Role of activator protein-1 (AP-1) family in RSV-transformed chicken embryonic fibroblasts (CEF)

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TITLE: Role of Activator Protein-1 (AP-1) Transcription Factor Family in RSV-Transformed Chicken Embryonic Fibroblast (CEF)

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Abstract

Proper gene expression programs cellular activities, while aberrant manipulation of transcription factors often leads to devastating consequences, such as cancer or cell death. The transcription factor family activator protein-1 (AP-1) plays an important role in many cellular activities including cell transformation, proliferation and survival (Shaulian and Karin 2002). However, little has been done to obtain a global view of the role of individual AP-1 members and how they cooperate in many cellular activities. We have discovered that blocking the AP-1 pathway by a c-Jun dominant negative mutant, TAM67, induced cell death in RSV-transformed primary chicken embryo fibroblasts (CEF), suggesting that AP-1 activity is vital for cell survival upon v-Src transformation. In addition, accumulation of cytoplasmic vesicles was observed in the cytoplasm of a proportion of RSV-transformed CEF expressing TAM67. Oil-red staining of these vesicles indicated the presence of lipid droplets in these cells, suggesting that the inhibition of AP-1 promotes the adipogenic conversion of v-Src transformed CEF. To understand the role of individual members of the AP-1 family, a retroviral-based shRNA expressing system was designed to stably downregulate individual AP-1 members. This retroviral-based RNAi system provided sustained gene downregulation of AP-1 family members. Reduction of the c-Jun protein level by shRNA induced senescence in normal CEF, while it modestly downregulated AP-1 activity in RSV-transformed CEF indicating that c-Jun is not the main component of the AP-1 complex in RSV-transformed CEF. Inhibition of JunD expression induced apoptosis and was deleterious to both normal and RSV-transformed CEF, suggesting that JunD is crucial for the survival of CEF. Transient

expression reporter-assays also showed that loss-of-function of JunD by shRNA dramatically repressed AP-1 activity. Hence JunD is the main component of the AP-1 complex that regulates the survival of CEF. Furthermore, we determined that loss of JunD expression resulted in an elevated level of tumour suppressor p53. Co-inhibition of p53 and JunD restored the transforming ability of *v*-Src transformed CEF, as indicated by foci formation in soft agar assays. Hence, repression of p53 induction was able to bypass the death signal released as a result of AP-1 inhibition in v-Src transformed CEF. Down-regulation of Fra-2 (Fos-related antigen 2) level by shRNA did not affect the proliferation of normal CEF. However, RSV-transformed CEFs expressing *fra-2* shRNA were transformation-defective with the presence of multiple vesicles in cytoplasm. Oil-red staining of these vesicles indicated the presence of lipid droplets, which resembles the effect of TAM67 in RSV-transformed CEF indicating that Fra-2 blocks differentiation. These findings help us to understand the role of individual members of the AP-1 transcription factor family in normal and RSV-transformed CEF. Importantly, global gene profiling of v-Src transformed CEF expressing shRNA for individual AP-1 members will improve our knowledge of the transformation process. Functional characterization of the cascade will rely on the use of retroviral-based shRNA expressing system as described above.

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List of Abbreviations

AMP	Adenosine Monophoshate
ANOVA	Analysis of Variance
Arf	ADP-ribosylation Factor
AP-1	Activator Protein-1
ATF	Activating Transcription Factor
ALV	Avian leukosis virus
BAD	BCL-x_L/BCL-2-associated death promoter;
BAK	Bcl-2 homologous antagonist/killer
BCL-x_L	Basal cell lymphoma-extra large;
Bmp2	Bone Morphogenetic Protein 2
bZIP	Basic Leucine Zipper
CDK	Cyclin-Dependent Kinase
C/EBP	CCAAT Enhancer Binding Protein
CEF	Chicken Embryo fibroblasts
CREB	cAMP-response element-binding
DAPK1	Death-associated protein kinase 1
CRE	cAMP responsive elements
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EGFR	Epidermal growth factor receptor
eIF2α	Eukaryotic Translation Initiation Factor 2 α
ERK	Extracellular signal-regulated kinase
Fra-1/2	Fos-related antigen 1/2
GAP	GTPase activating protein
GAS	Growth Arrest-Specific
GATE-16	Golgi ATPase Enhancer 16
GDP	Guanosine diphosphate

GTP	Guanosine triphosphate
GFP	Green Fluorescent Protein
GS3Kβ	Glycogen synthase kinase 3 β
IL	Interleukin
INK4	Inhibitors of Kinase 4
JNK	c-Jun N-terminal Kinase
LAP	Liver Activating Protein
LIP	Liver Inhibitory Protein
LTR	Long Terminal Repeat
MAPK	Mitogen Activated Protein Kinase
MDM2	Mouse Double Minute-2
MEF	Mouse Embryo Fibroblasts
mRNA	Messenger RNA
mTOR	Mammalian Target of Rapamycin
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PBS	Phosphate Buffer Saline
PDGF	Platelet-derived growth factor
PI3K	Phosphatidyl Inositol 3 Kinase
PCAF	p300/CBP Associated Factor
PLIER	Probe Logarithmic Intensity Error
PPARγ	Peroxisome Proliferator-Activated Receptor γ
pRb	Retinoblastoma Protein
PTPα	Protein tyrosine phosphotase-α
RCAS	Replication Competent ALV LTR with a Splice acceptor
RNA	Ribonucleic Acid
RNAi	RNA Interference
RSK	90 kDa ribosomal S6 kinase
RSV	Rous sarcoma virus
RHD	Rel-homology domain

SDS	Sodium Dodecyl Sulfate
SEK	SAPK/ERK kinase
SH	Src homology
shRNA	Short Hairpin RNA
Src	Sarcoma
SRU	Src-responsive unit
STAT	Signal transducer and activator of transcription
SU	Surface subunit
TAD	Transactivation domain
TAKU	Tsukushi
TdT	Terminal deoxynucleotidyl Transferase
TM	Transmembrane subunit
TNFα	Tumour necrosis factor α
TUNEL	Terminal deoxynucleotidyl Transferase dUTP Nick-End Labeling
VEGF	Vascular Endothelial Growth Factor

Chapter 1: Literature Review

1. Introduction

1.1 Role of c-Src in various cancers

The proto-oncogene cellular-Src (c-Src) encodes a 60 kDa non-receptor tyrosine kinase whose expression and activity have been implicated in the development, progression and metastasis of several human cancers including breast, lung, prostate, colorectal and head and neck cancer (Wheeler *et al.,* 2009). The activation and increased expression of c-Src are reported to modulate the initiation, size and metastatic potential of colon carcinomas (Cartwright *et al.,* 1990). The same events were also true for breast cancer where greater kinase activity and protein level were detected compared to normal tissues (Ottenhoff-Kalff *et al.,* 1992). Moreover, over 70% of the breast tumour samples exhibited increased Src activity (Biscardi *et al.,* 2000). Treatments based on Src inhibitors were capable of downregulating proliferation and invasion in cell lines derived from head and neck carcinoma (Wheeler *et al.,* 2009). Inhibition of Src resulted in cell growth disruption and apoptosis in human lung cancer cell lines (Song *et al.,* 2006). Collectively, Src activation is widely observed in human cancers highlighting the significance of investigations on the signaling cascades regulated by the Src kinase.

1.2 Structure and regulation of Src

The cellular counterpart of v-Src , the proto-oncogene c-Src, contains an N-terminal Src homology 4 (SH4) domain, a Src homology 3 (SH3) domain, a Src homology 2 (SH2) domain, an SH2-kinase linker, a Src homology 1 (SH1) kinase domain, and a C-

terminal regulatory domain [Figure 1; (Yeatman 2004)]. The SH4 domain of the Src protein contains a site for myristoylation that allows the translocation of c-Src to the inner plasma membrane and particularly in sites of focal adhesion and cell-cell adhesion (Courtneidge *et al.,* 1980). Translocation of Src to the inner plasma membrane is essential for transformation (Nigg *et al.,* 1982), implying that the localization is vital for full Src function where it can bind to signaling proteins on the inner plasma membrane. The SH3 domain binds to sequences that are rich in proline and the SH2 domain possesses high affinity for phosphotyrosine containing sequences (Roskoski Jr. R. 2004). Interactions of SH2 and SH3 domains with their ligands are important for the activation and function of Src.

The activity of c-Src is regulated by phosphorylation and dephosphorylation. Tyrosine 530 in human c-Src (Y527 in chicken) in the C-terminus is involved in the negative regulation of c-Src. Phosphorylation of this residue by Csk (C-terminal Src Kinase) results in an intramolecular interaction with the SH2 domain with a low affinity (Yeatman 2004). This interaction is stabilized by the interaction between the SH3 domain and proline residues within the SH2-kinase linker region, causing a folding conformation of c-Src and the subsequent inactivation of the protein (Roskoski Jr. R. 2004). The main mechanism of c-Src activation is through association with a phosphotyrosine containing protein such as ligand-activated receptor tyrosine kinases or activated focal adhesion kinase (FAK) that results in the unfolding of c-Src because these proteins generate higher affinity binding sites for the SH2 domain than the C-terminal regulatory domain of c-Src (Roskoski Jr. R. 2004, Yeatman 2004). Disruption of the

close conformation thus exposes Y530 for dephosphorylation, which is also an important mechanism for the full activation of c-Src (Roskoski Jr. Robert 2005). Zheng and co-workers reported that the activation process and persistent activation of c-Src can be achieved by overexpressing protein tyrosine phosphatase-α [PTPα; (Zheng *et al.,* 1992)]. In agreement with this finding, high level of c-Src activity accompanied by high level of protein tyrosine phosphatase was observed in some breast cancer cell lines (Bjorge *et al.,* 2000). Other candidate phosphatases have been discovered so far including cytoplasmic PTP1B, Shp1 (Src homology 2 domain-containing tyrosine phosphatase 1) and Shp2, and transmembrane enzymes including CD45, PTPα, PTPε, and PTPκ (Roskoski Jr. Robert 2005). Following the dephosphorylation, trans-phosphorylation of tyrosine 419 (Y416 in chicken) in the kinase domain leads to full activation of c-Src (Roskoski Jr. Robert 2005). Mutations in this residue have been reported to diminish the transforming activity in both c-Src and v-Src (Kmiecik *et al.,* 1988). In addition, Src activity can be regulated through proteasome-mediated degradation. Previous reports indicated that reduced level of CBL activity, a E3-like ubiquitin ligase, was observed in various cancers (Kamei T. *et al.,* 2000). In support of this report, CBL was found preferentially bound to activated Src, mediating ubiquitin-proteasome degradation of the protein (Kim Minsoo *et al.,* 2004). Hence, the activation and persistence of c-Src activity are tightly regulated by the availability of positive and negative regulators. V-Src differs in this structure in lacking the C-terminal regulatory tail and in possessing point mutations contributing to its constitutive activation and high transforming activity (Jove and Hanafusa 1987, Parsons and Weber 1989).

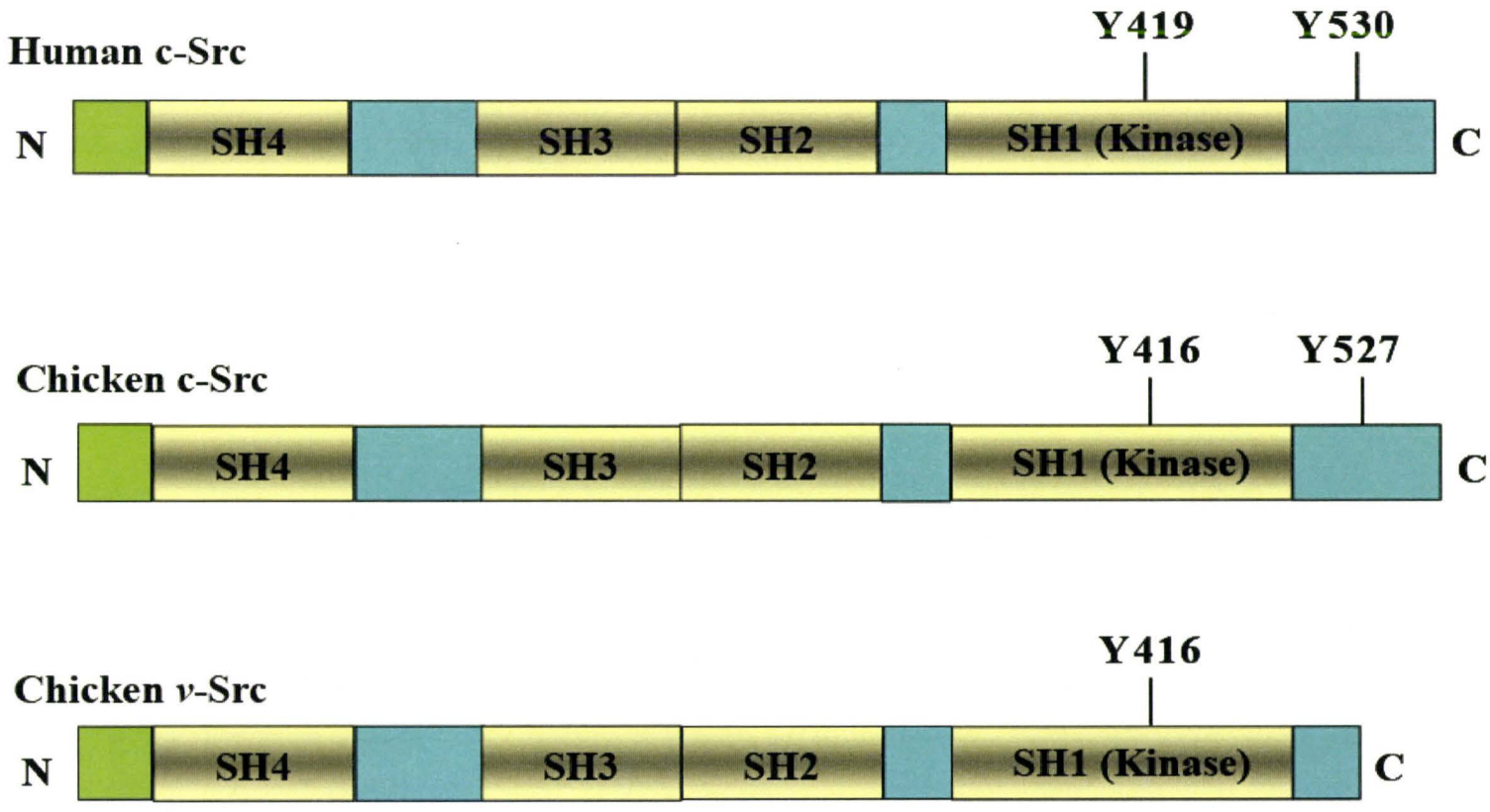


Figure 1. Structural comparison between c-Src and v-Src. Both human and chicken forms of c-Src possess the c-terminal regulatory tail while it is missing in v-Src, which contributes to constitutive activation of v-Src. The close conformation is a result of phosphorylation at the tyrosine located in the C-terminal tail.

2. Cellular activities regulated by Src

Although c-Src has a relatively lower transforming activity than its viral counterpart, c-Src was reported to be essential for angiogenesis and invasiveness of human cancers such as lung, skin, colon malignancies (Ishizawar R. and Parsons 2004). Increased activity of c-Src is frequently associated with lower level of negative regulators such as Csk or increased levels of positive regulators such as phosphatase PTPα (Irby and Yeatman 2000, Zheng *et al.,* 1992). In addition to the aberrant downregulation of negative regulators or upregulation of positive regulators, c-Src is also activated by extracellular signals and mediates cellular responses to activated receptors. Interaction of c-Src with receptor tyrosine kinases was observed in highly-metastatic cancers (Mao *et al.,* 1997). Another mechanism of c-Src activation is through naturally occurring mutational events. A rare c-Src activating mutation derived from the truncation of amino acid residues downstream of y530 has been described in highly metastatic colon carcinoma and endometrial cancer (Yeatman 2004). The deleted residues are critical for intra-molecular interaction with the c-Src SH2 domain and the subsequent inactivation of c-Src (Irby *et al.,* 1999). In this respect, this mutation is reminiscent of the deletion of the c-terminal regulatory region of v-Src. However, studies on other cancer populations failed to identify this mutation (Yeatman 2004), suggesting that naturally existing *Src* mutations are very rare and dysregulation of signaling pathways is a more common mechanism of Src activation. Hence, identification of the networks centered on Src appears to be critical in understanding the mechanism of tumour progression. The signaling pathways downstream of Src are summarized (**Fig.2**).

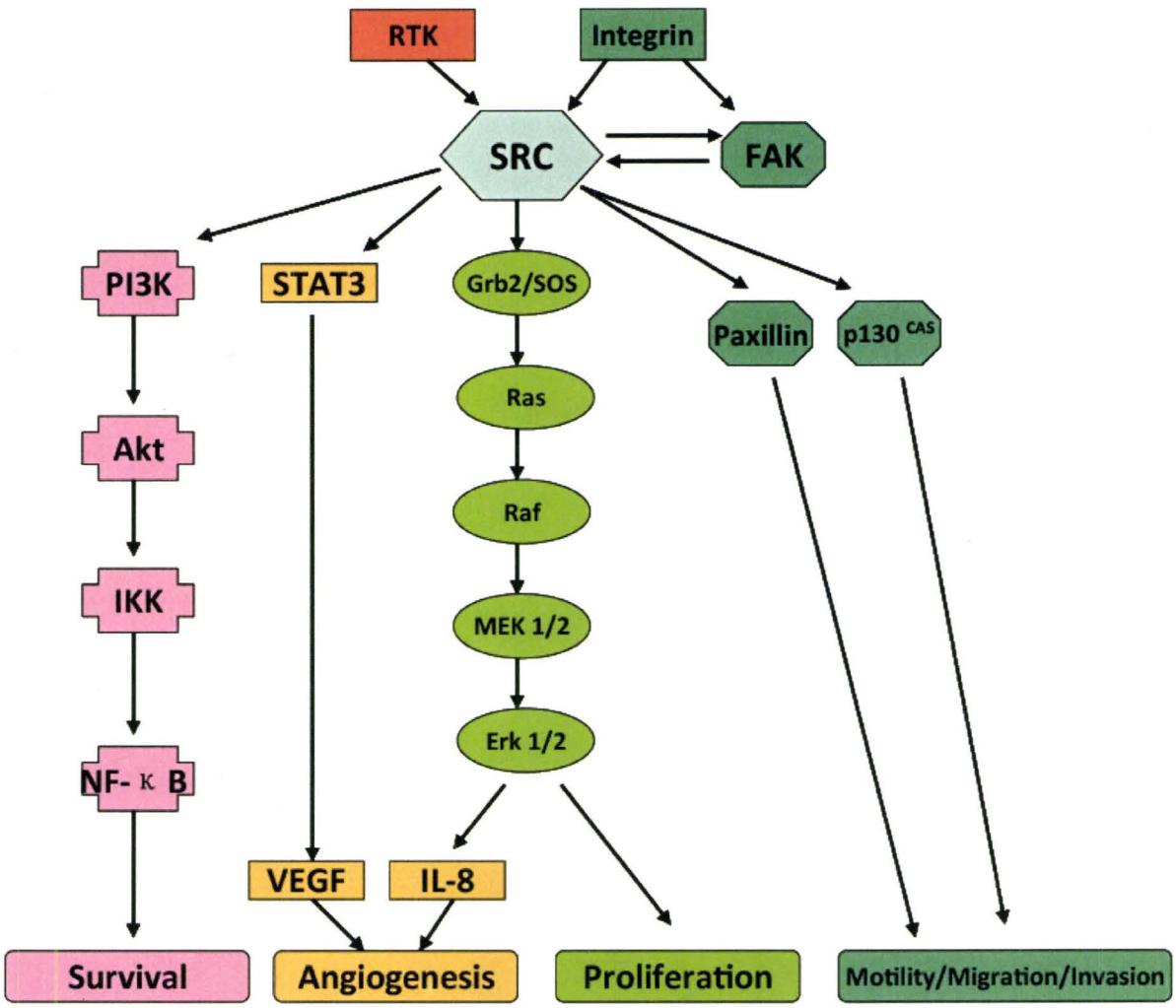


Figure 2. Signaling cascades of c-Src. Several downstream pathways activated by c-Src have been described. These pathways lead to different cellular activities that favor the development of aggressive tumors.

2.1 Signaling pathways lead to mitogenesis mediated by Src

2.1.1 Receptor tyrosine kinase (RTK)

The protein tyrosine kinase (PTK) family comprises proteins that possess the enzymatic activity to catalyze the transfer of a phosphate group from adenosine triphosphate (ATP) to the tyrosine residues of their substrate upon stimulation by growth factors. Depending upon membrane localization or their localization in a cellular compartment, PTKs are divided into two categories: Non-receptor tyrosine kinase (NRTK) and receptor tyrosine kinase (RTK). Receptor tyrosine kinases (RTK) are cell surface proteins embedded in the plasma membrane and capable of transducing extracellular signals. Common structure features of RTKs include an extracellular domain for ligand binding, a transmembrane domain and an intracellular kinase domain for phosphorylation of the tyrosine residue of specific substrates. Binding of a ligand to the extracellular domain of RTKs leads to phosphorylation of the tyrosines in their cytoplasmic domain and enzymatic activation (Bromann *et al.,* 2004). Association of SH2 domain of c-Src to the phosphotyrosine of RTK kinase domain alters the intramolecular conformation of c-Src and initiates the activation of Src (Bromann *et al.,* 2004). The recruitment of tyrosine phosphatase such as Shp2 to some activated RTKs has been reported to be involved in the activation process of c-Src. In response to PDGF, EGF and FGF, Shp2 promotes the Src activation by inducing the dephosphorylation of phosphotyrosine in the regulatory c-terminal of Src (Zhang *et al.,* 2004). Previous findings have demonstrated that association of Src to activated RTKs is required to transmit the extracellular signal to downstream pathways. Physical interaction of Src with

activated EGFR is essential for not only the activation of Src, but also for forming a positive feedback loop by Src to augment the EGFR-mediated tumour properties (Tice *et al.,* 1999, W.Mao *et al.,* 1997). In agreement with this finding, pharmacological inhibition of Src activity or expression of kinase-inactive form of Src result in activation defect of EGFR and leads to blockade of the downstream signaling pathway (Ishizawar RC *et al.,* 2007). The catalytic activity of Src is also elevated in platelet-derived growth factor (PDGF)-stimulated cells (Gould and Hunter 1988, Ralston and Bishop 1985), and association of Src is required for platelet-derived growth factor receptor (PDGFR)-dependent cell responses (Gelderloos *et al.,* 1998, Gould and Hunter 1988). Recruitment of Src to phosphorylated fibroblast growth factor receptor (FGFR) also initiates similar responses (Sandilands *et al.,* 2007).

Src activity is critical for growth factor-induced mitogenesis (Bromann *et al.,* 2004). EGF-induced augmentation in DNA synthesis has been reported in murine fibroblasts overexpressing Src (Luttrell *et al.,* 1988). In contrast, dominant negative Src was able to impair DNA synthesis in response to EGF (Wilson *et al.,* 1989). In addition, co-expression of Src and EGFR in immortalized murine fibroblast results not only in increased DNA synthesis, but also the ability to form colonies in soft agar (Maa *et al.,* 1995), a sign of cell transformation. Injection of a neutralizing antibody of Src inhibits DNA synthesis induced by PDGF and colony-stimulating factor [CSF; (Thomas and Brugge 1997)]. Therefore, Src plays an important role in propagating the extracellular growth signals to stimulate proliferation. One of the molecular mechanisms by which Src promotes proliferation induced by growth factors is to bypass the p53 activation

(Bromann *et al.,* 2004). Overexpression of a kinase-defective Src fails to inhibit the PDGF-triggered DNA synthesis in the presence of a dominant-negative p53 or in p53-null MEFs (Broome and Courtneidge 2000). Consistently, blockage of PDGF-induced DNA synthesis due to expression of a dominant-negative Src can be released by EIB-55K, an adenovirus protein that is able to repress p53 function (Furstoss *et al.,* 2002). A second mechanism of Src to promote proliferation relies on stabilization of the short-lived messenger-RNA of transcription factors such as Myc, whose expression and activation is required for mitogenesis in response to variety of growth factors (Bromann *et al.,* 2004). Previous findings have shown that the extracellular signal-regulated kinase/mitogen activated protein kinase (ERK/MAPK) pathway and phosphotidylinositol3'-kinase (PI3'K) pathway serve to stabilize the Myc protein via phosphorylation in response to Src activation (Sears *et al.,* 1999, Sears *et al.,* 2000). These pathways are known to be initiated upon RTK activation (Bromann *et al.,* 2004). Thus, Src mediates the signaling from RTK by downregulating anti-proliferative mechanisms or activating downstream signaling pathways leading to pro-proliferative gene transcription.

2.1.2 The Ras-MAPK pathway downstream of Src

The monomeric G protein Ras is one of the downstream targets of Src. First discovered as the transforming oncogene from the Harvey (the Ha-Ras oncogene) and Kirsten (Ki-Ras) murine sarcoma viruses (Chang *et al.,* 1982), cellular Ras and its activating mutants are found to be implicated in diverse biological processes (Malumbres

and Barbacid 2003). Ras is a guanosine nucleotide-binding protein (G protein) and acts as a switch that turns on the signaling pathway when interacting with guanosine triphosphate (GTP) and is off when interacting with guanosine diphosphate (GDP). The "on" phase is facilitated by guanine nucleotide exchange factors (GEFs) to increase the exchange rate of GDP for GTP on Ras due to a conformational change. Upon ligand binding, RTK is activated and interacts with the adaptor protein Grb2 (growth factor receptor-bound protein-2), which then recruits Sos [son of sevenless; (Tidyman and Rauen 2009)]. Sos is a guanosine nucleotide exchange factor (GEF) that exchanges GDP for GTP and results in an activated GTP-bound Ras. The "off" phase is promoted by a GTPase activating protein (GAP), which stimulates the intrinsic GTPase activity of Ras and converts the protein to the Ras-GDP bound form. Hence, GAP is a negative regulator of Ras activity. Interactions of GAP with both viral Src (v-Src) and cellular Src (c-Src) were observed in transformed rat fibroblasts (Brott *et al.,* 1991). This interaction leads to phosphorylation and inactivation of GAP (Moran *et al.,* 1991), which in turn activates Ras.

The requirement of Ras for *v*-Src induced transformation arises from the finding that microinjection of neutralizing antibodies against Ha-Ras suppressed transformation in NIH3T3 cells (Smith M. R. *et al.,* 1986). One of the key downstream mediators of Ras is the Raf/extracellular signal-regulated kinase/mitogen activated protein kinase (ERK/MAPK) pathway (Tidyman and Rauen 2009). The Raf/MAPK pathway comprises a series of activations of serine/threonine-specific protein kinases that lead to phosphorylation of downstream substrates. The highly conserved 90 kDa ribosomal S6

kinase (RSK) family of Ser/Thr kinases is an important downstream effectors of the Ras-MAPK signaling cascade that regulate diverse cellular activities such as proliferation, motility and survival (Anjum and Blenis 2008). For example, RSK phosphorylates and inhibits the glycogen synthase kinase-3 (GSK3) to promote the stabilization of cyclin D1 and Myc, leading to cell cycle progression and cell-survival (Balmanno and Cook 2009, Diehl *et al.,* 1998, Sears *et al.,* 2000). In chicken embryo fibroblast (CEF), expression of a dominant negative Ras (HRasN17) or addition of the MAPK kinase (MEK) inhibitor PD58095 were able to block several cellular responses induced by v-Src without interfering with transformation (Penuel and Martin 1999). This result suggested the presence of a Ras-independent pathway that works simultaneously with the Ras-MAPK pathway (Penuel and Martin 1999). However, overexpression of the dominant negative form of Ras, N17Ras, induced dramatic apoptosis in v-Src transformed murine pro-B cell lines likely through the downregulation of prosurvival protein Bcl-2 or elevation of caspase3 (Odajima *et al.,* 2000). In the same study, inhibition of caspase 3 or overexpression of Bcl-2 promoted cell survival but didn't rescue transformation: the *v*-Src-dependent proliferation was reduced by 85% (Odajima *et al.,* 2000), suggesting the action of Ras is pleiotropic to maintain survival and promote cell growth in response to *v*-Src transformation in these cells. Therefore, the role of Ras in *v*-Src transformation is cell type specific. Regardless, Ras is an important downstream effector of Src stimulated signaling.

2.1.3 The PI3K-Akt/PKB pathway

The PI3'K-Akt/PKB pathway participates in the regulation of many cellular processes such as cell growth and survival that are critical for tumorigenesis (Luo *et al.*, 2003). Activation of PI3'K is required for *v*-Src induced transformation because inhibitors of Phosphoinositide3'-kinase (PI3'K) attenuated the *v*-Src-dependent phenotypes in CEF (Penuel and Martin 1999). Dual inhibition of the PI3'K pathway and Ras-MAPK pathway dramatically repressed the *v*-Src-dependent transformation phenotype, suggesting that these two pathways are partly redundant in v-Src transformed CEF (Penuel and Martin 1999). PI3'K is a heterodimer comprised of a 85 kDa regulatory subunit (p85) and a 110 kDa catalytic subunit (p110). In Rous sarcoma virus-transformed CEF, a physical interaction between the proline-rich region of p85 subunit and the v-Src SH3 domain prior to phosphorylation was detected (Liu *et al.*, 1993). The interaction might lead to the tyrosine phosphorylation of PI3'K and activation of the kinase or, alternatively, facilitates the translocation of PI3'K to the inner plasma membrane where it has access to its substrates. At the membrane, PI3'K phosphorylates the hydroxyl groups on the inositol ring of phosphatidylinositol-4, 5-bisphosphate (PIP2) and converts it to PIP3, which in turns recruits pleckstrin-homology (PH) domain containing proteins to the membrane where they can be activated (Luo *et al.*, 2003). In v-Src transformed CEF, the serine-threonine kinases Akt/PKB and PI3'K-dependent kinase (PDK) are examples of PH-domain containing proteins that are recruited by activated PI3'K to the membrane, where Akt/PKB is phosphorylated and activated by PDK1 (Anderson *et al.*, 1998). Activation of Akt/PKB regulates a wide range of target proteins that regulate several cellular activities including cell growth and survival (Luo *et al.*, 2003). The mammalian

target of rapamycin (mTOR) kinase is a downstream target of Akt/PKB that regulates cell growth by stimulating protein translation through activation of the 70 kDa ribosomal S6 kinase (S6K1) and inhibition of the eukaryotic translation initiation factor 4E-binding protein1 (eIF4E-BP1) (Gingras *et al.,* 1998). Addition of the mTOR inhibitor, rapamycin, causes the same inhibitory effects as PI3'K inhibition by partially repressing v-Src-induced proliferation in CEF (Penuel and Martin 1999), suggesting that the PI3'K-mTOR pathway is one of the important pathways induced by v-Src in CEF. In addition, the PI3'K-Akt pathway can promote cell cycle progression in parallel with the Ras/MAPK pathway by phosphorylating and inactivating the kinase GSK3, leading to the stabilization of cyclin D1 and Myc (Diehl *et al.,* 1998, Sears *et al.,* 2000). Furthermore, activation of Akt/PKB is known to protect cells from programmed cell death induced by various stresses (Downward 1998). For example, activated Akt/PKB can phosphorylate BAD, a proapoptotic protein of Bcl-2 family, and facilitates its sequestration from the mitochondrial membrane by 14-3-3 proteins, resulting in the inhibition of cytochrome *c*-dependent apoptosis (Hennessy *et al.,* 2005). In *v*-Src transformed CEF, Akt/PKB is hyperphosphorylated, emphasizing the importance of PI3K-Akt pathway for v-Src induced transformation (Maślikowski *et al.,* 2010). Therefore, activation of the PI3'K-Akt pathway is able to promote cell growth and survival that are particularly important for tumorigenesis.

2.1.4 The JAK-STATs pathway downstream of Src

The signal transducers and activators of transcription (STAT) proteins activate gene

transcription upon activation by a variety of signals from cytokines to various growth factors, leading to cellular responses including differentiation, apoptosis and proliferation (Silva CM 2004). Phosphorylation of a tyrosine residue in the SH2 domain of STATs is modulated by non-tyrosine containing cytokine receptors via the family of Janus (JAK) family of tyrosine kinases. This STATs phosphorylation is required for homo- or heterodimer formation of STATs through the interaction of their tyrosine phosphorylated SH2 domains, and subsequent translocation to the nucleus. In the nucleus, the STAT dimer binds to the consensus DNA sequence via the DNA binding domain and activates gene transcription in responses to cytokines. Among the STAT members, only STAT1, STAT3, STAT5a and 5b have potential involvements in oncogenic signaling (Calò *et al.,* 2003). More importantly, these STATs are activated by Src or growth factors using a different mechanism from cytokines stimulation. Olayoiye *et al.* 1999 has reported that activation of the four members of STAT family by the epidermal growth factor receptor (EGFR), Erb1, is c-Src dependent and JAK-independent in NIH3T3 cells (Olayioye *et al.,* 1999). However, phosphorylation of STAT5a by Src does not cause translocation to the nucleus and therefore does not induce gene transcription (Silva CM 2004). These findings thus established a different activation and function pattern that might lead to a unique gene expressing profile in response to oncogenic stimulation. A previous finding has demonstrated that the DNA binding ability of STAT3 was enhanced upon *v*-Src transformation (Ihle 1996, Yu *et al.,* 1995). Moreover, *v*-Src mediates STAT3 phosphorylation through direct interaction (Cao X *et al.,* 1996). Expression of a dominant negative form of STAT3 resulted in dramatic reduction of *v*-Src-dependent cell

proliferation and transformation (Odajima *et al.,* 2000), supporting the essential role of STAT3 in *v*-Src transformation. Similarly, Src-mediated tyrosine phosphorylation of STAT5b is required to promote cell proliferation (Kabotyanski and Rosen 2003). In the Squamous Cell Carcinoma of the Head and Neck (SCCHN) model, EGFR mediates the STAT3 and STAT5b activation via Src, an event contributing to carcinogenesis (Xi *et al.,* 2003). Hence STAT has a role in promoting mitogenesis and oncogenesis in response to Src activation.

Interestingly, v-Src mediates the induction of vascular epithelial growth factor (VEGF) by activating the expression of STAT3 (Niu *et al.,* 2002), providing a role for Src in the regulation of the angiogenesis and tumorigenesis. STAT activation also results in transcription activation of genes involved in the inhibition of apoptosis and promotion of proliferation of cancer cells (Silva CM 2004). Thus STAT factors mediate a broad spectrum of cellular activities in tumorigenesis.

2.2 Src regulates adhesion, motility and invasion

In addition to rapid proliferation rates, *v*-Src transformed CEFs also display elongated and refractile morphology due to the loss of integrin-based attachments that are required for normal CEFs to grow in an ordered monolayer (Yeatman 2004). In addition, the fact that they are able to form foci in soft-agar assays indicates that they can proliferate in an anchorage-independent manner, a hallmark of cancer cells. Furthermore, *in vivo* injection of v-Src transformed cells results in the formation of tumours that are invasive and metastatic (Yeatman 2004). Consistently, highly-metastatic cancers are

frequently accompanied by high c-Src kinase activity, normally 5 fold higher than low-metastatic counterparts, indicating that high Src activity promotes the motility and invasiveness of cancer cells (Jones *et al.,* 2002). Thus, Src plays an important role in the regulation of the cell adhesion system during tumorigenesis. In the following sections, the action of Src in the regulation of two principal subcellular structures — focal adhesions and adherens junctions — will be reviewed.

2.2.1 The integrin-FAK signaling pathway

Focal adhesions are comprised of a large and dynamic protein complex that links the cell cytoskeleton to extracellular matrix (ECM) via clusters of integrins. Activation of integrin upon cellular interaction with ECM initiates signaling pathways involving tyrosine phosphorylation in many cytoskeleton- and signaling-proteins. One of the downstream effectors is focal adhesion kinase (FAK). The *fak* gene encodes a non-receptor protein tyrosine kinase of 120 kDa with little similarity to other tyrosine protein kinases (Owens *et al.,* 1995). FAK colocalizes with integrin at the focal adhesion through the carboxyl-terminal 'focal adhesion targeting' (FAT) domain, where its autophosphorylation and activation is stimulated by integrin to modulate cell migration and spreading (Playford and Schaller 2004). Phosphorylation on Y397 in FAK increases its affinity for binding the SH2 domain of Src (Schaller *et al.,* 1994). Furthermore, the proline-rich regions in FAK also attract c-Src via its SH3 domain (Thomas and Brugge 1997). The interaction between FAK and Src through the SH2 and SH3 domains leads to disruption of the intramolecular conformation of c-Src and then exposure of the C-

terminal regulatory domain and kinase domain (Cary *et al.,* 2002). Previous studies have demonstrated that the cell adhesion-regulated transmembrane protein tyrosine phosphatase α (PTPα) is required for dephosphorylation of the tyrosine residue in the C-terminal regulatory domain of c-Src, a mechanism to activate the protein (Pallen 2003, Playford and Schaller 2004). Other tyrosine phosphatases such as PTP1B and Shp2 have also been reported to participate in the activation of c-Src in focal adhesions (Playford and Schaller 2004). Once activated, c-Src then phosphorylates additional tyrosine residues on FAK, leading to full activation of FAK (Zhao and Guan 2009). The activated FAK/Src complex then initiates a series of phosphorylation events and triggers multiple downstream pathways to regulate cell survival, cell cycle progression, and focal adhesion dynamics and cell migration (Hsia *et al.,* 2003).

FAK was first identified as a key signaling molecule that is responsible for anchorage-independent growth (Guan *et al.,* 1991, Kornberg LJ *et al.,* 1991), and a key substrate of v-Src during transformation (Kanner *et al.,* 1990, Schaller *et al.,* 1992), implying the potential role of FAK in tumorigenesis. The FAK/Src complex might contribute to tumorigenesis by promoting cell survival. For example, overexpression of an activated Src was able to rescue 'anoikis', a process induced by detachment from the extracellular matrix, induced by a dominant negative form of FAK in breast cancer cell lines (Park *et al.,* 2004). Moreover, FAK/Src signaling also plays an important role in promoting cell proliferation. Phosphorylation of Y925 on FAK by activated Src can serve as a docking site for Grb2, leading to activation and initiation of the Ras-MAPK signaling pathway (Schlaepfer *et al.,* 1994).

In addition to its role in tumorigenesis, the FAK/Src complex is an important modulator of tumour progression by promoting cell motility and invasion. Hyper-phosphorylation of FAK was observed in *v*-Src transformed cells while FAK deficient mouse fibroblast exhibited less organized, more and enlarged focal adhesions (Ilić *et al.*, 1995). This increase in size and number of focal adhesions mimics the phenotype observed in response to the expression of kinase-defective *v*-Src and reflects the impairment of focal adhesion turnover, a process contributing to motility (Fincham and Frame 1998). Moreover, the FAK/Src complex has also been shown to promote the invasive ability of transformed cells by a number of mechanisms. For example, in v-Src transformed cells, FAK mediates invasion by promoting the activation and secretion of matrix metalloproteinases (MMPs), a group of enzymes that can break down the ECM and facilitate invasion (Hsia *et al.*, 2003). Collectively, the FAK/Src complex participates in multiple aspects of cancerogenesis.

2.2.2 Src downregulates cadherins to disrupt cell-cell adhesion

In epithelial cells, adherens junctions are formed by the cadherin-catenin system. Cell-cell adhesion is mediated by homotypic interactions of transmembrane E-cadherin protein, which is anchored intracellularly to the actin cytoskeleton by cofactors called catenins. Co-immunoprecipitation of activated c-Src with the E-cadherin-catenin complex in highly metastatic hepatocellular cancer cells suggested the colocalization of Src with the cell-cell junction system (Avizienyte *et al.*, 2002). This localization also resulted in disruption of the cell-cell adhesion system implying a vital role of activated

Src for metastasis of cancer cells. In addition, *v*-Src transformed Madin–Darby canine kidney (MDCK) epithelial cells exhibited loss of cell-cell adhesion and an invasive potential (Behrens J *et al.,* 1993). In these cells, tyrosine phosphorylation of N-Cadherin, E-Cadherin or β-catenin was shown to lead to instability of adherens junctions. A previous study reported that the tyrosine phosphorylation of E-cadherin by activated Src is required for the ubiquitination and endocytosis of E-cadherin via Hakai, an E3 ubiquitin protein ligase; this results in disruption of cell-cell contacts (Fujita *et al.,* 2002, Yeatman 2004). Similar findings have been reported in Ras-transformed breast epithelia cells (Frame *et al.,* 2002), indicating that the tyrosine phosphorylation of the cadherin-catenin complex is associated with the destabilization of the cell-cell junction. Furthermore, the addition of pharmaceutical inhibitors of c-Src tyrosine kinase activity stabilized the cell-cell junction and promoted the recruitment of E-cadherin to contact sites (Frame *et al.,* 2002). Hence, activated Src promotes cell migration in part by disrupting adherent junctions.

The *v*-Src substrate p120ctn, a member of the Armadillo catenin family, has emerged as a central regulator of E-cadherin stability and turnover in epithelia cancer cell lines (Reynolds and Roczniak-Ferguson 2004). As a major component of the epithelial adherens junctions, aberrant loss of E-cadherin (DNA methylation, direct mutation or downregulation) has been reported in many tumours and correlates with metastasis (Reynolds and Roczniak-Ferguson 2004, Yap 1998). Thus, E-cadherin is recognized as a tumour and metastasis suppressor. The restoration of p120 expression in the p120-deficient carcinoma cell lines SW48 was able to rescue the epithelial phenotype of poorly

organized cell-cell contact in a dose and phosphorylation level dependent manner (Ireton *et al.,* 2002), indicating that p120 is required for the stabilization of E-cadherin and cell-cell contact. In this study, the reconstituted p120 was less phosphorylated and functioned via direct interaction with E-cadherin to stabilize the protein itself (Ireton *et al.,* 2002). Conversely, p120 depletion via RNA interference led to degradation of E-cadherin and subsequent cell-cell contact turnover (Davis *et al.,* 2003). The phosphorylation of p120 by v-Src reduces the affinity between p120 and E-cadherin, a mechanism makes E-cadherin accessible for v-Src mediated phosphorylation and degradation via the ubiquitination pathway (Reynolds and Roczniak-Ferguson 2004). Hence, activated Src modulates the cell-cell interaction turnover by downregulating the cadherin-catenin complex. The role of p120 is not restricted to the regulation of cell-cell adhesion strength and stability; other functions are outside the scope of this study and therefore will not be reviewed here.

2.3 Src promotes tumour cell survival

Cell viability is a prerequisite of the development of the tumour, where survival signals are transduced by growth factors, cytokines, ECM and cell-cell contacts (Thomas and Brugge 1997). Expression of *v*-Src has been demonstrated to rescue cells from apoptosis induced by various stimuli such as irradiation, chemotherapeutic drugs and anoikis (Thomas and Brugge 1997). In addition, v-Src activates the expression of prosurvival genes such as p105 *nf-kb* and stimulates NFκB activity (Cabannes *et al.,* 1997). NFκB is a family of dimeric transcription factors sharing the conserved Rel-

homology domain (RHD) that is required for DNA-binding and dimerization (Papa *et al.*, 2004). Activation of NFκB has been linked to viral transformation and survival (Kucharczak *et al.*, 2003). Anti-apoptotic proteins including the *Bcl-2* family and cellular inhibitors of apoptosis [cIAP (Greten and Karin 2006)] are known targets of NFκB, which reflects the role of NFκB in survival. Hence, Src can also regulate cell survival by activating prosurvival genes. Consistently, specific inhibition of c-Src or the PI3'K inhibitor LY294002 increased the incidence of apoptosis incidence in vascular endothelial growth factor (VEGF)-stimulated human umbilical-vein endothelial cells [HUVECs; (Abu-Ghazaleh *et al.*, 2001)], suggesting that Src is involved in the VEGF-mediated anti-apoptotic activity, likely through the activation of the PI3'K-Akt pathway.

2.4 Src regulates angiogenesis

In addition to its role in cell proliferation, STAT activation by *v*-Src also leads to other activities that are important for tumour progression. Established tumours induce angiogenesis to obtain nutrients in order to support their rapid growth and promote metastasis. The VEGF is reported to be critical for angiogenesis in several types of tumours (Grunstein *et al.*, 1999). The expression of VEGF was shown to be activated by v-Src through direct binding of STAT3 to the promoter region of VEGF (Niu *et al.*, 2002). In addition, upregulation of Flit1/VEGFR was observed in our gene expression profile of v-Src transformed cells (Maślikowski *et al.*, 2010). Thus, activated Src may promote tumour progression by inducing angiogenesis via the STAT3 pathway. Transcriptional activation of the *IL8* gene (originally called *9E3/CEF4*) is tightly

associated with CEF transformation by v-Src (Bedard *et al.,* 1987, Sugano *et al.,* 1987). Like mammalian IL8, the chicken counterpart (originally designated chicken chemotactic and angiogenic factor, or cCAF) was shown to be angiogenic, suggesting that angiogenesis is also stimulated by constitutive expression and secretion of this chemokine by *v*-Src transformed CEF (Martins-Green and Hanafusa 1997). Thus, Src has a potential role in regulating angiogenesis and tumorigenesis in several cell types and species.

3. Control of gene expression by *v*-Src

3.1 Control of transcription factors by v-Src

Transformation by *v*-Src is characterized by profound changes in gene expression. Aberrant gene expression in cells transformed by *v*-Src is a result of constitutive activation of transcription factors. Work in our lab and others (Masker *et al.,* 2007, Maślikowski *et al.,* 2010) have shown that v-Src-transformation in CEF leads to changes in the expression of approximately 6% of the chicken genome. Numerous studies have identified several *v*-Src activated transcription factor families including Stats, NFκB, AP-1 and C/EBPβ (Cabannes *et al.,* 1997, Dehbi and Bedard 1992, Dehbi *et al.,* 1992, Gagliardi *et al.,* 2001, Silva CM 2004, Thomas and Brugge 1997). These studies provide potential targets of c-Src activation. As mentioned above, c-Src is known to activate the transcription of Myc in the presence of PDGF since inhibition of c-Src by an inhibitory antibody results in repression of *myc* transcription (Barone and Courtneidge 1995). In addition, expression of a kinase-defective Src mutant impairs the endothelin dependent

induction of the *fos* gene (Simonson *et al.,* 1996), a member of the AP-1 family. These findings reflect the importance of c-Src in the activation of transcription factors.

Of the many genes activated by Src, the *IL8* gene (originally called *9E3/CEF4*) has been shown to be tightly associated with CEF transformation by v-Src. In contrast, *il8* transcription is transient upon serum stimulation (Bedard *et al.,* 1987, Sugano *et al.,* 1987). Regulation of *IL8* relies on the Src-responsive unit (SRU) of its promoter which includes binding sites for AP-1, NFκB and C/EBPβ, all of which are known to be activated by *v*-Src in CEF (Cabannes *et al.,* 1997, Dehbi and Bedard 1992, Gagliardi *et al.,* 2001). We observe a dramatic increase in AP-1, NFκB and C/EBPβ activities in v-Src transformed CEF compared to normal CEF (Lizhen WANG, chapter 3). Investigating the role of these factors in IL8 activation and transformation by v-Src will shed light on the mechanisms by which v-Src regulates cellular transformation and tumorigenesis. My research focuses primarily on the role of AP-1 in *v*-Src transformation in CEF.

3.2 Transcriptional activation by transcription factors

Transcription is the first step leading to gene expression, a process known to be regulated by the interaction of transcription factors with the promoter region of the gene (Latchman 1997). The regulation is modulated via two major domains of transcription factors: the DNA-binding domain (DBD) and the transactivation domain [TAD; (Latchman 1997)]. The DBD is required to attach to specific sequences of DNA that are also known as response elements, while TAD upon activation is essential for the recruitment of the general transcriptional machinery to the promoter region (Ptashne and

Gann 1997). The general transcriptional machinery consists of components such as TFIIB, TFIID of general transcription factors (GTFs), transcription coregulators (co-activators or co-repressors) and the RNA polymerase II (Latchman 1997, Ptashne and Gann 1997, Wärnmark *et al.,* 2003). Interaction of the RNA polymerase II with the GTFs forms a preinitiation complex that is essential for the initiation of transcription (Kornberg RD 2007). Simultaneous interaction of co-activators stablizes the preinitiation complex and promotes the transcriptional initiation (Näär *et al.,* 2001). CREB1-binding protein (CBP)/p300 complex acts as a co-activator that widely participates in the activities of different transcription factors to control the expression of genes involved in cellular proliferation, apoptosis, and embryogenesis (Goodman and Smolik 2000). CBP and p300 share 63% similarity in amino acid sequence and are functionally identical in many physiological processes (Iyer *et al.,* 2004). These proteins function as histone acetyltransferases (HAT) complexes that catalyze the acetylation of the conserved lysine amino acids on histone or other proteins and promote the relaxation of chromatin allowing increased access of transcriptional machinery to the gene (Vo and Goodman 2001). Thus, as a part of transcription integrator , the amount of p300 is strictly limited (Ghosh and Varga 2007). Competition for limiting amounts of p300 between transcription factors directs the gene expression pattern and controls cell fate (Wang C *et al.,* 2001). Abnormal CBP/p300 function results in aberrant gene expression. Point mutations and heterozygous loss of human CBP gene causes the Rubinstein-Taybi syndrome, which is characterized by severe mental retardation and several physical abnormalities (Petrij *et al.,* 1995). Mutations in p300 have been identified in many human

cancers in colon and rectum, stomach, breast and pancreas (Iyer *et al.,* 2004). In our model, we proposed that competition for limiting amount of co-activators such as CBP/p300 directs the gene expression pattern and cell fate in v-Src transformed CEF. This will be further discussed in Chapter 6.

