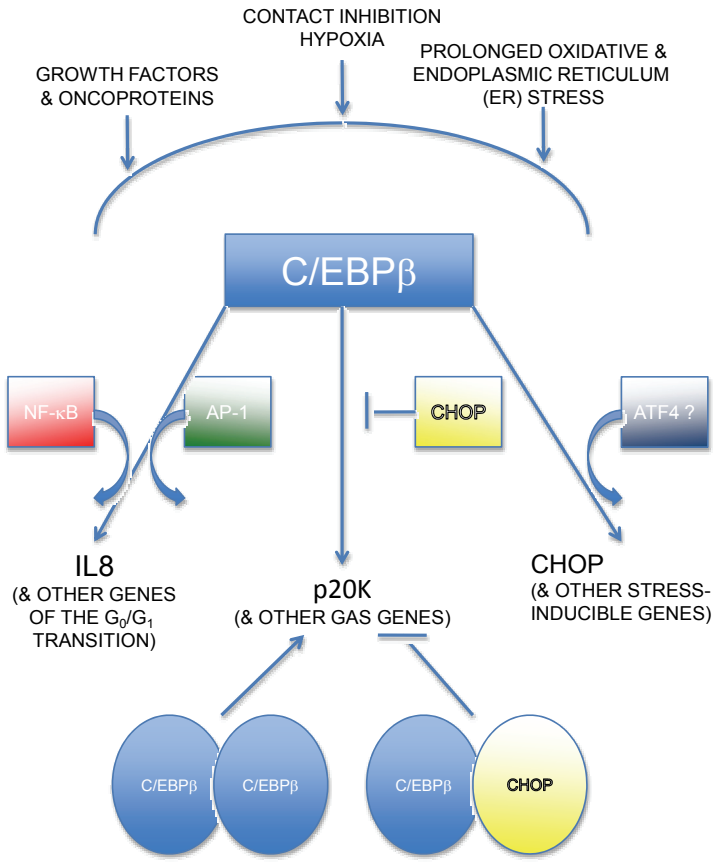
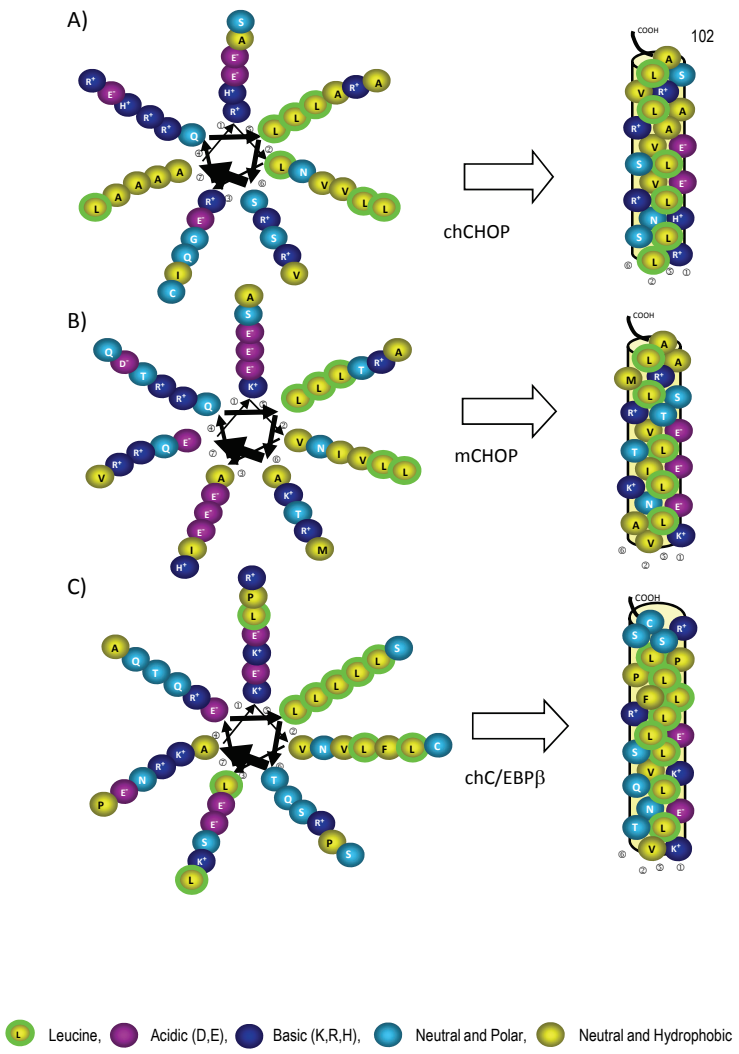


Figure 12. Model for the action of C/EBPβ as a switch factor determining cell fate.