4. Activator protein 1 (AP-1) family

The activator protein-1 (AP-1) transcription factors are homo- or hetero-dimers that regulate the expression of genes involved in cell proliferation, survival and transformation (Shaulian and Karin 2002). First identified as one of the components binding to the enhancer sequences of human metallothionein gene and SV40 promoter (Lee W *et al.,* 1987b), and the subsequent discoveries of viral oncogene counterparts led to investigations on its role in gene regulation and tumorigenesis (Eferl R and Wagner 2003). Several studies on the role of AP-1 orthologues in different model organisms suggest functional conservation and thus provide a basis for studies of AP-1 in human diseases (Wagner 2001). Activation of AP-1 controls a broad range of cellular activities. However, the following section will focus on the role of AP-1 in tumorigenesis.

The AP-1 family is comprised of the JUN, FOS, ATF (activating transcription factor) and MAF (musculoaponeurotic fibrosarcoma) protein families. AP-1 dimers form through the leucine zipper motif located in proximity to the basic DNA binding domain (Eferl R and Wagner 2003). The complexes recognize the TPA response element (TRE, 5' TGA$^{G}/_{C}$TCA-3'), which is responsive to the tumour promoting agent 12-O-tetradecanoylphorbol-13-acetate (TPA), or cAMP response elements (CRE, 5'-

TGACGTCA-3') and regulate gene transcription activation (Eferl R and Wagner 2003). Members of the MAF subfamily regulate different subsets of genes via binding to different consensus DNA sequences: the MAF-recognition elements (MAREs) and the antioxidant-response elements (AREs) , and (Eferl R and Wagner 2003). This review will focus on the regulation and function of the Jun and Fos families.

4.1 Structure and regulation of AP-1

The structure and functional domains of c-Jun and c-fos are shown in Figure 2. The identification of Jun as an AP-1 member stems from two major findings. First, c-Jun is the cellular counterpart of *v*-Jun, the oncogene of the transforming avian sarcoma virus 17 [ASV17; (Maki *et al.,* 1987)]. Second, Jun shares homology with the yeast transcription factor GCN4 that recognizes the same responsive element as AP-1 (Vogt P *et al.,* 1987). These two properties immediately linked Jun to the AP-1 family. The common structure of Jun proteins includes a N-terminal transactivation domain, a DNA binding domain and a C-terminal leucine zipper (Eferl R and Wagner 2003). Similar to c-Jun, c-Fos also has a viral counterpart that is the transforming gene of the Finkel-Biskis-Jinkins and the Finkel-Biskis-Reilly murine osteosarcoma virus (Foletta 1996).

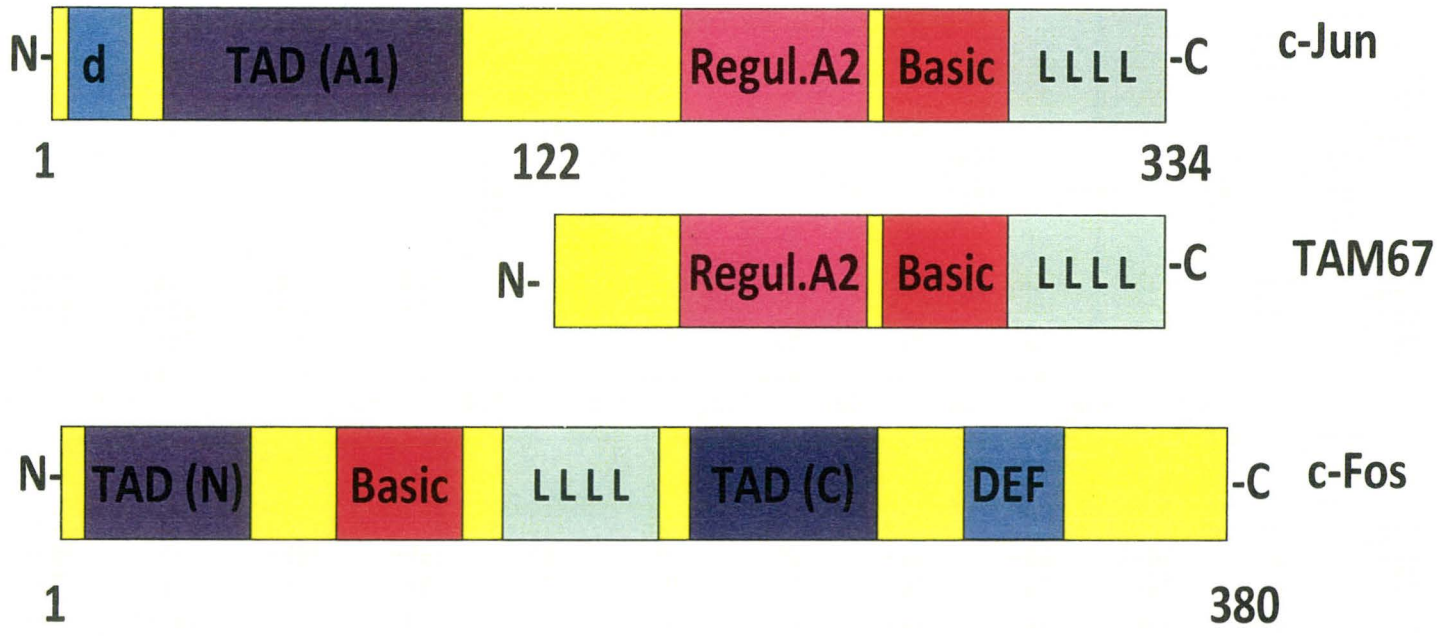


Figure 3. Structure of Jun and Fos proteins and the dominant negative mutant of c-Jun. The DNA backbone is shown in yellow. The Jun and Fos proteins exhibit several domains, including the bZIP domain (leucine zipper plus basic domain), transactivation domains and docking sites for several kinases, such as JNK (delta) or ERK (DEF). These kinases phosphorylate two serine and threonine residues and thereby modulate the activity of both proteins. JNK specifically phosphorylates serine residues within the transactivation domain of Jun at position 63 and 73 and thereby regulates its transactivation activity.

4.1.1 The transactivation domain and Delta domain

a. The TAD domain and Delta domain of the Jun family

The transactivation domain of Jun lacks definitive structural features that are usually found in other transcription factors. However, deletion of this domain results in a potent inhibitor of AP-1 activity (Brown PH *et al.,* 1993). This dominant negative form of Jun efficiently blocks AP-1-dependent transactivation and oncogenic transformation by activated Src (Brown M. T. and Cooper 1996). The essential role of Jun in the promotion of tumorigenesis is evident in the finding that overexpression of the dominant negative form is able to repress the proliferation and tumorigenesis of various human ovarian carcinoma cell lines (Neyns *et al.,* 1999). Thus this protein is widely used in studying the role of Jun in various cellular activities. Previous studies have shown that the activation of human c-Jun requires phosphorylation of two residues within the transactivation domain: Ser63 and Ser73(Vogt PK 2001). The Jun N-terminal kinase, also known as stress-activated protein kinase (SAPK), is responsible for phosphorylating c-Jun by interacting with c-Jun on the N-terminal delta domain once it is activated (May *et al.,* 1998). However, it has been observed that inactive JNK/SAPK remains constantly associated with the delta domain and mediates the ubiquitin-dependent degradation of c-Jun protein (Bohmann and Tjian 1989, Treier *et al.,* 1994). Hence, this delta domain serves as a docking site for JNK/SAPK. The delta domain is unique to c-Jun and is not found in other members of the AP-1 family (Vogt PK 2001). Unlike c-Jun, the viral Jun differs from c-Jun by lacking the N-terminal delta domain and some amino acids in the C-terminus (Nishimura et al., 1988). As a result, *v*-Jun is dissociated from the JNK/SAPK

pathway (May *et al.,* 1998), and the substitution of Ser63 and Ser73 with alanines does not interfere with the transforming ability of *v*-Jun in CEF (Vogt PK 2001). However, the function of the delta domain is cell type specific. One of the early findings demonstrated that mutation of the delta domain does not interfere with the transactivation of the c-Jun protein in F9 murine embryonic carcinoma cells (Havarstein *et al.,* 1992). Furthermore, mutation of the JNK phosphorylation sites does not affect the transforming ability of c-Jun in avian cells (Vogt PK 2001). These findings suggest that the JNK-Jun pathway is not critical in these cells or under certain conditions.

b. The TAD domain of Fos family

All members of the Fos family have an N-terminal acidic activation domain, whereas a C-terminal proline-rich and acidic activation domain is unique to c-Fos and FosB (Abate *et al.,* 1991). The acidic domain is important for transcriptional activation (Nakabeppu and Nathans 1991). Accordingly, Fra-1 and Fra-2 are not transforming in rat fibroblasts (Wisdom and Verma 1993). However, elevated levels of Fra-1 and Fra-2 have been observed in several tumour types (Milde-Langosch 2005). Moreover, Murakami and colleagues have determined that the transactivation activity of Fra-2 is highly activated by Erk-2 of MAPK family in *v*-Src transformed CEF (Murakami *et al.,* 1997). These findings suggest that Fra-1 and Fra-2 might play a unique role in facilitating, rather than inducing, tumorigenesis. In fact, upon serum stimulation, c-Fos and FosB are induced rapidly yet transiently, while Fra-1 and Fra-2 expression is delayed but sustained (Milde-Langosch 2005). Hence, Fra-1 and Fra-2 will be the major components to form

the Jun/Fos dimer and mediates cellular responses.

4.1.2 The bZIP domain

The leucine-zipper is formed by five leucine residues that are located seven amino acids apart from each other to form an X-shaped α-helical structure upon dimerization (Eferl R and Wagner 2003). Dimerization is critical for efficient nuclear translocation and DNA binding (Chida *et al.,* 1999). The basic domain is located adjacent to the leucine zipper and is required for DNA binding. This characteristic organization has been found in many transcription regulators that are collectively known as 'bZIP' factors (Vogt PK 2001). C-Jun is able to form homo- and heterodimers with various bZIP proteins, accounting for its pleiotropic functions (Vogt PK 2001). The preferential dimerization partner for Jun is a Fos family member. This dimer is thought to be more stable and exhibit higher affinity for the TRE sequences (Lee W. *et al.,* 1987a). In addition, c-Jun is able to form heterodimers with other bZIP proteins of the AP-1 family while dimerization of c-Jun with ATF or MAF shows preferential recognition to the cAMP responsive element (CRE): TGACGTCA (Vogt PK 2001). Thus, dimerization with diverse partners leads to transcriptional regulation of different genes and produces specific gene expression profiles and cellular responses. Moreover, previous experiments indicated that different AP-1/c-Jun complexes contribute to distinct regulation in the c-Jun-induced transformation programme. Indeed, c-Jun–Atf2 triggers proliferation independently of growth factors while the c-Jun–c-Fos activity causes anchorage-independent growth (van Dam and Castellazzi 2001). C-Jun is able to form homodimers via the leucine zipper and

form a potent oncogenic factor (Vogt PK 2001). Chimeric proteins consisting of the c-Jun transactivation domain and leucine zippers from bZIP proteins GCN4 and Epstein-Barr virus transcription factor EB1 that can only form homodimers, show strong ability to induce tumorigenesis in animals (Hartl and Vogt 1992, Vandel *et al.,* 1995, Vandel *et al.,* 1996). Therefore c-Jun is capable of inducing transformation independently of other bZip partners.

The DNA binding domain is an important determinant for the transcriptional regulation of a spectrum of genes and the subsequent cellular activities regulated by these genes. Mutation in the DNA binding domain has been found to be correlated with the impaired interaction with TRE and the complete abolishment of oncogenicity (Basso *et al.,* 2000). The DNA binding activity of Jun depends on two mechanisms: phosphorylation and reduction-oxidation (redox) regulation (Eferl R and Wagner 2003). For example, phosphorylation of the Ser247 and Ser249 residues adjacent to the basic domain of c-Jun by a nuclear protein kinase casein kinase II (CKII) and the kinases glycogen-synthase-kinase-3β (Gsk-3β) leads to severe reduction of DNA binding activity of c-Jun (Eferl R and Wagner 2003, Lin A *et al.,* 1992a, Plyte *et al.,* 1992). In addition, an *in vitro* study has shown that the DNA binding activity of c-Jun is also modulated by reduction-oxidation (redox) on a conserved cysteine residue, Cys252, located in the DNA-binding domains of c-Jun (Abate *et al.,* 1990). Substitution of cysteine to serine enhances the transforming potential of c-Jun when expressed *in vivo*, implying the importance of the reactive cysteine residue in the regulation of the oncogenic potential of c-Jun (Xanthoudakis and Curran 1992). This mutation is present in *v*-Jun and contributs

to its dissociation from the redox-dependent regulation and increase in DNA binding affinity (Oehler *et al.,* 1993). Reduction of this cysteine residue is mediated by a nuclear regulatory redox factor, Ref-1, which acts to activate the DNA-binding activity of c-Jun (Xanthoudakis and Curran 1992).

4.2 Regulation of AP-1 activity

The regulation of AP-1 is multi-faceted. It is responsive to a broad range of stimuli including cytokines, growth factors, stress signals and viral infection (Hess *et al.,* 2004). Collectively, the regulation of AP-1 occurs through several events: transcription and mRNA stabilization, post-translational modification, protein turnover and interaction with other transcription factors (Hess *et al.,* 2004).

4.2.1 Post-translational regulation of AP-1

The post-translational modification of Jun family by the MAPK pathway has been studied extensively. The Jun N-terminal kinases (JNK), also known as stress-activated protein kinases (SAPK), specifically phosphorylate serine residues (Ser 63 and 73) in the N-terminal transactivation domain of c-Jun in response to activated Ras, leading to enhanced transactivation activity of c-Jun (Pulverer *et al.,* 1991). The phosphorylation of c-Jun by JNK/SAPK requires docking to the N-terminal delta domain of c-Jun (Kallunki *et al.,* 1996). On the contrary, due to the absence of a JNK docking site, phosphorylation of JunD by JNK/SAPK is less efficient but occurs when JunD is heterodimerized with a docking competent partner, particularly c-Jun (Hess *et al.,* 2004, Kallunki *et al.,* 1996). In

addition, phosphorylation on Ser247 and Ser249 adjacent to the basic domain of c-Jun by CKII and Gsk-3β leads to severe reduction of DNA binding activity and inactivation of c-Jun (Eferl R and Wagner 2003, Lin A *et al.,* 1992b, Plyte *et al.,* 1992). JunD is phosphorylated and activated preferentially by ERK on Ser100 in its N-terminus (Vinciguerra *et al.,* 2004). Vinciguerra and colleagues identified two functional motifs, the D domain and the FLYP/DEF motif that contribute to the distinct regulation modes of c-Jun and JunD by MAPK (Vinciguerra *et al.,* 2004). The D domain is essential for targeting JNK to c-Jun and is part of the delta domain (Dérijard *et al.,* 1994, Fantz *et al.,* 2001). The FLYP/DEF motif located at the C-terminus of JunD has been found to function together with the D domain to increase the affinity for ERK for phosphorylation (Fantz *et al.,* 2001). JunD also contains both the N-terminal D domain and the C-terminal FLYP/DEF motif, which may contribute to its rapid phosphorylation on the N-terminus upon ERK activation (Gallo *et al.,* 2002, Vinciguerra *et al.,* 2004). However, the differential regulation of c-Jun and JunD by JNK and ERK is not fully elucidated. Except for the Jun family members, ERK also directly and indirectly phosphorylates and activates the fos family members in response to serum and growth factor stimulation (Shaulian and Karin 2002). For example, phosphorylation of Fra-1 and Fra-2 by ERK increases their DNA binding activity upon serum or growth factor stimulation (Gruda *et al.,* 1994). Moreover, the transactivation activity of Fra-2 is highly elevated by ERK in responsive to *v*-Src induced transformation (Murakami *et al.,* 1997).

4.2.2 Transcriptional and translational regulation of AP-1

Numerous studies revealed that several AP-1 members are regulated at the transcriptional and translational levels. In addition to posttranslational regulation, induction of c-Jun and JunD transcription is mediated through positive autoregulation by binding of the existing AP-1 complex to a high affinity AP-1 binding site located in the promoter region (Angel *et al.,* 1988, Berger and Shaul 1994), which accounts for enhance expression and activity of both Jun proteins. Moreover, the translation of JunD and its isoform is regulated by the usage of alternative start codons (Vesely *et al.,* 2009) . The resulting proteins are full length JunD and the N-terminally shortened version, JunD-delta, for which no functional relevance has been identified thus far (Short and Pfarr 2002). Translation of JunB protein is controlled by mTOR in the highly aggressive T-cell tumour, Anaplastic Large Cell Lymphoma [ALCL; (Vesely *et al.,* 2009)].

Growth factor or serum stimulated transcriptional activation of *c-fos* gene is mediated by the ERK pathway through the serum response element (SRE) required for the regulation of many cellular immediate early genes by growth factors, (Johnson GL and Vaillancourt 1994). Recent findings also indicated that translational regulation of c-fos can be achieved by naturally existing microRNA (Vesely *et al.,* 2009). miR-7b induces the decay of the c-fos mRNA by binding to complementary sequences within the 3'-untranslated region (UTR) upon hyperosmolar stimulation in murine hypothalamus (Lee HJ *et al.,* 2006). In addition, AU-rich elements are also present in the 3'UTR of c-fos mRNA and are known to interfere with the mRNA stability (Vesely *et al.,* 2009). Overall, AP-1 activity is regulated at different levels in a stimulus-dependent manner.

4.3 Actions of AP-1 in proliferation and transformation

Numerous studies have suggested a role of AP-1 in promoting proliferation and neoplastic transformation. Cell cycle related proteins such as cyclin D1, cyclin A, cyclin E, p53, $p21^{cip1}$, $p16^{INK4a}$ and $p19^{ARF}$ are targets of AP-1 that control cell proliferation (Sharulian and Karin. 2001, Shaulian and Karin 2002). Analyses of cell culture and mouse models have revealed that the Jun family plays a vital role in regulating proliferation in different cellular contexts with some functional redundancy of Fos proteins. These two families are reviewed individually below.

4.3.1 The role of c-Jun in proliferation

The Jun family consists of three members: c-Jun, Jun-D and Jun-B. c-*jun* was found to mediate many cellular processes such as proliferation and survival following the identification as the cellular homolog of *v-jun*, the oncogene of avian sarcoma virus 17 (Maki *et al.,* 1987). The *c-jun* gene product has a potent transactivation domain and is sufficient to induce transformation in tissue culture by overexpression (Eferl R and Wagner 2003). c-Jun directly regulates the transcription of *cyclin D1*, and hence positively regulates the G1-S phase transition in the cell cycle in fibroblasts (Albanese *et al.,* 1995). This process is p53-dependent since c-Jun can inhibit p53 expression by binding directly to the AP-1 site in the *p53* promoter (Schreiber *et al.,* 1999). In addition to fibroblasts, c-Jun is essential in regulating cell cycle in other cell types such as hepatocytes and keratinocytes (Hess *et al.,* 2004). Liver-specific deletion of the *c-jun* gene in mice results in reduced hepatocyte proliferation and impaired liver regeneration

(Behrens A *et al.,* 2002). However, unlike c-Jun-null fibroblasts, the levels of p53 and $p21^{CIP1}$ in these cells remain normal, and a delayed accumulation of cyclin D1 and cell cycle progression are observed. This is also true for mutant keratinocytes derived from mice with conditional inactivation of c-Jun (Zemz 2003), suggesting that the impaired cell proliferation is independent of p53 and $p21^{CIP1}$ expression in these cells. Furthermore, inactivation of *c-jun* in mouse hepatocellular carcinomas results in reduced tumour size with elevated p53 level (Eferl R. *et al.,* 2003). Collectively, c-Jun regulates cell cycle progression through different mechanisms in different cell types and cellular contexts.

4.3.2 The roles of JunB and JunD in proliferation

JunD and JunB of the Jun family are thought to possess relatively weaker transactivation activity compared to c-Jun (Eferl R and Wagner 2003). Overexpression of JunD in immortalized mouse fibroblasts inhibits not only cell proliferation, but also transformation stimulated by Ras (Pfarr *et al.,* 1994). In agreement with this notion, $JunD^{-/-}$ immortalized fibroblast displayed increased proliferation (Weitzman *et al.,* 2000). On the contrary, JunD deficient primary fibroblasts exhibit p53-dependent growth arrest and premature senescence (Weitzman *et al.,* 2000). This data suggests that the function of JunD in proliferation is cell context-dependent.

A previous study has demonstrated that fibroblasts derived from JunB overexpressing mice exhibit reduced proliferation due to an impaired G1-S phase transition and premature entry into senescence (Passegué and Wagner 2000). This effect is the result of

repression of *cyclin D1* and activation of the transcription of the cyclin-dependent kinase inhibitor $p16^{INK4a}$. In addition, the level of JunB is downregulated while c-Jun remains constant during the M-G1 transition and this promotes cell cycle progression to G1, while overexpression of JunB is able to block the c-Jun-mediated cyclin D1 expression (Bakiri *et al.,* 2000). Lack of JunB expression in mice results in a disease phenotype resembling human chronic myeloid leukemia [CML;(Passegue´ *et al.,* 2001)]. Similarly, hypermethylation of the JunB promoter and subsequent loss of JunB expression are evident in human CML patients (Yang *et al.,* 2003). Hence JunB is recognized as a negative regulator of cell proliferation at least in some cell types. However, substitution of JunB expression for c-Jun is able to rescue the proliferative defects and deregulated expressions of cyclinD1, p53 and $p21^{CIP1}$ in c-Jun deficient fibroblasts (Passegué *et al.,* 2002). In addition, direct transcriptional activation of cyclin A by JunB has been reported, suggesting a pro-proliferative role for JunB in fibroblasts (Andrecht *et al.,* 2002). Similarly, JunB deficient mouse shows downregulation of cyclin A expression in chondrocytes and osteoblasts, which is associated with reduced proliferation (Hess *et al.,* 2004). These results illustrate the multi-faceted role of JunB in the control of cell proliferation.

The dual role of JunB in proliferation might be cell-context dependent. In the absence of c-Jun, the conversion of JunB from growth inhibitor to growth promoter suggests that the Jun-JunB heterodimer possesses weaker transactivation activity that might attenuate the expression of cell cycle related genes, such as cyclinD1 (Passegué *et al.,* 2002).

Similarly to JunB, the switch from growth promoter to inhibitor of JunD occurs when interacting with menin, a tumour suppressor (Agarwal SK *et al.,* 2003). This interaction leads to repression of the transactivation activity of JunD (Agarwal S. K. *et al.,* 1999). The role of JunD in different cell contexts might rely on the availability of menin. In summary, the dual roles of JunB and JunD are cell type and cell context dependent.

4.3.3 The Fos Family

The *fos* family comprises 4 genes: *c-fos, fos-b, fra-1* and *fra-2*. The *fos* family is not able to form homodimers and can only form heterodimers with the Jun family (Foletta 1996, Robert E. 2003). Similarly to *c-jun*, *c-fos* also has a viral counterpart *v-fos*, the transforming gene carried by the Finkel-Biskis-Jinkins and the Finkel-Biskis-Reilly murine osteosarcoma viruses (Foletta 1996). As an immediate early gene, *c-fos* mRNA accumulates rapidly and transiently following serum or TPA induction, and its activation does not require *de novo* protein synthesis (Eferl R and Wagner 2003). Conditional expression of Fos in osteoblast cell lines reveals that c-Fos expression promotes the G1-S transition as a result of increased cyclin A and cyclin E expression (Sunters *et al.,* 2004), indicating that fos expression is vital for proliferation in these cells. Moreover, overexpression of *c-fos* is transforming and tumour promoting in human osteoblasts, while its expression is suppressive in human bronchial epithelial malignancies, suggesting that the transforming potential of *c-fos* relies on the cell type and pathways involved (Lee H *et al.,* 1998, Milde-Langosch 2005). Fra-1 and Fra-2 are found highly expressed in a variety of transformed and neoplastic cells (Tkach *et al.,* 2003). Compared

to *c-fos*, the activation of *fra-1* is delayed yet more sustained upon serum stimulation. By using transcriptional reporter assays, induction of the *fra-2* promoter by serum was observed in normal chicken embryonic fibroblasts (CEF), which was attributed mainly to the c-fos/c-jun complex (Sonobe *et al.,* 1995). Promoter analysis revealed that there are two AP-1 binding sites in the *fra-2* promoter, which contributes to its activation in *v*-Src transformed CEF (Murakami *et al.,* 1997). Consistent with these findings, Fra-2 expression is highly elevated in *v*-Src transformed CEF while loss of Fra-2 expression inhibits transformation by *v*-Src in CEF (Manuscript in Appendix 2). Hence, Fra-2 expression is required for *v*-Src induced transformation in CEF.

4.4 Actions of AP-1 in cell survival

Previous studies have demonstrated a dual role of AP-1 in regulating cell survival. The action of AP-1 in survival is complex and cell-context dependent.

4.4.1 Pro-apoptotic function of the Jun family

The JNK-AP-1 pathway has been reported to be involved in the Fas ligand (FasL) and its cell-surface receptor Fas-mediated apoptosis in lymphoid and fibroblast cells (Hess *et al.,* 2004). Phosphorylation by JNK enhanced the transactivation activity of c-Jun, where it is able to activate transcription of stress-related genes such as Fas ligand (FasL) and TNFα, whose expression are pro-apoptotic (Hess *et al.,* 2004). JNK-2 deficient mice or transgenic mice expressing dominant negative JNK display normal activation-induced cell death of peripheral T cells (Rincón *et al.,* 1998). Consistently, mice expressing a c-

Jun mutant with serines 63 and 73 mutated to alanines are resistant to Fas-mediated cell death in thymocytes (Behrens A *et al.,* 2001), revealing that JNK phosphorylation of c-Jun is essential for induction of apoptosis in these cells. In addition to the AP-1 site in the promoter region of FasL, c-Jun also binds to the human Fas promoter and potently activates its transcription, while c-Fos functions in an opposite way by binding to the transcriptional repressor element within the promoter region of Fas (Lasham *et al.,* 2000). The mechanism by which individual AP-1 member regulates the FasL mediated cell death remains unclear.

The pro-apoptotic role of JNK-Jun pathway is also observed in neurons (Hess *et al.,* 2004). Cells derived from sympathetic and cerebella granular neurons undergo a JNK-Jun induced apoptosis when deprived of growth factors (Hess *et al.,* 2004, Lei *et al.,* 2002). In agreement with this finding, mice expressing a c-Jun mutant at serines 63 and 73 are resistant to kainite (a powerful neurotoxin)-induced neuronal cell death of hippocampal and cortical neurons (Behrens A *et al.,* 1999). Furthermore, expression of TAM67, a dominant negative form of c-Jun, inhibits mitochondrial cytochrome *c* release and hence abrogates apoptosis (Whitfield *et al.,* 2001). This inhibition is a result of downregulation of Bim, a pro-apoptotic protein of the Bcl-2 family of apoptosis regulators (Bouillet and Strasser 2002). This JNK-Jun-Bim mitochondrial pathway has been established as a stress-responsive pathway in neuron death control. In pathology of Alzheimer's disease, Jun activation by JNK and subsequent Bim activation are associated with the apoptosis of cerebral endothelial cells (Yin *et al.,* 2002). Hence, the JNK-Jun pathway is pro-apoptotic in the stress response, at least in some cell types.

JunB also plays a part in regulating apoptosis. Cells lacking JunB expression during myelopoiesis results in activation of survival genes such as *bcl-2* and *bcl-x* and reduction of cell cycle inhibitor $p16^{INK4a}$, leading to the development of leukaemogenesis (Passegue´ *et al.,* 2001). Little is known about the role of JunD as an inducer of apoptosis.

4.4.2 Pro-apoptotic function of Fos family

The opposite function of c-Fos in FasL mediated cell death stems from the observation that c-Fos interacts with the transcriptional repressor element within the promoter region of Fas and abrogates the c-Jun mediated transcriptional activation of the *Fas* gene (Lasham *et al.,* 2000). On the contrary, FosB mediates the induction of human FasL expression in a complex with c-Jun and promotes the normal activation-induced cell death of human T cells (Baumann *et al.,* 2003). Hence the activation of the Fas-FasL pathway relies on the interaction of components of AP-1 with the promoter regions of both genes. Meanwhile, the role of c-Fos in controlling stress-induced cell death has been established. Light-induced apoptosis of retinal cells are associated with c-Fos induction (Hess *et al.,* 2004). Interestingly, the induction of apoptosis by Fra-1 expression in c-Fos deficient photoreceptor cells is believed to be achieved via suppression of anti-apoptotic genes since Fra-1 lacks a potent transactivation domain (Wenzel *et al.,* 2002). This finding provides a view of the pleiotropic regulation of apoptosis by the Fos family.

C-Fos is also pro-apoptotic in hepatocytes. Ectopic expression of c-Fos results in not only cell cycle inhibition, but also cell death in hepatocytes (Mikula *et al.,* 2003). Fos

also mediates the Myc-induced cell death in hepatoma cells in the absence of growth factors without inducing p53 (Kalra and Kumar 2004). This finding provides a mechanism under which overexpression of c-Fos might be able to suppress tumour formation induced by oncogenes. It has been reported that Fos expression inhibits the oncogenic Ras-induced anchorage-independent growth ability of hepatocytes *in vitro* and potently represses tumour formation *in vivo* (Hess *et al.,* 2004). Therefore, c-Fos, originally identified as an oncogene, can function as a tumour suppressor in different cell types or under certain conditions. In support of this notion, $Fos^{-/-}$ and $p53^{-/-}$ dual knockout mice develop aggressive rhabdomyosarcomas, a relatively rare form of cancer arising from skeletal muscle progenitors (Fleischmann *et al.,* 2003), further supporting the role of Fos as a tumour suppressor. A previous finding using *fos-LacZ* transgene as a reporter detected persistent c-Fos expression in cells undergoing terminal differentiation and at sites of naturally occurring cell death (Smeyne *et al.,* 1992, Smeyne *et al.,* 1993). Thus, c-Fos regulates cell death not only in response to stress stimuli, but also in normal development and tumour formation.

4.4.3 Pro-survival role of the Jun family

The pro-survival role of c-Jun expression stems from the observation that c-Jun antagonizes cell death induced by cytokines such as tumour necrosis factor alpha (TNFα) via the Jun N-terminal kinase (JNK) pathway in primary embryonic fibroblasts (Wisdom *et al.,* 1999). Overexpression of c-Jun rescues apoptosis in murine T-cell associated with IL-4 deprivation by directly activating the transcription of survival factor Bcl-3, a

member of the IκB family and act as a co-activator of NF-kB/Rel transcription factors (Rebollo *et al.,* 2000). In the liver system, c-Jun plays a dual role in regulating proliferation as well as survival, depending on the state of differentiation (Hess *et al.,* 2004). Accordingly, differentiated hepatocytes deficient for c-Jun expression exhibit impaired proliferation, not survival (Behrens A *et al.,* 2002). Foetal hepatocytes, however, undergo apoptosis in the absence of c-Jun expression (Eferl R *et al.,* 1999). Similar dual regulation mode of c-Jun has been reported in PC12 neuron cells. Under the condition of growth factor deprivation, c-Jun-JNK mediates apoptosis in differentiated PC12 cells, while in non-differentiated cells, c-Jun is prosurvival (Leppä *et al.,* 2001). The mechanism underlying these situations remains to be clarified. In addition to normal primary hepatocytes, c-Jun expression is believed to be critical for the development of liver tumours by antagonizing p53 activity and maintaining survival (Eferl R. *et al.,* 2003). However, the JNK pathway is not involved in this process, as demonstrated by the expression of mutant c-Jun (Jun-AA) that cannot be phosphorylated by JNK but protects liver tumour cells from apoptosis (Eferl R and Wagner 2003). Thus, c-Jun promotes cell cycle progression as well as survival in the liver system.

The anti-apoptotic function of c-Jun is also observed in keratinocytes and notochord cells. Expression of c-Jun promotes keratinocyte proliferation, survival and skin tumour formation by regulating the expression of EGFR and its ligand HB-EGF (Hess *et al.,* 2004). Different from liver tumours, JNK phosphorylation is involved in this process since expression of Jun-AA attenuates the formation of skin and bone tumour induced by activated Ras or a Fos transgene (Behrens A *et al.,* 2000).

Numerous studies have established the role of JunD as anti-apoptotic. Mouse embryonic fibroblasts (MEF) deficient of JunD expression undergo p53-dependent cell death in response to UV-irradiation (Weitzman *et al.,* 2000). Moreover, JunD$^{-/-}$mice exhibit enhanced TNF-α mediated hepatocyte apoptosis triggered by bacterial lipopolysaccharides (LPS), an inflammatory stimulus (Weitzman *et al.,* 2000). JunD was also reported to facilitate the Jun N-terminal kinase (JNK)-mediated cell survival upon TNF-α stimulation (Lamb *et al.,* 2003). In this case, the JunD-JNK pathway collaborates with NFκB to induce the expression of the anti-apoptotic *iap-2* (inhibitor of apoptosis) gene. Hence, JunD protects cells from stress-induced or cytotoxin-triggered cell death. JunB was originally recognized as a tumour suppressor. However, forced expression of JunB using a knock-in strategy rescues both liver and cardiac defects caused by apoptosis in c-Jun-null mice (Passegué *et al.,* 2002).

4.4.4 Pro-survival role of Fos family

In addition to mediating cell death in neuronal cells, c-Fos expression antagonizes the c-Jun induced FasL mediated cell death in human lymphoma cell lines (Lasham *et al.,* 2000). Moreover, mice deficient in c-Fos expression exhibit massive apoptosis during embryonic development (Roffler-Tarlov *et al.,* 1996). Furthermore, CD4/CD8 double positive thymocytes that lack c-Fos expression are hypersensitive to a variety of apoptotic stimuli such as dexamethasone and forskolin (Ivanov and Nikolić-Zugić 1997). In addition, c-Fos also protects cells against apoptosis in fibroblasts. Fos$^{-/-}$ fibroblasts are more sensitive to UV irradiation, as indicated by increased incidence of apoptosis and

prolonged cell cycle arrest (Schreiber *et al.,* 1999). Therefore, Fos expression is anti-apoptotic in response to various stress stimuli at least in the cell types mentioned above.

4.5 The role of AP-1 in tumorigenesis

AP-1 has been implicated in the regulation of multiple steps of tumorigenesis including oncogenic transformation, deregulated proliferation and apoptosis, invasive growth, metastasis and angiogenesis (Jochum *et al.,* 2001).

4.5.1 Oncogenic transformation

The viral homologue of c-Jun, *v*-Jun, is the oncogene of avian sarcoma virus 17 [ASV17. (Maki *et al.,* 1987)], and expression of *v*-Jun leads to formation of fibrosarcmoas at sites of wounding in transgenic mice carrying the *v*-Jun oncogene (Schuh *et al.,* 1990). In contrast, c-Jun does not induce tumours in young chicks and mice (Vogt PK 2001). *In vitro*, stable expression of c-Jun using the RCAS retroviral vector system is able to induce transformation and foci formation in soft agar in CEF (Wong *et al.,* 1992), suggesting that c-Jun is somewhat oncogenic *in vitro* yet less efficient than its viral counterpart. However, co-expression of c-Jun with other AP-1 member, in particular the Fos family members, promotes transformation and tumorigenesis both *in vitro* and *in vivo*. NIH3T3 cells can be transformed by co-expressing c-Jun and Fra-1 (Mechta et al., 1997), which is probably achieved by forming a potent AP-1 factor through dimerization of c-Jun and Fra-1 and acting like a single oncogenic factor. Double-transgenic mice overexpressing c-Jun and c-Fos exhibit more developed osteosarcomas with invasive potential than mice expressing Fos alone (Wang ZQ *et al.,* 1995). Furthermore, c-Jun can

facilitate transformation induced by oncogenes. Expression of c-Jun efficiently induces transformation in immortalizing primary rat embryo cells in cooperation with activated Ras (Schütte *et al.,* 1989a). CEFs or MEFs deficient for c-Jun expression or expressing the dominant negative form of c-Jun cannot be transformed by activated Ras or *v*-Src (Brown PH *et al.,* 1993, Johnson R. S. *et al.,* 1993, Lloyd *et al.,* 1991).

Ectopic expression of JunB or JunD repress the transformation induced by Ras in immortalized fibroblasts (Passegué and Wagner 2000, Pfarr *et al.,* 1994, Schütte *et al.,* 1989b), implying their roles as anti-oncogenic agents in these cells. Moreover, the anti-tumorigenesis role of JunB is further supported by the finding that cells lacking JunB expression during myelopoiesis are characterized by the activation of survival genes such as *bcl-2* and *bcl-x* and the reduction of cell cycle inhibitor $p16^{INK4a}$ (Passegue´ *et al.,* 2001). However, JunD can facilitate transformation and tumorigenesis. Johnson and colleagues have shown that c-$Jun^{-/-}$ immortalized mouse fibroblasts that cannot be transformed by activated Ras *in vitro* are still able to form tumours in mice with delayed kinetics by regaining high AP-1 activity as a result of enhanced expression of JunD, but never JunB (Johnson R. *et al.,* 1996). This finding is strong evidence that JunD can substitute for c-Jun and contribute to transformation and tumorigenesis induced by activated Ras. Collectively, the roles of Jun family members are complexed in tumorigenesis.

Like c-Jun, c-Fos also has a viral counterpart that is the transforming gene of Finkel-Biskis-Jinkins and the Finkel-Biskis-Reilly murine osteosarcoma virus (Foletta 1996). Overexpression of c-Fos alone is able to induce transformation in both chondroblasts and

osteoblasts (Grigoriadis *et al.,* 1993, Wang ZQ *et al.,* 1991). However, c-Fos appears to be dispensable in the action of *v*-Src, *v*-Raf and Ha-*ras* since these oncoproteins can induce transformation in both wild type and c-Fos deficient fibroblasts (Hu E. *et al.,* 1994). In addition, FosB expression is oncogenic *in vitro* upon activation via phosphorylation of serine residues in the transactivation domain at the C-terminus of the protein (Skinner *et al.,* 1997). In contrast, FosB expression is not transforming *in vivo* since transgenic mice expressing FosB do not develop tumours in bone tissue (Grigoriadis *et al.,* 1993). In addition, lack of a strong transactivation domain in Fra-1 and Fra-2 proteins accounts for their relatively weaker transforming ability. As a result, Fra-1 overexpressing does not cause transformation in fibroblast despite the induction of anchorage-independent growth, indicating that Fra-1 might be involved in other aspects of tumorigenesis. Overexpression of Fra-2 is transforming in chicken embryonic fibroblasts (CEF), but not in rat fibroblasts (Foletta 1996). Moreover, Murakami and colleagues have demonstrated that v-Src transformation leads to enhanced kinase activity of ERK2 and hyperphosphrylation of several Ser and Thr residues in the c-terminal region of Fra-2 mediated by ERK2 (Murakami *et al.,* 1997). This phosphorylation results in not only the transactivation activity of Fra-2, but elevated protein levels via two AP-1 binding sites within the Fra-2 promoter region. The same study also revealed the presence of two AP-1 binding sites within the promoter region of Fra-2 that might contribute to the elevation of Fra-2 transcripts through positive autoregulation (Murakami *et al.,* 1997). In agreement with this notion, overexpressing JunD leads to an increase in Fra-2 expression in CEF (Gagliardi *et al.,* 2003).

4.5.2 Actions of AP-1 in aggressive tumour phenotypes

AP-1 activity is required for development of aggressive tumour phenotypes. Inhibition of AP-1 activity by a dominant negative form of c-Jun leads to suppression of invasive growth of various tumour cells including murine and human squamous cell carcinomas and rat fibroblasts (Eferl R and Wagner 2003).

The induction of invasive growth of *v*-Fos-transformed rat fibroblasts is correlated with the upregulation of invasive growth related genes including CD44, cathepsin L, Mts and Krp1 (Jochum *et al.,* 2001), suggesting that the *v*-Fos promotes invasiveness in tumour progression. Expression of c-Fos in mammary epithelial cells induces the loss of cell polarization and invasion into collagen gels that resemble the epithelial-mesenchymal transition (EMT) during tumorigenesis, suggesting a potential function of c-Fos is modulating the invasive events of epithelial tumours (Reichmann *et al.,* 1992). Expression of c-Jun, on the other hand, only causes loss of polarization in the same cells (Fiaka, 1996). Furthermore, the role of c-Fos in promoting invasive growth is evident in the observation that mice deficient for c-Fos expression impairs the progression of papillomas to invasive Squamous cell carcinomas (Jochum *et al.,* 2001). The regulation of invasiveness is associated with the loss of cell- extracellular matrix (ECM) since loss of c-Fos expression downregulates the expression of several members of the matrix metalloproteinase (MMP) family of endopeptidases (Saez *et al.,* 1995, Werb 1997). Like c-Fos, expression of Fra-1 promotes motility and invasiveness in mammary adenocarcninoma cell lines (Kustikova *et al.,* 1998), implying a role for Fra-1 in aggressive tumour phenotype.

Comparison of the gene expression pattern between fibroblasts derived from c-Fos deficient mice and that from wild type mice revealed that the expression of vascular endothelial growth factor (VEGF) is downregulated in the absence of c-Fos (Orlandini *et al.,* 1996), indicating that VEGF is a c-fos-responsive gene. In addition to invasiveness, this finding immediately links AP-1 to angiogenesis, another feature of an aggressive tumour phenotype.

4.6 Interaction of AP-1 with other transcription factor families on SRU

Activation of *IL8* relies on the Src-responsive unit (SRU) of its promoter containing binding sites for AP-1, NFκB and C/EBPβ, all of which are known to be activated by *v*-Src in CEF (Cabannes *et al.,* 1997, Dehbi *et al.,* 1992, Gagliardi *et al.,* 2001). C/EBPβ is essential for the activation of the *IL-8* gene since mutation of the C/EBPβ binding sites leads to the disruption of *IL-8* transcription, (Gagliardi *et al.,* 2001). However, although overexpression of a dominant negative form of C/EBPβ, Δ184-C/EBPβ, decreases the accumulation of *IL-8* mRNA in *v*-Src transformed CEF, it does not interfere with transformation (Gagliardi *et al.,* 2001), suggesting that C/EBPβ activity is only required for *v*-Src induced gene expression but not transformation *in vitro*. In normal CEF, overexpression of Δ184-C/EBPβ induces the expression of three major AP- members (c-Jun, JunD and Fra2), enhances the expression of cyclinD1 and stimulates proliferation (Gagliardi *et al.,* 2003), indicating that AP-1 and C/EBPβ act antagonistically in the control of cell proliferation (Gagliardi *et al.,* 2003). Nullizygous C/EBPβ (-/-) mouse embryonic fibroblasts (MEF) also proliferate more rapidly than their C/EBPβ (+/+)

proficient counterparts, indicating that the role of C/EBPβ in the proliferation of primary fibroblasts is conserved (Gagliardi *et al.,* 2003). As shown in this thesis, the interplay between AP-1 and C/EBPβ is also important for the control of survival in *v*-Src transformed CEF (Chapter 3 and Appendix 1).

4.7 Structure and regulation of C/EBPβ

The CCAAT/enhancer-binding proteins (C/EBPs) are a family of leucine-zipper- and basic DNA binding domain transcription factors that regulate various cellular activities including proliferation, differentiation, and inflammation (Zahnow 2009). There are 6 members in this family, designated according to the chronological order of their discovery: C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε, C/EBPζ (also known as CHOP10, C/EBP homologous protein10) and Gadd153. In rodent and human, C/EBPα, C/EBPβ, C/EBPγ and C/EBPδ are encoded by intronless genes while C/EBPε and C/EBPζ are encoded by genes with introns (Zahnow 2009). C/EBPγ, C/EBPδ and C/EBPζ are translated as a single protein, while C/EBPα, C/EBPβ and C/EBPε have several isoforms (Zahnow 2009). C/EBPα is expressed as two isoforms (p42 and p30 in rodent) and plays a role of tumour suppressor in many cell types (Koschmieder *et al.,* 2009). C/EBPε has four isoforms (p32, p30, p27 and p14 in rodent) and is believed to be the result of alternative splicing and differential promoter usage (Yamanaka *et al.,* 1997). Regulated by differential use of in-frame AUG, C/EBPβ is expressed as three isoforms: LAP1 (38 kDa), LAP2 (34 kDa) and LIP (20 kDa) in rodents, whereas they are renamed as C/EBPβ1 (44 kDa), C/EBPβ2 (42 kDa) and C/EBPβ3 (20 kDa) in humans (Zahnow

2009). Full length C/EBPβ (LAP1) consists of an N-terminal transactivation domain (AD), a regulatory domain (RD) and the bZIP domain that contains a basic region for DNA binding and a leucine zipper for dimerization (Fig.4). LAP2 differs from LAP1 by lacking the N-terminal 21 amino acids as a result of alternative in-frame translation initiation, a modification that is important in the activity of these proteins. This N-terminal region is required for the recruitment of the SWI-SNF complex, whose function is to activate gene transcription via chromatin remodelling that increases access of transcription factors to the promoter region of the target gene (Kowenz-Leutz and Leutz 1999). Specifically, the cysteine 11 in this region of rodent LAP1 can form an intramolecular disulfide bridge with Cys33, leading to inhibition of the association with the SWI-SNF complex (Su *et al.,* 2003). Hence, differential structure on this region will lead to distinct regulatory mechanisms and functions. LIP lacks the entire transactivation region (AD1, AD2 and AD3), and acts as a dominant negative form to inhibit the transcriptional activity of C/EBPs by competing for the DNA binding sites or forming an inactive dimers with other C/EBPs (Fig.4). Hence, the LIP:LAP ratio is important for the transactivation potential of C/EBPβ and the activation of gene expression. Moreover, LIP can also function as a transcription activator when interacting with transcription factors. For example, interaction between the Rel-homology domain of NFκB and the leucine-zipper region of LIP forms a transcription activator on the IL-6 promoter in B lymphoblasts (Stein *et al.,* 1993a).

The regulatory domain 1 (RD1) is capable of interfering with the transactivation potential of C/EBPβ by forming a close conformation with the AD domain, whereas RD2

is capable of interfering with the bZIP domain to inhibit DNA binding of C/EBPβ (Williams *et al.,* 1995). These processes are inhibited by direct phosphorylation of both RD1 and RD2 via the Ras-MAPK pathway (Williams *et al.,* 1995), which in turns leads to the full activation of C/EBPβ. Activation of C/EBPβ is primarily regulated at the post-translational level. In addition, C/EBPβ can also be activated by acetylation within the AD domains, decreasing acetylation in this area abolishes the transactivation potential of C/EBPβ (Ceseña *et al.,* 2008).

There are two major post-translational modifications that negatively regulate the activation of C/EBPβ: methylation and sumoylation (Zahnow 2009). Methylation of Lys 39 by histone lysine N-methyltransferase has been identified as the mechanism repressing the transactivation potential of C/EBPβ (Pless *et al.,* 2008). This residue, however, also serves as a target for acetylation and the subsequent activation of C/EBPβ (Ceseña *et al.,* 2008). It is also conserved in mouse, rat and chicken C/EBPβ (Zahnow 2009), suggesting that modification on the Lys39 is a common mechanism in the regulation of C/EBPβ activity. A previous study demonstrated that the activity of C/EBPβ is also regulated by sumoylation, a reversible post-translational modification that often leads to decreased transactivation potential of transcription factors (Kim J *et al.,* 2002). Covalent interaction of the SUMO protein, a small ubiquitin-like modifier, to the RD1 of LAP1 impairs its ability to activate the transcription of cyclin D1 (Eaton and Sealy 2003, Melchior 2000).

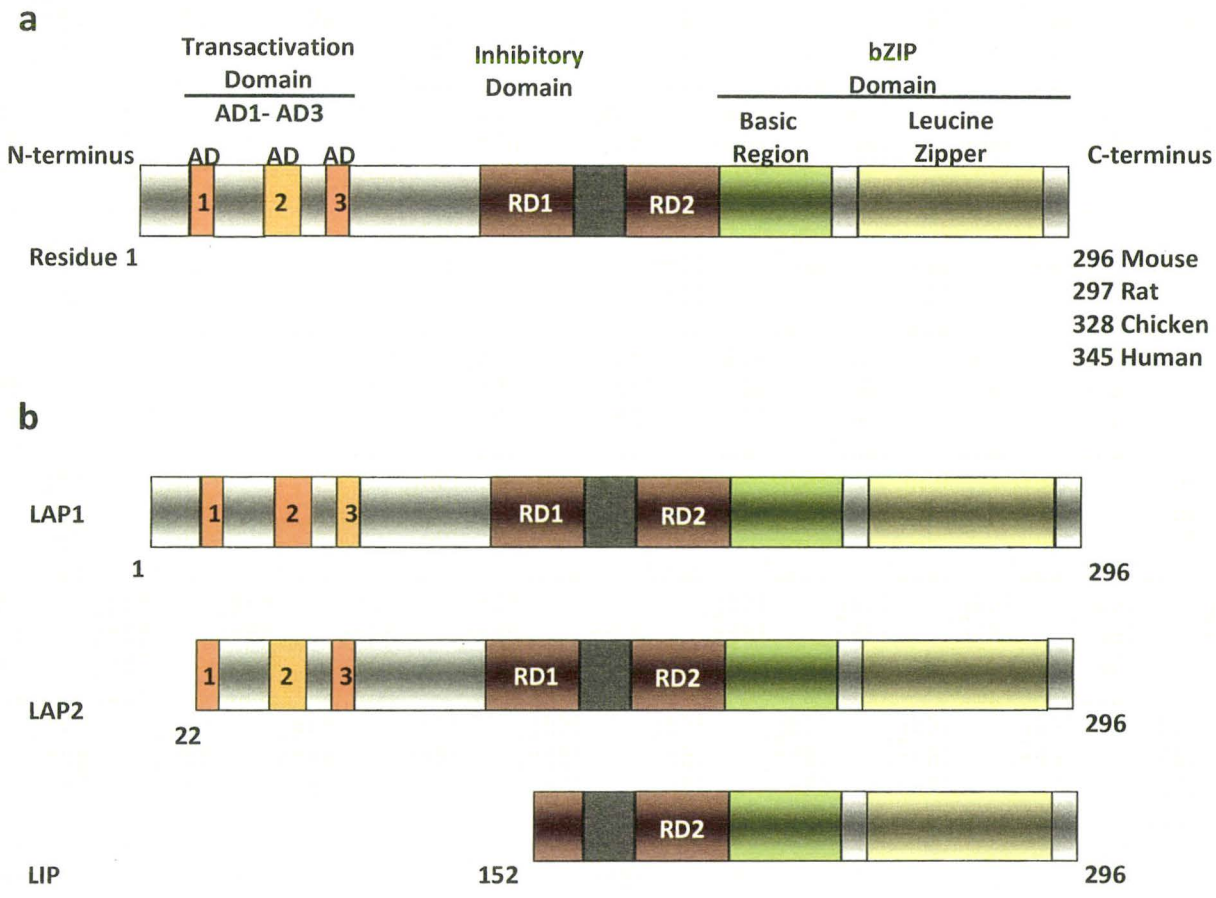


Figure 4. Structure of C/EBPβ and its isoforms. The structures of C/EBPβ and its isoforms are represented. **a)** Full length of C/EBPβ consists of the transactivation domain (AD), the regulatory domains (RD1 and RD2) and the bZIP domain (basic domain and leucine zipper); **b)** Different isoforms are generated through alternative usage of in-frame translational start sites.

4.8 Actions of C/EBPβ

Several investigations using C/EBPβ knockout mouse models have suggested a crucial role for C/EBPβ in the control of energy metabolism and differentiation. For example, C/EBPβ$^{-/-}$ mice exhibit impaired metabolism homeostasis in the liver and adipocytes and reduced proliferation and regeneration in the liver (Croniger *et al.,* 2001, Greenbaum *et al.,* 1998). In normal CEF, overexpression of C/EBPβ produced an anti-proliferative effect accompanied with reduced expression of AP-1 proteins (Gagliardi *et al.,* 2003). Moreover, MEF nullizygous for the C/EBPβ gene also proliferate more rapidly than their normal counterparts (Gagliardi *et al.,* 2003, Johnson PF 2005). These findings suggest that the function of C/EBPβ is cell type dependent.

4.7.1 C/EBPβ in survival, apoptosis and senescence

C/EBPβ has been implicated in oncogene-induced senescence (OIS). Two recent reports demonstrated that C/EBPβ-mediated induction of inflammatory cytokines or chemokines in response to oncogenes, in particular IL6 and IL8, generate DNA damage and contributes to OIS (Acosta *et al.,* 2008b, Kuilman *et al.,* 2008). Moreover, C/EBPβ activates the expression of death-associated protein kinase 1 (DAPK1), a protein critical in regulating apoptosis, by binding to the promoter region and thus contributes to the induction of apoptosis upon interferon gamma (IFN-γ) treatment in mouse MEF (Gade *et al.,* 2008).

In contrast, C/EBPβ is also involved in the promotion of cell survival in some cell types. For instance, Chojkier's group showed that phosphorylation of C/EBPβ on Thr^{217}

by activated RSK is essential for the survival of hepatocytes upon hepatotoxin CCl4 treatment (Buck *et al.,* 2001). The phosphorylation event creates a site of interaction for procaspase 1 and 8, resulting in the inhibition of their processing and subsequent induction of the apoptosis cascade. This finding indicates that C/EBPβ is a survival factor in hepatocytes. Moreover, C/EBPβ is also required for the survival of keratinocytes in response to the carcinogen DMBA treatment, likely through the Ras-ERK pathway and the phosphorylation of C/EBPβ on Tyr 188 by ERK (Zhu *et al.,* 2002). Further study revealed that this effect is also associated with the inhibition of p53 induction by C/EBPβ in response to DNA damage (Yoon *et al.,* 2007). In summary, C/EBPβ promotes cell survival in some cells while it induces cell cycle arrest or apoptosis in others, suggesting that the function of C/EBPβ is cell type and stimulus-dependent.

4.7.2 C/EBPβ and receptor tyrosine kinases

The EGFR receptor tyrosine kinase family comprises of 4 members: ErbB1/EGFR, ErbB2, ErbB3 and ErbB4. All four members are found frequently overexpressed in breast tumours and play a role in tumour progression (Zahnow 2009). Initiation of ErbB1/EGFR signaling upon extracellular ligand binding leads to increased LIP expression in cultured mammary epithelial cells and in transgenic mice (Baldwin BR *et al.,* 2004). This activation process involves the activation of the RNA-binding protein CUG-binding protein 1 (CUG-BP1) and its enhanced binding to the C/EBPβ mRNA (Baldwin BR *et al.,* 2004). Increased LIP expression results in increased LIP:LAP ratio, decrease C/EBPβ activity contributing to the proliferation and the development of a more aggressive

cancerous phenotype in these cells.

The FGFR family contains 4 members: FGFR1, FGFR2, FGFR3 and FGFR4, and 22 ligands have been found to bind and activate various receptor isoforms (Zahnow 2009). Aberrant expression of FGFR has been associated with hyperplastic growth and neoplasia development in mouse models (Tsukamoto *et al.,* 1988, Welm *et al.,* 2002). Specifically, activation of the FGFR2 locus was recently found to be involved in the development of breast cancer (Hunter *et al.,* 2007). LAP has been found to bind to the promoter region of *FGFR2* in a complex with Oct1/Runx2 and to increase the expression of FGFR2 (Meyer *et al.,* 2008). Interaction of LAP with the promoter region of the FGF-binding protein 1 (FGFBP1), a secreted carrier protein that delivers activated FGF to its receptor, is another means of promoting the FGF signaling upon EGF stimulation (Kagan *et al.,* 2003). On the contrary, expression of LIP inhibits the expression of FGFBP1 and blocks FGF signaling (Kagan *et al.,* 2003). However, how FGR regulates the LIP:LAP ratio in different cell context remains largely unknown. Moreover, how FGF signals to C/EBPβ is also unclear. Therefore, the action of C/EBPβ in the promotion or inhibition of mammary carcinomas may depend on the receptor tyrosine kinases expressed in these tumours.

4.7.3 Action of C/EBPβ in differentiation

Many CEBPs play a role in cell differentiation events. The involvement of the C/EBP family in the development of adipocytes has been studied extensively. Transcription of C/EBPβ and δ is first activated in cultured preadipocytic cell lines upon differentiation

induction (Cao Z *et al.,* 1991, Yeh *et al.,* 1995). It has been demonstrated that C/EBPβ is a potent inducer of adipogenesis since overexpression of C/EBPβ is sufficient to initiate the differentiation of 3T3-L1 cells even in the absence of hormonal inducer (Rosen *et al.,* 2000). Forced expression of C/EBPδ is able to accelerate the differentiation process in 3T3-L1 cells only in the presence of dexamethasone (DEX), a pro-differentiation agent that potently induces terminal differentiation of 3T3-L1 (Yeh *et al.,* 1995), indicating that C/EBPδ is a relatively weaker inducer of adipogenesis. In addition, mouse embryonic fibroblasts deficient for either C/EBPβ or δ expression show only modest impairment of cytoplasmic lipid accumulation, a sign of adipogenic conversion, whereas cells lacking both proteins were nonreactive to the pro-differentiation agents dexamethasone (DEX) and methylisobutylxanthine [MIX;(Tanaka *et al.,* 1997)], suggesting the presence of functional redundancy between these two proteins in fibroblasts. *In vivo* studies using mouse models show the same effect as in fibroblasts. Mice deficient for either one of the proteins develop normal white adipose tissues (WAT), while the development of brown adipose tissues (BAT) is slightly affected in accumulation of lipids (Tanaka *et al.,* 1997). However, the absence of both proteins leads to embryonic lethality in 85% of the experimental mice while survivors display abnormal WAT & BAT (Tanaka *et al.,* 1997). Thus, C/EBPβ and δ are critical for adipogenesis. C/EBPα, on the other hand, is induced by C/EBPβ and δ in the differentiation process, yet remains expressed until the formation of fat cells (Rosen *et al.,* 2000). Overexpression of C/EBPα in 3T3-L1 preadipocytes promotes the differentiation into mature fat cells (Lin FT and Lane 1994), whereas the expression of C/EBPα antisense RNA in these cells suppresses the differentiation process

(Lin FT and Lane 1992). C/EBPζ functions as a negative regulator of adipogenesis, whose expression is suppressed during the induction of differentiation, but returns when differentiation is almost completed (Darlington *et al.,* 1998). This protein is thought to act as an attenuator of the adipogenic program when the differentiation specific events are completed. The regulatory network of adipogenesis initiated by C/EBPβ and δ, induces the subsequent activation of the nuclear hormone receptor PPARγ via the C/EBP binding site within the PPARγ promoter region (Fajas *et al.,* 1999), PPARγ then activates C/EBPα. The activation of C/EBPα forms a positive feedback with PPARγ to maintain the active state and promotes the progression to adipogenesis (Rosen *et al.,* 2000). This relationship is evident in embryonic fibroblasts or embryonic stem cells derived from PPARγ-null mice, which fail to differentiate into adipocytes in the presence of a prodifferentiative regimen (Kubota *et al.,* 1999, Rosen *et al.,* 1999). Furthermore, C/EBPα expression is reduced in these cells despite normal levels of C/EBPβ and δ. Moreover, fibroblasts derived from C/EBPα$^{-/-}$ embryos exhibit reduced levels of PPARγ and are not able to differentiate into adipocytes when exposed to hormonal stimuli (Wu *et al.,* 1999). Hence, a positive feedback loop between PPARγ and C/EBPα is essential for successful adipogenesis.

5. The Rous sarcoma virus

5.1 RSV as a tool for studying cellular transformation

Introduction: The first demonstration of an oncogenic virus came from the discovery by Ellerman and Bang (1908) in Copenhagen that showed that a sub-bacterial filtrate

could transmit leukemia in chicken. However, the significance of this finding was unclear at the time because leukemia was not recognized then as a disease of cell proliferation, i.e. a form of cancer (Martin G. S. 2001). In 1911, Peyton Rous observed that a filtered extract from the tumour of sick chicks was able to cause sarcoma (tumours in connective tissues) in healthy chicks simply by muscle injection. The causative agent was smaller than bacteria and this was identified as an oncogenic virus, and named the Rous sarcoma virus [RSV;(Martin G. S. 2004)]. This finding suggested that viruses are capable of inducing cancers. The gene causing transformation and tumorigenesis of RSV, *v*-Src, was eventually identified and sequenced (Bernstein 1976, Martin GS 1970). The v-Src oncogene-containing RSV thus became valuable in the study of cell transformation, signaling and cancer.

RSV derivatives including the RCASBP vector system and temperature inducible v-Src mutants are widely used currently. The RSV-derived RCAS vectors are widely used to stably and efficiently express genes of interest in many host systems including chicken and mouse. The name RCAS is an abbreviation of Replication Competent ALV LTR with a Splice acceptor (Hughes SH 2004). RSV and ALV (avian leukosis virus) all belong to the avian sarcoma-leukosis virus (ASLV) family. ALV is genetically related to RSV and differs in structure by the fact that ALV doesn't contain an oncogene. As a result, ALV infection is not acutely transforming in CEF. RSV is believed to have originated from ALV upon infection, it acquired the cellular Src gene without losing any of the genes of the parental ALV genome (Hughes SH 2004, Swanstrom *et al.,* 1983). Therefore, RSV is a rare example of acutely transforming and replication-competent

retrovirus. The development and application of the RSV mutants as well as the RCAS system are discussed below.

5.1.1 The life cycle of retroviruses

The life cycle of a retrovirus can be summarized into three steps: entry, reverse transcription and integration, and expression assembly (Fig.5A). A typical replication-competent virus genome contains a *gag* gene encoding capsid proteins, *pol* gene for reverse transcriptase, RNase H and integrase activity, and *env* gene for envelope glycoprotein. The infection of the retrovirus is mediated by the interaction of the envelope glycoprotein with a receptor on the host surface (Hughes SH 2004). Retroviral envelop proteins are mostly trimers and each monomer of the trimer contains a surface subunit (SU) and a transmembrane subunit (TM). SU is required for binding with the host receptor to trigger the rearrangement of TM, which leads to fusion of the viral membrane with the host membrane resulting in the introduction of the viral core into the cytoplasm. There are two requirements for entry: membrane fusion and low-pH vesicles. Infection of human immunodeficiency virus (HIV) only requires receptor binding since low pH does not play a role in membrane fusion (Hughes SH 2004). Unlike HIV, low-pH is required for both receptor binding and TM rearrangement in infection by ALV, RSV and RCAS vectors (Melikyan *et al.,* 2004, Mothes *et al.,* 2000, Smith JG *et al.,* 2004). Reverse transcription and integration are two major events of the life cycle of retroviruses. After the viral core enters the host cytoplasm, the viral single-stranded RNA genome is then reverse-transcribed into double stranded DNA by the viral reverse

transcriptase and RNaseH encoded by the *pol* gene. The DNA form of viral genome then shuttles through the host nuclear membrane and eventually inserts into the host genome with the help of integrase. Nuclear membrane dissolution at mitosis is also required for the integration of the ALV/ASV genome into the host chromosome. Once integrated into the host genome, the viral genome is referred to as a provirus and is expressed like any other gene of the host genome. The unspliced virus genomic RNA is then packaged by retroviral proteins and part of the plasma membrane, and an infectious viral particle is formed. The newly formed virus then can infect the adjacent cells and start another life cycle. The integration process is essentially random with the viral genome inserting randomly into intra- and intergenic regions (Hughes SH 2004). It is then reasonable to assume that an insertion event may either enhance the expression of an oncogene or disrupt a tumour suppressor gene. In fact this acquired "side-effect" of retroviruses has been reported and has raised great concerns in gene therapy approaches commonly using retroviral vectors (McCormack and Rabbitts 2004). Insertion of a viral genome can also lead to the acquisition of a cellular gene (Hughes SH 2004). Most of the cellular gene-containing retroviruses are replication-defective because the acquisition events are accompanied by the loss of viral genes. The Rous sarcoma virus is an exception by the fact that it bears an oncogene and is replication-competent. It is therefore a powerful tool in the study of cell transformation and the biology of retroviruses.

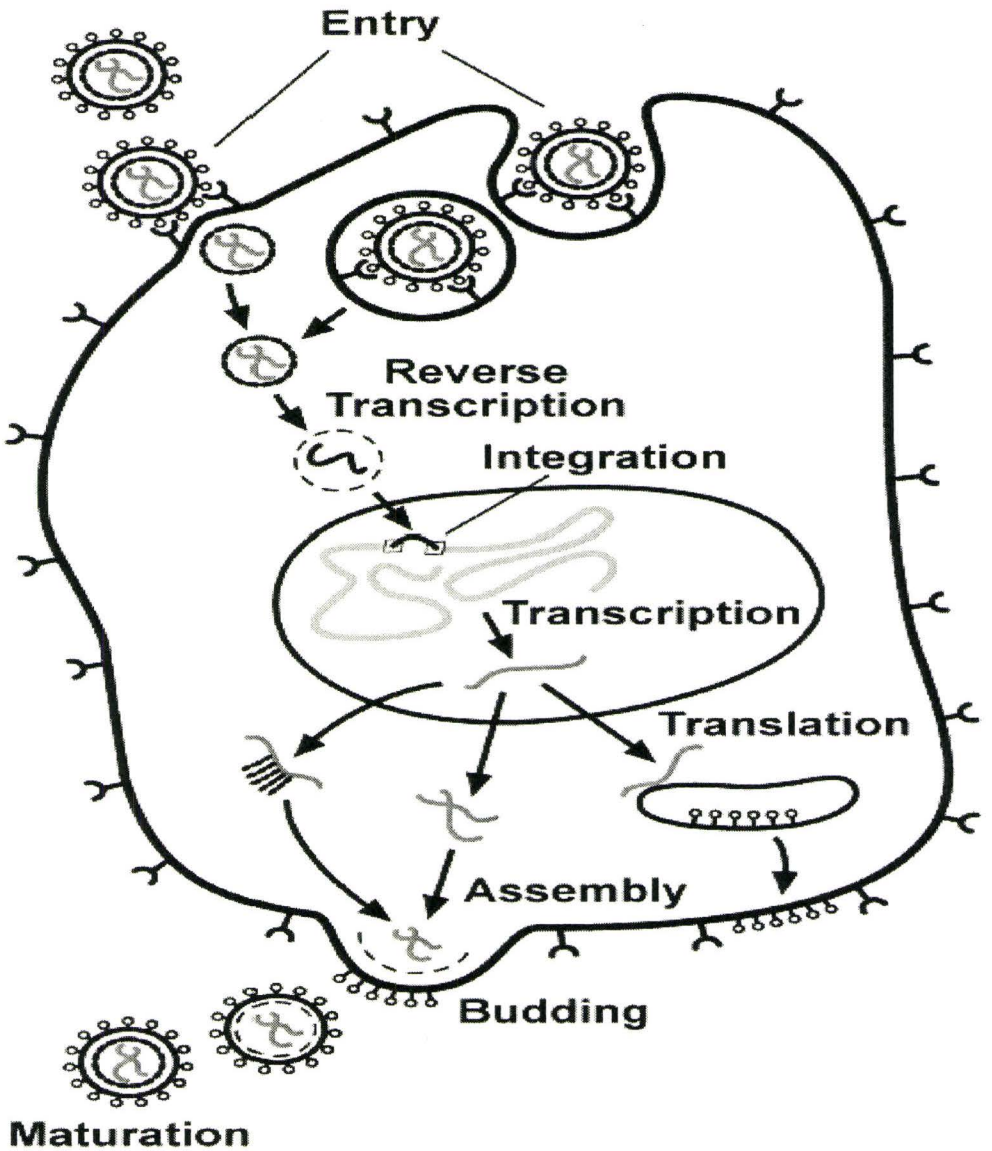


Figure 5A. The life cycle of a retrovirus. This figure shows a simplified diagram of the life cycle of a retrovirus. Two modes of entry are shown including the membrane fusion and low-pH dependent vesicles engulfment. The ASLV and RCAS viruses use the low-pH dependent vesicles route to introduce the viral core into the cytoplasm of the host cell. Reverse transcription and integration into the host genome are two major events of the life cycle. Successful assembly of the viral particles leads to infection of adjacent cells and results in viral gene expression in the whole cell population. *Adapted from: Hughes SH (2004), 'The RCAS vector system', Folia Biologica (Praha), 50, 107-19.*

5.1.2 RSVs bearing v-Src mutants

The Rous sarcoma virus (RSV) of the ASLV family contains the v-Src oncogene and possesses replicative ability. Schmidt-Ruppin A (SR-A) RSV is a wild type strain encoding a constitutively active *v*-Src kinase capable of transforming CEF at either 41.5°C or 37°C. Several mutants encoding a conditionally-active *v*-Src protein are available for studies of cell transformation. A temperature-sensitive (ts) strain of RSV, tsNY72-4, is a group A Rous sarcoma virus (RSV) variant bearing two specific alterations within the C-terminal kinase domain of *v*-Src: valine-461 to methionine and proline-503 to serine (Garber *et al.,* 1987). Another thermolabile strain tsLA90, which belongs to the group B Rous sarcoma virus (RSV), has 4 mutated amino acids in the *v*-Src protein compared to that of a wild type SR-A RSV (Maroney *et al.,* 1992). These two types of ts strains, currently used in our lab, are transformation-deficient at 41.5°C and transforming at 35.5°C or 37°C. In addition to these conditionally-active strains of RSV, a transformation-defective mutant, NY315, was also used in our lab as a negative control for SRA in the study of transformation-relative gene expression (Maślikowski *et al.,* 2010). The NY315 virus is not able to anchor to the inner plasma membrane of infected cells since the amino acids 2 to 15 in the N-terminus are substituted by a tripeptide Asp-Leu-Gly, a mutation which was previously generated to study the membrane association of RSV (Cross *et al.,* 1984).

5.1.3 The RCAS system

Derived from ASLV, RCAS viruses are replication-competent and capable of

expressing a gene of interest in various cell types expressing the appropriate receptors. In the RCAS system, the v-Src gene is replaced by a single *ClaI* site for cloning purposes (Hughes SH 2004). In addition to the introduction of a cloning site, some modifications on the original RSV system have been made in order to obtain more stable expression. Firstly, one of the direct repeats (DRs) has been removed from the RSV genome (Fig.5B). In the RSV genome, two copies of DRs are lying upstream and downstream of the v-Src gene. This organization may result in the loss of the v-Src gene during viral replication since the reverse transcriptase can skip the 3'DR and jump to the 5'DR (Hughes SH 2004). To prevent the loss of the inserted gene, the 5'DR was removed. While the DR sequence is critical in virion packaging and replication, a mutant virus with a single copy of DR remains replication-competent (Sorge *et al.,* 1983). Therefore, retaining one copy of DR in RCAS still allows the vector to express and replicates properly while preventing the loss of the inserted gene.

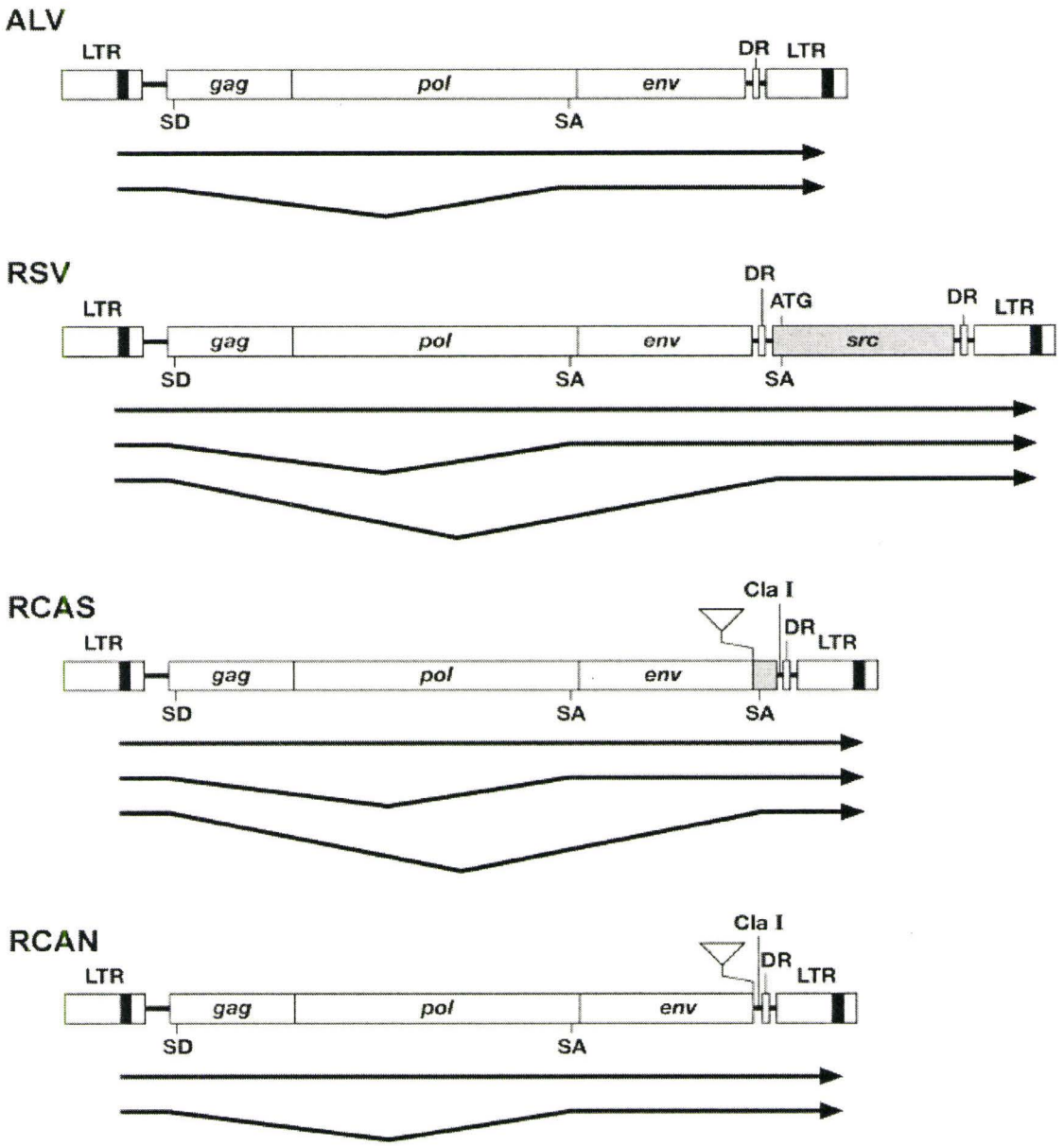


Figure 5B. Genome structure of ALV and its derivative RCAS system. In the RCAS system, the v-Src gene is replaced by a single ClaI site for cloning. Other modifications such as removal of the upstream DR (direct repeat) and retaining of the SA (splice acceptor) of the v-Src gene are also indicated in the diagram. These modifications are valuable for the use of the RCAS system in order to obtain successful and stable expression of inserted genes. In the RCAS system, the transgene is expressed as a sub-genomic transcript generated by splicing. *Adapted from: Hughes SH (2004), 'The RCAS vector system', Folia Biologica (Praha), 50, 107-19.*

Adaptor plasmids were also designed with multiple cloning sites for convenient cloning within flanking *ClaI* sites, allowing convenient excision of the insert (Hughes SH *et al.,* 1987). These modifications allow a gene of interest to be expressed as part of the viral genome. In order to achieve successful and stable expression of the inserted gene, factors that would hinder the stable expression of the gene deserve extra consideration. If the inserted gene encodes a protein that interferes with expression of the viral genome, or is toxic to cell growth, adequate expression might not be achieved since viral assembly could be blocked. Attempts to express an inhibitory gene of cell proliferation often results in deletion of the inserted gene during viral replication [Our unpublished observations and (Hughes SH 2004)]. In this case, high transfection efficiency is extremely important in order to obtain infection in the entire culture in a short time (Hughes SH 2004). In addition, large genes also affect the function of the vector indicating size limit to viral genome being packaged into the virion (Hughes SH 2004). To solve this issue, a RCAS derivative suitable for caring large inserts (over 4.5kb), BBAN, has been prepared. The ASLV family contains various envelope subtypes from A to J. The cell surface receptor for type A envelope is designated 'tva', a protein with unknown function while structurally related to ligand-binding repeat of the low density lipoprotein receptor (LDLR) super family that mediates the endocytosis of cholesterol-rich low density lipoprotein [LDL;(Bates *et al.,* 1993)]. Type B, D and E all shared the same receptor, 'tvb', which belongs to the fas ligand receptor family (Brojatsch *et al.,* 1996). The receptor for type C has not been identified and type C ASLV vector system has yet to be established. Among all these subtypes, A to E are commonly used in the laboratory

(Hughes SH 2004). The RCAS system is then categorized into 4 types: RCAS (A), RCAS (B), RCAS (D) and RCAS (E). These different types of RCAS vectors express different envelope glycoproteins allowing the simultaneous infection of a particular cell with over 2 types of vectors carrying different genes of interest. This application alleviates the problem of viral interference: when an infected cell is expressing the viral envelope glycoprotein, the expression of cell surface receptor is blocked and the infection of virus with the same type of envelope is prevented. The widely used RCAS vectors are the RCASBPs, where the original *pol* gene is substituted by Bryan polymerase (BP) from the Bryan high-titre strain of RSV (Hughes SH 2004). The replacement enhances the long terminal repeat (LTR) promoter activity by a mechanism that is poorly understood, leading to a higher viral titre (Petropoulos and Hughes 1991). RCASBPs are categorized into 4 genetic types according to the *env* gene: RCASBP-A, B, D and E. Other RCASBP vector derivatives are also available, including vectors that are capable of infecting mammalian cells (Hughes S Webpage).

6. Rationale of the study and organization of the thesis

Several studies have implicated AP-1 in cell transformation by *v*-Src (Bojovic *et al.*, 1996, Dehbi *et al.*, 1992, Lloyd *et al.*, 1991). Inhibition of AP-1 activity blocks the transformation induced by v-Src (Lloyd *et al.*, 1991), suggesting that AP-1 is required for *v*-Src-dependent transformation. AP-1 is also required for induction of the IL-8 gene in *v*-Src transformed CEF, a gene induced in the "aggressive tumour cell signature" defined recently in our laboratory (Maslikowski *et al.*, 2010). In this work, we employ different

approaches to investigate the activity and/or expression of the AP-1 factors and define the role of these factors in CEF transformation by *v*-Src. We provide evidence that AP-1 facilitates transformation by several processes including inhibition of senescence and differentiation, and acts as a survival factor in *v*-Src transformed CEF. The thesis are organized as follows (unless specify, the author fully contributes to the work from Chapter 3 to Appendix 1):

Chapter 2: Materials and methods that used in these studies.

Chapter 3: Studies on the role of AP-1 in *v*-Src transformation CEF by overexpressing a dominant negative form of c-Jun, TAM67 revealed that AP-1 is required for many aspects of v-Src transformation. Non-specific regulation of the activities of NFκB and C/EBPβ suggested that the function of TAM67 is pleiotropic, which prompted us to utilize the RNA interference technique to down-regulate individual AP-1 members.

Chapter 4: Modification of an existing shRNA expressing vector leading to stable inhibition of gene expression. The NFκB1 p105 is used as a model to characterize this vector.

Chapter 5: Investigations on the role of AP-1 in *v*-Src transformed CEF by downregulating the expression of individual AP-1 members using the shRNA vector described in Chapter 4.

Chapter 6: Discussion and perspectives.

Appendix 1: Preliminary data on the mechanism of the induction of apoptosis by *junD* shRNA.

Appendix 2: Manuscript based on the findings described in chapters 3 and 5.

Chapter 2: Materials and Methods

1. Cell Culture and virus infection: The chicken embryo fibroblasts were isolated from day 10 chicken embryos. Eearly passages (less than 13th passage) of chicken Embryo Fibroblasts (CEF) were cultured at 41.5°C in DMEM medium supplemented with 5% heat-inactivated newborn bovine serum (Cosmic calf serum, Hyclone), 5% Tryptose Phosphate Broth (TPB), and 1% Penicillin, Streptomycin, and L-Glutamine (Pen-Strep-Glut) (Gibco-BRL Lifesciences Technology). CEF were infected with the non-transforming RSV mutant strain New York-315 (NY315), or with the wild type transforming RSV strain Schmidt-Ruppin A (SR-A), or with the temperature sensitive (ts) RSV strains New York 72-4 (NY72-4) or Los Angeles 90 (LA90). LA90 and NY72-4 infected CEF were cultured at the non-permissive temperature of 41.5°C for non-transformed samples and were shifted to the permissive temperature of 37°C in order to activate v-Src transformation as required. All the experiments are conducted using early passage CEF. The CEFs can be completely infected after 1 passage of culture (Refer to Fig.13 in Chapter 4. EGFP expression was completely abolished by a virus expressing *egfp* shRNA).

2. Generation of retroviral vectors for shRNA expression: Synthetic microRNA hairpins (miRNA) have been used widely for RNA interference. MicroRNA have been shown to be more effective than small interfering RNA duplexes (Gregory *et al.,* 2005, Schwarz *et al.,* 2003, Siolas *et al.,* 2005, Zeng Y. and Cullen 2005). Raman and colleagues have developed a retroviral-based shRNA system for chicken cell using a

microRNA operon driven by the chicken U6 promoter and designated RCASARNAi and RCASBRNAi, which has been used to successfully silence up to 90% of the targeted gene's expression (Das *et al.,* 2006). We adapted this system and removed the U6 promoter from the microRNA operon. The chicken pri-miRNA operon was amplified and sub-cloned to delete the chicken U6 promoter and leader sequence in the transfer plasmid pRFPRNAiC (Das *et al.,* 2006). This was completed with forward (5' atactagcggccgcataaagt 3') and reverse oligos (5' ggatccatcgataaaaaagct 3') bordering the pri-miRNA operon. The resulting transfer plasmid was then designated as pRFPRNAi(-), and corresponding RCASRNAi vector is designated as RCASBP(A)-shRNA-ΔU6 and RCASBP(B)-shRNA-ΔU6 We selected several targets for components of the chicken AP-1 factor or NFκB1 p105 sub-unit including nt842-863 of c-jun, 821-842 of jun-d, 409-422 of fra-2 and 608-629 of p105 nfk-kb1 (Table I). These target sequences were subcloned into the modified microRNA operon on the transfer plasmid and later on into the RCAS vectors following the original supplier's instructions (ARK Genomics; (Das *et al.,* 2006)). All the constructs were designated using the number of the first nucleotide of the targeted sequence with a "minus" sign to indicate the removal of U6 promoter. For example, construct containing nt842-863 of c-jun is termed RCASBP(B)-shRNA-ΔU6-*cjun*842 or RCASBP(B)-shRNA-ΔU6-*cjun228*. These constructs were tested by transfection into normal CEF using the calcium phosphate transfection method. Temperature sensitive mutants of RSV were added to superinfect the transfected cells 24 hours after transfection. Suppression of gene expression was ascertained by western blotting analysis of the corresponding proteins.

3. Construction of mismatched and scrambled mutants: We designed scrambled forms of microRNA targets of AP-1 and NF-kB p105 (Table I). These target sequences were subcloned into the modified microRNA operon following the original supplier's instruction (ARK Genomics). All the constructs were designated using the number of the first nucleotide of the targeted sequence with a minus sign "(-)" to indicate the removal of U6 promoter and annotated with "scr" to indicate a scrambled sequence. For example, the target sequence of JunD microRNA construct was chosen from the No. 821 nucleotide to No.842 nucleotide of the cDNA. So the JunD microRNA construct was designated as RCASBP(B)-shRNA- ΔU6-*junD*821 and its scrambled form was designated as RCASBP(B)-shRNA- ΔU6-*junD*Scr. We also designed mismatched forms of each target by substituting 2 central nucleotides of the stem region in each target sequence (Table I).

Table 1. Target sequences used for shRNA constructs

miRNA Constructs	Target Sequence	Gene
C-Jun target	AAGAACAGGTTGCACAGCTTAA	**C-Jun**
C-Jun scrambled	TCAACTAACCAGGTAGAGAACG	
C-Jun mismatch	AAGAACAGGAAGCACAGCTTAA	
JunD target	CGAAGAGCCTCAAGAGCCAGAA	**Jun-D**
JunD scrambled form	AGAAGCGACACACGAACTATGT	
JunD mismatch form	CGAAGAGCCTTGAGAGCCAGAA	
Fra-2 target	ACAGCTGCTAAATGTCGTAACA	**Fra-2**
Fra-2 scrambled form	TCAAGAGATCATTATCGGCAGT	
Fra-2 mismatch form	ACAGCTGCTATTTGTCGTAACA	

4. Western Blotting Analysis: Whole cell protein lysates were prepared by harvesting non-transformed and transformed CEF washed and pelleted in cold 1X PBS. Cells were

resuspended and lysed in 1X Sodium Dodecyl Sulphate (SDS) buffer consisting of 2.3% SDS, 5% β-mercaptoethanol, 10% glycerol, and 62.5 mM Tris pH 6.8 by boiling samples for 5 minutes. 20-40μg of proteins were separated by electrophoresis through a denaturing 10% polyacrylamide gel, transferred electrophoretically onto a nitrocellulose membrane (Schleicher and Schuell, BA85) and probed with various antibodies. Commercial antibodies made against c-Jun (SC-45X), Jun D (SC-74X), Fra-2 (SC-604X) (all used at a dilution of 1:2000), p53 (SC-99) and ERK-1 (SC-93) (used at a dilution of 1:2000) were obtained from Santa Cruz Biotechnology (Santa Cruz, California). A commercially available p105NFkB1 antibody (1:150 dilution) was used in some experiments (ABCAM. Cat. ab54162). Immune complexes were visualized by chemiluminescent radiography using a horseradish peroxidase-conjugated secondary antibody and a chemiluminescent substrate (ECL system, Amersham). Densitometry analysis of down-regulation of specific protein levels by shRNAs is measured by Image J software (NIH).

5. Proliferation Assay: CEFs were transfected with shRNA expressing constructs and cultured for 2 passages to ensure sufficient virus replication and infection. The cells were then seeded into 24-well plates at 12,000-cells/per well to allow proliferation for 6 days. Each transfection was seeded in quadruple for statistical analysis (standard error). We monitored cell proliferation every 24 hours for 6 days.

6. Transient Expression Reporter Assay: The methodology and reporter gene constructs

have been described before (Dehbi *et al.*, 1992, Gagliardi *et al.*, 2001). The DEAE-dextran method was used for all transient expression experiments. In brief, normal CEF, SR-A RSV transformed CEF and NY72-4-infected CEF kept at the non-permissive temperature of 41.5˚C, were seeded on 100 mm dishes to a density between 70-80% prior to transfection. Variable amounts of the effector expression plasmid (see Figure legends for details) were co-transfected with 2μg of the CAT reporter plasmid and 2 μg of pRSV-βgal along with variable amounts of carrier salmon sperm DNA made up to a total of 30 μg of DNA. The pRSV-βgal plasmid contains the lac Z gene under the control of RSV's long terminal repeat enhancer sequence and β-galactosidase activity was determined for each transfected plate as an internal control of transfection efficiency for all transient expression assays. For transformed samples, NY72-4 or LA90 infected CEF transfected at the non-permissive temperature (41.5°C) were shifted to the permissive temperature (37°C) the day after transfection and left for a minimum of 24 hours. An in vitro assay for CAT activity using acetyl-CoA and radioactive 14C-Chloramphenicol was carried out for each plate of transfected cells. Acetylated products were separated by thin layer chromatography and analyzed in a PhosphoImager to quantify the levels of incorporated radioactivity. All transient expression assays were conducted with triplicate samples for each experiment and each assay was repeated two or more times. Error bars reflect the maximal and minimal standard deviation of the average CAT activity calculated for each sample. CAT reporter constructs were co-transfected with a control vector or TAM67 expressing vector into CEF infected by temperature sensitive strain of RSV, NY72-4, using DEAE-DEXTRAN transfection. Plasmid pRSV β-Gal was also co-transfected and

used as an internal control. Transfected cells were harvested at 36-48 hours to check the β-Gal activity. Cell lysates were normalized by β-Gal activity and incubated with C14 labeled chloramphenicol and co-factor Acetyl-CoA. CAT activities were quantitated by calculating acetylated spots and analyzed using statistical methods.

7. Senescence-associated β-galactosidase (SAβG) assays: All singly and doubly infected cells in 60mm or 100mm dishes were subjected to the SA-β-Gal stain provided in the senescence β-Gal staining kit from Cell Signaling Technology (Cat. 9860) containing the following reagents: 10X Fixative Solution (20% formaldehyde, 2% glutaraldehyde in 10X PBS), X-gal, 10X Staining Solution (400mM citric acid/sodium phosphate at pH 6.0, 1.5M NaCl, 20mM MgCl2), 100X Staining Solution Supplement A (500mM potassium ferricyanide) and 100X Staining Solution Supplement B (500mM potassium ferricyanide). Volumes listed below are given for 60mm dishes; volumes were double for 100mm dishes. Medium was removed from sub-confluent cells and washed once with 5ml 1xPBS. The cells were fixed in 2.5ml 1X Fixative Solution for 15 minutes at RT. Cells were washed twice with 2.5ml 1X PBS. After the washes, the cells were placed overnight at 37°C with 2.5ml of the staining solution mix which contained 930μl Staining Solution, 10μl of each Staining Supplement A and B, and 50μl of 20mg/ml X-Gal in DMF per ml required. For example, 5-60mm dishes would require 12.5ml of staining solution mix. Cells were checked the following day for appearance of blue precipitate and then stored at 4°C. Staining was performed in triplicate for statistical analysis. Induction of senescence is measured by proportion of senescent cells of the whole

population by counting 500 cells per plate (~50 cells/field and 10 fields per plate).

8. Cell Death Analysis: The *in situ* Cell Death Detection Kit (Roche, Cat. 12156792910) was used to identify apoptotic cells according to the instructions of the manufacturer. Cells were fixed with 4% Paraformaldehyde in 1X PBS for 1 hour at room temperature and permeablized with 0.1% Triton X-100 in 0.1% sodium citrate. After washing extensively in 1X PBS, specimens were incubated with Terminal deoxynucleotidyl Transferase (TdT) and TMR red labeled dUTP-containing solution for 1 hour at 37°C. Cell Nuclei was stained by using DAPI (Sigma, Cat. D9542). All the samples were mounted with 10μl of Aquapolymount (Polyscience, Cat. 18606). Apoptotic cells were visualized under fluorescence microscopy. 15 fields (~100 cells/field) of each coverslip were counted. Cell nuclei were stained using DAPI (Sigma, Cat. D9542) for quantitative purpose.

9. Soft Agar Assays and Colony Formation Assays: The assay was performed in 60mm dishes with lower layer of 0.5% low-melting-point agar in 1 X DMEM medium containing 5% cosmic calf serum, 4% chicken serum, 5% tryptose phosphate broth , and 1% penicillin and streptomycin. 10,000 cells were resuspended in the same medium with 0.35% low-melting-point agar and then were overlayed on the lower agar. The dishes were then incubated in 37˚C incubator containing 5% CO2 for 5 days or until colonies were visible. Images of colonies were documented with 10X magnification using inverted microscope (Magnification 2X). Colony formation assays were quantified using ImageJ

software. The colony formation assays were performed by Bart Maslikowski, a graduate student in our laboratory (Dr. Bedard's Laboratory).

10. Lipid Staining: Lipids were stained with the lipophilic dye Oil Red O, as described before (Kim S. *et al.,* 1999). Staining was performed in triplicate for statistical analysis. Induction of adipogenic conversion is measured by proportion of lipid-rich vesicles containing cells of the whole population by counting 500 cells per plates (~50 cells/field and 10 fields per plates).

11. Immunofluorescence: Cells were grown on coverslips, fixed in 3.7% formaldehyde in 1x Phosphate buffered saline (PBS) for 15 min at room temperature followed by a permeabilizing treatment for 5 min using 0.1% Triton X-100 in 1xPBS. The coverslips were then incubated with primary antibody diluted in 10% fetal calf serum in 1xPBS at a final concentration of 100ng/μl during 1 hr at room temperature in a humidified chamber. After washing for 5 times with 1xPBS (5 minutes each time) with gentle shaking. Coverslips were then incubated for at least 1 hr with a fluorescein-conjugated secondary antibody (Cappel Research Products, Durham, NC) at 1:100 dilution mixed with 1:150 dilution of DAPI (Sigma, Cat. D9542) to stain the cell Nuclei. 1xPBS containing 10% fetal calf serum. All the samples were mounted with 20μl of Aquapolymount (Polyscience, Cat. 18606).

Chapter 3: Investigation of the role of AP-1 in v-Src transformed CEF

1. Introduction

The activator protein-1 (AP-1) is a transcription factor family that functions by forming homo- or hetero-dimers to regulated the expression of genes involved in cell proliferation, survival and transformation (Shaulian and Karin 2002). TAM67 is an N-terminal transactivation domain deletion mutant of c-Jun that retains a functional DNA binding domain and dimerization domain known as the bZIP domain (basic region and leucine zipper). It dimerizes with wild type AP-1 and binds to the TPA responsive element (TRE) in responsive promoters and inhibits gene expression (Brown PH *et al.,* 1994, Brown PH *et al.,* 1993). The inhibition of AP-1 by TAM67 results in cell cycle inhibition, implying a role for AP-1 in mitogenesis. Previous studies has shown that TAM67 induces cell cycle arrest by impairing the cyclin-CDK formation and increasing the level and activity of cell cycle arrest factors such as $p21^{CIP1/WAF1}$ (Gagliardi *et al.,* 2003, Hennigan and Strambrook 2001). In addition, the cell cycle inhibitor pRB is hypophosphorylated in these cells, an event that promotes cell cycle arrest. In immortalized cell lines, TAM67 also blocks transformation by various oncoproteins such as activated Ha-Ras or *v*-Src (Lloyd *et al.,* 1991). More recently, Leaner and co-workers reported that TAM67 blocks the migration and invasive potential of K12 and K7M2 murine osteosarcoma cells, suggesting that AP-1 plays a role in mediating aggressive tumour cell properties (Leaner *et al.,* 2009).

Previous studies have identified several *v*-Src activated transcription factor families including Stats, NFκB, AP-1 and C/EBPβ (Cabannes *et al.,* 1997, Dehbi and Bedard

1992, Dehbi *et al.,* 1992, Gagliardi *et al.,* 2001, Silva CM 2004, Thomas and Brugge 1997). In *v*-Src transformed CEF, the *IL8* gene is induced constitutively (Bedard *et al.,* 1987, Sugano *et al.,* 1987), and is part of the "aggressive tumour cell gene signature" recently described in our laboratory (Maślikowski *et al.,* 2010). The induction of *IL8* relies on the Src-responsive unit (SRU) of its promoter which includes binding sites for AP-1, NFκB and C/EBPβ (Dehbi *et al.,* 1992). In agreement with this finding, previous work in our laboratory revealed that the expression of several components of AP-1 and the DNA binding activity of this factor are elevated upon *v*-Src transformation (Manuscript in Appendix 2). We over-expressed the dominant negative mutant of c-Jun, TAM67, to examine the role of AP-1 in the transformation of primary cells, namely chicken embryonic fibroblasts (CEF), by *v*-Src. We report that TAM67 induces premature entry into senescence in normal CEF and CEF infected with a temperature sensitive mutant of RSV at the non-permissive temperature. In contrast, three distinct phenotypes were apparent at the permissive temperature, including senescence, apoptosis and the accumulation of lipid vesicles, a process that may represents the adipogenic conversion of the fibroblast. Therefore, AP-1 facilitates v-Src transformation by several mechanisms.

2. Results

Control of AP-1 activity in v-Src transformed CEF

Expression of TAM67 inhibited markedly the activity of AP-1 in v-Src transformed CEF (Fig. 6A). This inhibition reflected, in part, the down-regulation of all three

components of the AP-1 factors, namely c-Jun, JunD and Fra-2, expressed in these cells (Fig. 6B; Manuscript in Appendix 2). This effect of TAM67 is consistent with previous observations indicating that the expression of these genes is positively auto-regulated at the transcriptional level (Angel *et al.,* 1988, Berger and Shaul 1994).

Hybrid proteins consisting of the trans-activation domain (TAD) of either c-Jun or JunD fused to the Gal4 DNA binding domain (DBD) were also co-expressed with a reporter gene controlled by Gal4 biding elements in normal and SR-A RSV transformed CEF. *V*-Src had little effect on the activity of the c-Jun TAD. On the contrary, a strong stimulation of the activity of the JunD TAD was observed (Fig.6C), suggesting that JunD is an important target of *v*-Src transformation in CEF. The same result was obtained when these constructs were expressed in CEF infected by the ts mutant NY72-4 RSV (A. Bédard, unpublished results).

The potency of the c-Jun and JunD TAD is regulated by phosphorylation/dephosphorylation mediated by JNK/SAPK or ERK MAPKs (Eferl R and Wagner 2003). In order to gain some insight on the role of these pathways, the ERK pathway was inhibited with PD98059, a specific inhibitor of MEK, while expression of a dominant negative mutant of SAPK/ERK kinase (SEK), SEKAL, was used to interfere with the activity of the JNK/SAPK pathway in transient expression assays. Expression of SEKAL had little effect on the activity of the TRE-controlled reporter gene in normal and *v*-Src transformed CEF (Fig. 6D). In contrast, the inhibition of MEK by PD98059 inhibited markedly the activity of AP-1 in these cells, suggesting that v-Src controls AP-1, at least in part, through the ERK pathway.