In response to growth factors or oncoproteins, C/EBPβ cooperates with AP-1 and NF-κB in the induction of IL8 and other genes of the G_0/G_1 transition (Gagliardi *et al.*, 2003; Gagliardi *et al.*, 2001). Basal levels of CHOP, expressed in cycling CEF, promote the formation of the C/EBPβ-CHOP heterodimer and repression of gas genes such as p20K. Upon contact inhibition and/or moderate hypoxia, AP-1, NF-κB and CHOP are repressed promoting the formation of C/EBPβ homodimers, the induction of gas genes such as p20K, and the entry into reversible growth arrest. Excessive oxidative stress or ER stress, created by prolonged starvation, results in the activation of members of the ATF family, such as ATF4, the formation of C/EBPβ-ATF heterodimers, marked induction of CHOP, repression of gas genes such as p20K, and cell death.



Supplementary Figure 1. 3D structure of the leucine zipper domain of C/EBP proteins.
The leucine zipper domain of ch-CHOP A), murine CHOP B) or chicken C/EBPβ C) are compared and are represented according to the heptad repeat α-helix model.

Table 2. Summary table indicating the number of probe-sets found to be differentially expressed between any pair-wise comparison indicated.
The proportion of genes repressed during contact inhibition (C.I.) is dramatically high with respect to cycling conditions, while nearly half of that proportion of genes is activated. Gene expression in the subconfluent and confluent starvation conditions are regulated in similar patterns with respect to contact inhibition. Both starvation conditions show a higher portion of activated genes in comparison to the contact-inhibited state. A similar proportion of genes are up-regulated or down-regulated by the subconfluent-starved state in comparison to the cycling state. Whereas, the confluent-starved condition has about double the number of genes that are differentially regulated by the subconfluent-starved versus cycling state. Minor number of genes are differentially regulated between the two starvation conditions.

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

Table 3. Selected examples of genes regulated during contact inhibition.

Changes in gene expression in contact inhibition (C.I.) with respect to cycling conditions are expressed as Log2 difference of means (C.I./Cycling) and linear fold-change values. The negative difference of means and linear fold-change values indicate genes that are repressed during starvation, whilst positive values indicate activation of gene expression by serum-depletion. Genes highlighted in orange represent starvation-specific genes. The one highlighted in green is a marker of contact inhibition and those highlighted in yellow indicate the signature for hypoxia.