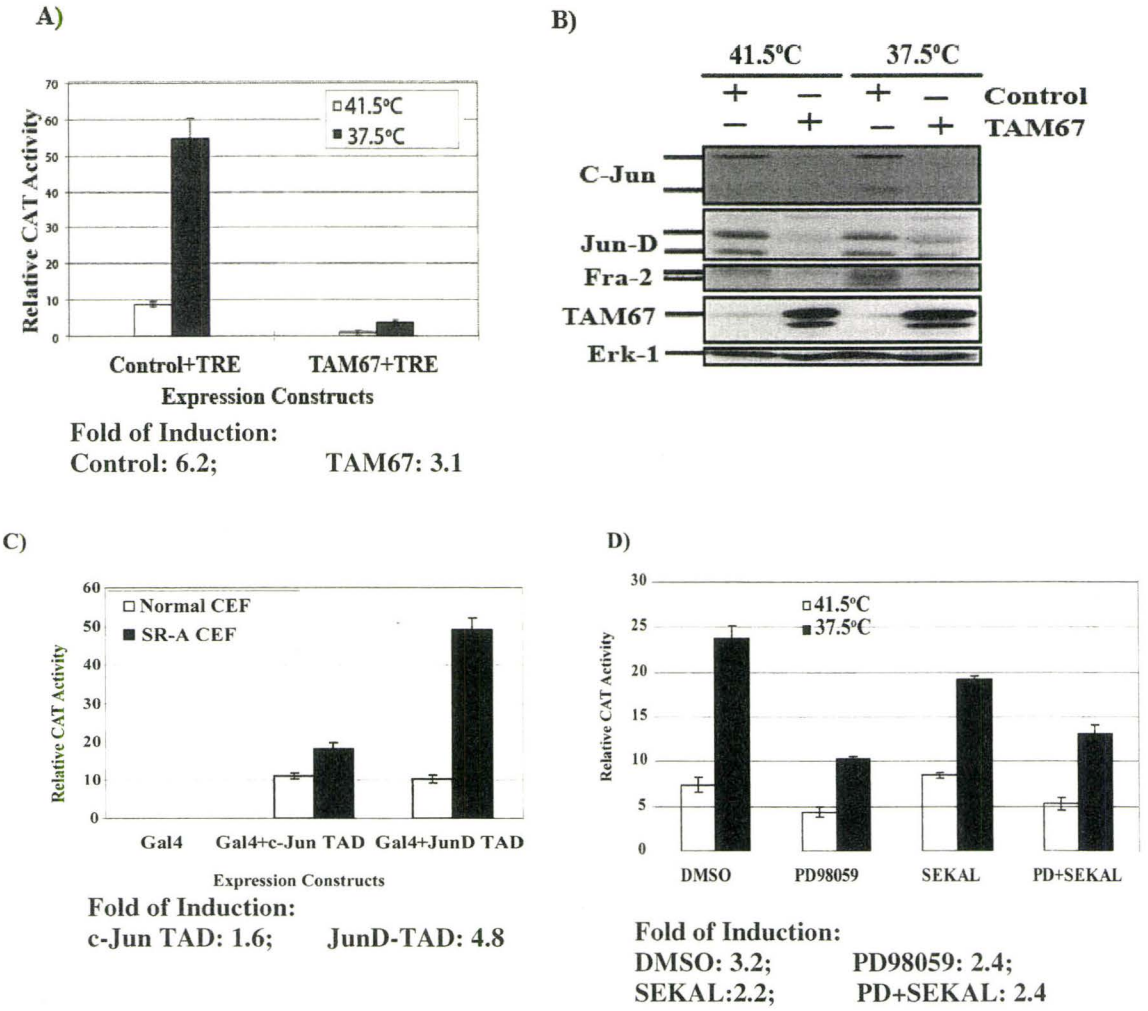


Figure 6. Regulation of AP-1 by v-Src and TAM67. To investigate the effect of TAM67 expression on v-Src induced transformation, CEFs expressing TAM67 or a control vector were superinfected with a temperature sensitive strain (ts) of RSV virus, NY72-4 RSV (Panel A) or LA90 RSV (Panel B). **A.** Transient expression assays were performed to examine the activity of AP-1 in the presence or absence of TAM67; **B.** Western blotting analysis of c-Jun, JunD and Fra-2 expression in CEF co-infected by the ts mutant LA90 RSV and a virus expressing the dominant negative mutant TAM67 or a control virus (RCASBP(A)). ERK was used as a loading control; **C.** Potentiation of the transactivation domain (TAD) of c-Jun and JunD in normal and wt SR-A RSV transformed CEF. Transformation induced by v-Src enhanced the activity of JunD TAD but had little effect on the corresponding domain of c-Jun; **D.** CEFs infected with temperature sensitive virus transiently transfected with a CAT reporter gene controlled by three copies of TRE were treated with PD98059 or DMSO as a control at a final concentration of 50mM for 14 hours as suggested previously (Alessi et al., 1995).

Effect of TAM67 expression in normal CEF

Gagliardi and co-investigators reported previously that TAM67 inhibits the proliferation of normal CEF, a process that may reflect the down-regulation of cyclin D1 in these cells (Gagliardi *et al.,* 2003). Flat and bi-nucleated cells were observed in populations of cells expressing TAM67, suggesting the presence of senescent cells. In agreement with this notion, staining for senescence-associated β galactosidase (SAβG) activity, described by Dimri *et al* (Dimri *et al.,* 1995), indicated that a significant proportion of the TAM67 expressing CEFs underwent senescence (Fig. 7A-C). Hence, TAM67 impairs the proliferation of normal CEF by inducing premature entry into senescence in a fraction of the CEF population.

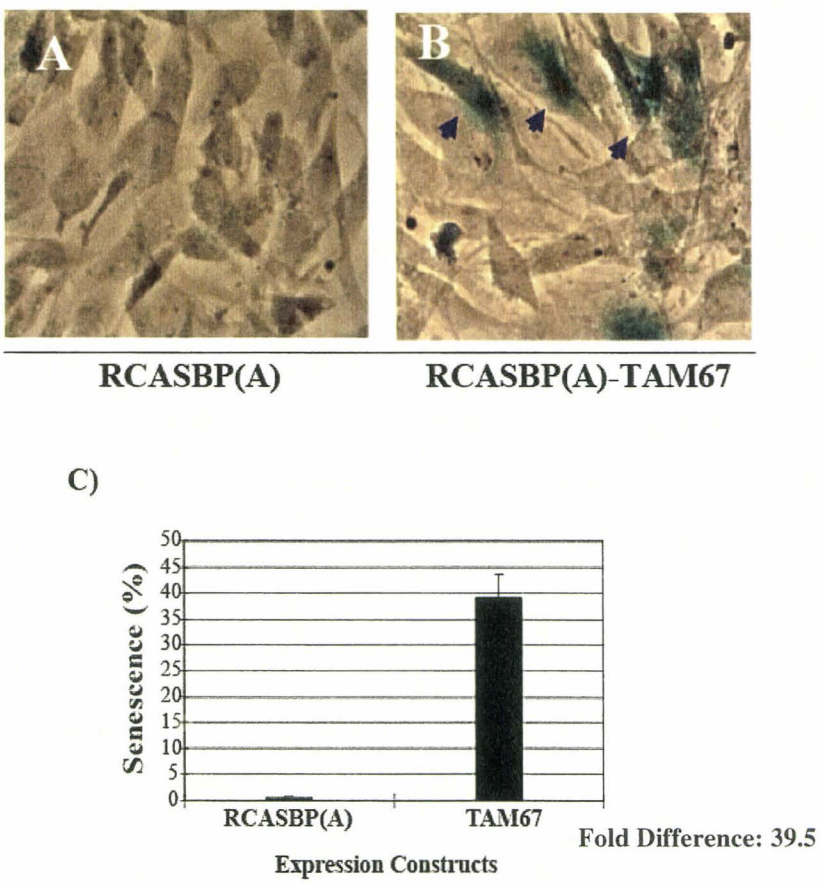
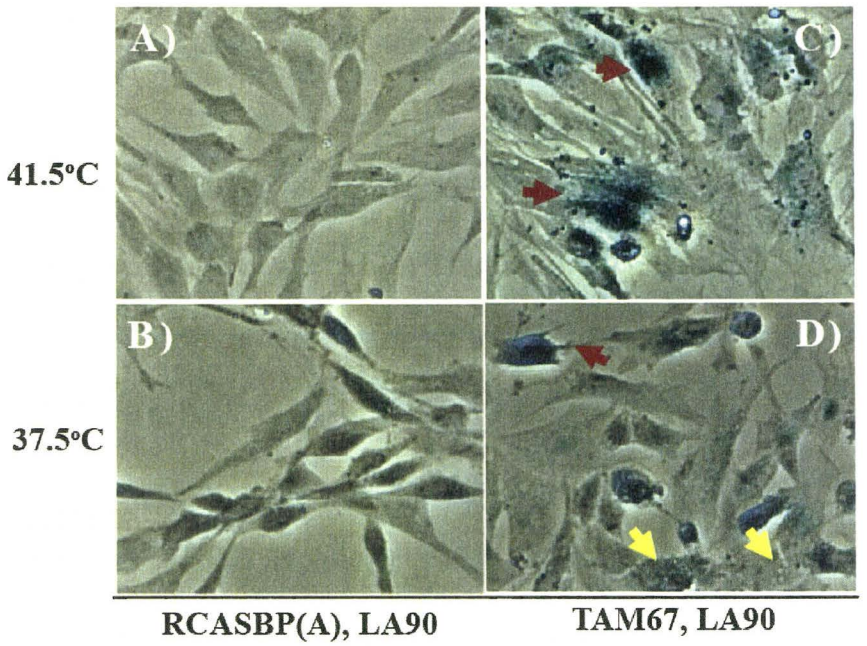


Figure 7. Expression of TAM67 induces senescence in normal CEFs. CEFs expressing TAM67 or control construct were monitored for phenotypic alteration. **A-B.** CEFs stably expressing TAM67 or control vector were stained for senescence associated β-galactosidase. Senescent cells were observed in the presence of TAM67 (blue arrows). CEFs expressing control vector did not show any senescence phenotype; **C.** Incidence of senescent cells in control and TAM67 expressing CEF. TAM67 markedly increased senescence.

TAM67 induces three distinct phenotypes in v-Src transformed CEF

To investigate the role of AP-1 in *v*-Src transformation, control and TAM67-expressing CEF were super-infected with ts LA90, a temperature sensitive mutant of RSV, and the phenotype of these cells was characterized at the non-permissive (41.5°C) and permissive temperature (37.5°C). As observed in normal CEF, a high proportion of the TAM67-expressing CEF expressed the SAβG activity. This was true at the permissive and non-permissive temperatures (Fig.8). CEF expressing TAM67 remained flat even at the permissive temperature, indicating that the expression of the dominant negative mutant of c-Jun also impaired the morphological transformation of CEF.



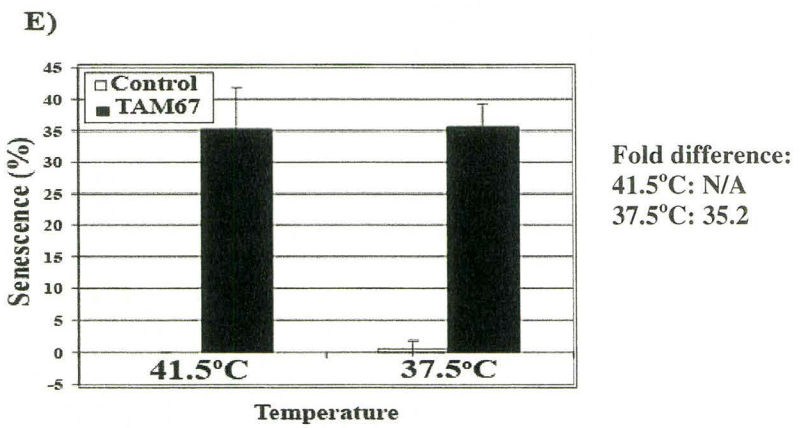


Figure 8. Expression of TAM67 induces senescence in LA90 infected CEFs. **A-D.** ts.LA90 RSV-infected CEFs stably expressing TAM67 or control vector were stained for senescence associated β-galactosidase. A high proportion of senescent cells was observed in the presence of TAM67 (Red arrows in panels C & D). Control CEFs did not show any senescence phenotype. Accumulation of vesicles in the cytoplasm was observed in some cells (Yellow arrows in panel D), this phenotype will be discussed later; **E.** Quantitation of senescence is provided in a graph, indicating that senescence was induced in the presence of TAM67 in ts.RSV infected CEF at both non-permissive and permissive temperatures. Image magnification: 100X.

We also observed that cell numbers of LA90-infected CEF expressing TAM67 were significantly reduced by about 60% over a 16 hr period at the permissive temperature (Wang *et al.*, Manuscript in Appendix 2). Furthermore, in addition to the senescent phenotype (Fig.8), some of the cells displayed membrane blebbing, suggesting that they were undergoing apoptosis. To determine if senescence or cell death accounts for the loss of transformed CEF, we repeated the experiment with cells transferred to the permissive temperature for a shorter duration (12-14 hrs). The apoptotic phenotype was then confirmed by *in-situ* TUNEL assays, indicating that TAM67 also induces apoptosis in ts.LA90 RSV-infected CEF expressing TAM67 at the permissive temperature (Fig.9A-H). On the contrary, apoptosis was nearly undetectable at the non-permissive temperature of 41.5°C. Interestingly, ts.LA90 RSV-infected CEF that did not express TAM67 also showed signs of apoptosis when transferred to the permissive temperature, suggesting that v-Src activity induces apoptosis, albeit modestly.

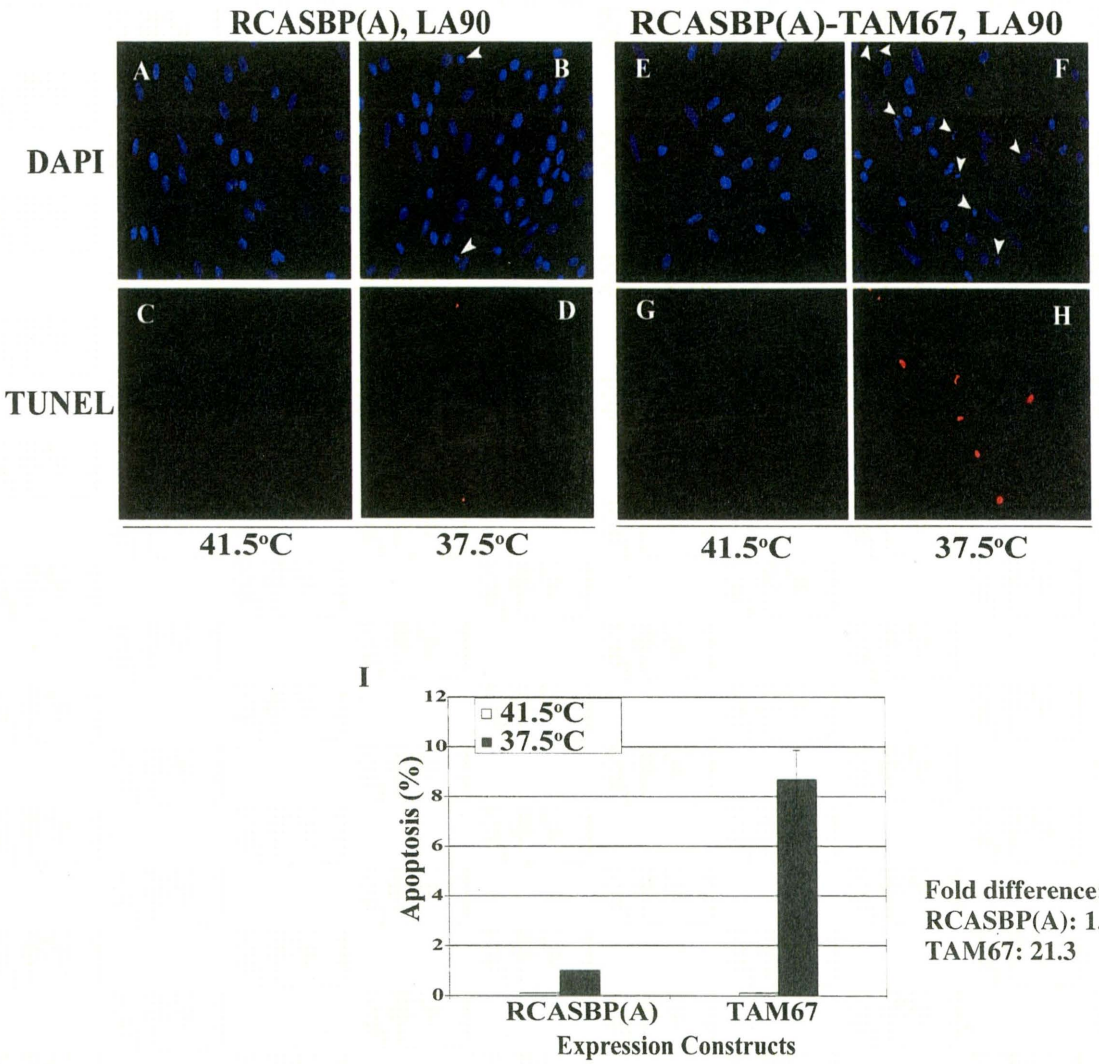


Figure 9. TAM67 induces apoptosis in v-Src-transformed CEF. Tunel assay of LA-90 RSV-CEF co-infected with RCASBP(A)-TAM67 or the control virus RCASBP(A) at the permissive (panels **B,D,F,H**) and non-permissive temperatures (panels **A,C,E,G**). Nuclei were stained with DAPI. Arrowheads indicate the position of apoptotic cell nuclei; **I.** Quantitation of the incidence of apoptosis as determined in the experiment described in panel A. The error bars represent the standard deviation. All photos were taken at a magnification of 40X.

To rule out the possibility that the phenotype caused by the expression of TAM67 is a non-specific effect of having two viruses replicating in the cell and to determine the extent of apoptosis, we expressed TAM67 transiently by transfecting plasmid CMV-TAM67 or a control vector in LA90 RSV infected CEF. A plasmid expressing GFP was included in the experiment to identify cells that were effectively transfected. Cells were seeded into coverslips 24 hours after transfection and half of the coverslips were shifted to the permissive temperature of 37.5°C for 12 hours. As described in Fig. 10, the expression of TAM67 did not induce apoptosis when the cells were maintained at the non-permissive temperature. In contrast, the activation of the ts *v*-Src kinase induced apoptosis in 50% of LA90 RSV infected CEF transfected with the TAM67 expression construct (Fig.10M). A lower incidence of apoptosis was also detected in CEF transfected with the control vector, in agreement with our previous observations that v-Src transformation caused cell death in a small fraction of the cells. The transfection efficiency was calculated by measuring the ratio of the GFP expressing cells over the whole cell population. The transfection efficiency was comparable (15-20%) in CEF transfected with the control vector or TAM67 expression constructs (Fig. 10N). Therefore, the induction of apoptosis was observed in a high proportion of the v-Src transformed cells indicating that cell death is the predominant phenotype caused by the expression of TAM67. Moreover, this result confirmed that cell death was not caused by the presence of multiple retroviruses replicating in the cell.

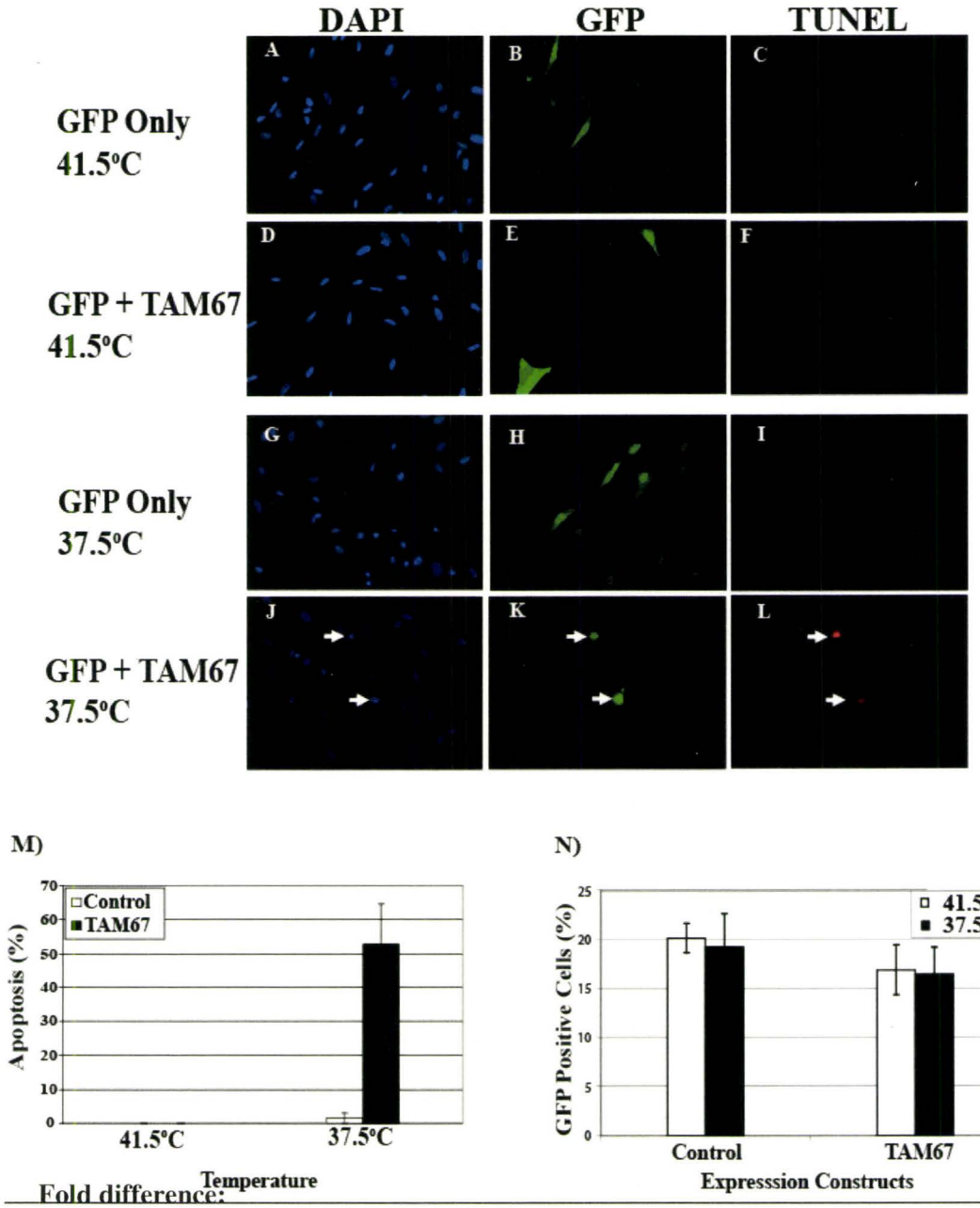
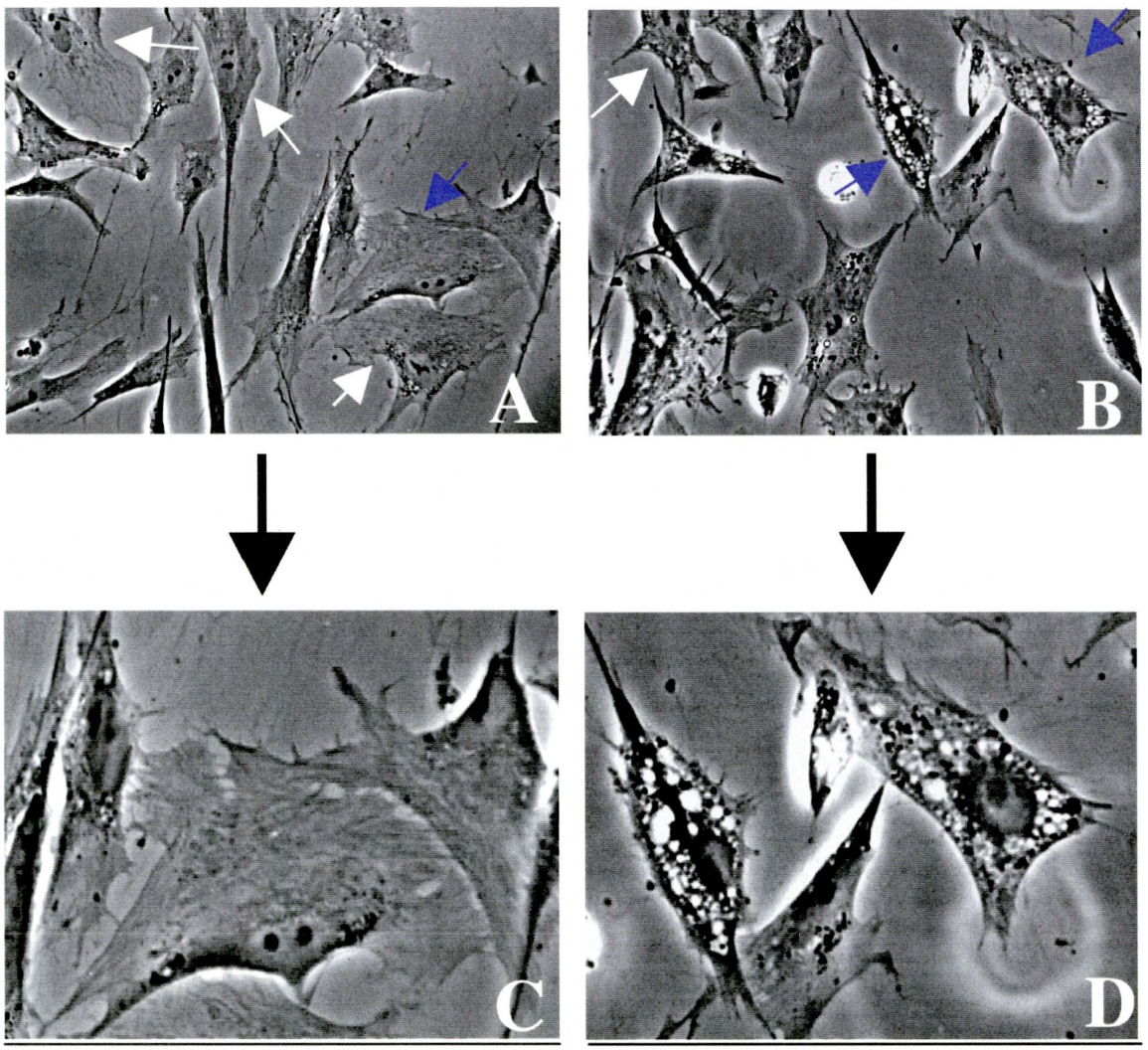


Figure 10. Transient expression of TAM67 induces apoptosis in v-Src-transformed CEF. **A.** Induction of apoptosis by transient expression of TAM67 in ts LA90 RSV-infected CEF. TUNEL analysis was performed to determine the incidence of apoptosis in GFP-positive CEFs at both non-permissive and permissive temperatures. Transiently expressed TAM67 is able to induced apoptosis in transformed CEF, but not at the non-permissive temperature. Quantitation of apoptosis (**panel B**) and transfection efficiency (**panel C**) in transfected CEF are shown below. All photos were taken at a magnification of 40X.

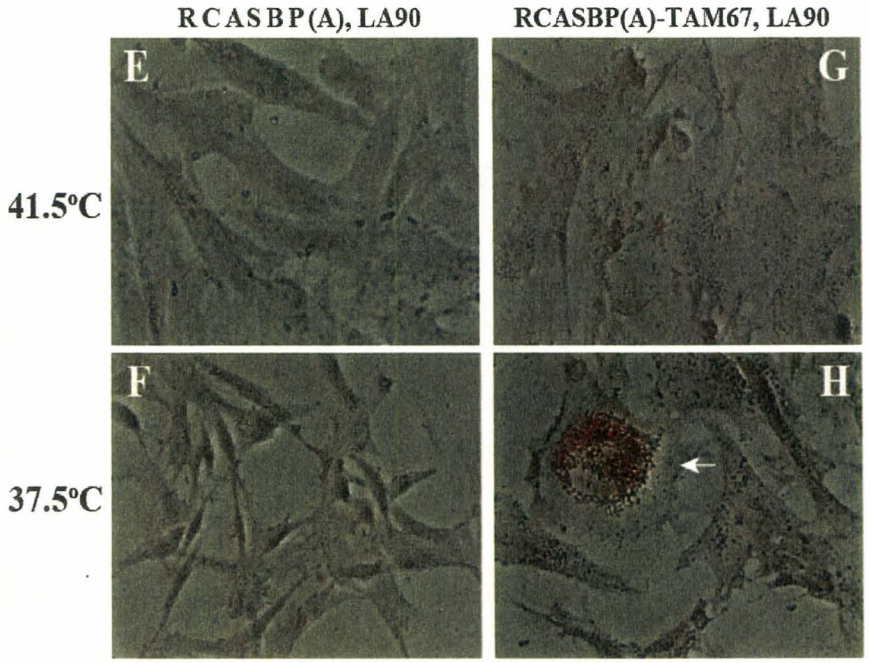
We also observed that a fraction of the cells were highly vesiculated in CEFs co-infected by ts.LA-90 RSV and TAM67 at the permissive temperature, but not at the non-permissive temperature (Fig.11A-D, but also see yellow arrowheads in Fig.8D). These cells were negative in the TUNEL, MAPLC3 processing/autophagy (T Yorimitsu 2005), macropinocytosis [known to be triggered by *v*-Src in rat fibroblasts; (Mettlen *et al.,* 2006, Veithen *et al.,* 1996)] and SAβG assays (data not shown). However, staining with the lipophylic dye oil-red O indicated that these vesicles are lipid-rich (Fig.11E-I). This phenotype was not observed in CEF co-infected by ts.LA90 RSV and the control RCASBP(A) virus.

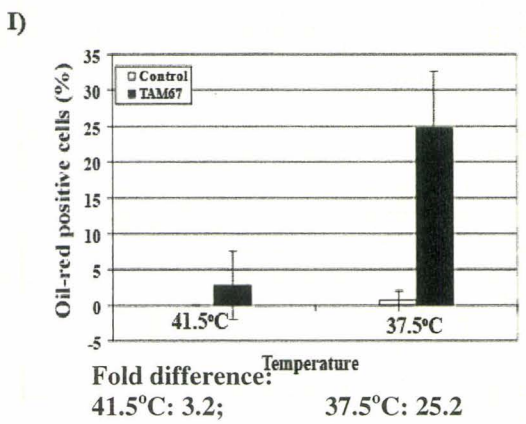
We also asked if TAM67 induced the expression of the lipid-binding p20K lipocalin, a marker of contact inhibition and reversible growth-arrest but not adipogenesis in CEF (Bedard *et al.,* 1989, Kim S. *et al.,* 1999). As shown in Fig.11J, the activation of the temperature-sensitive *v*-Src kinase did not enhance but, in fact, reduced the expression of p20K. This was true in control and TAM67 expressing CEF. Therefore, the inhibition of AP-1 did not induce a state of reversible growth arrest in these cells. Taken together, these results suggest that a fraction of the TAM67 expressing CEF had undergone differentiation upon ts.*v*-Src activation. Therefore, AP-1 facilitated transformation by preventing the entry into senescence, acting as a pro-survival factor and inhibiting differentiation in v-Src transformed CEF.



TAM67+ LA90
41.5°C

TAM67+ LA90
37.5°C





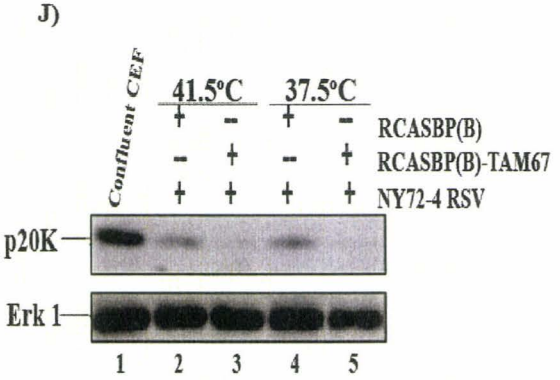


Figure 11. TAM67 triggers the accumulation of lipid-rich vesicles in v-Src transformed CEFs. **A-D.** The presence of TAM67 caused accumulation of vesicles in the cytoplasm in RSV-transformed CEF, but not in the non-transforming state. White arrows point to representative cells, while blue arrows represent cells that are enlarged below; **E-H.** Lipophylic dye oil-red O was used to stain for lipid containing vesicles (white arrows in Panel H). CEFs co-infected by ts.LA90 RSV and TAM67 or control vector were stained with oil red O. Lipid-containing vesicles stain red under microscope (Arrows); **I.** Quantitation of the incidence of oil red O-positive cells from Panels E-H is provided below. Downregulation of Fra-2 expression induced lipid-rich vesicles accumulation upon v-Src activation; **J.** Cell lysates were investigated for the induction of p20K. Lysate from contact-inhibited CEFs was used as a positive control. Erk-1 was used as loading control. Images of cell phenotype were taken on ZEISS inverted IM35 microscope under 200X magnification.

TAM67 regulates the activity of several factors of the v-Src-responsive unit (SRU)

In addition to members of the AP-1 family, c-Jun is capable of interacting with NFκB and C/EBPβ (Stein *et al.,* 1993a, Stein *et al.,* 1993b). In addition, previous studies have shown that TAM67 blocked not only the activity of AP-1 but also that of NFκB by direct interaction in human keratinocytes (Li *et al.,* 2000). Therefore, TAM67 may alter the activity of all three factors binding to the v-Src Responsive Unit (SRU) of the IL8 promoter. This was confirmed in transient expression assays. As expected, the activation of a TRE-controlled promoter by v-Src was strongly impaired by the expression of TAM67 (Fig. 12A). However, a significant inhibition was also observed for the NFκB-controlled promoter while, in contrast, C/EBP activity was stimulated by TAM67 (Fig.12A&B). The resulting effect was a partial inhibition of the IL8 promoter in v-Src transformed CEF (Fig.12C). Thus, the expression of the dominant negative mutant of c-Jun was not restricted to the inhibition of AP-1, and exerted positive and negative effects on the activity of factors binding to the SRU.

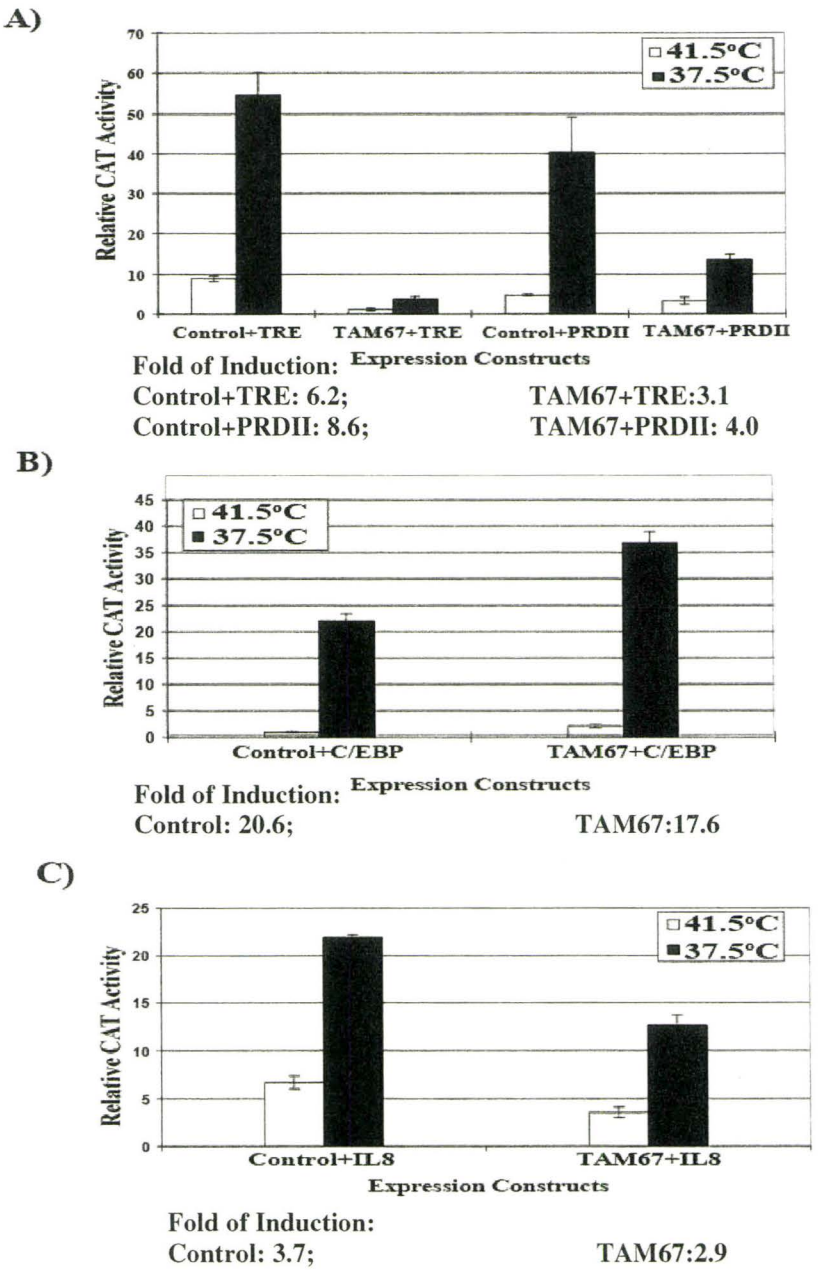


Figure 12. Effect of TAM67 on the IL8 promoter and SRU regulatory elements. CEFs infected with a temperature sensitive strain of RSV, NY72-4 RSV, were co-transfected with either TAM67 or control vector with a construct bearing a CAT promoter construct controlled by AP-1 (TRE; **panel A**), NF-κB (PRDII; **panel A**), CEBP/β (CAAT; **panel B**) or the IL8/CEF-4 promoter (**panel C**). CAT activity was determined at the permissive (transformed CEF) and non-permissive temperature (non-transformed CEF). Error bars indicate standard deviation from the mean.

3. Summary

The inhibition of AP-1 reduces markedly the proliferation of normal CEF (Gagliardi *et al.,* 2003). In this study, we showed that expression of a dominant negative mutant of c-Jun, TAM67, induces premature entry into senescence in normal CEF but three distinct phenotypes in *v*-Src transformed CEF. In these conditions, senescence was observed as well as cells undergoing apoptosis or displaying the formation of lipid vesicles. Therefore, TAM67 impaired the transformation and survival of CEF expressing the *v*-Src oncogene.

TAM67 lacks any transactivation domain (TAD) but retains the basic region and leucine zipper domain. Therefore, in addition to dimerizing with members of the AP-1 family, TAM67 has the potential to interact with C/EBPβ and NFκB (Stein *et al.,* 1993b). In agreement with this notion, TAM67 reduced the expression of a reporter construct controlled by AP-1 or NFκB. In contrast, C/EBPβ activity was enhanced by TAM67, indicating that the dominant negative mutant has both positive and negative effects on factors of the IL8 SRU. The resulting effect was a modest inhibition of the activity of the IL8/CEF-4 promoter in *v*-Src transformed CEF. Therefore, TAM67 generates a non-specific effect when blocking AP-1 activity. In this case, direct abolishment of an AP-1 member by RNA interference is necessary to study the role of AP-1 in RSV-transformed CEF.

Chapter 4: Construction of a retroviral-based RNA interference system

1. Introduction

The definitive description of RNA interference came from the work of Andrew Fire and his colleagues in 1998. Fire *et al* showed that the addition of artificial double stranded RNA into *Caenorhabditis elegans* was able to block the expression of mRNA containing the complementary sequence (Fire *et al.,* 1998). This exogenous double-stranded RNA provided stronger inhibition of gene expression than the conventional anti-sense RNA, which is unstable and has only modest effects. Endogenous interfering RNAs were also shown to have critical roles in many aspects of development and the immune response (Carthew and Sontheimer 2009, Pedersen and David 2008). These small RNAs execute the repression of gene expression by using the mechanism described for artificial siRNA. Based on their origins, structures and associated effector proteins, known small non-coding RNAs (ncRNAs) are categorized into three groups: short interfering RNA (siRNA), microRNA (miRNA) and piwi-interacting RNA (piRNA) (Carthew and Sontheimer 2009). Among these small RNAs, siRNA and miRNA are known to be double-stranded and are widely distributed in many cell types. In contrast, the piRNAs are found to be in a single-stranded structure (Bartel 2009). MicroRNAs consist of a large family of endogenous non-coding RNAs of 21-25nts long that function as regulators of gene expression (Bartel 2009). The *lin-4* RNA from *Caenorhabditis elegans* was the first identified miRNA (Ambros 1993). It has a role in development by repressing *lin-14* gene expression by interacting with the 3'UTR and interfering with *lin-14* translation (Lee R. *et al.,* 1993). These RNAs are characterized by a hairpin structure

with a stem of 21 to 23 nucleotide pairs and flanking sequence.

The mature microRNA is generated from a self-folding transcript rather than a longer hairpin RNAs or small RNA duplex (Bartel 2009). The primary microRNA (pri-miRNA) is processed by a RNase III type endonuclease Drosha. The microRNA is then exported into the cytoplasm, where it is further processed by Dicer into a small interfering RNA (siRNA)-like structure (Filipowicz *et al.,* 2008, Kim VN *et al.,* 2009b). miRNA represses gene expression by either interfering with the mRNA translation machinery or destabilizing the mRNA, or both (Bartel 2009, Filipowicz *et al.,* 2008). Recently, several investigators have taken advantage of the unique structure and expression features of microRNAs to express shRNA (short-hairpin RNA) and induce gene downregulation. Due to the difference in biogenesis process, shRNA is capable of generating more persistent RNA interference with less off-target effects when compared to small artificial RNA duplexes [siRNA (Rao *et al.,* 2009)]. Zeng and colleagues provided a successful example using the human mir-30 miRNA. Mir-30 was able to inhibit the translation of an artificial mRNA containing a mir-30 target site (Zeng Y *et al.,* 2002). Moreover, in transient expression, an artificial ncRNA mimicking the mir-30 precursor was able to induce RNA interference to efficiently repress the translation of mRNA containing a complementary target sequence (Zeng Y *et al.,* 2002). This was then improved by using mir-30-based shRNA to stably repress gene expression (Dickins *et al.,* 2005, Silva JM *et al.,* 2005, Stegmeier *et al.,* 2005). Following these findings, a retroviral-based mir-30-like microRNA operon driven by the chicken U6 promoter was developed to successfully downregulate ectopic gene expression in chicken embryos or cells (Das *et al.,* 2006). In

this vector, the microRNA cassette can carry two target sequences simultaneously, which is convenient for silencing of two independent gene targets. The chicken U6 promoter is a RNA polymerase III (*pol* III) promoter that has been widely used to express shRNA and repress target genes in chicken (Kudo and Sutou 2005, Wise *et al.,* 2007). However, it has been reported that the *pol* III-driven cassette does not transcribe efficiently when placed within a retroviral genome that is under control of the functional long terminal repeats [LTRs (Ilves *et al.,* 1996)]. In agreement with this finding, this microRNA cassette did not generate efficient silencing of endogenous genes in CEF (our unpublished data). Moreover, shRNA under the control of the viral LTRs (*pol* II) of the Rous sarcoma virus (RSV)-derived RCAS system was convincingly shown to provide prolonged and specific RNA interference when compared to *pol* III-driven shRNA operon (Chen M *et al.,* 2007). In this study, we modified a retroviral-based mir-30-like microRNA operon by removing the U6 promoter from the microRNA cassette and subcloning it back into the RCAS system. This modified viral-based microRNA system is distinct from the original one since shRNA expression is driven directly from the viral LTR. We use this vector to down-regulate the expression of p105NFκB and show that it is required for the survival of *v*-Src transformed CEF.

2. Results

Avian replication competent retroviruses for stable RNA silencing

Avian retroviruses for expression of shRNA have been described before (Bromberg-White *et al.,* 2004, Chen M *et al.,* 2007, Das *et al.,* 2006, Harpavat and Cepko 2006).

However, little information exists on the efficacy of these vectors and their capacity to silence gene expression in a sustained manner, a condition required for studies on cell proliferation and transformation. Using existing RCASBP vectors expressing shRNA from an internal *Pol*III promoter, we failed to observe prolonged down-regulation of several transcription factors of the AP-1 or NF-κB family in chicken embryonic fibroblasts (CEF; our unpublished results). Similar conclusions were reached by other investigators (Chen M *et al.,* 2007). In the vector designed by Das and co-investigators, a shRNA is expressed in the context of a chicken microRNA under the control of a U6 promoter. We reasoned that transcriptional interference caused by the strong viral LTR or hindrance by the splicing machinery interfered with the expression of the shRNA. Since microRNA genes are transcribed by RNAPII, we deleted the U6 promoter and leader sequence to generate plasmid RCASBP(A)-shRNA-ΔU6 and RCASBP(B)-shRNA-ΔU6. In this vector, the shRNA sequence is cloned downstream of a splicing acceptor site and *egfp* gene (Fig.13A). Since expression of the viral genome can be monitored through GFP expression, we used the modified RCASBP vector to express a shRNA sequence targeting this gene and followed GFP expression in CEF. As shown in Fig. 13B-C, GFP was detected broadly in the cell population transfected with the control parental plasmid but was undetectable in cells expressing the shRNA for *egfp*. This was confirmed by Western blotting analysis (Fig.13D). Gag expression was reduced in vectors containing the microRNA cassette but was detected with both the parental RCASBP(A)-RNA-ΔU6 vector and its derivative construct harbouring the shRNA sequence for *egfp*. Infection assays confirmed that functional retroviral particles were

released in the medium of these cells (data not shown). Thus, while the vector was able to effectively silence the expression of GFP, viral protein expression and virus replication were not completely impaired by the presence of the shRNA sequence in the retroviral genome.

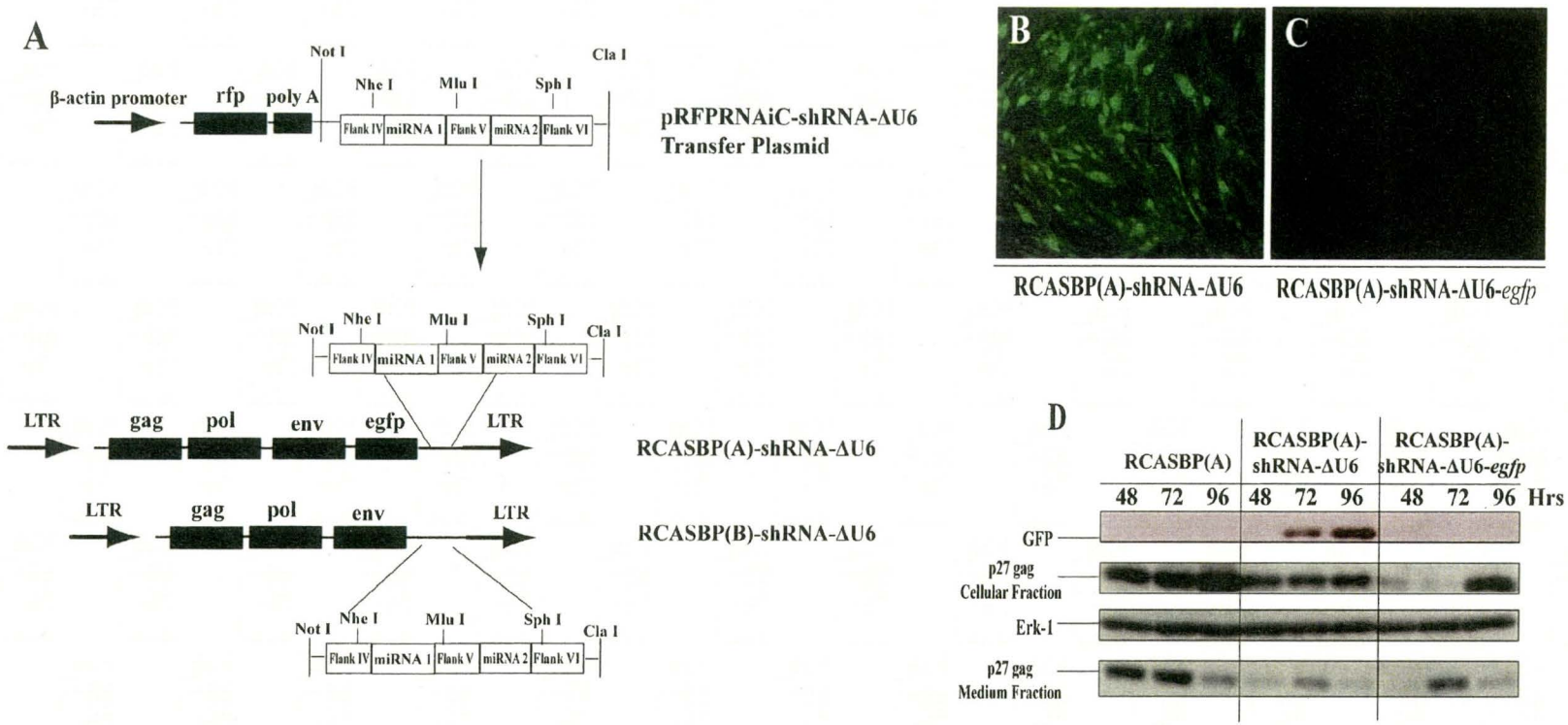


Figure 13. Avian replication competent retroviruses for shRNA expression. **A.** Schematic representation of the viral-based shRNA interference system. The U6 promoter and leader sequences have been removed from the original microRNA operon described by Das and co-workers (Das *et al.*, 2006). Sequences generating the hairpin structure are cloned at the NheI/MluI, MluI/SphI or at both of these locations in the transfer vector pRFPRNAiC-shRNA-ΔU6. The microRNA cassette, harbouring the hairpin sequences, is then sub-cloned in the retroviral vectors. The RCASBP(A)-shRNA-ΔU6 vector carries an *egfp* gene missing in the RCASBP(B) counterpart; **B-C.** GFP expression was examined by fluorescence microscopy four passages after transfection of the control RCASBP(A)-shRNA-ΔU6 or RCASBP(A)-shRNA-ΔU6 vector carrying the *egfp* shRNA (RCASBP(A)-shRNA-ΔU6-GFP) inserted at the NheI/MluI site (magnification: 40X). GFP expression was completely suppressed by the GFP shRNA; **D.** Western blotting analysis of GFP expression in CEF infected with the control viruses RCASBP(A) and RCASBP(A)-shRNA-ΔU6 or the *egfp* shRNA expressing virus. The expression of p27 gag in the cell or recovered from the medium was also examined. Erk-1 was used as a loading control.

The expression of EGFP and Gag was followed for several passages after transfection of these retroviral constructs (Fig.14A). EGFP expression was sustained in cells transfected with the parental vector but remained undetectable in CEF expressing the *egfp* shRNA sequence. Gag expression was detected for the duration of the experiment with both retroviral vectors. We then determined if EGFP expression could be inhibited in these cells when expressed transiently from an unrelated vector (pGFP-N1). CEF infected with RCASBP (A) were used as control in this experiment to allow for detection of the transiently expressed EGFP in the absence of the shRNA. As shown in Fig. 14B, EGFP expression was reduced considerably in CEF infected by RCASBP-shRNA-ΔU6-*egfp* for more than six passages, suggesting that sustained inhibition was provided by the shRNA. This result also suggests that EGFP down-regulation was the result of RNA interference and was not limited to the retrovirally encoded GFP gene of RCASBP(A)-shRNA-ΔU6-*egfp*. We also examined the effect of shRNA expression on cell proliferation. CEF infected with RCASBP(A)-shRNA-ΔU6-*egfp* accumulated at levels and with kinetics comparable to those of RCASBP (A) or RCASBP-shRNA-ΔU6 infected CEF (Fig.14C). Therefore, the induction of RNA interference did not impair CEF proliferation.

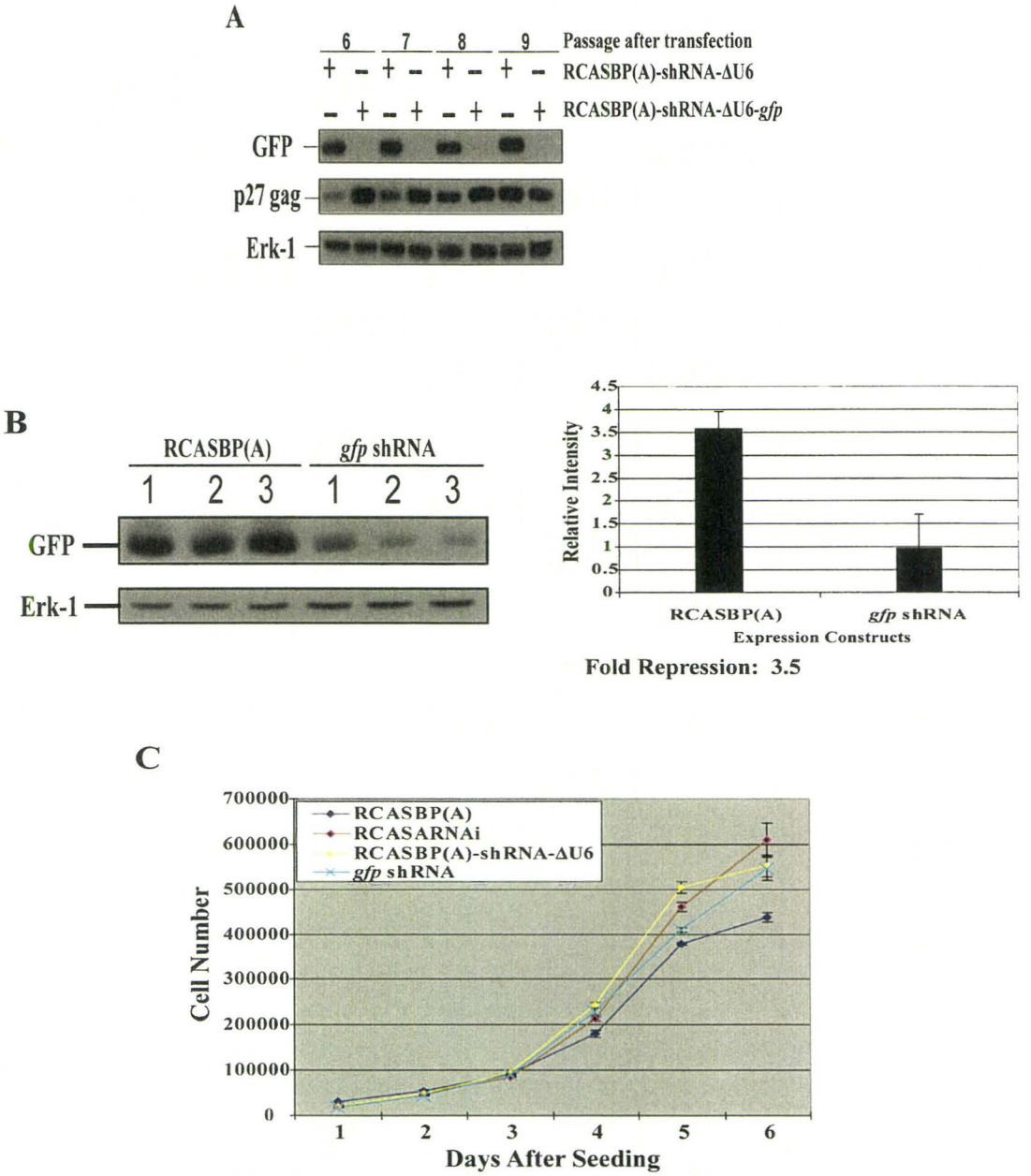


Figure 14. Sustained inhibition of EGFP expression by shRNA. **A.** EGFP expression in control and *egfp* shRNA expressing CEF. Protein lysates were prepared from the 6th passage after transfection of the control and retroviral vector expressing the *egfp* shRNA. Levels of EGFP and p27 gag, recovered from the tissue culture medium, were analyzed by Western blotting analysis. Erk-1 was used as a loading control; **B.** A EGFP expression plasmid (pGFP-N1) was transfected with pRSV-βGal at the 5th passage into CEF infected with the RCASBP(A) control vector or *egfp*-shRNA expression virus. pRSV-βGal was used to control for transfection efficiency. The results of triplicate samples are shown and quantitated. The inhibition of transiently expressed GFP was over 70% in CEF expressing the e*gfp* shRNA. **C.** The proliferation of CEF infected with the control vectors RCASBP(A), RCASBP(A)RNAi (Das *et al.*, 2006) and RCASBP(A)-shRNA-ΔU6 is compared to that of CEF expressing the *egfp* shRNA. Error bars represent the standard error from the mean.

Effect of Down-regulation of p105 NFκB1 by shRNA in normal and v-Src transformed CEF

NFκB controls the expression of gene products promoting cell survival and proliferation (Baldwin A. S. 2001, Guttridge *et al.,* 1999, Hinz *et al.,* 1999, Papa *et al.,* 2004). Previous studies indicated that two NFκB1 gene products accumulate in the v-Src transformed CEF: a full length protein p105 NFκB1 and a small form p50 (Cabannes *et al.,* 1997). To determine the role of this factor in CEF, we constructed a shRNA vector targeting p105 NFκB1. Lin and colleagues has demonstrated that p105 and p50 are generated by co-translational processing from the same mRNA, and thus they do not exhibit a precursor-product relationship (Lin L *et al.,* 1998). As a result, levels of p105/p50 NFκB1 were significantly down-regulated by *p105* shRNA in normal CEF (Fig.15A). Moreover, the down-regulation of p105/p50 NFκB1 decreased CEF proliferation (Fig.15B). Flat and often binucleated cells were observed in cultures of CEF expressing the *p105* shRNA, suggesting the presence of senescent cells (Fig.15C). This was confirmed with the senescence-associated β-galactosidase assay described by Dimri et al [SAβG; Fig.3D-E; (Dimri *et al.,* 1995)]. Therefore, the down-regulation of p105/p50 NFκB1 reduced normal CEF proliferation by causing, at least in part, premature entry into senescence.

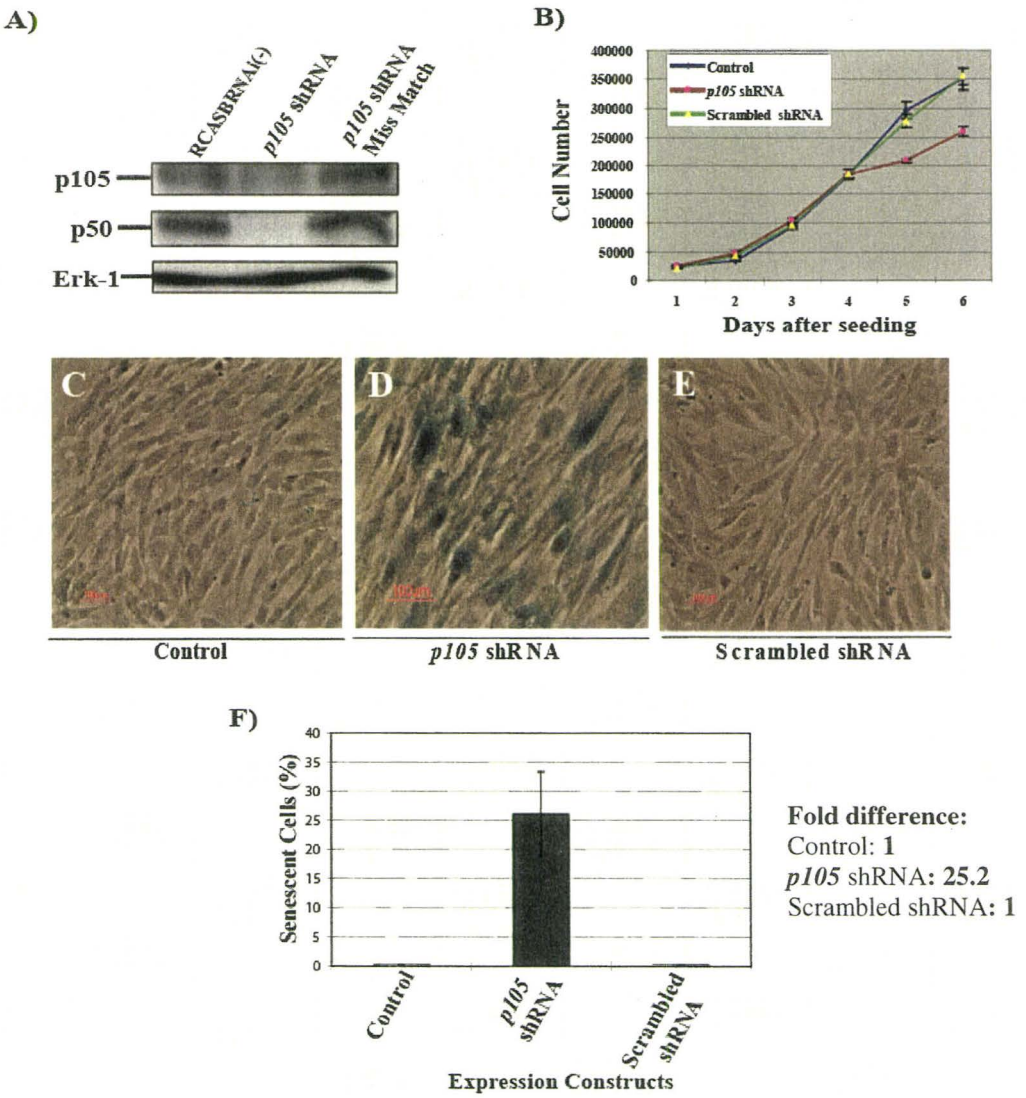


Figure 15. Effect of p105 NF-κB1 silencing in normal CEF. CEF expressing a *p105* shRNA were examined for proliferation and senescence-associated β-galatosidase (SA-βG) activity. **A.** Inhibition of p105/p50 by shRNA was examined by Western blotting analysis. The level of p105 and the processed form p50 were reduced by the shRNA, but not by the mismatch form of shRNA. Erk-1 was used as the loading control; **B.** Proliferation assays were carried out to investigate the effect of p105 inhibition on cell proliferation. Cells were seeded into 24-well plates 48 hours after transfection and counted every 24 hours for up to 6 days. Each transfection was counted in triplicates; **C-E.** CEFs expressing the *p105* shRNA were examined for the expression of SAβG activity. Quantitation shown below indicated that senescence was dramatically induced in the presence of the *p105* shRNA. CEFs expressing control vector or scrambled form of the *p105* shRNA didn't show any senescence phenotype. Image Magnification: 400X.

Transformation by the v-Src tyrosine kinase induces the activity of NFκB in CEF (Cabannes *et al.,* 1997). To determine the role of p105/p50 NFκB1 in v-Src transformation, we generated CEF co-infected with a temperature sensitive mutant of the Rous sarcoma virus (ts NY72-4 RSV) and a virus expressing the shRNA for *p105/p50 nfκb1*. Down-regulation of p105 and p50 NFκB1 was confirmed by Western blotting analysis (Fig.16A). This reduction in the level of the p105/p50 proteins decreased but did not abrogate the overall level of NFκB activity in the cells, suggesting that other members of the NFκB family are expressed and contribute to the activity of this factor in CEF (Fig. 16B). Upon transfer to the permissive temperature of 37.5˚C, control CEF displayed the features of transformed cells with a more refractile or round-up morphology (Fig. 16D). Senescent cells, positive for the SAβG assay, were detected in CEF expressing the *p105* shRNA at the non-permissive temperature of 41.5°C (Fig.16E). While some cells also appear to stain positively for SAβG at the permissive temperature, they lacked some of the features of senescent cells such as a flat morphology (Fig. 16F).

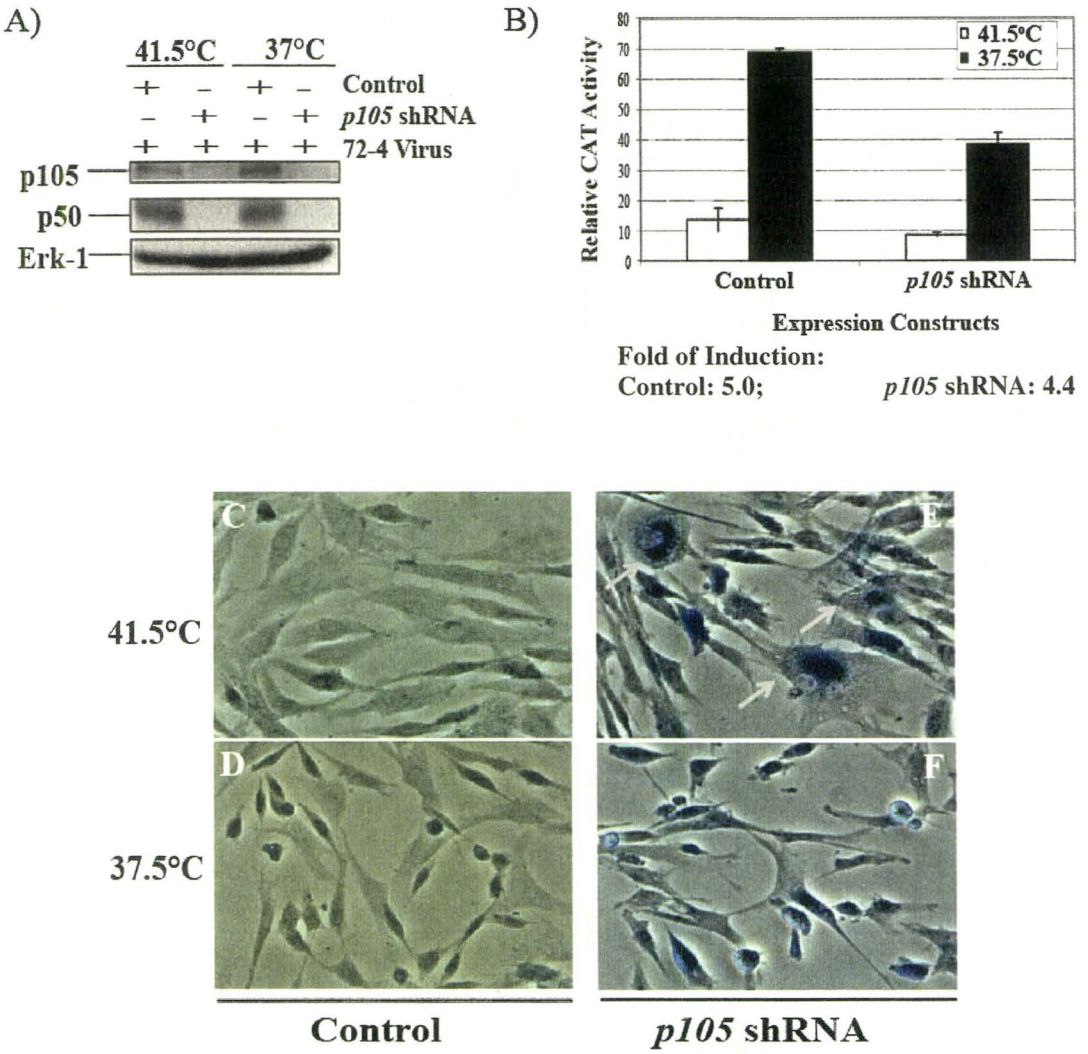


Figure 16 Effect of *p105* shRNA in RSV-transformed CEF. CEFs expressing a control virus or *p105* shRNA were superinfected with NY72-4 RSV, a temperature sensitive (ts) strain of RSV. **A.** Western blotting analysis of p105/p50 expression in these cells. Erk-1 was used as loading control. The expression of p105 and the processed form p50 was impaired by the shRNA; **B.** Transient expression assays were used to study the effect of p105 inhibition on NF κB activity in RSV-infected CEF; **C-F.** The SAβG activity of CEF expressing *p105* shRNA following v-Src induced transformation was examined under the same culture condition as panel A. Image Magnification: 100X.

Within 8-12 hours of transfer to the permissive temperature, cell numbers began to decline in CEF populations expressing the *p105/p50* shRNA (data not shown). As indicated by the TUNEL assay, this decline was the result of a high incidence of apoptosis (Fig. 17H). Indeed, apoptotic cells were detected primarily in CEF expressing the *p105/p50* shRNA at the permissive temperature but not in CEF maintained at the non-permissive temperature. These results suggest that p105 NFκB1 is required for the survival of v-Src transformed CEF.

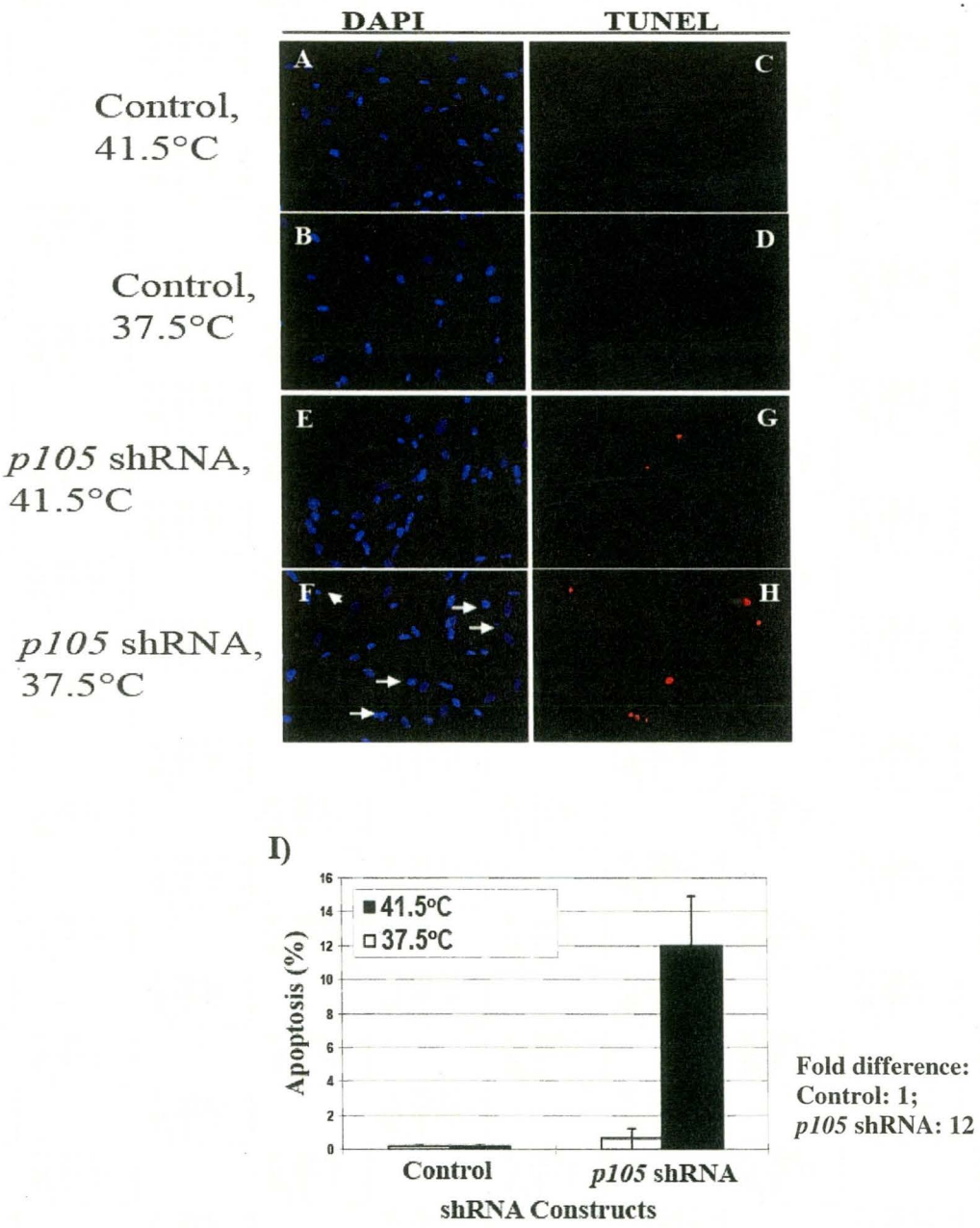


Figure 17. Effect of p105/p50 inhibition on the survival of v-Src transformed CEF. CEF expressing the control virus or *p105* shRNA were superinfected with NY72-4 virus, a temperature sensitive (ts) strain of RSV. **A-H.** TUNEL assay was performed to quantitate apoptosis. The inhibition of p105/p50 caused an increase in apoptosis at the permissive temperature. Nuclei of TUNEL positive cells showed condensation (arrowhead); **I.** Quantitation of TUNEL assays. Image Magnification: 40X

3. Summary

Gene down-regulation provided by RNA interference is an invaluable approach in the study of gene function. However, several biological processes, such as cell transformation, are required to alter gene expression in a sustained manner i.e. over several cell divisions. In this report, we describe the modification and validation of an avian replication-competent retroviral vector that is able to express a shRNA over several passages and the use this vector to define an essential role for p105 NFκB1 in the survival of primary cells transformed by *v*-Src. We showed that p105 down-regulation induces premature entry into senescence in normal chicken embryo fibroblasts (CEF) but causes apoptosis upon activation of a temperature sensitive mutant of v-Src. Therefore, p105 NFκB1 is required for the survival of v-Src transformed CEF. The availability of replication competent viruses for expression of shRNA opens several avenues of research on primary cells without requiring extensive period of selection in culture.

Chapter 5: Loss-of-function analysis of AP-1 members in v-Src transformed CEF

1. Introduction

Activated Ras and v-Src induce profound changes in the pattern of gene expression (Dehbi and Bedard 1992, Maślikowski *et al.,* 2010). These changes are regulated at multiple levels but are often dependent on the activation of transcription factors working cooperatively on promoter/enhancer regions. The significance of transcription factor activation is highlighted by the inhibitory effects that dominant negative mutants of Ets, Stat3 or AP-1 exert on cell 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). Separate groups reported that the inhibition of AP-1 by the expression of a deletion mutant of c-Jun lacking a trans-activation region (TAM67) interferes with transformation by RasV12 or v-Src. In addition, immortalized fibroblasts nullizygous for c-Jun cannot be transformed by these oncoproteins (Johnson R. *et al.,* 1996). However, these cells are still capable of inducing tumours in animals albeit with delayed kinetics. Cells recovered from these tumours were characterized by high levels of AP-1 activity resulting from transcriptional activation of the *junD* but never *junB* gene (Johnson R. *et al.,* 1996). These observations stress the importance of AP-1 activation in cell transformation by oncogenic Ras and tyrosine kinases.

Several mechanisms of AP-1 activation have been described before. Herschman and co-workers reported that *v*-Src controls the trans-activation potential of c-Jun by inducing the activity of the JNK/SAPK pathway in murine fibroblasts (Lu *et al.,* 1995). However, the activity of this pathway is high in normal chicken embryo fibroblasts (CEF)

and is only enhanced modestly in response to v-Src transformation, indicating that other pathways of AP-1 regulation are activated in these cells (Bojovic *et al.,* 1996). The Ras-dependent stimulation of ERK modulates the phosphorylation and activity of Fra-2 in RSV transformed CEF, in agreement with the notion that v-Src controls several aspects of the regulation of AP-1 (Murakami *et al.,* 1997). Similar studies performed on NIH 3T3 fibroblasts concluded that transformation by activated Ras is dependent on c-Jun/Fra-1 and the displacement of weaker trans-activators of the Jun family by the more potent c-Jun protein (Mathas *et al.,* 2002). Significantly, Yaniv and co-workers reported that the over-expression of Jun B and Jun D inhibits the transformation of NIH 3T3 cells by Ras, implying that these factors function as negative regulators or poor activators of AP-1 in these cells (Pfarr *et al.,* 1994). However, a different view is depicted by the studies of primary mouse embryo fibroblasts (MEF) harboring a disruption of the *c-jun* or *junD* gene. Indeed, *c-jun* or *junD* (-/-) MEF proliferate poorly and rapidly undergo premature senescence *in vitro*, suggesting that both gene products are required for normal proliferation of primary embryonic fibroblasts (Weitzman *et al.,* 2000, Wisdom *et al.,* 1999). Consistent with this notion, MEFs lacking JunD express elevated levels of p19Arf and p53, which likely triggers the entry into senescence (Weitzman *et al.,* 2000). The role of JunD is not restricted to the control of cell proliferation since *junD* (-/-) MEF are also hyper-sensitive to the action of TNFα and rapidly undergo apoptosis in response to this factor (Weitzman *et al.,* 2000).

We showed previously that AP-1 consists of three proteins, c-Jun, JunD and Fra-2 in RSV-transformed CEF (Wang *et al*, Manuscript in Appendix 2). JunD/Fra-2 is the

predominant form of the AP-1 complex in RSV-transformed CEF (N. Rodrigues, unpublished results; Wang *et al*, Manuscript in Appendix 2). In agreement with these results, both proteins were recruited to the v-Src responsive unit (SRU) of the IL8 promoter in response to v-Src transformation while c-Jun was not detected by ChIP assays in normal (RCASBP infected) and v-Src transformed CEF (SR-A RSV infected), confirming that the JunD/Fra-2 dimer is the predominant form of AP-1 in these cells (B. Maślikowski, unpublished results; Wang *et al*, Manuscript in Appendix 2). In this study, we characterize the effects of loss-of-function of individual AP-1 members, in particular c-Jun, JunD and Fra-2, in RSV-transformed CEF by using the modified shRNA expressing vector (RCASBP(B)-shRNA-ΔU6) described in Chapter 4. We also showed that the trans-activation potential of JunD was stimulated markedly in response to v-Src transformation, suggesting that JunD is a relevant target of v-Src transformation. The inhibition of AP-1 by a dominant negative mutant of c-Jun resulted in a high incidence of apoptosis in RSV-transformed CEF but not in their normal counterparts (Chapter 3). Down-regulation of c-Jun by shRNA induced senescence but no apoptosis in normal and v-Src transformed CEF. In contrast, the disruption of JunD expression caused a high incidence of apoptosis in *v*-Src transformed CEF, suggesting that the pro-survival activity of AP-1 depends on JunD. Down-regulation of Fra-2 expression by shRNA had no visible phenotype in normal CEF but induced the accumulation of lipid-rich vesicles in v-Src transformed cells. Therefore, AP-1 promoted cell transformation by acting as a survival factor, by inhibiting premature entry into senescence and by antagonizing the differentiation of v-Src transformed CEF.

2. Results

Different phenotypes are observed in response to the down-regulation of c-Jun, JunD and Fra-2 by shRNA in normal CEF

a. *Downregulation of c-Jun by shRNA*

It has been reported previously that activation of the *c-jun* gene is involved in cell cycle progression by directly activating *cyclinD1* gene expression and inhibiting the expression of p53 level (Schreiber *et al.,* 1999). In order to characterize the role of c-Jun in normal CEF, we generated 4 constructs expressing different *c-jun* shRNAs to test the inhibition efficiency of c-Jun expression. Expression of one of these *c-jun* shRNAs, RCASBP(B)-shRNA-ΔU6-*c-jun*842, caused a greater reduction in the level of the c-Jun protein, albeit incomplete (Fig.18A). Expression of *junD* shRNA did not interfere with the expression of c-Jun, indicating that the *c-jun* shRNA was specific. CEFs expressing this *c-jun* shRNA were flat and proliferated poorly. SAβG staining of CEFs expressing the *c-jun* shRNA revealed that CEFs underwent senescence when c-Jun expression was downregulated (Fig.18C). As a result, cell proliferation was significantly impaired upon c-Jun inhibition caused by this *c-jun* shRNA (Fig.18F). On the contrary, CEFs expressing a control vector or scrambled form of the *c-jun* shRNA were not positive for senescence staining (Fig.18B&D). Hence our results demonstrate that c-Jun is a positive regulator of cell proliferation by restricting the entry into premature senescence of primary CEF.

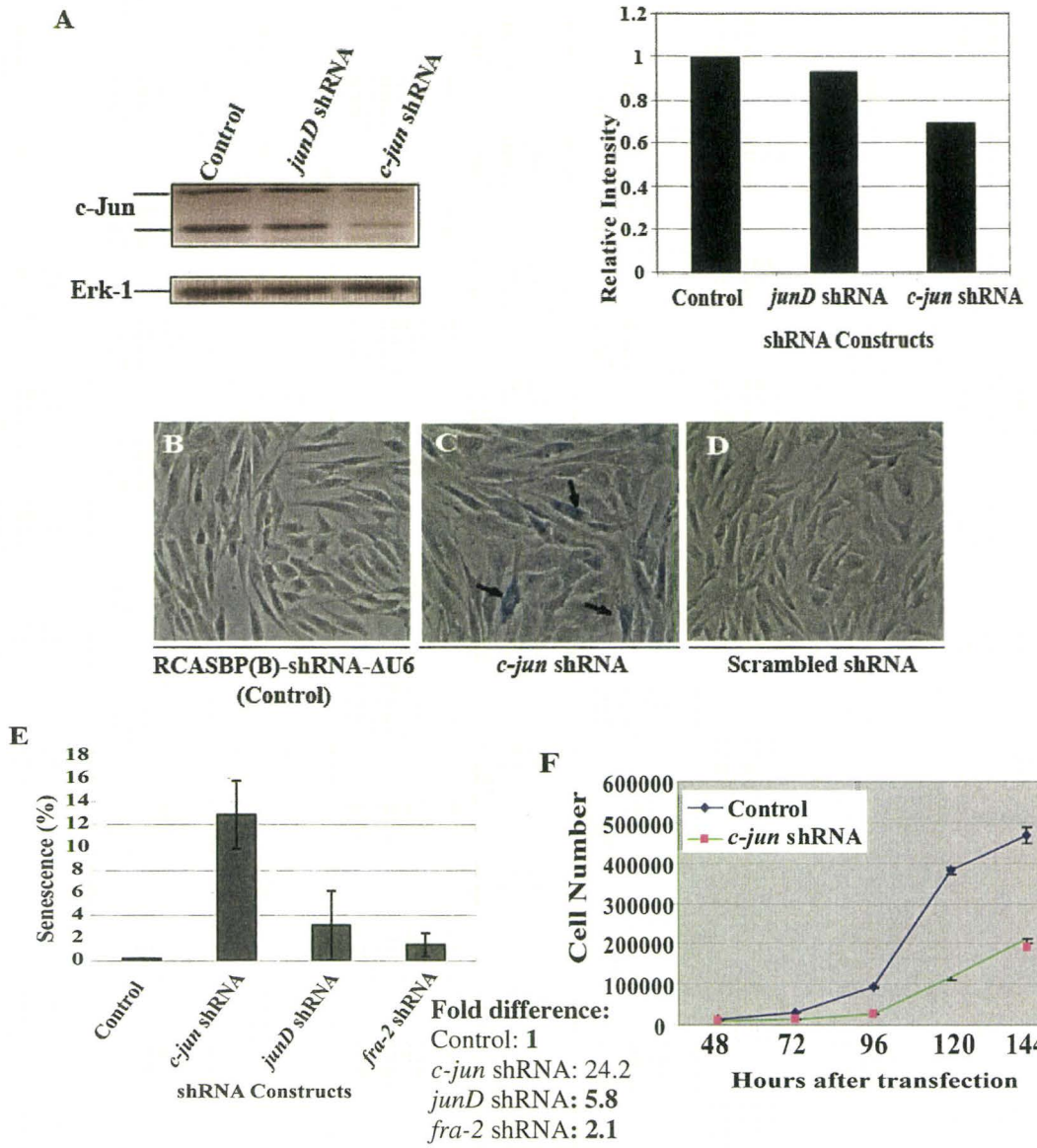


Figure 18. Effect of a *c-jun* shRNA in normal CEF. **A.** Western blotting analysis was performed using anti-c-Jun antibody (Santa Cruz). The level of c-Jun inhibition was determined by densitometry. The level of c-Jun in normal CEF was only partially down-regulated by *c-jun* shRNA. The Erk-1 was used as loading control; **B-D.** Senescence associated β-galatosidase (SAβG) assays were used to examine the senescence phenotype induced by *c-jun* shRNA (Arrowheads). Inhibition of c-Jun expression induced senescence in normal CEF (Panel D), while expression of a control vector or the scrambled form of *c-jun* shRNA did not express any SAβG activity. Image magnification: 100X; **E.** Incidence of senescence from panels B-D. Over 12% of normal CEFs underwent senescence upon c-Jun inhibition. The control vector or scrambled form of *c-jun* shRNA did not have an effect on cell morphology, and no induction of senescence was observed in these cells indicating the *c-jun* shRNA was specific; **F.** Proliferation assays showed that cell proliferation was impaired when c-Jun expression was reduced by shRNA.

b. *Characterization of the effect of JunD downregulation in normal CEF*

Four *junD* target sequences were tested and RCASBBP(B)-shRNA-ΔU6-*junD*821 was found to have the greatest efficiency of inhibition in normal CEF (Fig.19A and data not shown). We also observed that CEFs expressing this *junD* shRNA were more refractile and often displayed signs of membrane blebbing but were negative for SAβG staining (Fig.19B-D). TUNEL assays confirmed that the down-regulation of JunD increased the incidence of apoptosis (Fig. 19E-H). As a result, cell proliferation was impaired in these (Fig.19J). In contrast, little apoptosis was detected in CEF expressing the control vector or a shRNA for *c-jun* or *fra-2* (Fig.19I). CEF expressing the control shRNA vector or a scrambled form of *junD* shRNA sequence proliferate normally (Fig.19B and data not shown), suggesting that the effects of JunD down-regulation were specific.

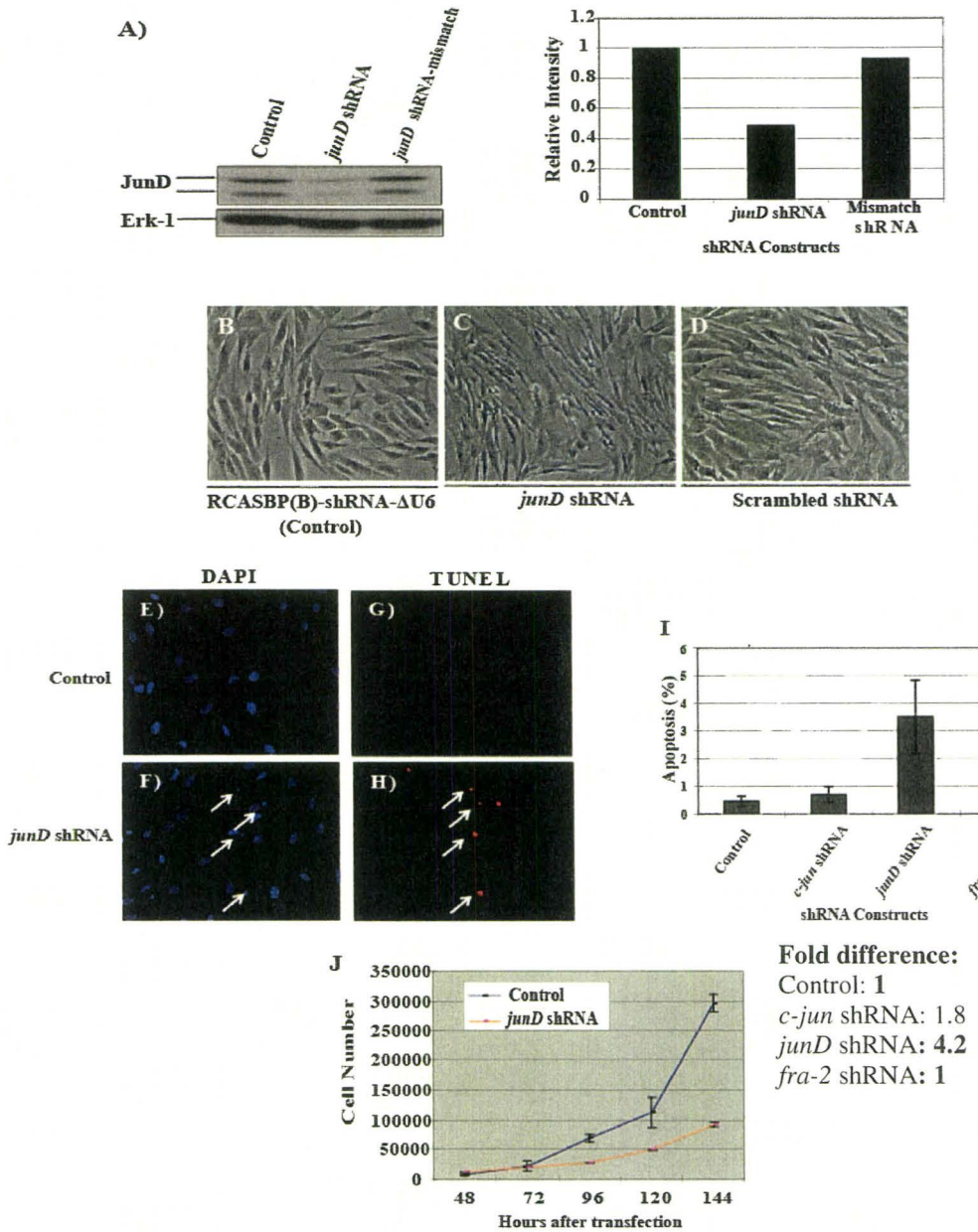


Figure 19. Loss of JunD expression promotes apoptosis in normal CEF. **A.** Western blotting was performed to examine the downregulation of JunD by shRNA. The level of JunD inhibition was determined by densitometry. Erk-1 was used as loading control; **B-D.** Senescence associated β-galatosidase (SAβG) assays were used to monitor the senescence phenotype induced by the *junD* shRNA. No senescence was observed in these cells; however, some cells displayed membrane blebbing; **E-H.** TUNEL assays were used to examine the incidence of apoptosis in normal CEF with or without expression of the *junD* shRNA. Nuclei of TUNEL positive cells showed condensation under the DAPI staining (Arrowheads); **I.** TUNEL-positive cells were quantitated and illustrated in the bar graph. Error bars represent the standard deviation from the mean; **J.** Proliferation assay was carried out to investigate the effect of JunD inhibition on cell proliferation.

c. *Effect of Fra-2 downregulation in normal CEF*

Unlike c-Jun and JunD, the reduction of Fra-2 expression in normal CEF exhibited modest effects on cell proliferation (Fig. 20A-B). These cells were also negative for the SAβG assays (Wang *et al.*, manuscript in Appendix 2) and TUNEL assays (Fig.19J and data not shown). Hence, Fra-2 expression does not appear to play a critical role in the proliferation of normal CEF.

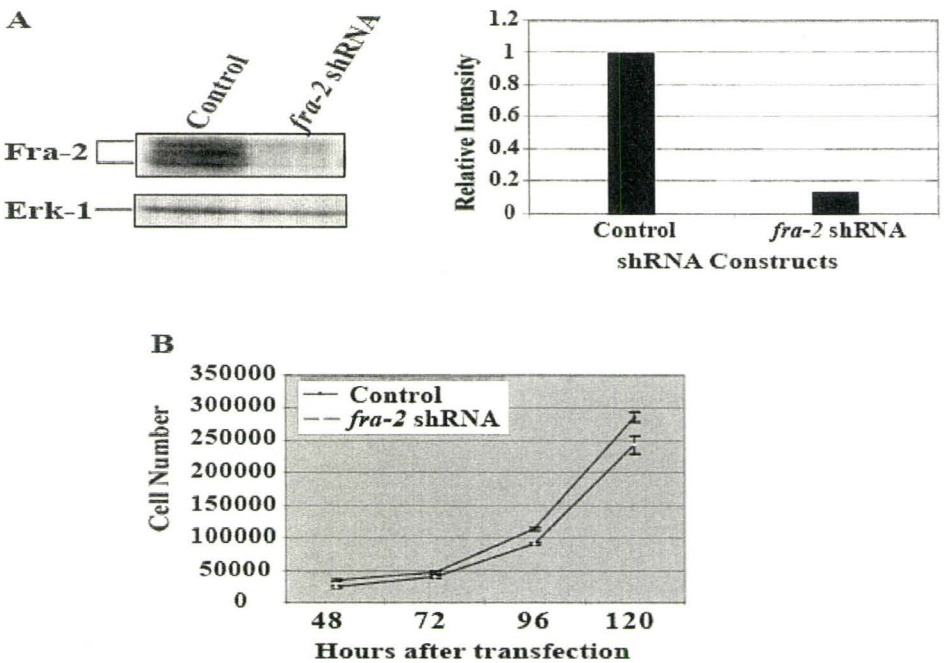


Figure 20. *fra-2* shRNA is not critical for proliferation of normal CEF. **A.** Western blotting analysis was performed to examine the effects of Fra-2 inhibition by shRNA. Erk-1 was used as loading control; **B.** Proliferation assay indicated that the loss of Fra-2 expression had modest effects on normal CEF proliferation.

Down-regulation of individual components of AP-1 is associated with a different phenotype in v-Src transformed CEF

To investigate the role of AP-1 in v-Src transformation, CEF expressing a shRNA for a component of AP-1 were super-infected with ts NY72-4, a temperature sensitive mutant of RSV, and the phenotype of these cells was characterized at the non-permissive (41.5°C) and permissive temperature (37°C).

a. Downregulation of c-Jun by shRNA

We have observed that c-Jun inhibition by shRNA impairs cell proliferation by inducing senescence in normal CEF. In ts.NY72-4 infected CEF, a partial inhibition of c-Jun expression was observed in CEF expressing the corresponding shRNA (Fig.21A). Morphologically, ts.NY72-4 infected CEF expressing *c-jun* shRNA were flat at the non-permissive temperature. SAβG assays confirmed the presence of senescence with fewer cells expressing the SAβG activity at the permissive temperature (Fig.21B-F). Since apoptosis was barely detected in these cells (date not shown), we reasoned that the reduced proportion of senescent cells at the permissive temperature was a result of increased proliferation and thus diluting the ratio of senescent cells (Fig.21F). Consistently, soft-agar assays confirmed that expression of *c-jun* shRNA did not interfere with the anchorage-independent proliferation of *v*-Src transformed CEF (Fig.21G-H). Moreover, transient expression assay showed that the *c-jun* shRNA only modestly affected the level of AP-1 activity (Fig.21I). This effect (or lack of) may reflect the fact that c-Jun is only a minor component of AP-1 or that the down-regulation is too partial to

have any effects. Several other target sequences were also examined but we consistently failed to see a significant down-regulation of c-Jun expression by these shRNA constructs (data not shown).

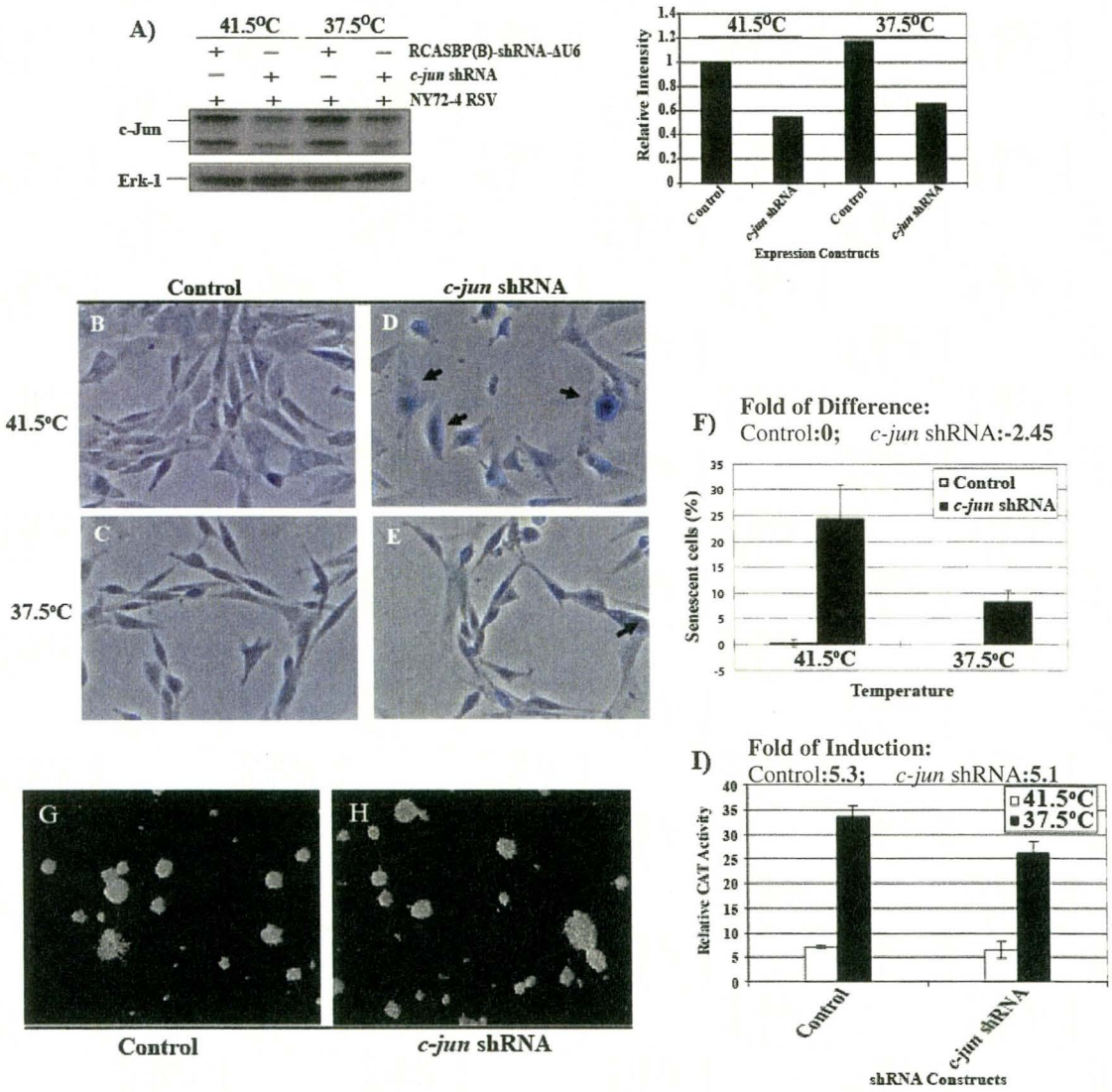
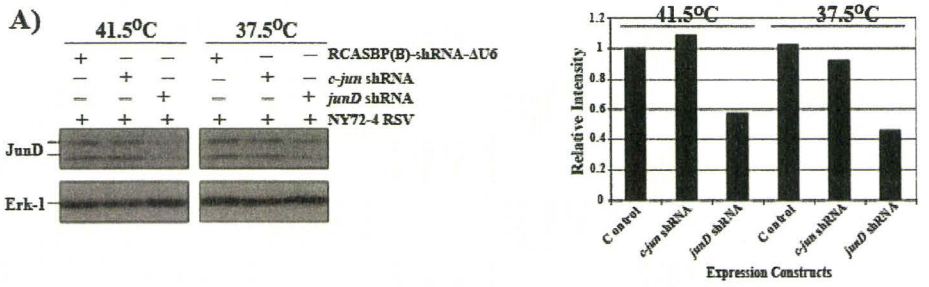
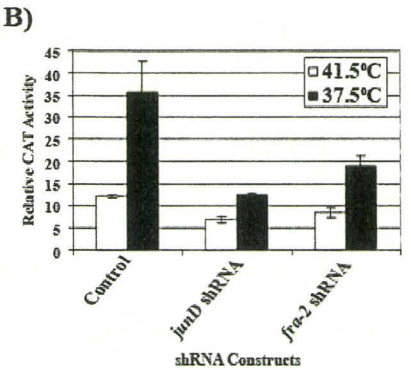


Figure 21. Reduction of c-Jun expression in ts.RSV-infected CEF. CEFs infected with a control virus or a virus expressing a *c-jun* shRNA were superinfected with 72-4 virus, a temperature sensitive (ts) strain of RSV, to examine the role of c-Jun downregulation in v-Src transformed CEF. **A.** Downregulation of c-Jun expression at both non-permissive temperature (41.5°C) and permissive temperature (37°C) was examined by Western blotting analysis. Densitometric analysis indicated that the expression of c-Jun was partially inhibited by shRNA. Erk-1 was used as a loading control; **B-E.** SAβG analysis was performed to examine the induction of senescence by *c-jun* shRNA at both non-permissive and permissive temperature. Senescence was induced by *c-jun* shRNA at the non-permissive temperature (**panel C**; Arrowheads); **F.** Quantitative results of cells expressing SAβG activity from panels C-F; **G&H.** Soft agar assays were carried out to study the effect of *c-jun* shRNA on the transformation ability of CEFs transformed by v-Src. Image magnification: 100X; **I.** Transient expression assays were used to study the effects of c-Jun inhibition on AP-1 activity in RSV-infected CEF.