Contact Inhibited/Cycling Regulated Genes			
Gene	Difference of Means	Linear Fold-Change	P-value
CA9: Carbonic anhydrase IX	5.79	55.33	0.0001
Mpp8: M-phase phosphoprotein 8	5.59	48.20	0.0069
FABP4: fatty acid binding protein 4, adipocyte	5.34	40.50	0.0005
p20K: quiescence-specific protein	5.33	40.22	0.0002
ANGPTL5: angiopoietin-like 5	4.82	28.25	0.0048
CA12: carbonic anhydrase XII	4.64	24.93	0.0000
PROK2: Prokenicitin 2	4.58	23.90	0.0001
ENO2: enolase 2	3.31	9.92	0.0000
PRKCH: Protein Kinase C Eta	3.08	8.46	0.0075
SULT1E1: Sulfotransferase 1E1	2.91	7.52	0.0001
TPD52: tumor protein D52	2.01	4.03	0.0018
HIF1A: hypoxia-inducible factor 1, alpha subunit	1.75	3.36	0.0024
PDGFRL: platelet-derived growth factor receptor-like	1.62	3.07	0.0059
GOLGA4: golgi autoantigen, golgin subfamily a, 4	1.45	2.73	0.0020
CDKN2C: cyclin-dependent kinase inhibitor 2C (p18)	1.18	2.27	0.0067
CBS: Cystathionine beta-synthase	1.10	2.14	0.0023
CDKN1B: cyclin-dependent kinase inhibitor 1B (p27, Kip1)	1.06	2.08	0.0000
BIRC2: baculoviral IAP repeat-containing 2	1.01	2.01	0.0006
ID1: inhibitor of DNA binding 1	-5.88	-58.89	0.0004
ID2: inhibitor of DNA binding 2	-4.11	-17.27	0.0000
APOA1: apolipoprotein A-I	-4.07	-16.80	0.0002
BMP2: bone morphogenetic protein 2	-3.70	-13.00	0.0000
CDKN2B: cyclin-dependent kinase inhibitor 2B (p15)	-3.24	-9.45	0.0000
DDX47: DEAD (Asp-Glu-Ala-Asp) box polypeptide 47	-2.16	-4.47	0.0005
RBL1: Retinoblastoma-like 1 (p107)	-1.58	-2.99	0.0023
MAP1LC3C: microtubule-associated protein 1 light chain 3 g	-1.43	-2.69	0.0052
RORA: RAR-related orphan receptor A	-1.27	-2.41	0.0069
DAPK1: death-associated protein kinase 1	-1.26	-2.39	0.0001
HES1: hairy and enhancer of split 1, (Drosophila)	-1.09	-2.13	0.0006
CEBPZ (CHOP10): CCAAT/enhancer binding protein zeta	-1.01	-2.01	0.0010

Table 4. Selected examples of genes regulated in response to serum-starvation.
Changes in gene expression between starvation and cycling conditions are expressed as Log2 difference of means (Starved/Cycling) and linear fold-change values. The negative difference of means and linear fold-change values indicate genes that are repressed during starvation, whilst positive values indicate activation of gene expression by serum-depletion. Genes highlighted in orange represent starvation-specific genes and the one highlighted in green is a marker of contact inhibition.

Gene	Difference of Means	Linear Fold-Change	P-value
FGF18: fibroblast growth factor 18	6.61	97.68	0.0059
ANGPTL5: angiopoietin-like 5	4.07	16.80	0.0081
SFRP2: secreted frizzled-related protein 2	3.02	8.11	0.0007
p20K: quiescence-specific protein	2.85	7.21	0.0014
RORA: RAR-related orphan receptor A	2.37	5.17	0.0024
PTN: pleiotrophin	1.88	3.68	0.0016
DDX4: DEAD (Asp-Glu-Ala-Asp) box polypeptide 4	1.88	3.68	0.0065
SFRP1: secreted frizzled-related protein 1	1.74	3.34	0.0006
APOA1: apolipoprotein A-I	1.69	3.23	0.0015
PDGFRL: platelet-derived growth factor receptor-like	1.65	3.14	0.0053
ARF tumor suppressor	1.65	3.14	0.0001
CDKN2B: cyclin-dependent kinase inhibitor 2B (p15)	1.47	2.77	0.0003
BIRC2: baculoviral IAP repeat-containing 2	1.39	2.62	0.0065
Cell cycle progression 1	1.38	2.60	0.0008
CAPN2: calpain 2, (m/II) large subunit	1.35	2.55	0.0010
MAP1LC3C: microtubule-associated protein 1 light chain 3 gamma	1.32	2.50	0.0029
TP53INP1: tumor protein p53 inducible nuclear protein 1	1.11	2.16	0.0035
DKK1: dickkopf homolog 1 (Xenopus laevis)	-8.00	-256.00	0.0005
ID1: inhibitor of DNA binding 1	-5.83	-56.89	0.0000
ID2: inhibitor of DNA binding 2	-2.90	-7.46	0.0000
TGFB3: transforming growth factor, beta 3	-2.78	-6.87	0.0041
TRAIP: TRAF interacting protein	-2.69	-6.45	0.0005
FGFR2: fibroblast growth factor receptor 2	-2.05	-4.14	0.0001
SMAD9: SMAD family member 9	-1.95	-3.86	0.0002
BMP2: bone morphogenetic protein 2	-1.94	-3.84	0.0005
SMAD6: SMAD family member 6	-1.87	-3.66	0.0018
RASL11B: RAS-like, family 11, member B	-1.66	-3.16	0.0045
BIRC5: baculoviral IAP repeat-containing 5 (survivin)	-1.56	-2.95	0.0005
CCNB2: cyclin B2	-1.40	-2.64	0.0034
BCLAF1: BCL2-associated transcription factor 1	-1.37	-2.58	0.0059
CDCA8: cell division cycle associated 8	-1.01	-2.01	0.0039