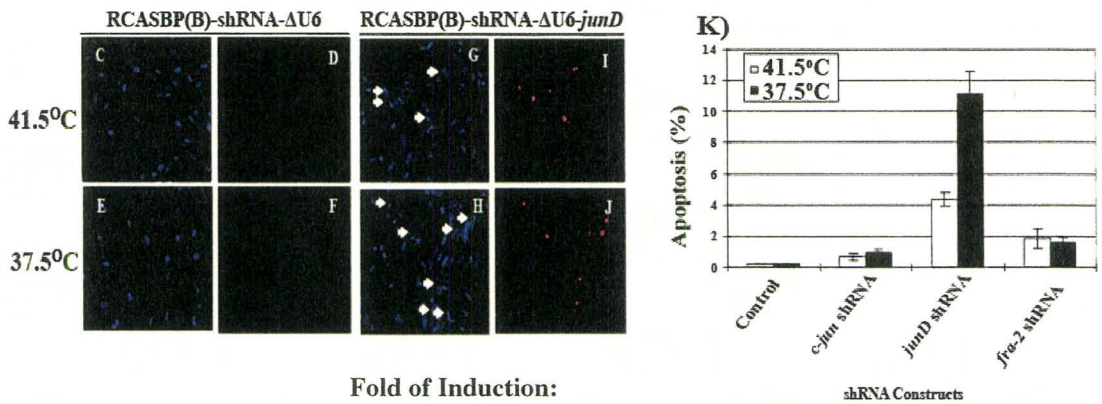
b. Characterization of the effect of JunD inhibition in RSV-transformed CEF

We also super-infected CEFs expressing the *junD* shRNA with a temperature sensitive strain of RSV, NY72-4, to investigate the role JunD in v-Src mediated transformation. The expression of JunD was partially reduced in CEF expressing the *junD* shRNA but not the c-Jun shRNA (Fig.22A). Transient expression assays demonstrated that reduced JunD expression repressed markedly the AP-1 activity of RSV-transformed CEF (Fig.22B). Importantly, JunD expression was also required for survival of *v*-Src transformed CEF since downregulation of Jun-D expression by shRNA caused apoptosis in ts.NY72-4 RSV-infected CEF (Fig.22C-J). This phenotype was enhanced by the activation of the temperature sensitive *v*-Src kinase at the permissive temperature (37.5°C), indicating that the requirement for JunD/AP-1 functions is greater in *v*-Src transformed CEF. A lower incidence of apoptosis was also detected in conditions of Fra-2 and c-Jun inhibition (Fig.22K). Therefore, JunD was important for the pro-survival activity conferred by AP-1 in v-Src transformed CEF.





Fold of Induction:
Control: **2.9**
JunD shRNA: **1.8**
fra-2 shRNA: **2.2**



Fold of Induction:
Control: **2.9**; *c-jun* shRNA:**1.1**; *junD* shRNA: **3.6**; *fra-2* shRNA: **1.1**

Figure 22. Reduced JunD expression is deleterious to ts.RSV-infected CEF. CEF infected with a control virus or a virus expressing the *junD* shRNA construct were superinfected with NY72-4 virus, a temperature sensitive (ts) strain of RSV. **A.** Western blotting was performed to verify the downregulation of JunD expression at both non-permissive and permissive temperatures. The expression of JunD was partially inhibited (Bar graph of band density). Erk-1 was used as a loading control; **B.** Transient expression assays were used to study the effects of JunD inhibition on AP-1 activity in RSV-infected CEF. Expression of the *junD* shRNA significantly downregulated AP-1 activity in v-Src transformed CEF; **C-J.** TUNEL assays were used to examine the incidence of apoptosis in ts.NY72-4 RSV-infected CEF expressing the *junD* shRNA construct. Nuclei of TUNEL positive cells showed condensed phenotype with the DAPI staining (white arrowheads); **K.** The percentage of TUNEL positive cells was determined and is presented for CEF expressing the *c-jun*, *junD* or *fra-2* shRNA. JunD down-regulation is characterized by a high incidence of apoptosis.

c. Downregulation of Fra-2 expression causes the appearance of lipid-rich vesicles in v-Src transformed CEF

Down-regulation of Fra-2 expression by shRNA did not interfere with the proliferation of primary CEFs, suggesting that Fra-2 does not contribute significantly to the activity of AP-1 in these cells. However, JunD and Fra2 are the major components of the AP-1 complex in *v*-Src transformed CEF, implying that Fra-2 might play a role in v-Src transformation (N. Rodrigues, unpublished observations; Wang *et al.,* Manuscript in Appendix 2). The expression of Fra-2 was significantly inhibited in CEF co-infected with ts.NY72-4 RSV and the *fra-2* shRNA (Fig. 23A). These cells were flat at the non-permissive and permissive temperature, and were not positive for the TUNEL and SAβG assays (data not shown), indicating that they were neither senescent nor undergoing apoptosis. However, cytoplasmic vesicles were observed at the permissive but not at the non-permissive temperature (Fig.23B-E). Oil-red-O staining confirmed that these vesicles were lipid-rich (Fig.23F-I). Transient expression assays indicated that AP-1 activity was significantly repressed by *fra-2* shRNA (Fig.22B). Therefore, Fra-2 was required for morphological transformation and to prevent the formation of lipid-rich vesicles in *v*-Src transformed CEF.

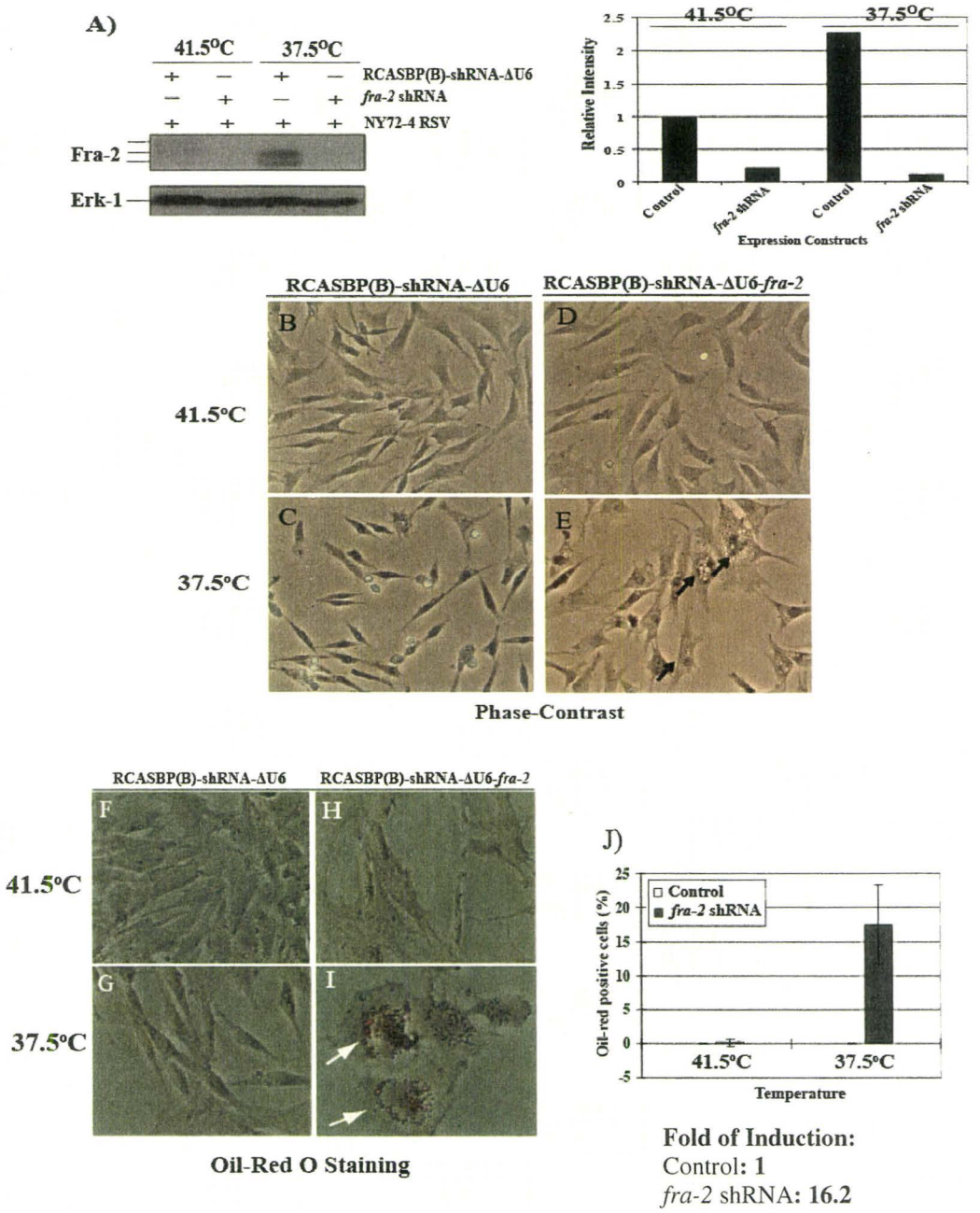
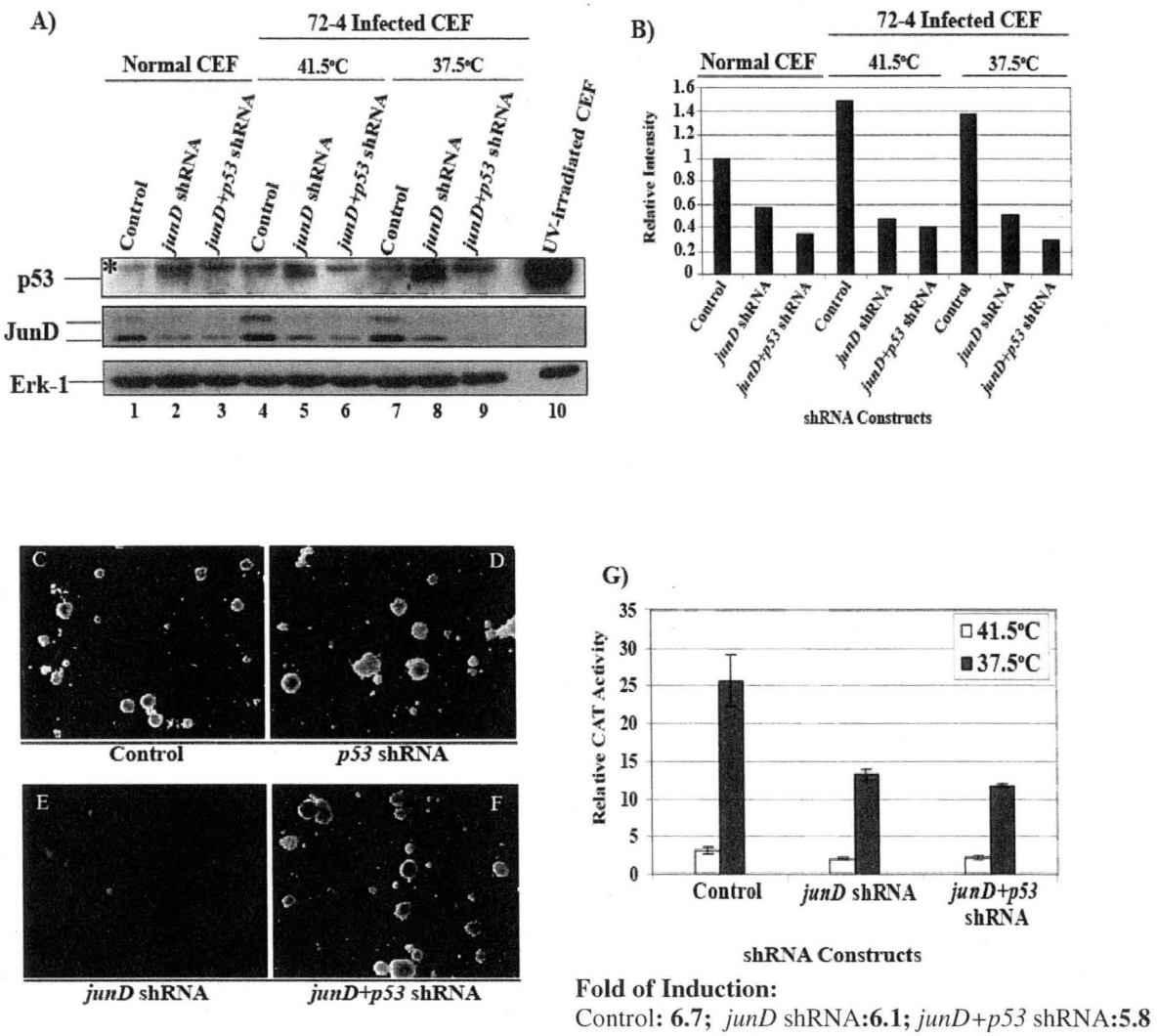


Figure 23. *fra-2* shRNA causes the accumulation of lipid-rich vesicles in v-Src transformed CEF. **A.**Western blotting analysis indicated that Fra-2 expression was significantly downregulated by shRNA. Erk-1 was used as a loading control; **B-E.** Accumulation of vesicles in the cytoplasm was observed in ts.NY72-4 RSV infected CEF expressing the Fra-2 shRNAi at the permissive temperature (37°C), but not in non-permissive temperature (41.5°C). Image magnification: 100X.; **F-I.** Oil-red-O staining confirmed the presence of lipid-rich vesicles in cells of panel E. Image magnification: 20X.; **J.** Quantitation of the fraction of cells with lipid-rich vesicles is provided in the bar graph.

Role of p53 in the apoptosis induced by JunD downregulation

Weitzman and colleagues have shown that mouse primary fibroblasts lacking JunD expression exhibits p53-dependent cell cycle arrest and senescence, and displays increased sensitivities to UV- or TNF-alpha treatment (Weitzman *et al.,* 2000). Consistently, we observed that down-regulation of JunD expression by shRNA induced apoptosis upon *v*-Src transformation (Fig.22). We then asked whether p53 (officially known as tp53) is also involved in the apoptosis caused by JunD inhibition. Western blotting analysis revealed that p53 accumulated in normal and ts.NY72-4 RSV-infected CEF in the presence of *junD* shRNA. This induction was enhanced upon *v*-Src activation at the permissive temperature (Fig.24A, Lane 8). The induction of p53 expression by UV-irradiation was used as a positive control in this experiment. These findings suggest that the apoptosis induced by JunD inhibition is p53-dependent. To address this question, we asked whether the inhibition of p53 could suppress the cell death triggered by the loss of JunD expression and re-establish transformation by *v*-Src. The microRNA operon expression cassette (MOEC) of the retroviral-based RNA interference system provides cloning sites for two separate target sequences, which is convenient to obtain the down-regulation of two different gene products in the same cell (Das *et al.,* 2006). Accordingly, we constructed a RCASBP(B)-shRNA-ΔU6 vector carrying target sequences of both *junD* and *p53*. Then we examined the role of p53 by co-infecting CEF with a virus expressing the shRNA for both *junD* and *p53* species and a temperature sensitive mutant NY72-4 RSV. The concomitant inhibition of JunD and p53 expression was successful in both normal and NY72-4-infected CEF (Fig. 24A). Importantly, CEFs with inhibition of

JunD and p53 were capable of forming colonies in soft agar, suggesting that they are able to growth in an anchorage-independent manner, an indicator of transformation (Fig.24C-F). AP-1 activity remained low in conditions of co-inhibition of JunD and p53 (Fig.24G). Therefore, the down-regulation of p53 expression protects *v*-Src transformed CEF from cell death induced by AP-1 inhibition.



Fold of Induction:
Control: **6.7;** *junD* shRNA:**6.1;** *junD+p53* shRNA:**5.8**

Figure 24. The apoptosis induced by *junD* shRNA is p53-dependent. **A.** Western blotting analysis was carried out to examine the induction of p53 by *junD* shRNA. The induction of p53 expression by UV-irradiation was used as a positive control (Lane 10). The asterisk points to a protein of lower mobility whose expression is not induced by UV irradiation or *p53* shRNA and therefore appears to be unrelated to p53; **B.** The inhibition efficiency of both the *junD* shRNA and the shRNA for JunD and p53 species was determined by densitometry; **C-F.** Soft agar assays were carried out to examine the transformation capability of v-Src transformed CEF in conditions of JunD and p53 inhibition. Colony formation was observed in v-Src transformed CEF expressing a control virus, *p53* shRNA or shRNA for JunD and p53 species (Panels C, D and F), but not in cells expressing *junD* shRNA (Panel E); **G.** Transient expression assay was used to study the effect of co-inhibition of JunD and p53 on AP-1 activity in CEFs co-infected by ts.NY72-4 RSV and a control virus, a virus expressing *junD* shRNA or a shRNA for JunD and p53 species. A similar inhibition of AP-1 activity was observed in v-Src transformed CEF expressing the *junD* shRNA and JunD/p53 shRNA.

3. Summary

Using a modified retrovirus system, we were able to investigate the function of individual components of AP-1 by stable down-regulation with shRNA. Loss of c-Jun expression impaired normal CEF proliferation, in part by inducing premature entry into senescence. This finding is in agreement with the previous conclusion that c-Jun is required for cell cycle progression in mouse embryo fibroblasts isolated from *c-jun* nullizygous embryos (Schreiber *et al.,* 1999). However, downregulation of c-Jun expression did not interfere with the transformation induced by v-Src, possibly as a result of insufficient down-regulation by shRNA. Silencing of JunD induced apoptosis even in normal CEF, albeit modestly. This incidence of apoptosis rapidly increased upon activation of ts.*v*-Src, indicating a pro-survival role of JunD in *v*-Src transformed CEF. On the contrary, lack of Fra-2 expression had little effect on the proliferation of normal CEF. This was also true in CEF infected with a temperature sensitive mutant of RSV at the non-permissive temperature, suggesting that Fra-2 expression is dispensable for normal CEF proliferation. On the contrary, upon *v*-Src transformation, the *fra-2* shRNA caused the accumulation of lipid-containing vesicles, but no senescence or apoptosis. Thus, c-Jun provided a block to senescence, JunD was required for cell survival and Fra-2 antagonized the formation of lipid vesicles, suggesting that each component of AP-1 fulfills a different function in v-Src transformed cells.

Weitzman and colleagues have shown that mouse primary embryo fibroblasts lacking JunD expression exhibit p53-dependent cell cycle arrest and senescence, and display increased sensitivities to UV- or TNF-treatment (Weitzman *et al.,* 2000).

Consistently, we observed an accumulation of p53 upon JunD inhibition and the accumulation was enhanced upon ts.*v*-Src activation at the permissive temperature (Fig.24A). This induction of p53 was correlated with the onset of apoptosis, suggesting that JunD is a survival factor in these cells. Significantly, co-inhibition of JunD and p53 re-established the capacity of v-Src transformed CEF to grow in soft-agar, suggesting that p53 is the inducer of apoptosis when JunD expression is inhibited. Therefore, the absence of JunD/AP-1 causes the induction of a p53-dependent pathway of apoptosis in *v*-Src transformed CEF.

Chapter 6: Discussion and perspective

1. AP-1 is regulated at multiple levels in *v*-Src transformed CEF

Electrophoretic mobility shift assays (EMSA) indicated that JunD and Fra-2 are the main components of AP-1 while c-Jun accounts for a minor fraction of this factor in v-Src transformed CEF (Wang *et al.*, Manuscript in Appendix 2). The expression of individual AP-1 members, in particular JunD and Fra2, was stimulated by *v*-Src. This accumulation accounted for the increase in DNA binding activity of AP-1 in v-Src transformed CEF observed in chromatin immunoprecipitation (ChIP) assay (Manuscript in Appendix 2). Hybrid proteins consisting of the c-Jun or JunD trans-activation domain (TAD) fused to the Gal4 DNA binding domain (DBD) were then expressed to examine the trans-activation potential of these factors (Fig.6). Our result showed that *v*-Src transformation had little effect on the c-Jun TAD but stimulated markedly the activity of the corresponding domain in JunD. In addition, the AP-1 activity was significantly enhanced in *v*-Src transformed CEF (Fig.6). Hence, v-Src stimulates the activity of AP-1, e.g. JunD/AP-1, by increasing the DNA binding capacity and transactivation potential of its constituents.

The AP-1 family is a major downstream effector of the MAPK pathways (Mechta *et al.*, 1997). Inhibition of the SAPK/JNK pathway by overexpression of a dominant negative mutant of SEK (SEKAL) reduced partially the activity of AP-1 in *v*-Src transformed CEF. A more significant suppression of AP-1 activity was observed when v-Src transformed CEF were treated with a specific inhibitor of MEK (PD98059), suggesting that the induction of AP-1 was dependent primarily on the ERK pathway

(Fig.6). The control AP-1 activity by ERK-dependent pathway remains to be elucidated (also see section 8.1).

2. Role of AP-1 in normal and *v*-Src transformed CEF

TAM67 is an N-terminal transactivation domain deletion mutant of c-Jun that retains a functional DNA binding domain and dimerization domain known as the bZIP domain (basic region and leucine zipper). Inhibition of proliferation of normal CEF by TAM67 is correlated with the down-regulation of cyclinD1 level (Gagliardi *et al.,* 2003), suggesting a mechanism by which AP-1 likely promotes cell proliferation. Our results indicated that CEFs stably expressing TAM67 exhibit reduced level of individual AP-1 members, e.g. c-Jun, JunD and Fra-2 and undergo senescence (Fig.7). It has been reported previously that c-Jun (-/-) MEF undergo premature entry into senescence *in vitro* (Schreiber *et al.,* 1999). Likewise, JunD (-/-) MEF undergo premature entry into senescence in culture (Weitzman *et al.,* 2000), suggesting that AP-1 is pro-proliferative in primary fibroblasts. Moreover, the induction of senescence in these cells is p53-dependent. We failed to detect the p53 expression in normal CEFs expressing TAM67 (data not shown). However, we cannot rule out the possibility that there is an induction in p53 levels in senescent cells since only a fraction of the whole cell population underwent senescence. Therefore, the increase in p53 levels may be insufficient to be detected. The same reason may be also true for the observation that a reduction of c-Jun expression by shRNA did not induce p53 in CEF (data not shown). On the contrary, we detected the induction of p53 in CEF when JunD expression was inhibited by shRNA (Fig.24). These results will

be discussed extensively in section 6.

CEFs co-expressing TAM67 and a temperature sensitive strain of RSV, LA90, was used to study the effects of AP-1 inhibition upon *v*-Src activation. Transient expression assays confirmed that AP-1 activity is dramatically repressed by TAM67 at both non-permissive (41.5°C) and permissive temperature (37.5°C). In addition, senescence was induced primarily by TAM67 at the non-permissive temperature (Fig.8). Interestingly, three distinct phenotypes were present at the permissive temperature. First, SAβG assays revealed that a proportion of the cells were undergoing senescence, suggesting that AP-1 is essential for the proliferation of either normal or v-Src transformed CEF. Second, a high incidence of apoptosis was observed upon activation of a temperature sensitive *v*-Src kinase (Fig.9&10). Finally, in addition to apoptosis, a proportion of the RSV-transformed CEFs expressing TAM67 were transformation defective and developed vesicles in the cytoplasm, but were negative in the TUNEL or SAβG assays. Instead, this phenotype was reminiscent of the lipid droplets found in adipocytes. Staining these cells with Oil Red O confirmed that the vesicles were lipid-rich (Fig.11). We failed to detect the induction of p20K (Fig.11), a lipid-binding protein of the lipocalin family and a quiescence-specific protein (Bedard *et al.,* 1989), in these cells suggesting that these cells were not in a state of reversible growth arrest. Collectively, AP-1 activity was required for not only maintaining the survival and proliferative capacity of *v*-Src transformed CEF, but also for inhibiting differentiation. Previously, Iba's group reported that expression of TAM67 impairs the transformation induced by *v*-Src without addressing the mechanism (Suzuki *et al.,* 1994). Our results showed that AP-1 is required to maintain cell survival upon v-

Src-induced transformation in CEF. Therefore, the reason why Iba and co-workers failed to report any senescence or apoptosis may be due to the poor expression of TAM67 controlled by older and weaker RCAS vectors and a lack of a sufficient repression of AP-1 activity.

3. The pleiotropic effects of TAM67 in *v*-Src transformed CEF

TAM67 interacts with all proteins of the AP-1 family and leads to inhibition of AP-1 activity (Brown PH *et al.,* 1994). Consistently, expression of TAM67 dramatically inhibited the AP-1 activity in *v*-Src transformed CEF (Fig.6). In addition, it can associate with C/EBPβ and proteins of the NFκB family via the bZIP domain (Stein *et al.,* 1993b). This direct interaction results in the down-regulation of the NFκB activity (Li *et al.,* 2000). NFκB is a family of dimeric transcription factors sharing a conserved Rel-homology domain. Activation of NFκB has been linked to many aspects of oncogenesis and, in particular, cell survival (Kucharczak *et al.,* 2003). In addition, NFκB activity was elevated upon *v*-Src transformation in CEF (Fig.12) and was required for the survival of these cells (Fig.19). Moreover, expression of TAM67 repressed NFκB activity in *v*-Src transformed CEF. Thus, induction of cell death by TAM67 in RSV-transformed CEF might be, in part, due to the loss of an effective NFκB complex.

Surprisingly, overexpression of TAM67 enhanced the C/EBPβ activity in v-Src transformed CEF (Fig.12). C/EBPβ is a key regulator of adipogenesis when overexpressed in fibroblasts including CEF (Kim S. *et al.,* 1999, Mandrup and Lane 1997). In our study, we observed the presence of lipid-rich vesicles in the cytoplasm of

CEFs co-expressing activated *v*-Src and TAM67 (Fig.9). Recent profiling analyses revealed that known targets of C/EBPβ, including IL6 and PPARγ, are induced in TAM67 expressing v-Src transformed CEF (our unpublished results). Since PPARγ is an important modulator that positively regulates the adipogenesis process (Kim S. *et al.,* 1999, Mandrup and Lane 1997), the inhibition of AP-1 may lead to the induction of PPARγ expression and adipogenesis by C/EBPβ in a fraction of the CEF population. Whether or not the induction of PPARγ expression and formation of lipid vesicles is dependent on C/EBPβ in v-Src transformed cells remains to be confirmed.

Collectively, we observed that TAM67 inhibits both AP-1 and NFκB activity but stimulates the expression of the C/EBP reporter gene (Fig.12). As a result, the activity of the *IL8* promoter, which contains one AP-1, one NFκB and two C/EBP binding sites (Bojovic *et al.,* 1996), was partly reduced by TAM67 in RSV-transformed CEF. Hence, the pleiotropic effects of TAM67 may reflect the interaction with different transcription factors including AP-1, NFκB and C/EBPβ. Loss-of-function studies on individual members of AP-1 are thus required for the characterization of a precise role of AP-1 in *v*-Src transformation.

4. Gene inhibition by virus-based shRNA expression system

Avian retroviruses for expression of shRNA have been described before (Bromberg-White *et al.,* 2004, Chen M *et al.,* 2007, Das *et al.,* 2006, Harpavat and Cepko 2006). However, little information exists on the efficacy of these vectors and their capacity to silence gene expression in a sustained manner, a condition required for studies on cell

proliferation and transformation. Using existing RCASBP vectors expressing shRNA from an internal PolIII promoter, we failed to observe prolonged down-regulation of several transcription factors of the AP-1 or NFκB family in chicken embryo fibroblasts (CEF; Our unpublished results). Similar conclusions were reached by other investigators (Chen M *et al.,* 2007). In the vector designed by Das and co-investigators, a shRNA is expressed in the context a chicken microRNA under the control of a U6 promoter. We reasoned that transcriptional interference caused by the strong viral LTR or hindrance by the splicing machinery interfered with the expression of the shRNA. Since microRNA genes are transcribed by RNAPII, we deleted the U6 promoter and leader sequence to generate plasmid RCASBP(A)-shRNA-ΔU6 and RCASBP(B)-shRNA-ΔU6. In this vector, the shRNA sequence is cloned downstream of a splicing acceptor site and *egfp* gene (Fig.13). Since expression of the viral genome can be monitored through GFP expression, we used the modified RCASBP vector to express a shRNA sequence targeting this gene and followed GFP expression in CEF. Silencing of GFP expression was observed with limited effects on p27 gag protein expression and viral propagation. This observation suggests that the genomic viral RNA, which includes the *egfp* hairpin, was at least partially protected from degradation perhaps as a result of the interaction with viral proteins and efficient packaging into viral particles. This was observed in conditions where GFP was no longer detectable by either immunofluorescence or Western blotting analysis, indicating that the production of viral particles was not the result of inefficient RNA silencing (Fig.13). The persistence of the silencing effect lasted up to nine passages after transfection, i.e. for the entire duration of the experiment,

demonstrating that RNA interference was stable. Ectopic expression of GFP, following transfection of a CMV promoter/enhancer expression plasmid at the fifth passage after transfection of the RCASBP(A)-shRNA-ΔU6-*egfp* vector that expressing a *egfp* shRNA , also resulted in a reduction in GFP expression, indicating that gene silencing was a consequence of RNA interference (Fig.14). Moreover, induction of RNA interference, provided by the RCASBP(A)-shRNA-ΔU6-*egfp* vector, had no effects on CEF proliferation and did not interfere with morphological transformation by ts NY72-4 RSV (Fig.15 and data not shown). Patterns of phospho-tyrosine containing proteins were also unaltered by the induction of RNA interference, indicating that the presence of a shRNA-expression retroviral vector in the cell did not affect cell transformation by RSV (unpublished data). The development of the modified vector RCASBP(A)-shRNA-ΔU6 and RCASBP(B)-shRNA-ΔU6 proved to be an invaluable tool in the study of gene expression in primary cells, such as CEF. Thus far, it has been used extensively in our laboratory to induce gene-specific down-regulation.

5. NFκB1 p105 is required for proliferation in normal CEF and survival for *v*-Src transformed CEF

The NFκB transcription factor is a dimeric complex of RelA/p65, RelB, cRel, p52 NFκB2, p50 NFκB1 or Bcl-3 proteins. Activation of NFκB has been linked to transformation and survival (Kucharczak *et al.,* 2003). It has been reported that pp60*v*-src stimulates the transcription of two gene products of *NFκB1*, the full length p105 and smaller form p50 in CEF (Cabannes *et al.,* 1997), implying a role of NFκB1 in *v*-Src

induced transformation. Since p105 and p50 are generated by co-translational processing from the same messenger RNA (Lin L *et al.,* 1998), inhibition of p105 expression by shRNA would also result in the down-regulation of p50 level. Our results indicated that the expression of both proteins was dramatically inhibited (Fig.15&16). The inhibition of p105/p50 impaired cell proliferation by inducing senescence in normal CEF, whereas cell proliferation was not affected by the scrambled form of this shRNA (Fig.15). Transient expression assays revealed that the NFκB activity was reduced by the *p105* shRNA in *v*-Src transformed CEF (Fig. 15). Consistently, we observed that the inhibition of NFκB1 p105 expression efficiently induced apoptosis in *v*-Src transformed CEF (Fig.16). Hence, NFκB1 p105 was also important for maintaining the survival of *v*-Src transformed CEF. Genes induced by *v*-Src in an NFκB dependent manner remain to be identified. Anti-apoptotic proteins such as Bcl-xL, NR13 and cellular inhibitors of apoptosis (cIAP) are known targets of NFκB (Coll *et al.,* 2002, Gillet *et al.,* 1995, Greten and Karin 2006), which reflects a positive role of NFκB in cell survival. However, these factors are not induced by *v*-Src in CEF (Maślikowski *et al.,* 2010). Therefore, other genes controlled by this factor must account for the pro-survival activity of NFκB in v-Src transformed CEF.

6. Distinct functions of individual AP-1 members in v-Src transformed-CEF

The expression of TAM67 caused the appearance of several phenotypes in v-Src transformed CEF. Surprisingly, each phenotype was ascribed to a different component of AP-1 as the downregulation of c-Jun caused senescence, the inhibition of JunD induced apoptosis and the reduction of Fra-2 levels resulted in the accumulation of lipid-rich

vesicles but no senescence or apoptosis. These results imply a certain specificity in the action of each component of AP-1. Alternatively, the phenotypes may simply reflect a different degree of AP-1 inhibition and a different contribution to the activity of this factor by c-Jun, JunD and Fra-2.

6.1 Expression c-Jun is required for normal CEF proliferation

c-Jun is involved in cell cycle progression by directly activating the *cyclinD1* gene and inhibiting p53 (Schreiber *et al.,* 1999). Accordingly, inhibition of c-Jun expression by shRNA may induce growth arrest and senescence due to prolonged cell division inhibition and p53 activation. Senescence associated β-galactosidase (SAβG) staining confirmed that CEFs undergo senescence when c-Jun expression was down-regulated in normal CEF (Fig.18), while CEFs expressing a control vector or scrambled form of the shRNA were not positive for senescence staining. Hence our results are in accordance with the previous finding that c-Jun is a positive regulator for cell proliferation. However, we failed to detect the induction of p53 in these cells and in CEFs expressing TAM67 (data not shown), which might be a result of the partial down-regulation of c-Jun or insufficient induction of p53. Interestingly, Gillespie's group has reported that c-Jun (-/-) MEF could proliferate normally when cultured in conditions of mild hypoxia [3% O_2; (MacLaren *et al.,* 2004)]. DNA damage was evident in these cells, suggesting that the elevated level of DNA damage is the result of hyperoxic stress caused by the laboratory conditions. Thus it would be interesting to ask whether CEFs expressing the *c-jun* shRNA also behave similarly. In this case, level of DNA damage will also be investigated in

these cells. This result may reveal a role of c-Jun in the hyperoxic stress response in CEF.

We also studied the effect of c-Jun inhibition in RSV-transformed CEF. Transient expression assays showed that loss of c-Jun expression weakly interfered with AP-1 activity (Fig.21). Moreover, soft-agar assays indicated that expression of the *c-jun* shRNA did not interfere with the anchorage-independent growth of *v*-Src transformed CEF (Fig.21). These findings may be the results of a partial, inefficient down-regulation of c-Jun (Fig.21). Since c-Jun down-regulation induced senescence in normal CEF, the partial inhibition may reflect the counter-selection (elimination) of CEF with strong c-Jun inhibition. Significantly, a partial inhibition was consistently observed with 4 other *c-jun* shRNA constructs, suggesting that the absence of c-Jun gene function is not tolerated in CEF. For this reason and the fact that c-Jun is not the predominant component of AP-1 in *v*-Src transformed CEF, we decided not to pursue the study of this factor in v-Src transformed CEF.

6.2 JunD is required for the survival of both normal and v-Src transformed CEF

In normal CEF, the reduction in JunD expression was characterized by the inhibition of cell proliferation and the onset of apoptosis in a small fraction of the cells (Fig.19). In addition, we have been able to show that inhibition of JunD expression resulted in a marked reduction of AP-1 activity in RSV-infected CEF (Fig. 22), implying that JunD is an important component of AP-1 in RSV-infected CEF. Moreover, JunD inhibition was deleterious in these cells and the incidence of apoptosis was greater than that in normal CEF (Fig.22), suggesting that the expression of JunD was critical for the survival of *v*-Src

transformed cells. We also showed that the expression of TAM67 down-regulated NFκB activity in these cells, an effect which may also account for the induction of apoptosis by TAM67. However, transient expression assay confirmed that the expression of *junD* shRNA did not interfere with the NFκB activity in *v*-Src transformed CEF (data not shown), suggesting that the effects of *junD* shRNA were specific. Taken together, JunD was crucial to maintain cell survival in both normal and *v*-Src transformed CEF. Mouse primary fibroblasts lacking JunD expression led to the accumulation of $p19^{ARF}$ and p53, resulting in cell cycle arrest and senescence (Weitzman *et al.,* 2000). These cells were also hypersensitive to UV- or TNF-alpha-treatment. Consistently, we observed the induction of p53 in CEFs expressing the *junD* shRNA (Fig.24). The induction of p53 as well as the incidence of apoptosis became prominent in the presence of a transforming *v*-Src. However, we did not observe senescence in CEFs upon JunD inhibition. This discrepancy may reflect species-specific differences or the conditions of culture of MEF and CEF. Furthermore, we failed to detect the accumulation of chicken ARF ($p7^{ARF}$) using antibodies generated by other investigators (data not shown). Since these antibodies are the only ones available for chicken Arf, it is difficult to conclude if the failure to detect $p7^{ARF}$ indicates a lack of induction of this protein or reflects the poor quality of this reagent. It is important to note that ARF is poorly conserved and that chicken encodes an ARF protein that is significantly smaller (7kDa) than the human ($p14^{ARF}$) and murine counterparts [$p19^{ARF}$; (Kim SH *et al.,* 2003)]. However, this chicken form of ARF is functionally similar to its human counterpart playing a role in the regulation of p53 (Kim SH *et al.,* 2003, Moulin *et al.,* 2008). Therefore, the involvement of chicken ARF in the

p53-dependent apoptosis induced by JunD inhibition in CEF remains to be investigated. Dual inhibition of JunD and p53 by shRNA restored the anchorage-independent growth property of *v*-Src transformed CEF (Fig.24), as indicated by colony formation in soft-agar. Therefore, the apoptosis observed in conditions of JunD inhibition was p53-dependent. This is also supported by the observation that the inhibition of JunD is more prominent in CEFs expressing shRNA for both *junD* and *p53* than that expressing the *junD* shRNA alone (Fig.24), suggesting that a high degree of JunD inhibition is not tolerated in CEF. Therefore, the co-inhibition of p53 allows for a more efficient repression of JunD in these cells. Hence, JunD is a survival factor for CEF.

6.3 Fra2 provides a block to differentiation

Loss of Fra-2 expression did not affect the proliferation and survival of normal CEF (Fig.20), which is in agreement with the observation that Fra-2 is poorly expressed in these cells. A previous study has demonstrated that the transactivation domain of Fra-2 is strongly activated in response to *v*-Src transformation (Murakami *et al.*, 1997). In addition, we observed that the expression of Fra-2 was strongly stimulated by v-Src (N. Singh, personal communication and manuscript in Appendix 2), suggesting that increased Fra-2 activity is important for *v*-Src transformation. In agreement with this notion, we observed that the down-regulation of Fra-2 expression by shRNA reduced AP-1 activity in *v*-Src transformed CEF (Fig.22). This result suggests that the predominant form of AP-1 of v-Src transformed CEF is the JunD/Fra-2 heterodimer. Moreover, v-Src transformed CEF expressing the *fra-2* shRNA were flat and morphologically non-transformed

(Fig.23). Soft-agar assays revealed that these cells were not able to form colonies in soft-agar, indicating that inhibition of Fra-2 expression impairs the capacity of *v*-Src transformed CEF to grow in an anchorage-independent manner, a marker of transformation (Appendix 1, Fig. 27). However, apoptosis was not detected in these cells (Fig. 22), probably due to the presence of JunD and the formation of AP-1 complexes containing JunD and c-Jun. Instead, lipid-rich vesicles accumulated in the cytoplasm of these cells (Fig.23), but not in *v*-Src transformed CEF expressing a shRNA for other transcription factors such as JunD, c-Jun and NFκB1 p105 (data not shown). In addition, Fra-2 is unlikely to bind DNA in the conditions of JunD inhibition due to the fact that Fra-2 is unable to form homodimers and must interact with a member of the Jun family to bind DNA (Eferl R and Wagner 2003). However, lipid-rich vesicles were not observed in condition of JunD inhibition (our unpublished results). This observation may reflect the fact that cells are committed to apoptosis in the absence of JunD and are therefore unable to differentiate in the absence of Fra-2. Interestingly, we detected vesicles formation in a fraction of the *v*-Src transformed CEF with co-inhibition of JunD and p53 by shRNA (data not shown). Gene profiling studies in our laboratory led to the observation that PPARγ expression is induced by TAM67 upon *v*-Src transformation, an observation that might account for the lipid-vesicle formation (data not shown). Whether or not the induction of PPARγ expression is involved in the formation of lipid vesicles upon Fra-2 inhibition in v-Src transformed cells requires further investigation. More importantly, these results suggest that v-Src can stimulate pathways controlling lipid vesicles formation, a process antagonized by the presence of Fra-2/AP-1.

7. Consequences of AP-1 inhibition in *v*-Src transformed CEF

7.1 Involvement of C/EBPβ in the response to AP-1 inhibition

v-Src transformed CEF stably expressing TAM67 underwent apoptosis, implying that AP-1 is required for v-Src-dependent viability in CEF (Chapter 3). We have demonstrated that in addition to apoptosis, a proportion of the *v*-Src-transformed CEFs expressing TAM67 were flat and developed lipid-containing vesicles in the cytoplasm. Transient expression assays indicated that C/EBPβ activity was elevated in RSV-transformed CEF in the presence of TAM67. Therefore, it is possible that the formation of lipid-rich vesicles reflects the stimulation of C/EBPβ activity in these conditions. A mutual antagonism between AP-1 and C/EBPβ has been reported previously. Indeed, overexpression of a dominant negative form of C/EBPβ, Δ184-C/EBPβ, induced the expression of three major AP-1 members (c-Jun, JunD and Fra2) and stimulated the expression of cyclinD1 in CEF (Gagliardi *et al.,* 2003). In quiescent CEF, AP-1 activity is reduced markedly while C/EBPβ activity remains high and is required for the expression of p20K, a marker for reversible growth arrest in CEF. Moreover, overexpression of c-Jun, JunD and Fra-2 blocks the induction of the p20K lipocalin by C/EBPβ at high cell density (Gagliardi *et al.,* 2003). In these conditions, CEF are no longer contact-inhibited and rapidly undergo apoptosis, possibly as a result of starvation. Therefore, AP-1 and C/EBPβ cooperate in the induction of genes of the G0-G1 transition, such as IL8, but antagonize each other in the control of cell proliferation and quiescence-specific gene expression. The mechanism underlying this phenomenon is unknown but may reflect the competition for limiting amounts of co-activators (Gagliardi *et al.,* 2003).

The elimination of AP-1 may redirect the action of C/EBPβ in gene expression and, as a result, change the fate of the cell. This may account for the formation of lipid-rich vesicles in *v*-Src transformed CEF, a process that suggests that CEF in these conditions have undergone adipogenic conversion. Further studies are required to test this hypothesis.

7.2 Gene profiling revealed a C/EBPβ signature in conditions of AP-1 inhibition

The inhibition of individual AP-1 members by shRNA suggests different functions for individual members in proliferation and survival in normal and RSV-infected CEF. With the availability of the chicken genome and the "gene chips", we have obtained a gene profile of RSV-transformed CEF expressing TAM67 or a *junD* shRNA by microarray analysis. Gene profiling studies of v-Src transformed CEF expressing TAM67 or the *junD* shRNA identified 22 genes induced aberrantly by *v*-Src when AP-1/JunD is inhibited (Table 2 in Appendix 1). Many of these genes are components of the IFN pathway and/or are known targets of C/EBPβ (DAPK1, IL6).

Two recent reports demonstrated that the C/EBPβ-mediated induction of inflammatory cytokines or chemokines in response to oncogenes, in particular IL6 and IL8, generates DNA damage and contributes to oncogene-induced senescence [OIS; (Acosta *et al.,* 2008b, Kuilman *et al.,* 2008)]. Moreover, C/EBPβ activates the expression of death-associated protein kinase 1 (DAPK1), a protein critical for the regulation of apoptosis by interferon gamma (IFN-γ) in mouse MEF (Gade *et al.,* 2008). Induction of DAPK1 represses transformation induced by Ras or SV40 large T-antigen

by activating a $p19^{ARF}$/p53 apoptotic checkpoint in MEF (Raveh *et al.,* 2001). Interestingly, our result demonstrated that *v*-Src transformed CEF expressing the *junD* shRNA have elevated levels of p53 (Fig.24). Dual inhibition of JunD and p53 bypassed apoptosis and restored *v*-Src induced transformation (Fig.24). Finally, DAPK1 expression was stimulated in conditions of AP-1 inhibition in *v*-Src transformed CEF (Appendix 1, Fig.27). Co-inhibition of JunD and DAPK1 expression by shRNA in *v*-Src transformed CEF re-established cell survival and the ability to form colonies in soft agar. Significantly, colonies formed in these conditions were more numerous and larger than colonies formed by control *v*-Src transformed CEF with normal levels of AP-1/JunD and DAPK1 (Appendix 1, Fig.27). Hence, these results suggest that the loss of JunD/AP-1 in *v*-Src transformed CEF leads to transcriptional activation of C/EBPβ targeted genes, which might sensitize the cell to apoptosis as a result of DAPK1 activation. However, a direct link between C/EBPβ and DAPK1 in *v*-Src transformed CEF remains to be demonstrated. Taken together, the results of our studies suggest that the induction of AP-1 serves, in part, to restrict the induction of C/EBPβ controlled genes such as DAPK-1. The inhibition of AP-1/JunD may activate a latent C/EBPβ-DAPK1-p53 pathway that functions as oncogene-checkpoint in these cells. It is interesting that DAPK1 is tyrosine-phosphorylated and inhibited by v-Src (Wang WJ *et al.,* 2007). The accumulation of DAPK-1 in conditions of AP-1 inhibition may overcome the inhibitory effects of *v*-Src and induce apoptosis. Therefore, DAPK-1 may be a relevant target of *v*-Src, whose activity is normally restricted by direct phosphorylation and a limited expression determined by the level of AP-1 in the cell.

7.4 The interplay between C/EBPβ and AP-1

Collectively, AP-1 and C/EBPβ appear to function in an opposite manner in regulating normal cell growth and *v*-Src induced transformation *in vitro*. Two possible mechanisms may account for the induction of C/EBPβ activity and its controlled genes such as IL-6 and DAPK1 in response to AP-1 inhibition. In the first mechanism, AP-1 competes with C/EBPβ on a responsive-promoter region and exerts a negative effect on gene transcription. In this scenario, the DAPK1 promoter can be studied since its murine counterpart includes a functional CRE targeted by C/EBPβ in response to IFN-γ (Gade *et al.,* 2008). Since JunD has been shown to interact with CRE sequences in some promoters and block transcription (Guberman *et al.,* 2003), it may function directly by binding to the DAPK1 promoter region containing CRE sequences. In the second mechanism, competition for limiting amount of co-activators such as p300/CBP between AP-1 and C/EBPβ might contribute to defects in the transcription activation of C/EBPβ targets in *v*-Src transformed CEF. A similar mechanism has been demonstrated previously to explain how nuclear receptors downregulates AP-1 activity by competing for limited amount of p300/CBP (Kamei Y *et al.,* 1996). Therefore, the inhibition of AP-1 would allow for the preferential recruitment of p300/CBP by other transcription factors present in the cell such as C/EBPβ. In this situation, overexpression of p300/CBP might be able to enhance the expression of C/EBPβ targets in *v*-Src transformed CEF. Further investigation is required to test these hypotheses.

7.4 The gene regulatory network of v-Src transformed CEF

We propose a functional gene regulatory network involving three families of transcription factors (AP-1, C/EBPβ and NFκB) that promote the induction of genes of the G0-G1 transition, such as IL8, and cell transformation (Fig.25). Impairment of a component of this network, such as AP-1 or NFκB, results in a reorganization of this network, the global pattern of gene expression and the fate of the cell. Investigations of these transcription factors will provide novel insights on the role of this cooperative network in the CEF transformation by *v*-Src. More importantly, novel therapeutic approaches may be uncovered by characterizing the AP-1 gene regulatory network of v-Src transformed cells.

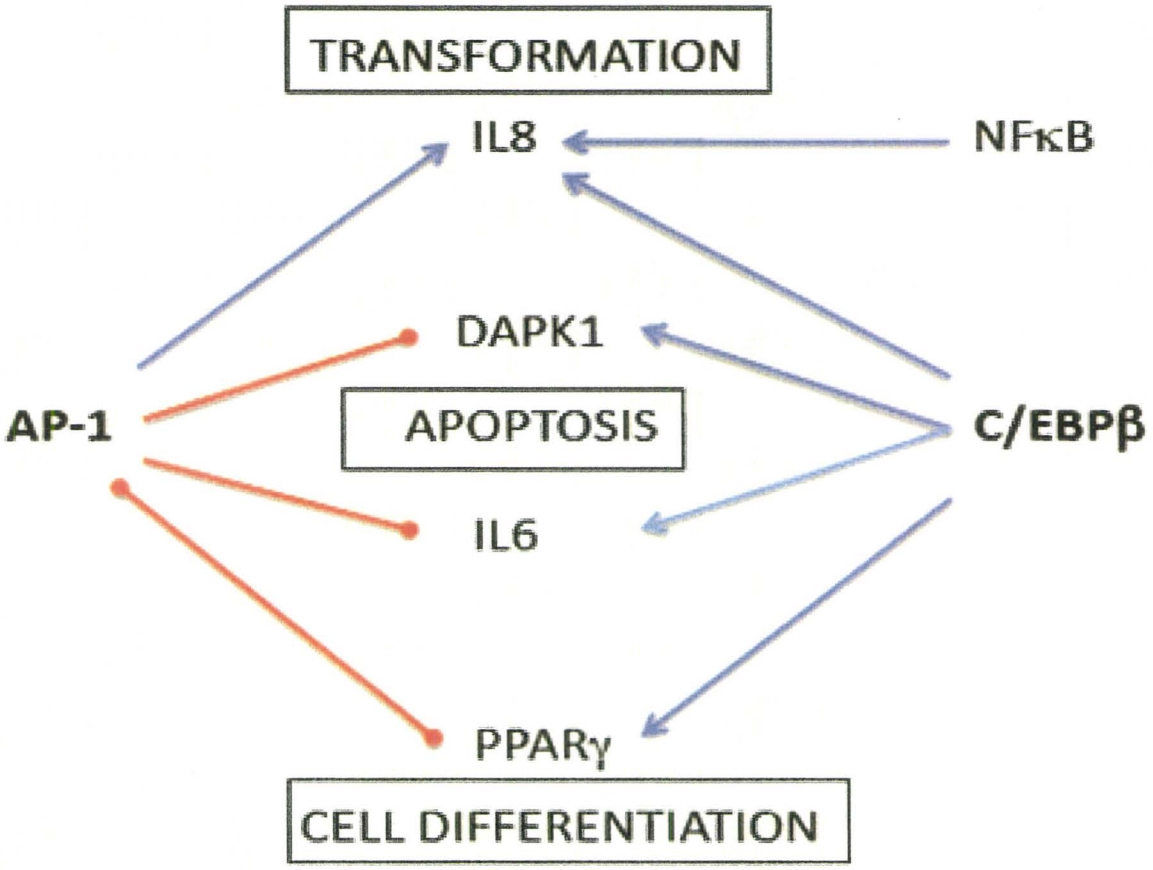


Figure 25. Proposed AP-1 gene regulatory network. AP-1, NFκB and C/EBPβ cooperate in the induction of the IL8 gene and cell transformation. V-Src activated AP-1 antagonizes the activity of C/EBPβ and represses DAPK1, IL6 and PPARγ. The inhibition of AP-1 in v-Src transformed CEF allows the induction of DAPK1, IL6 and PPAR γ by the activated C/EBPβ thus committing cells to apoptosis or cell differentiation (adipogenesis).

8. Future Directions

8.1 The involvement of ERK2 in the activation of the JunD TAD

Our data suggests that AP-1 is activated by *v*-Src via a pathway dependent on MEK/ERK (Chapter 3, Fig.1). Transient expression assays confirmed that the activity of the JunD TAD is highly stimulated by *v*-Src (Fig.6). EMSA analysis and ChIP assays revealed that JunD/Fra-2 is a major AP-1 complex in v-Src transformed CEF (Wang *et al.,* Manuscript in Appendix 2). Consistently, down-regulation of JunD expression by shRNA significantly repressed AP-1 activity in *v*-Src transformed CEF (Fig.22), suggesting that activation of JunD TAD contributes, in part, to the increased AP-1 activity in response to *v*-Src transformation. Previous studies demonstrated that the TAD domain of Fra-2 is known to be activated by ERK in *v*-Src transformed CEF (Murakami *et al.,* 1997) . Moreover, the JunD TAD is activated preferentially by ERK (Fantz *et al.,* 2001, Vinciguerra *et al.,* 2004). Thus it is worthy to investigate whether the stimulation of AP-1 activity by *v*-Src depends on an ERK-dependent phosphorylation of the JunD TAD domain.

8.2 The interplay of ERK2 and DAPK1 in the regulation of survival of v-Src transformed CEF

Recently, Hu and co-workers reported that ERK2 functions as a DNA binding and transcriptional repressor of IFN-responsive genes controlled by the GATE element bound by C/EBPβ (Hu S *et al.,* 2009). If we confirm a role for ERK2 in the control of the JunD TAD, this kinase may function as a repressor of GATE-controlled genes by two

separate mechanisms (direct DNA binding and control of JunD TAD activity). Since DAPK1 inhibits ERK signaling by sequestering ERK in the cytoplasm (Chen CH *et al.*, 2005), a long-term objective of this project is to study the interplay between ERK and DAPK1 in the control of GATE regulated genes and apoptosis by *v*-Src. Particular attention will be devoted to genes of the IFN pathway included in Table1 of Appendix 1.

8.3 Characterization of AP-1 or NFκB dependent gene expression

The observation that the down-regulation of individual AP-1 members yielded different phenotypes may reflect different degrees of AP-1 reduction and a different contribution to the activity of this factor by each component (Chapter 5). Particular attention was devoted to JunD and Fra-2 since these are the major components of AP-1 in v-Src transformed CEF (Wang *et al.*, manuscript in Appendix 2). Inhibition of JunD induced apoptosis in v-Src transformed CEF, while lipid-rich vesicles accumulated in the cytoplasm of v-Src transformed CEF expressing the *fra-2* shRNA (Chapter 5). Thus far, we have obtained the profiles of CEF co-expressing a ts v-Src kinase (NY72-4 RSV) with either the dominant negative mutant of c-Jun (TAM67) or a shRNA for JunD at the non-permissive and permissive temperatures. Comparison between these two gene profiles revealed 22 genes that are commonly regulated by TAM67 and JunD inhibition (Table 2 in Appendix 1). Some of these genes belong to the IFN pathway and are targets of C/EBPβ. Co-inhibition of these genes and JunD will shed light on pathways that are involved in the apoptosis induced by JunD inhibition. Similar approaches will be applied to CEF co-expressing ts *v*-Src and the *fra-2* shRNA. The results of these analyses may

reveal a pathway responsible for the formation of lipid-rich vesicles.

Expression of TAM67 resulted in the down-regulation of NFκB activity in *v*-Src transformed CEF (Fig.10). Inhibition of *nfκb1 p105/p50* by shRNA led to the induction of senescence in normal CEF. CEFs co-infected with a temperature sensitive mutant of RSV, NY72-4, and a virus expressing the *p105/p50* shRNA also exhibited a senescence phenotype at the non-permissive temperature while the incidence of apoptosis was dramatically increased at the permissive temperature (Fig.15). Transient expression assays revealed that the NFκB activity was reduced by the *p105/p50* shRNA by 50% (Fig. 15), suggesting the presence of other members of the NFκB family accounting for the remaining activity of NFκB in *v*-Src transformed CEF. Hence in order to investigate the pathways regulated by NFκB1 p105 upon *v*-Src transformation, we plan to perform gene profiling analysis following the same methodology described above. The comparison between the gene profiles of CEF co-expressing a ts v-Src kinase (NY72-4 RSV) with either the dominant negative mutant of c-Jun (TAM67) or a shRNA for *p105/p50* will reveal genes that are under the control of v-Src and NFκB1. Loss-of-function studies using the shRNA vector modified in our laboratory will be needed for the investigation of these genes. While v-Src is known to activate several targets of NFκB in mammalian cells or in quail neuro-retinal cells (Coll *et al.,* 2002, Gillet *et al.,* 1995), genes such as Bcl-xL, IAP2 and NR-13 (also a pro-survival member of the Bcl-2 family) are not regulated by *v*-Src in CEF (Maślikowski *et al.,* 2010). Therefore, the proposed gene profiling studies may uncover novel pro-survival targets of *v*-Src controlled by NFκB in CEF.

Inhibition of individual AP-1 members using shRNA expression vectors modified in our laboratory led to the discoveries of the role of individual AP-1 members in response to *v*-Src transformation. Gene profiling analyses revealed valuable information for future studies on pathways promoting transformation and latent pathways functioning as oncogene checkpoints. The characterization of these pathways will provide new opportunities for the development of novel strategies in the treatment of cancer patients with aggressive tumours characterized by high Src kinase activity.

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Appendix 1: Mechanism of apoptosis induced by JunD down-regulation in *v*-Src-transformed CEF

1. Introduction

The proliferation and survival of cancer cells are restricted by several "oncogene checkpoints". Several mechanisms of tumour suppression have evolved to limit the proliferation of cancer cells. Seminal studies by Serrano and co-workers showed that activated Ras induces the expression of $p16^{INK4A}$ and $p21^{WAF1}$, and promotes senescence in MEF (Serrano *et al.,* 1997). Inactivation of the *p53* and *Rb* genes bypassed the Ras-induced senescence, highlighting the importance of these pathways in oncogene-induced senescence (OIS). Recent studies have uncovered novel pathways of cellular senescence. For instance, prolonged treatment with IFN-β or -γ causes senescence in normal human fibroblasts, a process that is dependent on the activation of p53 (Kim KS *et al.,* 2009a, Moiseeva *et al.,* 2006). Hence, p53 plays an important role in the induction of OIS. The role of the C/EBPβ-p53 pathway in the induction of oncogene checkpoint came from the recent findings that C/EBPβ mediates the induction of inflammatory cytokines or chemokines in response to oncogenes, in particular IL6 and IL8, resulting in DNA damage and OIS via a p53-dependent manner (Acosta *et al.,* 2008b, Kuilman *et al.,* 2008). However, the exact mechanism causing OIS in this pathway remains largely undefined.

In previous studies, we have shown that the down-regulation of AP-1 activity by overexpressing TAM67 in v-Src transformed CEF induces three distinct phenotypes

contributing to the impairment of the cell proliferation, survival and transformation (Chapter 3). In chapter 5, we also uncovered a role for AP-1 and, in particular, for JunD in the survival of *v*-Src transformed CEF. Inhibition of JunD by shRNA also resulted in down-regulation of AP-1 activity (Chapter 5), suggesting that AP-1 is essential for cell survival upon *v*-Src transformation. In addition, we also observed that the apoptosis induced by JunD inhibition is p53-dependent (Chapter 5, Fig.24). In this section, we present preliminary data on the target(s) of JunD/AP-1 inhibition and apoptosis in these cells. We performed the gene profiling analysis of *v*-Src transformed CEF expressing the dominant negative mutant TAM67 or the *junD* shRNA and identified a cohort of 22 genes induced aberrantly by *v*-Src in conditions of AP-1 inhibition. Four of these genes (Serpin B2, IFIT5, OASL and DAPK1) are known components of the IFN-γ pathway and two (DAPK1 and IL6) are well characterized targets of C/EBPβ. Concomitant down-regulation of JunD and C/EBPβ or DAPK1 by shRNA suppressed the apoptosis observed in conditions of JunD/AP-1 inhibition and restored the capacity of *v*-Src transformed CEF to grow in an anchorage-independent manner. These results suggest that the inhibition of AP-1/JunD causes the activation of a latent pathway involving C/EBPβ, DAPK1 and p53 that functions as oncogene checkpoint in *v*-Src transformed CEF.

2. Results

Identification of target genes commonly regulated by TAM67 and JunD inhibition

In order to identify critical targets of AP-1, we performed a gene profiling analysis of CEF co-infected with ts NY72-4 RSV and a group B virus expressing TAM67 or the

junD shRNA. Since JunD inhibition causes apoptosis upon ts *v*-Src activation and the same phenotype is also prominent when AP-1 activity is impaired by expression of TAM67, we reasoned that genes mis-regulated in a *v*-Src- and AP-1-dependent manner would include candidate genes involved in the induction of apoptosis in these cells. In this analysis, the control cells were CEF co-infected with NY72-4 RSV and a RCASBP(B) virus expressing *gfp* and a shRNA for this gene product. We have shown in the past that GFP expression is silenced in these conditions thus providing an essential control for any non-specific effects dependent on the induction of RNA interference (Wang *et al,* Manuscript in Appendix 2). The inhibition of AP-1 altered markedly the pattern of gene expression of *v*-Src transformed CEF (data not shown) A complete analysis of these results will be presented elsewhere (Wang and Maślikowski, in preparation). Interestingly, a set of 21 non-redundant genes, normally not regulated by *v*-Src, were induced in response to TAM67 expression or downregulation of JunD expression by shRNA. These genes are described in Table 2. TSKU, which encodes a secreted protein involved in development of chick embryo, is a target of PPARα in human hepatocytes based on microarray analysis (Rakhshandehroo *et al.,* 2009). However, its function is not fully elucidated. The *cathepsin K* gene predominantly expressed in osteoclasts encodes a lysosomal cysteine protease involved in bone remodeling and resorption (Troen 2006). Most interestingly, some of the genes (IFIT5, Serpin B2, OASL and DAPK1) are part of the interferon pathway. OASL (2'-5'-oligoadenylate synthetase-like) is a distant member of the OAS family lacking any catalytic activity and known function (Andersen *et al.,* 2004). SerpinB2 is a protease

inhibitor with pleiotropic actions. Its role and that of IFIT5 in the control of the cell cycle is unclear (Tonnetti *et al.,* 2008). In contrast, Death-Associated Protein Kinase 1 (DAPK1) is involved in the activation of the ARF/p53 pathway leading to apoptosis (Gade *et al.,* 2008, Raveh *et al.,* 2001). Over-expression of c-Myc or E2F-1 in MEF is known to activate DAPK1 and contribute to OIS caused by these factors (Raveh *et al.,* 2001). Both DAPK1 and IL6 are well-known targets of C/EBPβ (Acosta *et al.,* 2008a, Gade *et al.,* 2008).

Table 2: Identification of genes activated by v-Src in conditions of AP-1 inhibition reveals the Interferon and C/EBPβ signatures. The microarray data is analyzed by Bart Maslikovski, a graduate student in our laboratory.

			NY72-4 JunD KD		NY72-4 RCAS(B)		NY72-4 TAM67	
probe set	Symbol	Name	NP	P	NP	P	NP	P
Gga.10034.2.S1_a_at	SERPINB2	Serpin peptidase inhibitor, clade B (ovalbumin), member 2	9.5	12.49	8.84	9.61	9.54	11.12
Gga.10930.1.S1_at	TSKU	tsukushin	7.27	9.23	8.15	7.85	11.01	11.49
Gga.13110.1.S1_at	Gga.13110.1	---	5.75	6.91	4.36	5.07	4.65	6.64
Gga.1442.1.S1_at	Gga.1442.1	---	7.76	9.18	6.61	6.7	8.17	9.73
Gga.18046.1.S1_at	CTSK*	cathepsin K	5.46	6.34	5.33	4.67	5.55	6.78
Gga.389.1.S1_at	HBEGF	heparin-binding EGF-like growth factor	7.52	9.49	7.27	7.58	9.4	10.45
Gga.4502.1.S1_at	SFRS5	splicing factor, arginine/serine-rich 5	9.23	9.58	7.87	8.47	8.9	10.14
Gga.536.1.S1_a_at	OASL	2'-5'-oligoadenylate synthetase-like	7.75	10.64	7.96	8.2	8.14	10.71
Gga.5553.1.S1_at	GPS1	G protein pathway suppressor 1	6.5	7.59	6.47	6.3	8.61	8.8
Gga.6121.1.S1_at	Gga.6121.1	---	8.95	10.14	8.95	8.98	9.06	10.14
Gga.6815.1.A1_at	IL6	interleukin 6	6.67	7.42	6.26	6.08	6.75	7.59
Gga.7920.1.S1_at	LOC770939	hypothetical protein LOC770939	6.53	7.99	6.1	5.4	9.35	9.51
Gga.9179.1.S1_at	RCJMB04_1b10	death-associated protein kinase 1	7.3	8.04	6.82	7.01	8	8.83
GgaAffx.20664.1.S1_at	GgaAffx.20664.1	---	5.53	6.55	5.73	5.44	6.99	8.29
GgaAffx.20888.1.S1_at	CBLB	Cas-Br-M (murine) ecotropic retroviral transforming sequence b	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	6.66	9.1	7.16	7.21	8.22	9.93
GgaAffx.23472.1.S1_at	CHN2	chimerin (chimaerin) 2	9.39	10.49	9.48	9.29	10.19	10.79
GgaAffx.24929.1.S1_at	GgaAffx.24929.1	---	8.99	9.69	8.88	8.64	8.9	9.84
GgaAffx.26005.1.S1_s_at	CACNA1E	calcium channel, voltage-dependent, R type, alpha 1E subunit	6.61	7.94	6.49	6.26	8.45	8.98
GgaAffx.5026.1.S1_at	LIF	leukemia inhibitory factor (cholinergic differentiation factor)	5.73	6.64	5.03	5.29	8.58	9.62
GgaAffx.6896.1.S1_at	LOC776184	similar to RASGEF1B protein	5.84	6.76	5.91	5.66	6.9	7.57
GgaAffx.9237.1.S1_s_at	PPP1R3C	protein phosphatase 1, regulatory (inhibitor) subunit 3C	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	8.64	9.94	8.69	8.41	9.73	9.99

Effect of co-inhibition of JunD and C/EBPβ on the transformation of CEF by v-Src

As shown in Table I, DAPK1 (death associated protein kinase 1) and IL6 are among the target genes. These two genes are also known targets of C/EBPβ (Gade *et al.,* 2008). In particular, the induction of DAPK1 by IFN-γ is dependent on C/EBPβ and does not rely on the canonical Jak-STAT pathway (Gade *et al.,* 2008). Since C/EBPβ activity is enhanced in response to TAM67 expression in *v*-Src transformed CEF (Chapter 3), we asked whether the inhibition of *c/ebpβ* by shRNA would alter the survival and capacity of *v*-Src transformed CEF to grow in soft agar in conditions of JunD inhibition. Two shRNA constructs for *c/ebpβ* have been tested and shRNA #99 was selected based on its capacity to efficiently inhibit the expression of C/EBPβ in normal CEF (Fig. 26A). The shRNA operon in the retroviral vector provides two separate sites allowing the expression of two hairpin sequences simultaneously. Therefore, vectors were generated to express shRNA species for *junD* and *c/ebpβ*, and co-infection of CEF was performed with a temperature sensitive mutant of RSV, NY72-4. As shown in Fig.1B, expression of shRNA species for *junD* and *c/ebpβ* was able to restore the capacity of *v*-Src transformed CEF to grow in an anchorage-independent manner. This finding indicates that blocking C/EBPβ activity was able to bypass the inhibitory effect caused by JunD inhibition, implying a role of C/EBPβ in the apoptosis induced in these conditions.

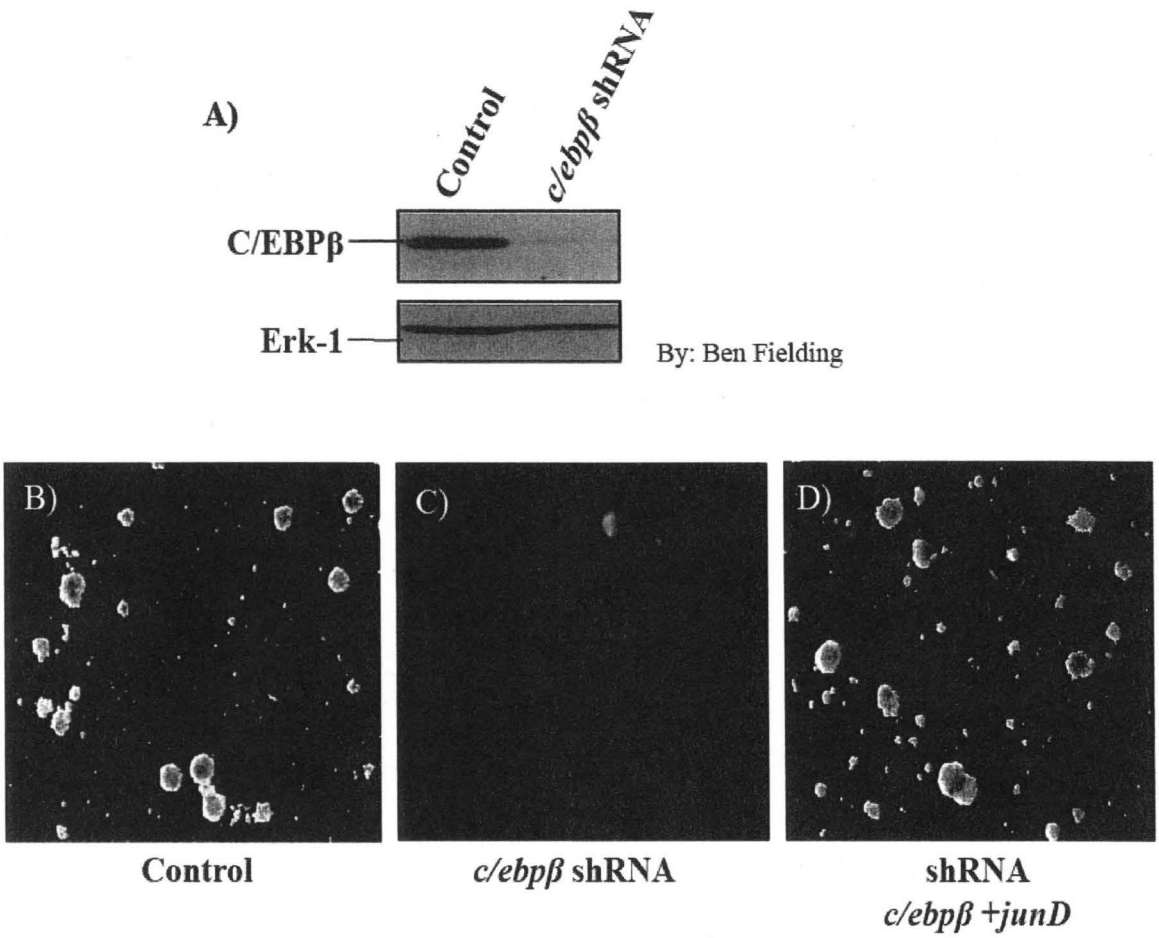
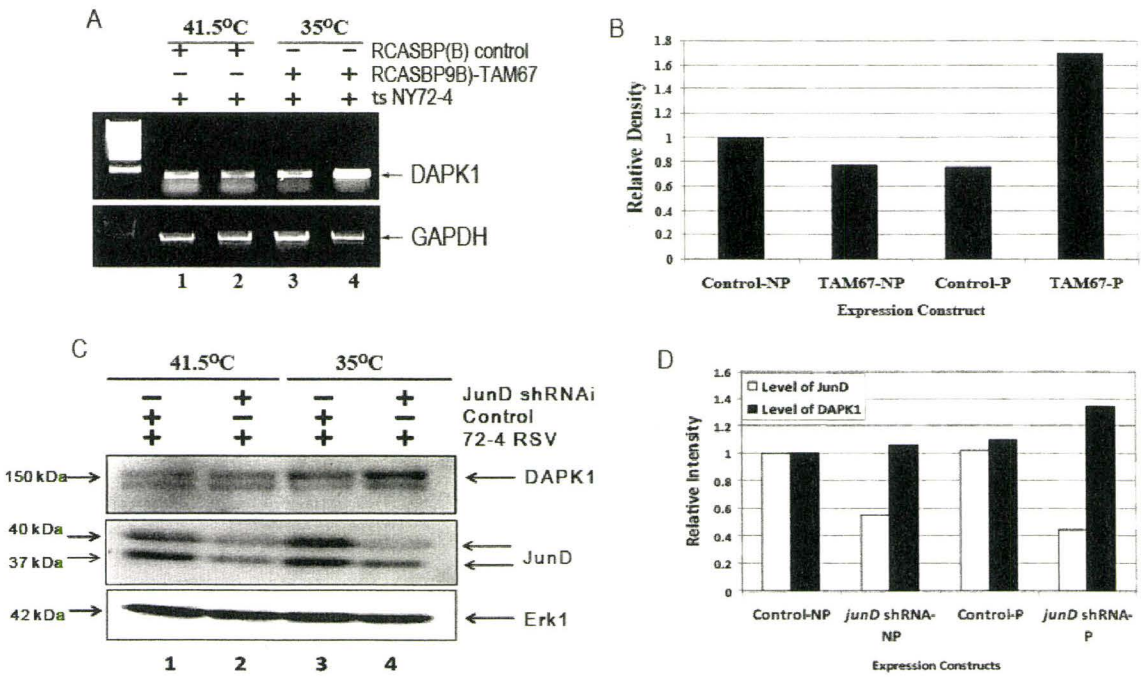


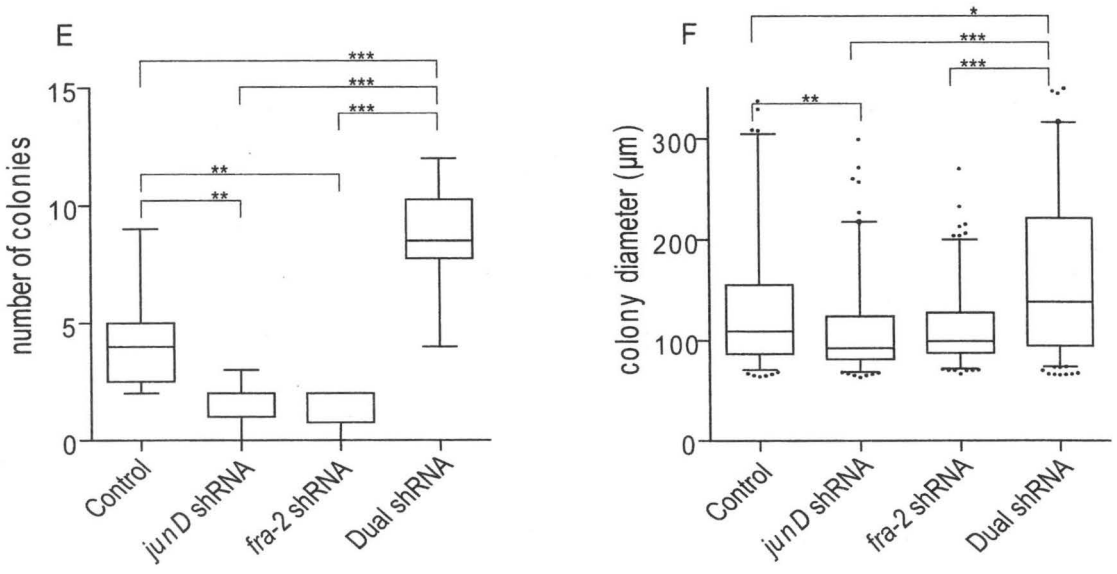
Figure 26. Co-inhibition of JunD and C/EBPβ expression restores colony formation in soft agar. **A.** Western blotting analysis was carried out to examine the inhibition of C/EBPβ expression by shRNA in normal CEF. The level of C/EBPβ was significantly reduced in normal CEFs. Erk1 was used a loading control; **B.** Soft agar assays were carried out to study the transformation capability of v-Src transformed CEF upon co-inhibition of JunD and C/EBPβ. Colonies formation was observed in v-Src transformed CEFs expressing a control virus (**Panels B&D**), or a virus expressing shRNA species for *junD* and *c/ebpb*, but not that expressing *junD* shRNA (**Panel C**).

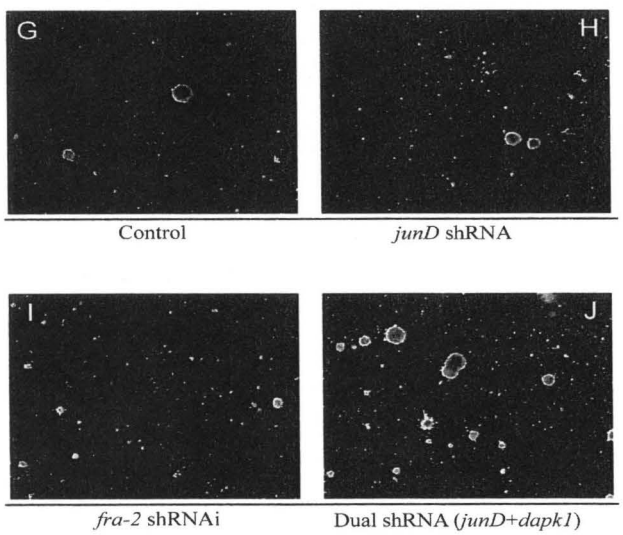
Following the finding that the activity of C/EBPβ is critical for the apoptosis induced by JunD inhibition, and since DAPK-1 is a known target of C/EBPβ (Gade *et al.*, 2008), we then asked whether DAPK1 plays a similar role in these cells. Semi-quantitative RT-PCR and Western blotting analyses confirmed the induction of DAPK1 upon ts *v*-Src activation and TAM67 or *junD* shRNA expression in CEF, respectively (Fig.27A-D). More importantly, the concomitant repression of DAPK1 restored the capacity of v-Src transformed CEF to form colonies in soft agar in conditions of JunD down-regulation (Fig.27J). Colonies formed in these conditions (JunD and DAPK1 co-inhibition) were more numerous and significantly larger than control v-Src transformed CEF (Fig.27 E&F). This result suggests that DAPK1 limits the proliferation of v-Src transformed cells, a process antagonized in part by the activation of AP-1. Collectively, these results also imply that C/EBPβ, DAPK1 and p53 form a negative regulatory pathway limiting the proliferation and survival of these cells.

Figure 27. *Role of DAPK1 in the apoptosis induced by JunD inhibition.* **A.** CEFs co-infected with temperature sensitive mutant of RSV, NY72-4 and a control virus or virus expressing TAM67. Semi-quantitative RT-PCR confirmed that DAPK1 is induced in the presence of TAM67 at the permissive temperature (Lane 4). GAPDH was used as a loading control; **B.** Densitometric analysis revealed that the transcription of DAPK-1 is increased by 1.7 fold in Lane 4 of Panel A; **C.** CEFs were co-infected with temperature sensitive mutant of RSV, NY72-4 and a control virus or virus expressing *junD* shRNA. Western blotting analysis verified that the expression of DAPK1 is stimulated upon *v*-Src activation and JunD inhibition (Lanes 2&4); **D.** Densitometric analysis was performed to examine the down-regulation of JunD expression and stimulation of DAPK1. The level of JunD expression is reduced by 50% at both non-permissive (41.5°C) and permissive temperature (35°C), while DAPK1 is stimulated at the permissive temperature upon JunD inhibition; **E-F.** Soft-agar assays were performed to show that co-repression of *dapk1* and *junD* by shRNA restores the ability of *v*-Src transformed CEF to form colonies in soft agar. Number of colonies per field greater than 100 μm in each condition is shown. Mean number of colonies formed in the presence of dual shRNA for *junD* and *dapk1* species is greater than control (RCASBP(B)-shRNA-ΔU6), *junD* shRNA or *fra-2* shRNA (one-way ANOVA followed by Tukey post-hoc test, $p<0.05$; panel E). Mean number of colonies per field for *v*-Src transformed CEF expressing the control, *junD* shRNA, *fra-2* shRNA or dual shRNA (*junD* + *dapk1*) are 4.22, 1.40, 1.40 and 8.70, respectively. As shown in panel F, mean colony diameter of cells in the presence of dual shRNA (*junD* + *dapk1*) is greater than the control (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 *v*-Src transformed CEF expressing the control, *junD* shRNA, *fra2* shRNA or dual shRNA (*junD* + *dapk1*) are 138.6 μm, 113.7 μm, 111.5 μm and 163.5 μm, respectively. Whiskers in both plots indicate 5th -95th 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; **G-J.** Representative fields from colony formation assays quantified in panels E and F (10X magnification). Only a few colonies were observed in *v*-Src transformed CEF expressing *junD* or *fra-2* shRNA (Panels H and I); NP: Non-permissive temperature; P: Permissive temperature.




E
number of colonies
15
10
5
0
***
***
***
**
**
Control
junD shRNA
fra-2 shRNA
Dual shRNA
F
colony diameter (μm)
300
200
100
0
*
***
***
**
Control
junD shRNA
fra-2 shRNA
Dual shRNA


G
H
Control
junD shRNA
I
J
fra-2 shRNAi
Dual shRNA (junD+dapk1)

3. Summary

We have shown that the down-regulation of JunD expression caused the induction of a p53-dependent pathway of apoptosis (Chapter 5, Fig.24). In this study, we performed gene profiling studies and identified 22 genes that are commonly regulated by TAM67 and JunD inhibition. Some of these genes (IFIT5, Serpin B2, OASL and DAPK1) are part of the interferon pathway, which also functions as oncogene checkpoint. Indeed, prolonged treatment with IFN-β or γ causes senescence in normal human fibroblasts, a process that may also involve DNA damage and the activation of p53 (Kim KS *et al.*, 2009a, Moiseeva *et al.*, 2006). We paid particular attention to the death associated protein kinase 1 (DAPK1), whose expression is directly activated by C/EBPβ and contributes to the induction of apoptosis upon interferon gamma (IFNγ) treatment in MEF (Gade *et al.*, 2008), implying a potential role of C/EBPβ-DAPK1 in the induction of apoptosis. Colony formation and survival of *v*-Src transformed CEF were restored when C/EBPβ was down-regulated concomitantly with JunD (Fig.1 and data not shown), suggesting that C/EBPβ mediates the apoptosis observed in condition of JunD inhibition. Likewise, co-inhibition of *junD* and *dapk1* by shRNA relieved the effects of JunD inhibition and restored the capacity of v-Src transformed CEF to form colonies in soft agar. Significantly, colonies formed in these conditions were more numerous and larger than colonies formed by control v-Src transformed CEF with normal levels of AP-1/JunD and DAPK1 (Fig.27). This result suggests that DAPK1 limits the anchorage dependent proliferation of v-Src transformed cells. However, the RT-PCR and western blotting analyses revealed a modest induction of DAPK1 in v-Src transformed CEF in the

presence of TAM67 or *junD* shRNA (Fig.27). This might be due to fact that these analyses were based on samples from short incubation (6-8 hours) at the permissive temperature (37.5°C), where the ts.*v*-Src mutant is activated to induce transformation. We reasoned that longer incubation at the permissive temperature may reveal more considerable induction of DAPK1 expression and enzymatic activity in these cells. Further studies are required to confirm this hypothesis. Moreover, whether or not C/EBPβ is directly responsible for the induction of DAPK1 in conditions of AP-1 inhibition remains to be investigated. Since DAPK1 induces apoptosis in a p53-dependent manner (Raveh *et al.,* 2001), these results suggest that C/EBPβ, DAPK1 and p53 form a pathway restricting the proliferation of *v*-Src transformed cells.

Appendix 2 (This manuscript has been submitted to the Journal of Virology)

Pleiotropic action of AP-1 in v-Src transformed cells

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Running title: Role of AP-1 in v-Src transformation

Key Words: v-Src , AP-1, JunD, shRNA, Apoptosis

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ABSTRACT

The activation of AP-1 is a hallmark of cell transformation by tyrosine kinases. In this study, we characterize the role of AP-1 proteins in the transformation of chicken embryo fibroblasts (CEF) by v-Src. In normal CEF, the expression of a dominant negative mutant of c-Jun (TAM67) induced senescence. In contrast, three distinct phenotypes were observed when TAM67 was expressed in v-Src transformed CEF. While senescent cells were also present, the inhibition of AP-1 caused apoptosis in a fraction of the v-Src transformed cells. In addition, cells containing lipid-rich vesicles accumulated, suggesting that a sub-population of the v-Src transformed cells underwent differentiation in response to the inhibition of AP-1. JunD and Fra-2 were the main components of this factor while c-Jun accounted for a minor fraction of AP-1 in v-Src transformed CEF. The down-regulation of c-Jun expression by shRNA induced senescence in normal and v-Src transformed cells. In contrast, a high incidence of apoptosis was caused by the down-regulation of JunD, suggesting that the pro-survival function of AP-1 was determined by this factor. Levels of the p53 tumour suppressor were elevated in conditions of JunD inhibition. Repression of p53 by shRNA enhanced the survival and anchorage-independent proliferation of v-Src transformed CEF with JunD/AP-1 inhibition. The inhibition of Fra-2 had no visible phenotype in normal CEF but caused the appearance of lipid-rich vesicles in v-Src transformed CEF. Therefore, AP-1 facilitated transformation by acting as a survival factor, by inhibiting premature entry into senescence and by blocking the differentiation of v-Src transformed CEF.

INTRODUCTION

Activated Ras and v-Src induce profound changes in the pattern of gene expression (9). These changes are regulated at multiple levels but are often dependent on the activation of transcription factors working cooperatively on promoter/enhancer regions. The significance of transcription factor activation is highlighted by the inhibitory effects that dominant negative mutants of Ets, Stat3 or AP-1 exert on cell transformation (5, 16, 29, 42-44). Separate groups reported that the inhibition of AP-1 by the expression of a deletion mutant of c-Jun lacking a trans-activation region interferes with transformation by RasV12 or v-Src. In addition, immortalized fibroblasts nullizygous for c-Jun cannot be transformed by these oncoproteins (22). However, these cells are still capable of forming tumours in animals albeit with delayed kinetics. Cells recovered from these tumours are characterized by high levels of AP-1 activity resulting from increased expression of JunD (22). These observations underline the importance of AP-1 activation in cell transformation by oncogenic Ras or tyrosine kinases.

Several mechanisms of AP-1 activation have been described before. Herschman and co-workers reported that v-Src controls the trans-activation potential of c-Jun by inducing the activity of the JNK/SAPK pathway in murine fibroblasts (30). In contrast, the activity of this pathway is enhanced modestly in v-Src transformed CEF, indicating that other pathways of AP-1 regulation are activated in these cells (3). The Ras-dependent stimulation of ERK modulates the phosphorylation and activity of Fra-2 in RSV transformed CEF, in agreement with the notion that v-Src controls several aspects of the regulation of AP-1 (36). Similar studies performed on NIH 3T3 fibroblasts

concluded that transformation by activated Ras is dependent on c-Jun/Fra-1 and the displacement of weaker trans-activators of the Jun family by the more potent c-Jun protein. Significantly, Yaniv and co-workers reported that the over-expression of JunB and JunD inhibits the transformation of NIH 3T3 cells by Ras, implying that these factors function as negative regulators or poor activators of AP-1 in these cells (38). However, a different view is depicted by the studies of primary mouse embryo fibroblasts (MEF) harboring a disruption of the c-jun or junD gene. Indeed, c-jun or junD (-/-) MEF proliferate poorly and rapidly undergo premature senescence in vitro, suggesting that both gene products are required for normal proliferation of primary embryonic fibroblasts (46, 47). Consistent with this notion, MEF lacking JunD express elevated levels of p19Arf, which triggers the entry into senescence (46). The role of JunD is not restricted to the control of cell proliferation since junD (-/-) MEF are also hyper-sensitive to the action of TNF□ and rapidly undergo apoptosis in response to this factor (46).

In this study, we characterize the activation of AP-1 in CEF transformed by the Rous sarcoma virus (RSV). We show that the JunD/Fra-2 heterodimer accounts for most of the AP-1 activity in normal and v-Src transformed CEF. The increase of AP-1 activity was dependent on the accumulation of JunD, Fra-2 and, to a lesser extent, c-Jun. Moreover, the trans-activation potential of JunD was stimulated markedly in response to v-Src transformation, suggesting that JunD is a key target of v-Src transformation. The inhibition of AP-1 by a dominant negative mutant of c-Jun resulted in a high incidence of apoptosis in RSV-transformed CEF but not in their normal counterparts. Down-regulation of c-Jun by shRNA induced senescence but no apoptosis. In contrast, the

disruption of JunD expression caused a high incidence of apoptosis, suggesting that the pro-survival activity of AP-1 depends on JunD. Down-regulation of Fra-2 expression by shRNA had no visible phenotype in normal CEF but induced the accumulation of lipid-rich vesicles in v-Src transformed cells. Therefore, AP-1 promoted cell transformation by acting as a survival factor, by inhibiting premature entry into senescence and by antagonizing the differentiation of v-Src transformed CEF.

MATERIALS AND METHODS

Cell culture. Chicken Embryo Fibroblasts (CEF) were cultured at 41.5°C in DMEM medium supplemented with 5% heat-inactivated newborn bovine serum (Cosmic calf serum, Hyclone), 5% Tryptose Phosphate Broth (TPB), and 1% Penicillin, Streptomycin, and L-Glutamine (Gibco-BRL Lifesciences Technology). CEF were infected with the non-transforming myristylation-deficent strain NY315 RSV, the wild type transforming Schmidt-Ruppin A (SR-A), or with the temperature sensitive (ts) strains NY72-4 (a group A virus) or LA90 RSV (a group B virus). Results pertaining to the role of AP-1 in the transformation and survival of v-Src transformed CEF were confirmed using both temperature sensitive strains of RSV. LA90 and NY72-4 RSV-infected CEF were cultured at the non-permissive temperature of 41.5°C and transferred to the permissive temperature of 37.5°C to activate the temperature sensitive v-Src kinase. CEF were also treated with a 800 nM dose of Tetradecanoyl Phorbol Acetate (TPA) (Sigma) or 1% ethanol diluent in some experiments.

Generation of retroviral vectors for shRNA expression. Using existing avian

RCASBP vectors for shRNA expression, we failed to observe prolonged down-regulation of several transcription factors of the AP-1 or NFκB family in CEF (our unpublished results). In vectors designed by Das and co-investigators, a shRNA is expressed in the context a chicken microRNA under the control of the chicken U6 promoter (8). Since microRNA loci are transcribed by RNAP II, we deleted the U6 promoter and leader sequence to generate vectors expressing one or two shRNA species under the control of the strong viral LTR. This was done by replacing the Not1-Cla-1 expression cassette of the transfer plasmid pRFPRNAiC with a DNA fragment consisting of the microRNA operon sequences but lacking the chicken U6 promoter and leader region of the same vector (8). This was completed by amplifying the microRNA region by PCR using forward (5' ATACTAGCGGCCGCATAAAGT 3') and reverse primers (5' GGATCCATCGATAAAAAAGCT 3') bordering the region of interest. Cloning of sequences for shRNA expression in the modified transfer vector, designated pRFPRNAiC-shRNA-□U6, was performed as described by Das and co-workers (8).

Target sequences for components of the chicken AP-1 factor (c-Jun, JunD, Fra-2) are described in Table 1. While two or more targets were generated of each factor included in this study, the results described below were obtained with the RCASBP-shRNA vector providing the most efficient down-regulation of the factor of interest (Table 1). However, the phenotype associated with the down-regulation of c-Jun, JunD or Fra-2 was observed with two or more independent targets of the same gene (data not shown). All shRNA constructs were designated by the number of the first nucleotide of the targeted sequence, subcloned into the sub-group A or B RCASBP retroviral vectors

and tested by transfection into normal CEF using the calcium phosphate method (15). Following transfection of the shRNA retroviral vector, in some experiments CEF were super-infected 24 hrs later with the temperature sensitive mutants NY72-4 or LA90 RSV.

Construction of mismatch and scrambled shRNA control constructs. Two types of retroviral constructs were generated to examine the specificity of a shRNA species. In the first type, the sequence forming the stem structure of the shRNA was scrambled to eliminate the targeting of the mRNA of interest. Alternatively, we substituted two nucleotides of the stem structure disrupting the complementarity of the hairpin sequence to the target mRNA; these were designated "mismatch constructs". Results obtained with scrambled shRNA constructs were confirmed with the corresponding mismatch constructs. Sequences of these constructs are also described in Table 1.

Since the over-expression of JunD reduces the expression of c-Jun and the over-expression of c-Jun impairs JunD expression in CEF (14), we also looked at the effects of a shRNA on the expression of other members of the AP-1 family but did not see any stimulatory effects of the down-regulation of c-Jun, JunD or Fra-2 (see Fig.10A and data not shown).

Transactivation Domain Constructs and Transient Expression Assays. DNA fragments corresponding to amino acids 1-193 of chicken c-Jun and 1-220 of chicken JunD were amplified by PCR and sub-cloned in the HindII-SalI sites of plasmids pCDNA3-Gal4 DBD or pRC/RSV-Gal4 DBD. These constructs encode fusion proteins consisting of the trans-activation domain (TAD) of c-Jun or JunD at the N terminus and the DNA binding domain (DBD) of the yeast Gal4 transactivator at the C-terminus. The

activity of the Gal4 fusion proteins was assessed in NY72-4 RSV infected CEF co-transfected with a plasmid consisting of a CAT reporter gene controlled by five Gal4 binding sites fused a minimal promoter (pJFCAT-TATA-Gal4). The same results were obtained when the c-Jun or JunD Gal4 fusion proteins were expressed with the pCDNA3-Gal4 or pRC/RSV-Gal4 backbone vectors.

The methodology and CAT reporter gene constructs consisting of the IL8 (also designated CEF-4) promoter or a minimal promoter fused to TPA-response elements (TRE), NFκB binding sites (PRDII) or C/EBP binding sites have been described before (10, 13). The DEAE-dextran method was used for all transient expression experiments. In brief, normal CEF, SR-A RSV transformed CEF , NY72-4 or LA90 RSV-infected CEF kept at the non-permissive temperature of 41.5°C, were seeded at a confluence of 70-80% in 100 mm dishes. Variable amounts of the effector expression plasmid (see Figure legends for details) were co-transfected with 2 μg of the CAT reporter plasmid and 2 μg of pRSV-βgal along with variable amounts of carrier salmon sperm DNA to make up to a total of 30 μg of DNA. Transfection efficiency was determined by the level of β-galactosidase activity recovered in lysates of transfected cells. Transformation was obtained by transferring NY72-4 or LA-90 RSV infected CEF to the permissive temperature of 37.5°C for a 24 hr period. All transient expression assays were conducted in triplicate samples in two or more independent experiments. Error bars represent the standard error.

Proliferation and soft agar assays. CEFs were transfected with shRNA retroviral constructs and cultured for 2 passages to ensure infection of the entire CEF population. In

some cases, the CEF population was super-infected with NY72-4 or LA90 RSV, as described below. For proliferation assays, cells were seeded into 24-well dishes at a density of 12,000 cells/per well and cell numbers were determined over a six day period by counting quadruplicate samples of each cell type in a Coulter counter. Anchorage-independent proliferation was determined in soft agar assays performed at the permissive temperature (37.5°C), as described before (13).

Lipid staining, senescence-associated β-galactosidase (SAβG) and Tunel assays. Lipids were stained with the lipophilic dye Oil-Red O, as described before (24). All singly and doubly infected cells were assayed for SAβG activity using commercially available reagents and protocols provided by the supplier (Cell Signaling Technology; #9860). Apoptosis was quantitated by the Tunel assay using commercially available reagents (Roche, #12156792910). In brief, cells were fixed with 4% Paraformaldehyde in 1X PBS for 1 hour at room temperature and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After washing extensively in PBS, specimens were incubated with Terminal deoxynucleotidyl Transferase (TdT) and TMR red labeled dUTP containing solution for 1 hour at 370C. All the samples were mounted in 10 ul of 0.1% phenylene diamine in 70% glycerol. Apoptotic cells were visualized under fluorescence microscopy. Chromatin was stained with DAPI (Sigma, #D9542) and nuclei were counted to quantitate the number of cells in each field. A minimum of 500 cells in an average of 15 fields were counted on each slide. The percentage of Tunel positive cells and standard errors were determined for each condition and cell type.

Electrophoretic mobility shift assays (EMSA). Nuclear extracts were prepared

from normal and v-src transformed CEF, as described before (3). Briefly, cells were scraped and pelleted in 1X Phosphate Buffered Saline (PBS) and lysed in a buffer containing 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM Dithiothreitol (DTT), 0.5 mM PhenylMethylSulphonylFluoride (PMSF), 2 μg/mL leupeptin, 5 μg/mL antipain, 5 μg/mL aprotinin, 1 mM NaF, 1 mM Na3VO4, and 10% NP-40. The nuclear pellet was isolated by centrifugation and solubilized in a solution containing 20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 10 μg/mL leupeptin, 5 μg/mL antipain, 5 μg/mL aprotinin, 1 mM NaF, and 1 mM Na3VO4. Insoluble debris was removed by centrifugation and the remaining supernatant containing the nuclear protein fraction was used in a gel shift assay. Gel shifts were performed as follows: 2 μg of nuclear protein were incubated on ice at 4°C for 3 hours in 20 μL of 1X interaction buffer consisting of 10 mM Tris pH 7.5, 1 mM DTT, 1 mM EDTA, 2 μg poly dI-dC (for non-specific DNA competition), 100 mM NaCl, and 25% glycerol. 1 μg of the appropriate Jun or Fos antibody or antibody pre-incubated with 5X amount of peptide antigen were also included in reactions for supershifting of binding complexes and as a control for supershifted complexes, respectively. For competitive binding analyses, a 50 molar excess of a cold wild type or mutant TRE oligonucleotide was included in the binding reaction. All protein reactions were subsequently incubated with approximately 0.1 ng (10,000cpm) of a α32P-dCTP or α32P-dATP radioactively labelled TRE probe for 15 minutes at room temperature prior to loading. Reactions were run under non-denaturing conditions at 4°C on a pre-run 4.8% polyacrylamide gel in 0.5X TBE for 2.5 hours at 200 volts. Gels were dried under

vacuum and exposed to Kodak film overnight at -80°C.

Chromatin immunoprecipitation. Chromatin immunoprecipitation (ChIP) protocol was based on the EZ ChIP protocol by Upstate Biotechnology (Millipore, Massachusetts, USA). All recipes are provided in the EZ ChIP documentation. Briefly, CEFs infected with RCASBP(A) or SR-A RSV were grown to sub-confluence, fixed in 1% formaldehyde for ten minutes and then quenched in 125mM glycine for 5 minutes. Cells were collected, centrifuged at 200g and washed in PBS twice to remove media and fixation solution. Cells were lysed in SDS lysis buffer in the presence of protease inhibitors. Lysates were sonicated on ice using a Branson 350 probe-sonicator for 6 minutes at 60% intensity, 50% duty cycle. An aliquot of DNA from each sample was reverse cross-linked and assayed for shearing by agarose gel electrophoresis. 10% of each sample was reserved for input controls and not subjected to immunoprecipitation. One mg of protein was immunoprecipitated overnight at 40C in the presence of 30µl (packed volume) blocked sepharose beads and 1µg of relevant antibody. Anti-c-Jun (sc-44), JunD (sc-74), and Fra-2 (sc-604) antibodies were obtained from Santa Cruz Biotechnology. Following precipitation, beads were washed twice in low salt buffer, once in high salt buffer, once in LiCl buffer and twice in TE. Protein/DNA complexes were eluted twice with elution buffer, reverse cross-linked overnight and then treated with RNAse A and proteinase K the following day. DNA was isolated by phenol/chloroform extraction, precipitated and resuspended in TE. Precipitated DNA was PCR amplified using standard protocols. Primer pairs for the v-Src responsive unit (SRU) of the promoter/enhancer of IL8/CEF-4 gene and upstream negative control were:

5'AAGAAACTAGTCTGCATGGGCA 3' (forward SRU)

5' CCAAACACTCCTAACCATGAACG 3' (reverse SRU)

5' GCTGCTTCAGGATCATCTTCTAGGA 3' (forward negative control region)

5' CACTCAGTCGGCTGATTCAGAGA3' (reverse negative control region)

Western blotting analysis. Whole cell protein lysates were prepared by harvesting non-transformed and transformed CEF washed and pelleted in cold 1X PBS. Cells were resuspended and lysed in 1X Sodium Dodecyl Sulphate (SDS) buffer consisting of 2.3% SDS, 5% β-mercaptoethanol, 10% glycerol, and 62.5 mM Tris pH 6.8 by boiling samples for 5 minutes. 20-40 μg of proteins were separated by electrophoresis through a denaturing 10% polyacrylamide gel, transferred electrophoretically onto a nitrocellulose membrane (Schleicher and Schuell, BA85) and probed with various antibodies. Commercial antibodies made against c-Jun (SC-45X), JunD (SC-74X), Fra-2 (SC-604X) (all used at a dilution of 1:2000), p53 (SC-99) and ERK-1 (SC-93) (used at a dilution of 1:2000) were obtained from Santa Cruz Biotechnology (Santa Cruz, California). The c-Fos antibody (#2000752) used in EMSA analysis was purchased from Geneka Biotechnology Inc. (Montréal, Québec). Immune complexes were visualized using horseradish peroxidase-conjugated secondary antibody and a chemiluminescent substrate (ECL system, GE Healthcare).