Table 5. Selected examples of genes regulated in response to high cell density and serum-starvation.

Changes in gene expression between the contact-inhibited and starved conditions versus the cycling condition (C.I &.Starved/Cycling) are expressed as Log2 difference of means and linear fold-change values. The negative difference of means and linear fold-change values indicate genes that are repressed during starvation, whilst positive values indicate activation of gene expression by serum-depletion. Genes highlighted in orange represent starvation-specific genes. The one highlighted in green is a marker of contact-inhibition and those highlighted in yellow indicate the signature for hypoxia.

Gene	Difference of Means	Linear Fold-Change	P-value
SFRP2: secreted frizzled-related protein 2	4.55	23.43	0.0000
p20K: quiescence-specific protein	4.35	20.39	0.0005
CA12: carbonic anhydrase XII	3.83	14.22	0.0000
CAPN9: calpain 9	2.76	6.77	0.0004
RORA: RAR-related orphan receptor A	2.17	4.50	0.0062
PTN: pleiotrophin	2.12	4.35	0.0004
APOA1: apolipoprotein A-I	1.91	3.76	0.0018
PDGFRL: platelet-derived growth factor receptor-like	1.90	3.73	0.0010
MAP1LC3C: microtubule-associated protein 1 light chain 3 gamma	1.75	3.36	0.0006
SFRP1: secreted frizzled-related protein 1	1.60	3.03	0.0009
Cell cycle progression 1	1.34	2.53	0.0031
TP53INP1: tumor protein p53 inducible nuclear protein 1	1.31	2.48	0.0064
ARF tumor suppressor	1.27	2.41	0.0002
ERO1L: ERO1-like (S. cerevisiae)	1.23	2.35	0.0011
PDCD4: programmed cell death 4	1.09	2.13	0.0004
Dishevelled, dsh homolog 1 (Drosophila)	1.03	2.04	0.0004
GOLGA4: golgi autoantigen, golgin subfamily a, 4	1.02	2.03	0.0067
DKK1: dickkopf homolog 1 (Xenopus laevis)	-8.05	-265.03	0.0067
ID1: inhibitor of DNA binding 1	-6.35	-81.57	0.0000
TGFB3: transforming growth factor, beta 3	-3.41	-10.63	0.0017
TRAIP: TRAF interacting protein	-3.33	-10.06	0.0002
ID2: inhibitor of DNA binding 2	-3.05	-8.28	0.0011
BCLAF1: BCL2-associated transcription factor 1	-1.98	-3.94	0.0016
BIRC5: baculoviral IAP repeat-containing 5 (survivin)	-1.95	-3.86	0.0069
BMP2: bone morphogenetic protein 2	-1.91	-3.76	0.0003
CCNB2: cyclin B2	-1.75	-3.36	0.0009
SMAD6: SMAD family member 6	-1.66	-3.16	0.0031
SMAD9: SMAD family member 9	-1.56	-2.95	0.0008
AATF: apoptosis antagonizing transcription factor	-1.47	-2.77	0.0015
NLE1: notchless homolog 1 (Drosophila)	-1.44	-2.71	0.0027
FGFR2: fibroblast growth factor receptor 2	-1.28	-2.43	0.0022
CDCA8: cell division cycle associated 8	-1.23	-2.35	0.0019
BCL2-antagonist/killer 1	-1.20	-2.30	0.0002
RBL1: Retinoblastoma-like 1 (p107)	-1.20	-2.30	0.0019
DDX47: DEAD (Asp-Glu-Ala-Asp) box polypeptide 47	-1.14	-2.20	0.0082

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