RESULTS

The inhibition of AP-1 induces multiple phenotypes in v-Src transformed chicken embryo fibroblasts. Cells transformed by tyrosine kinases or activated Ras have elevated

levels of AP-1 activity (9, 22). In immortalized cell lines transformed by these oncogenes, the inhibition of AP-1 results in a more normal morphology and the inability to proliferate in the absence of anchorage (16, 29). AP-1 is also activated in chicken embryo fibroblasts (CEF) transformed by the Rous sarcoma virus (3). To characterize the role of AP-1 in primary embryonic fibroblasts, we expressed TAM67, a mutant of c-Jun deleted of the N-terminal trans-activation domain, with the RCASBP retroviral system (1, 14). As reported previously, the proliferation of normal CEF was markedly inhibited by the expression of TAM67, a process that may reflect the down-regulation of cyclin D1 (14). Flat, bi-nucleated cells were often found in populations of normal CEF expressing TAM67, suggesting that the dominant negative mutant of c-Jun induces senescence. In agreement with this notion, a significant proportion of the TAM67 expressing CEF were positive for the senescence-associated β–galactosidase activity (SAβG) described by Dimri et al (Fig.1A-B; (12)). Likewise, CEF co-infected with a temperature sensitive mutant of RSV, ts LA-90, and RCASBP-TAM67 expressed the SAβG activity, indicative of cellular senescence. This was seen at both the permissive and non-permissive temperature (Fig.1C-F). In addition, CEF populations expressing the ts v-Src kinase and TAM67 were significantly depleted and cell numbers decreased by 60% when transferred to the permissive temperature for a 16 hr period (data not shown). Cells floating in the medium were abundant. Finally, a fraction of the cells were highly vesiculated but did not express the SAβG activity (asterisk in Fig.1F). These phenotypes were not observed in CEF co-infected by ts LA-90 RSV and the RCASBP(A) control virus (Fig.1C-D).

To determine if senescence or cell death accounts for the loss of transformed CEF, we repeated the experiment with cells transferred to the permissive temperature for a shorter duration (12 hrs). The quantitation of SAβG activity confirmed that TAM67 induces cellular senescence to similar extents at the permissive and non-permissive temperatures (Fig.1G). A high incidence of apoptosis was also detected in CEF expressing TAM67. However, this incidence was at least one order of magnitude greater at the permissive temperature. In fact apoptosis was nearly undetectable at the non-permissive temperature of 41.5°C (Fig.2). Interestingly, control LA90 RSV-infected CEF that did not express TAM67 also showed signs of apoptosis when transferred to the permissive temperature, suggesting that v-Src activity induces apoptosis, albeit modestly.

Vesiculated CEF, appearing in response to TAM67 expression and activation of the temperature-sensitive v-Src, did not stain in the Tunel and SAβG assays, indicating that they were neither senescent nor undergoing apoptosis. We also looked at processing of MAPLC3, a marker of autophagy, in conditions of AP-1 inhibition and v-Src transformation but did not see any difference between control and TAM67 expressing cells (data not shown). Finally, an assay based on the incorporation of Lucifer yellow ruled out macropinocytosis as a source of the vesicles detected in the TAM67 expressing RSV infected CEF [data not shown; (35, 37)].

Transient expression of TAM67 induces apoptosis in a high fraction of the v-Src transformed CEF. To rule out the possibility that the phenotype caused by the expression of TAM67 is a non-specific effect of having multiple viruses replicating in the cell and to determine the extent of apoptosis, we expressed TAM67 transiently by transfecting

plasmid CMV-TAM67 or a control vector in LA90 RSV infected CEF. A plasmid expressing GFP was included in the experiment to identify cells that were effectively transfected. The transfection efficiency was comparable (15-20%) in CEF transfected with the control vector or TAM67 expressing construct (data not shown). As described in Fig.3, the expression of TAM67 did not induce apoptosis when the cells were maintained at the non-permissive temperature. In contrast, the activation of the ts v-Src kinase induced apoptosis in 50% of the LA90 RSV infected CEF transfected with the TAM67 expression construct. A lower incidence of apoptosis was also detected in CEF transfected with the control vector, in agreement with our previous observations that v-Src transformation causes cell death in a small fraction of the cells (Fig.3). Therefore, the induction of apoptosis was observed in a high proportion of the v-Src transformed cells indicating that cell death is the predominant phenotype caused by the expression of TAM67. Moreover, this result confirmed that cell death was not caused by the replication of multiple viruses in the cell.

JunD and Fra-2 are the main components of AP-1 in RSV-transformed CEF. Elevated levels of AP-1 DNA binding activity were detected in nuclear extracts of v-Src transformed CEF (Fig.4A). The results of gene profiling studies indicated that c-Jun, JunD and Fra-2 are expressed in CEF (34). Using antibodies and EMSA, we confirmed the presence of all three proteins in the AP-1 complex of uninfected and SR-A RSV-transformed CEF. While c-Fos was found in nuclear extracts of TPA-stimulated cells, it could not be detected in v-Src transformed CEF (Fig.4A, lanes 4,9,15). Significantly, the same constituents, JunD and Fra-2, predominated in the AP-1 complex of normal and

transformed CEF. Therefore, constitutive activation of AP-1 was not characterized by the expression and recruitment of different factors induced during v-Src transformation.

JunD and Fra-2 were also detected on the IL8 promoter/enhancer by chromatin immunoprecipitation (ChIP). In agreement with the results of EMSA, both proteins were recruited to the IL8 promoter/enhancer region in response to v-Src transformation (Fig. 4B). In contrast, c-Jun was not detected by ChIP assay in normal (control RCASBP infected) and v-Src transformed CEF (SR-A RSV infected), confirming that the JunD/Fra-2 dimer is the predominant form of AP-1 in these cells.

v-Src controls the activity of AP-1 at multiple levels. To characterize the increase in AP-1 DNA binding activity, we first looked at the expression of the three components of AP-1 identified above, by Western blotting analysis. As shown in Fig.5A, c-Jun, JunD and Fra-2, were more abundant in CEF transformed by the wt SR-A strain of RSV. This was more striking for Fra-2 and JunD. CEF infected with the transformation deficient virus NY 315 RSV or with the control RCASBP(A) virus expressed similar levels of Fra-2, c-Jun and JunD. As reported by other investigators, multiple forms of these proteins were detected in this analysis. To a large extent, the v-Src dependent increase in AP-1 DNA binding activity can be accounted by the accumulation of c-Jun, JunD and Fra-2 in CEF stably transformed by SR-A RSV.

Pathways controlling the activity of several MAPKs, including ERK and JNK/SAPK, are activated in v-Src transformed cells (3, 30). Since JNK/SAPK targets the activation domain of c-Jun and JunD (11, 19, 23, 26, 48), we examined the potency of these domains fused to the DNA binding region of the Gal4 transactivator [Fig.5B; (39)].

Using this approach, we observed the potentiation of the JunD trans-activation function in v-Src transformed CEF (Fig.5C). Potentiation of the corresponding domain of c-Jun was also observed in these cells. However, the effects on the c-Jun domain were modest. A fraction of the JunD trans-activation function was sensitive to the action of a dominant negative mutant of SEK but a significant fraction of this activation was still detected in these conditions. Collectively, these results suggest that v-Src controls the activity of AP-1 at several levels and targets JunD and Fra-2, primarily, to enhance the activity of this factor in CEF.

TAM67 regulates the activity of AP-1, NFκB and C/EBPβ. The leucine zipper mediates the interaction of c-Jun with members of the Jun, Fos and ATF families. The same region promotes the interaction with NFκB and C/EBPβ (17, 20, 41). Therefore, TAM67 may alter the activity of all three factors binding to the v-Src Responsive Unit (SRU) of the IL8 promoter (6, 10, 13). This was confirmed in transient expression assays. As expected, the activation of a TRE-controlled promoter by v-Src was strongly impaired by the expression of TAM67 (Fig. 6A). However, a significant inhibition was also observed for the NFκB-controlled promoter while, in contrast, C/EBP activity was stimulated by TAM67 (Fig.6B). The resulting effect was a partial inhibition of the IL8 promoter in v-Src transformed CEF (Fig.6C). Thus, the effect of the dominant negative mutant of c-Jun was not restricted to the inhibition of AP-1.

TAM67 promotes the accumulation of lipid-rich vesicles in v-Src transformed CEF. The over-expression of C/EBPβ induces adipogenesis in fibroblasts, including CEF (24, 33). Therefore, the observation that TAM67 stimulates the activity of the C/EBP-

controlled promoter in v-Src transformed CEF raised the possibility that lipids accumulate in these cells. This was confirmed by staining CEF with Oil-Red O. As shown in Fig.7, the vesicles observed in a fraction of the TAM67 expressing cells accumulated the lipophilic dye, indicating that they contain lipids. Oil-Red O positive cells were detected primarily at the permissive temperature indicating that the response was v-Src-dependent and that, on its own, TAM67 was unable to promote a marked accumulation of lipids (Fig.7E).

We also asked if TAM67 induces the expression of the lipid-binding p20K lipocalin, a marker of contact inhibition and reversible growth-arrest but not adipogenesis in CEF (14, 24). As shown in Fig.7F, the activation of the temperature-sensitive v-Src kinase did not enhance the expression of p20K. This was true in control and TAM67 expressing CEF. Therefore, the inhibition of AP-1 did not induce a state of reversible growth arrest in these cells. Cells accumulating lipids at the permissive temperature were flat and viable for extended periods of time, suggesting that they had undergone differentiation.

Generation of retroviral vectors for shRNA expression in CEF. RNA interference provides the means to inhibit gene expression in a sequence-specific manner. Avian retroviruses for expression of shRNA have been described before (4, 7, 8, 18). However, little information exists on the efficacy of these vectors and their capacity to silence gene expression in a sustained manner, a condition required for studies on cell proliferation and transformation. Using existing RCASBP vectors expressing shRNA from an internal PolIII promoter, we failed to observe prolonged down-regulation of several transcription

factors of the AP-1 or NFκB family in chicken embryo fibroblasts (CEF; our unpublished results). Similar conclusions were reached by other investigators (7). In the original vector designed by Das and co-investigators, a shRNA is expressed in the context a chicken microRNA locus under the control of a U6 promoter. We reasoned that transcriptional interference between the viral LTR and the U6 promoter impairs the replication or expression of the viral genome. Since microRNA genes are transcribed by RNAPII, we deleted the U6 promoter and leader sequence to generate plasmid RCASBP-shRNA-ΔU6. In this vector, the shRNA sequence is cloned downstream of a splicing acceptor site and egfp gene (Fig.8A). Since expression of the viral genome can be monitored through GFP expression, we used the modified RCASBP vector to express a shRNA sequence targeting this gene and followed GFP expression in CEF. As shown in Fig.8B-C, GFP was detected broadly in the cell population transfected with the control retroviral vector but was undetectable in cells expressing the shRNA for GFP. This was confirmed by Western blotting analysis (Fig.8D). The expression of p27 gag was reduced in vectors containing the microRNA cassette but was detected with both the parental RCASBP-shRNA-ΔU6 vector and its derivative construct harbouring the shRNA sequence for GFP. Infection assays confirmed that functional retroviral particles were released in the medium of these cells (data not shown). Thus, while the vector was able to effectively silence the expression of GFP, viral protein expression and virus replication were not completely impaired by the presence of the shRNA sequence in the retroviral genome.

The expression of GFP and p27 gag was followed for several passages after

transfection of these retroviral constructs (Fig.9A). GFP expression was sustained in cells transfected with the parental vector but remained undetectable in CEF expressing the shRNA sequence. Gag expression was detected for the duration of the experiment with both retroviral vectors. We then determined if GFP expression could be inhibited in these cells when expressed transiently from an unrelated vector (CMV-GFP). CEF infected with RCASBP (A) were used as control in this experiment to allow for the detection of the transiently expressed GFP in the absence of the shRNA. As shown Fig.9B, GFP expression was reduced considerably in CEF infected by RCASBP-shRNA-ΔU6-*egfp* for more than six passages, suggesting that sustained inhibition was provided by the shRNA. This result also suggests that GFP down-regulation was the result of RNA interference and was not limited to the retrovirally encoded GFP gene of RCASBP-shRNA-ΔU6- *egfp*. We also examined the effect of shRNA expression on cell proliferation. CEF infected with RCASBP-shRNA-ΔU6-GFP accumulated at levels and with kinetics comparable to those of RCASBP(A) or RCASBP-shRNA-ΔU6 infected CEF (Fig.9C). Therefore, the induction of RNA interference did not affect CEF proliferation.

JunD mediates the pro-survival function of AP-1 in v-Src transformed CEF. Retroviral constructs expressing shRNA were generated to inhibit the expression of a single component of AP-1. A partial down-regulation of c-Jun expression led to a significant inhibition of CEF proliferation and a high incidence of senescent cells but no apoptosis (Fig.10A-F, R-S). The inhibition of JunD expression decreased CEF proliferation but did not cause senescence, as indicated by the absence of SAβG-positive

cells in these conditions (Fig. 10G-L). However, apoptotic cells were detected in CEF population expressing the junD shRNA (Fig. 10S). Despite a marked reduction in protein expression, the down-regulation of Fra-2 had little effect on the proliferation of normal CEF. Moreover, the inhibition of Fra-2 had no visible phenotype, causing no senescence or apoptosis (Fig.10M-S). In summary, c-Jun inhibition caused premature entry into senescence while JunD inhibition triggered apoptosis in a small fraction of normal CEF (3%; Fig.10).

We generated CEF infected with a temperature-sensitive mutant of RSV (NY72-4 RSV) and a virus expressing a shRNA for *c-jun*, *junD* or *fra-2*. A partial inhibition of c-Jun and JunD expression was observed in CEF expressing the corresponding shRNA. In contrast, Fra-2 expression was severely impaired at the permissive and non-permissive temperature (Fig.11). Transient expression assays confirmed the inhibition of AP-1 activity in v-Src transformed CEF expressing the shRNA for junD or fra-2 (Fig.12A-B). In contrast, the partial down-regulation of c-Jun did not dramatically reduce the activity of AP-1 in these cells.

As described in normal CEF, SAβG activity was observed in 72-4 RSV infected CEF expressing the c-jun shRNA at the permissive and non-permissive temperature, but was not detected in conditions of JunD or Fra-2 inhibition (data not shown). However, apoptotic cells were observed in cells expressing the junD shRNA. This phenotype was enhanced by the activation of the temperature sensitive v-Src kinase (Fig. 12I-J). A lower incidence of apoptosis was also detected in conditions of Fra-2 and c-Jun inhibition (Fig.12K). Therefore, JunD was important for the pro-survival activity conferred by AP-

1 in v-Src transformed CEF.

Lipid vesicles accumulate in response to Fra-2 inhibition in v-Src transformed cells. NY72-4 RSV infected CEF expressing the fra-2 shRNA were flat at the non-permissive and permissive temperature, indicating that Fra-2 expression was required to achieve morphological transformation. In addition, lipid-rich vesicles accumulated at the permissive but not at the non-permissive temperature (Fig.13D, H-I). This was not observed in conditions of c-Jun or JunD down-regulation (data not shown). Therefore, Fra-2 expression was required to block the accumulation of lipids in v-Src transformed CEF.

In summary, the pleiotropic action of TAM67 can be ascribed to individual components of the AP-1 factor. Thus, c-Jun inhibition caused a premature entry into senescence while JunD was required for the survival of v-Src transformed CEF. Finally, the inhibition of Fra-2 blocked morphological transformation and induced the appearance of lipid-rich vesicles upon activation of the temperature-sensitive v-Src kinase.

The down-regulation of p53 restores the viability and transformation of v-Src transformed CEF in conditions of JunD inhibition. The induction of p53 was observed in cells expressing the junD shRNA. This induction was enhanced by the activation of the temperature sensitive v-Src kinase (Fig. 14A, lane 8). UV-irradiated CEF served as positive control in this experiment. To determine the role of p53 in the apoptosis induced by JunD inhibition, we constructed a retroviral vector expressing separate shRNA for p53 and JunD (Fig. 14A, lanes 3,6,9). The down-regulation of p53 restored the proliferation

and morphology of v-Src transformed CEF (data not shown). Anchorage-dependent proliferation was examined in soft agar assays. While RSV infected CEF were unable to form colonies in conditions of JunD inhibition, the concomitant down-regulation of p53 restored the ability of these cells to grow in an anchorage-independent manner. JunD expression and the activity of AP-1 remained low in these cells (Fig.14A-B). Therefore, the induction of apoptosis observed in conditions of JunD/AP-1 inhibition was p53-dependent. These results imply that in the absence of p53, high AP-1 activity is no longer required to ensure the survival of v-Src transformed CEF.

DISCUSSION

Sustained gene down-regulation provided by the RCASBP-shRNA retroviral system. Two methods were employed to assess the role of AP-1 in v-Src transformed CEF. The expression of TAM67 with the RCASBP vector provided the most potent inhibition by reducing the activity of a TRE-controlled promoter by over 90% (Fig.6A). However, the effects of the dominant negative mutant of c-Jun were not limited to AP-1 as a considerable inhibition of NFκB was also observed, in agreement with the results of other investigators (28). In contrast, TAM67 enhanced the expression of a reporter gene controlled by C/EBP binding sites, indicating that it can exert positive or negative effects on the factors binding to the v-Src responsive unit of the IL8 promoter/enhancer. We showed previously that the expression of a dominant negative mutant of C/EBPβ enhances the activity of AP-1 in CEF (14). Therefore, AP-1 and C/EBPβ cooperate in the induction of genes of the G0/G1 transition, such as IL8, but antagonize each other in

different promoter/enhancer contexts. The mechanism underlying this phenomenon is unknown but may reflect the competition for limiting amounts of co-activators in the cell.

To alleviate the non-specific effects of the dominant negative mutant of c-Jun, we employed a second strategy based on the expression of shRNA by the RCASBP retroviral system (21). To this end, we modified an existing vector developed by Das and co-investigators by removing the internal U6 promoter and leader sequence and expressing the shRNA as a spliced sub-genomic transcript controlled by the strong LTR of RCASBP. Gene down-regulation by the modified vector was sustained over several passages and thus adequate for biological assays such as colony formation in soft agar (Fig.14).

The efficiency of the RCASBP-shRNA vectors varied in a gene-dependent manner. Efficient down-regulation was observed for gene products, such as Fra-2, that did not reduce cell viability or markedly impair cell proliferation. In contrast, a partial inhibition was observed for gene products that provided a survival advantage, such as JunD, or were required for cell proliferation, such as c-Jun (Fig.10). The same result was observed with shRNA vectors targeting four different regions of the c-jun or junD mRNA, making it unlikely that the limited down-regulation of these gene products reflects the secondary structure and inaccessibility of these transcripts (our unpublished results). The partial inhibition may be the result of the selective pressure exerted against cells with a high degree of c-Jun or JunD down-regulation. In support of this idea, we observed that the modified RCSABP-shRNA-ΔU6 vector provides near complete inhibition of GABARAP (γ-aminobutyric acid receptor A associated protein) or

CHOP10/gadd153, two proteins promoting apoptosis in conditions of ER stress in CEF (our unpublished results). Thus, further modification of the vector to provide inducibility of the shRNA may enhance the efficiency of this retroviral system (7).

The inclusion of a short hairpin structure in the retroviral genomic RNA did not abolish replication and the release of infectious particles, suggesting that the genomic transcript is less susceptible to degradation. Other investigators have reported similar observations using different retroviral constructs (18). It is possible that the rapid assembly and release of retroviral particles limit the accessibility of genomic transcripts to the RNA interference machinery or that the secondary structure masks or disrupts the formation of the hairpin in the genomic and env sub-genomic transcripts, as proposed by Harpavat and Cepko (18). This feature of the RCASBP-shRNA system promotes the rapid and stable expression of the shRNA in the vast majority of the CEF population.

AP-1 is required for the survival of v-Src transformed CEF. The inhibition of AP-1 reduces markedly the proliferation of normal CEF (14). In this study, we show that AP-1 is also required for the survival of v-Src transformed cells. The expression of a dominant negative mutant of AP-1 (TAM67) or the down-regulation of JunD by shRNA resulted in a high incidence of apoptosis in these cells (Fig.2 and 12). Other studies have shown that v-Src transformed cells are hypersensitive to the action of PI3K inhibition and suggested that v-Src generates conditions promoting apoptosis (2, 45). In agreement with this notion, we found that the activation of v-Src was sufficient to increase the incidence of apoptosis, albeit modestly, even in the absence of AP-1 inhibition (Fig. 2I).

The nature of the pro-apoptotic pathway(s) involved in this response is unknown

but may function as a p53-dependent "oncogene checkpoint". Wagner and co-workers reported that c-Jun(-/-) MEF undergo premature entry into senescence in vitro and that c-Jun inhibits the transcription of the p53 tumour suppressor by a mechanism that remains poorly understood (40). Likewise, JunD(-/-) MEF undergo premature entry into senescence in culture, the result of the accumulation of ARF and the induction of the p53 pathway (46). Again, the mechanism by which JunD inhibits the expression of ARF is unclear. More recently, Gillespie and co-investigators provided a different explanation for the response of primary MEF with inhibition of AP-1 (31). These authors showed that the normal in vitro proliferation of c-Jun(-/-) MEF is restored by culturing the cells in conditions of moderate hypoxia (3%). They also showed that the c-Jun(-/-) MEF display the formation of γH2AX foci and evidence of DNA damage in normal laboratory conditions (i.e. 20% oxygen). Thus, they concluded that MEF with reduced c-Jun/AP-1 activity are more susceptible to DNA damage in conditions of hyperoxia. The activation of the DNA damage response has also been described in the senescence induced by activated Ras (RasV12; (32)). Ferbeyre and co-workers showed that the senescence caused by RasV12 can be bypassed by the down-regulation of ATM in primary human fibroblasts. We observed the accumulation of p53 in v-Src transformed CEF over-expressing TAM67 or the JunD shRNA (Fig.14 and data not shown). Significantly, the concomitant inhibition of p53 and JunD by shRNA restored the ability of v-Src transformed CEF to grow in soft agar, indicating that they were transformed. Using antibodies for chicken Arf (p7Arf);(25) or RT-PCR, we failed to detect the induction of p7Arf protein or transcript in normal and v-Src transformed CEF expressing TAM67 or

shRNA for junD (our unpublished results). Species-dependent differences may account for this discrepancy in the induction of p53 in MEF and CEF (46). Therefore, the pathway leading to the activation of p53 remains to be investigated in v-Src transformed CEF. JunD provides a survival signal in MEF treated with TNFα (27, 46). Therefore, the pro-survival function of JunD is conserved in CEF and MEF.

TAM67 inhibited NFκB activity in v-Src transformed CEF (Fig.6). Preliminary results revealed that the inhibition of p105/p50 NFkB1 by shRNA induces senescence in normal CEF and apoptosis in their v-Src transformed counterparts (our unpublished results). Therefore, like JunD, p105/p50 NFkB1 is required for the survival of v-Src transformed cells.

Specificity of action of AP-1 proteins. The expression of TAM67 caused several phenotypes in v-Src transformed CEF. Surprisingly, each phenotype was ascribed to a different component of AP-1 as the down-regulation of c-Jun caused senescence, the inhibition of JunD induced apoptosis and the reduction of Fra-2 levels resulted in the accumulation of lipid-rich vesicles but no senescence or apoptosis. These results imply a certain specificity in the action of each component of AP-1. Alternatively, the phenotypes may simply reflect a different degree of AP-1 inhibition and/or a different contribution to the activity of this factor by c-Jun, JunD and Fra-2. Since complete inhibition of c-Jun (and JunD) was not achieved in these studies, it is difficult to distinguish between these models. Fra-2 must also dimerize with a member of the Jun family to bind to the TRE. Therefore, it is expected that the down-regulation of JunD, the predominant member of the Jun family expressed in CEF, would also impair the

function of Fra-2. However, no lipid vesicles were observed in conditions of JunD inhibition. It is possible that the expression of genes associated with the accumulation of lipids is also regulated in conditions of JunD inhibition but that the induction of apoptosis interferes with this process. As described in other fibroblasts, the over-expression of C/EBPβ promotes adipogenesis in CEF (24). It is tempting to speculate that the increase in C/EBPβ activity caused by the expression of TAM67 promotes cell differentiation (adipogenesis) and is responsible for the formation of lipid-rich vesicles in v-Src transformed CEF (Fig.7). Studies to evaluate the role of C/EBPβ and determine if markers of adipogenesis are induced by v-Src in conditions of Fra-2/AP-1 inhibition are in progress.

CONCLUSION

In these studies, we demonstrate that AP-1 facilitates transformation by several processes, blocking premature entry into senescence, functioning as a survival factor and antagonizing cell differentiation. We identify JunD and Fra-2 as the main components of AP-1 in v-Src transformed CEF and provide evidence that these proteins fulfill different functions in the cell. Using a modified RCASBP system for shRNA expression, we implicate JunD as an essential factor of cell transformation and show that JunD/AP-1 is required for the survival of v-Src transformed CEF.

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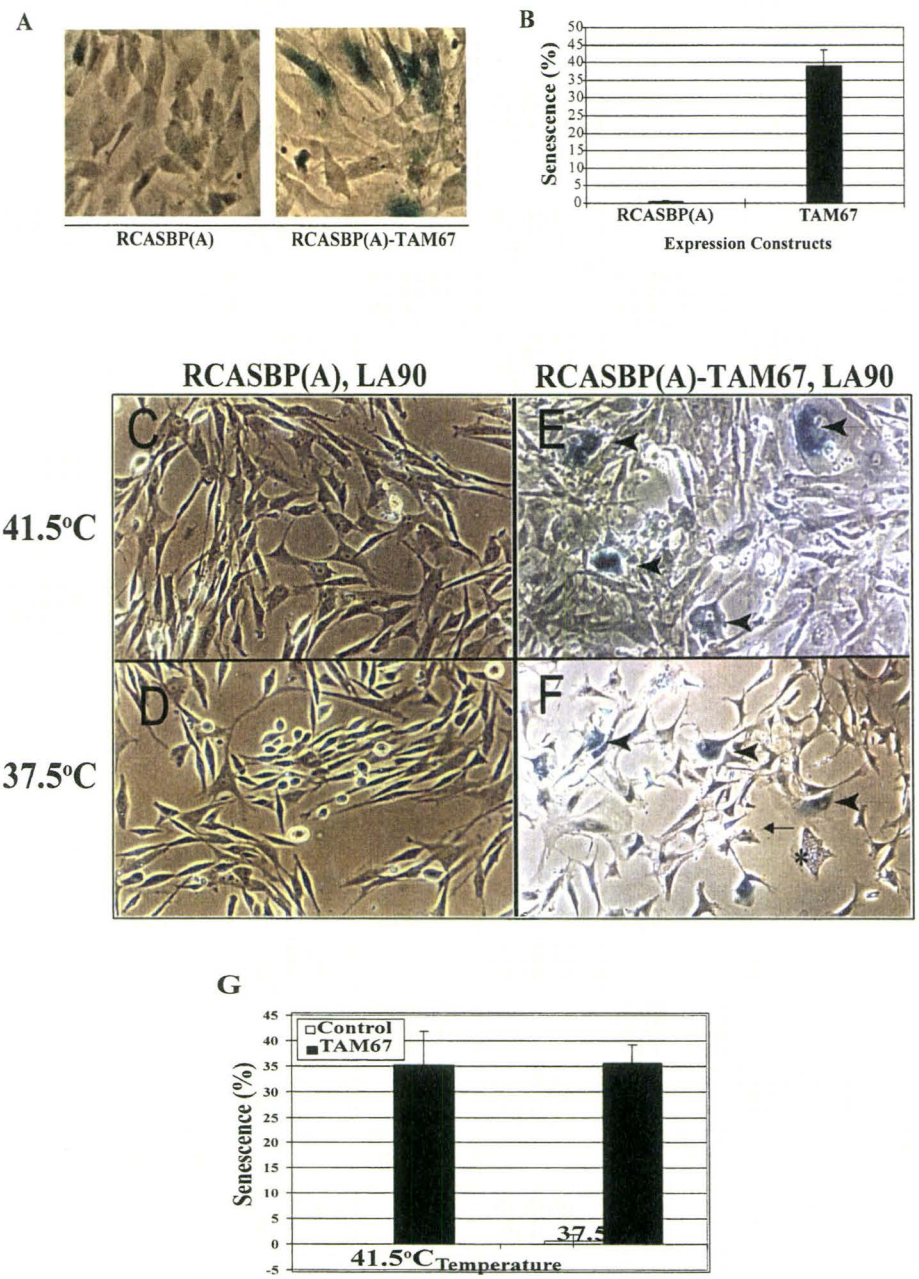
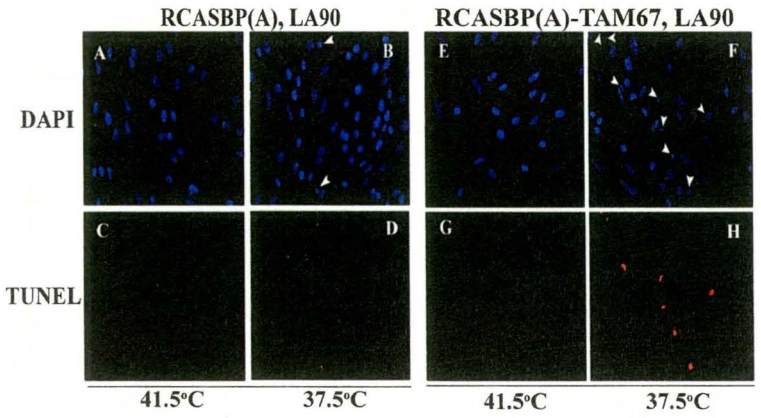


Figure 1. Determination of senescence-associated β galactosidase activity (SAβG) in CEF infected with RCASBP(A) or RCASBP(A)-TAM67 (panels A-B), or co-infected with the temperature sensitive mutant LA90 RSV (panels C-F). SAβG was determined at the permissive and non-permissive temperature. Arrow-heads point to SAβG positive cells while the asterisks identifies cell that is highly vesiculated but negative for SAβG activity. The arrow points to a cell displaying membrane blebbing. All photos were taken at a 400x magnification. **G)** Quantitation of SAβG activity in CEF co-infected with LA90 RSV and RCASBP(A)-TAM67 or the control virus RCASBP(A) at the permissive and non-permissive temperature.



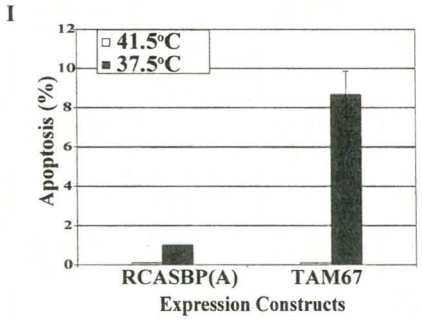


Figure 2. Tunel assays of CEF co-infected with LA90 RSV and RCASBP(A)-TAM67 or the control virus RCASBP(A) at the permissive **(panels B,D,F,H)** and non-permissive temperature **(panels A,C,E,G)**. Nuclei were stained with DAPI. Arrowheads indicate the position of apoptotic cell nuclei. **I)** Quantitation of the incidence of apoptotic cells as determined in the experiment described in panels A-G. The error bars represent the standard error. All photos were taken at a magnification of 400x.

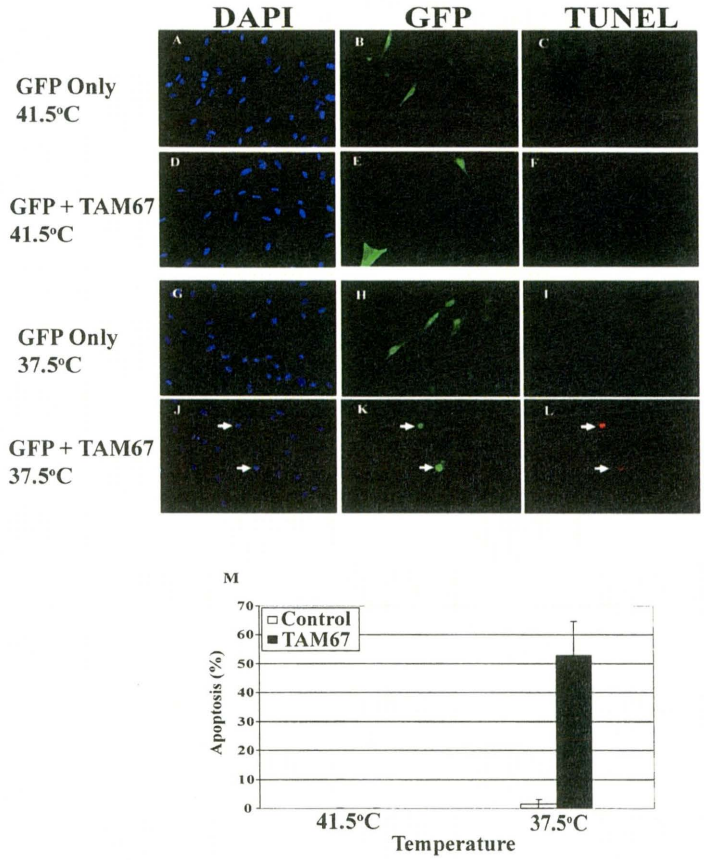


Figure 3. The induction of apoptosis by transient expression of TAM67 in LA90 RSV-infected CEF at the non-permissive (41.5˚C) and permissive temperature (37.5˚C) was determined by the Tunel assay **(panels A-L). M)** Quantitation of apoptosis. The error bars represent the standard error. All photos were taken at a magnification of 400x.

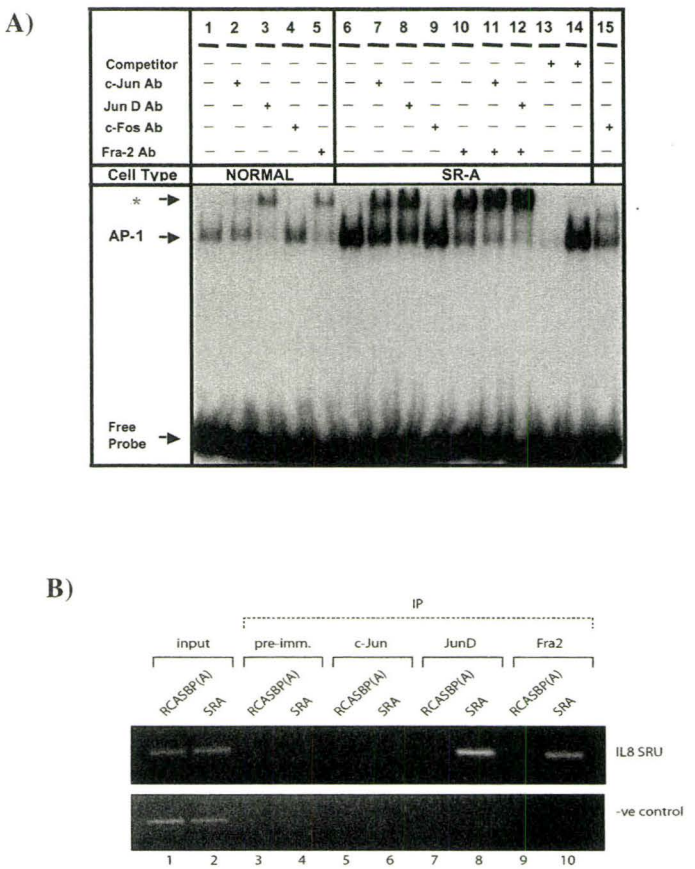


Figure 4. A) Identification of the components of AP-1 by EMSA and specific antibodies for c-Jun, Jun D, Fra-2 and c-Fos. Nuclear extracts were prepared from normal CEF **(lanes 1-5)**, SR-A RSV transformed CEF **(lanes 6-14)** and TPA treated CEF **(lane 15)**. The position of the nucleoprotein complex bound by the antibody is indicated by the asterisk. The competitor was a 50 molar excess of the unlabelled TRE oligonucleotide (lane 13) or mutant form of the TRE (lane 14), added to the binding reaction. **B)** Identification of JunD and Fra-2 as factors recruited to the SRU region of the IL8 promoter by ChIP assays. RCASBP(A) infected CEF served as controls and were compared to wt SR-A RSV transformed CEF. An upstream promoter region was studied in parallel and used as negative control.

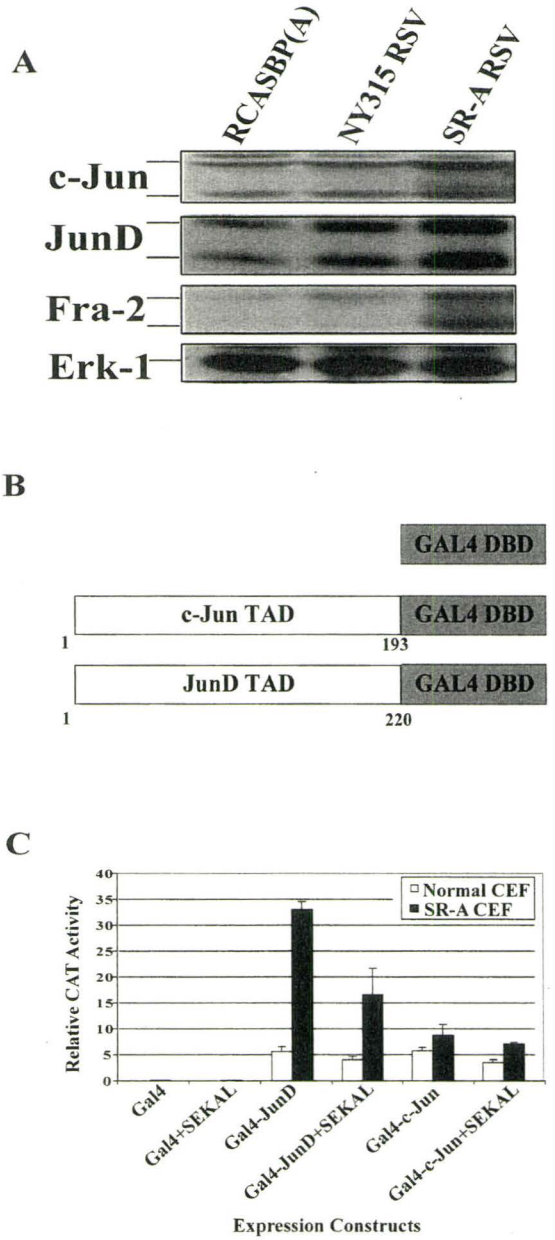


Figure 5. A) Western blotting analysis of c-Jun, JunD and Fra-2 expression in CEF infected with the transformation-deficient viruses RCASBP(A) and NY315 RSV or transformed by wt SR-A RSV. ERK was used as a loading control. **B)** Schematic representation of Gal4 fusion constructs. The transactivation domain (TAD) of chicken c-Jun (amino acids 1-193) or JunD (amino acids 1-220) was fused to the DNA binding domain (DBD) of the Gal4 protein. **C)** Potentiation of the TAD of c-Jun and JunD in normal and wt SR-A RSV transformed CEF. Partial inhibition of the c-Jun and JunD trans-activation function is observed in response to the expression of a dominant negative mutant of SEK (SEKAL).

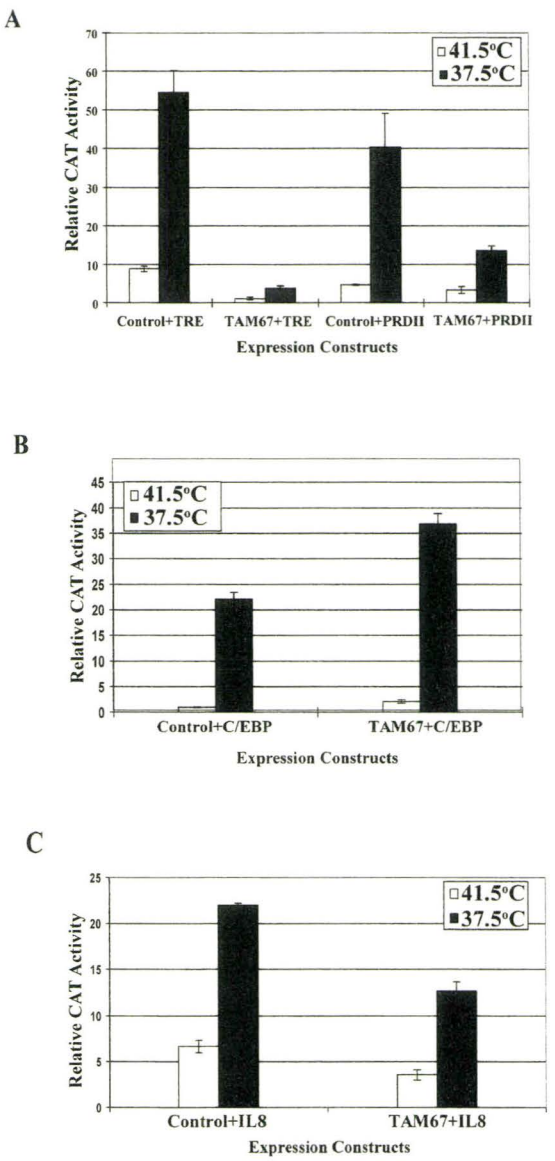


Figure 6. Effect of TAM67 on the IL8 promoter/enhancer and regulatory elements. CEF infected with the temperature sensitive strain NY72-4 RSV were co-transfected with either a TAM67 expression construct or the control vector along with CAT reporter constructs consisting of a minimal promoter controlled by (A) the TRE for AP-1 or PRDII for NFκB, (B) C/EBP binding sites for CEBP/β or (C) the IL8 promoter/enhancer. CAT activity was measured for cells at the non-permissive temperature of 41.5˚C (non-transformed) and permissive temperature of 37.5˚C (transformed).

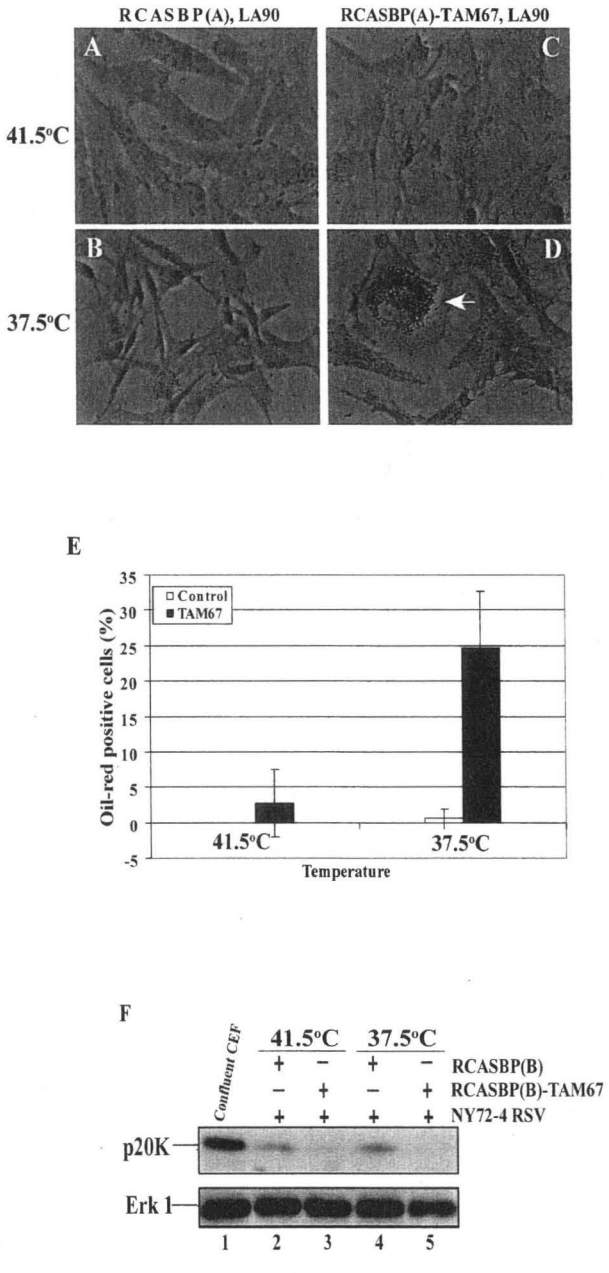


Figure 7. Lipid-containing vesicles (arrow) were identified at the permissive temperature by Oil-Red O staining of CEF co-infected with ts NY72-4 RSV and RCASBP(A)-TAM67 (panels A-D). **E)** Quantitation of cells with Oil-Red O positive vesicles. **F)** Expression of the growth arrest-specific p20K lipocalin in control and TAM67 expressing CEF. Contact-inhibited CEF were used as a positive control for p20K expression. Erk-1 was used as loading control.

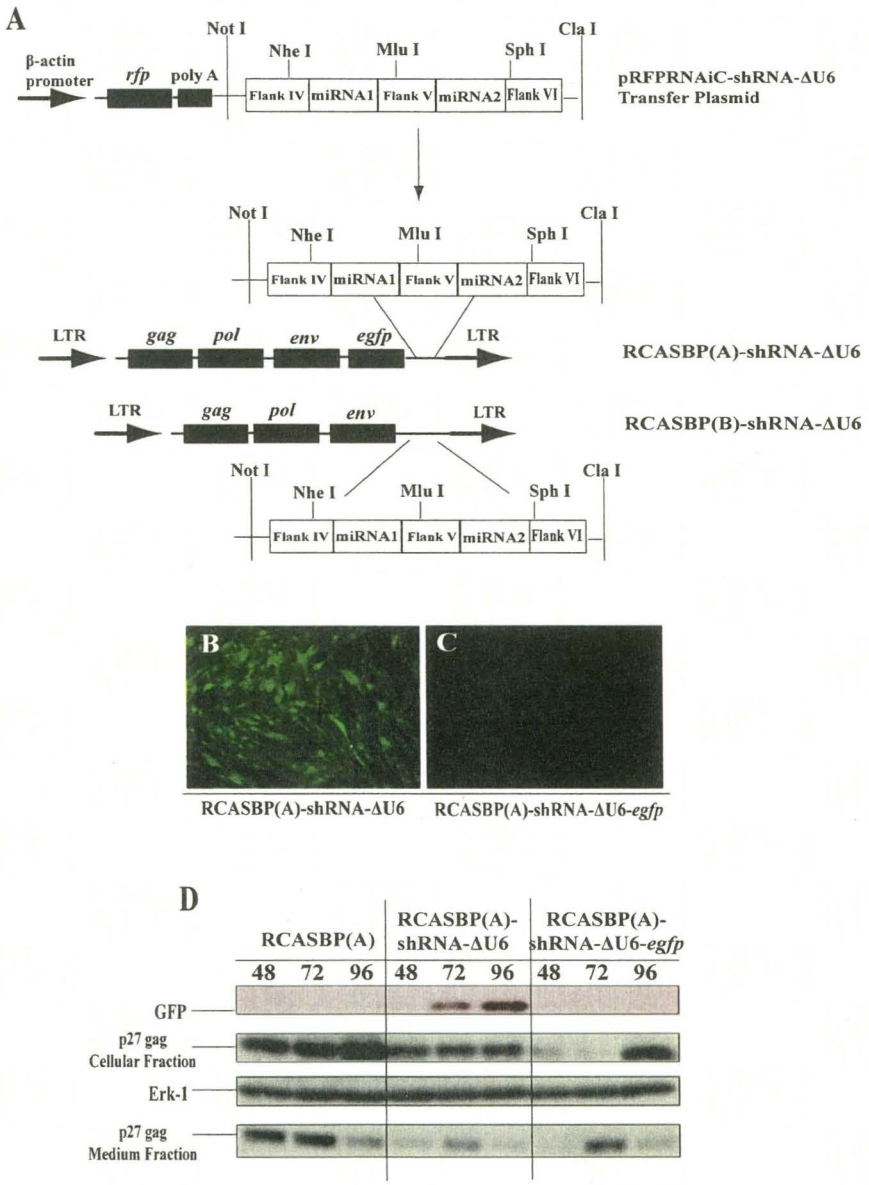


Figure 8. Avian replication competent retroviruses for shRNA expression. **A)** Schematic representation of the virus-based shRNA expression system. The U6 promoter and leader sequences have been removed from the original microRNA operon described by Das and co-workers {Das, 2006 #6360}. Sequences generating the hairpin structure are cloned at the NheI/MluI, MluI/SphI or at both of these locations in the transfer vector pRFPRNAiC-shRNA-ΔU6. The microRNA cassette, harbouring the hairpin sequences, is then sub-cloned in the retroviral vectors. The RCASBP(A)-shRNA-ΔU6 vector carries an *egfp* gene missing in the RCASBP(B) counterpart; **B-C)** GFP expression was examined by fluorescence microscopy four passages after transfection of the control RCASBP(A)-shRNA-ΔU6 or RCASBP(A)-shRNA-ΔU6 vector carrying the *egfp* shRNA (RCASBP(A)-shRNA-ΔU6-GFP) inserted at the NheI/MluI site (magnification: 200X). GFP expression was completely suppressed by the GFP shRNA; **D)** Western blotting analysis of GFP expression in CEF infected with the control viruses RCASBP(A) and RCASBP(A)-shRNA-ΔU6 or the *egfp* shRNA expressing virus. The expression of p27 gag in the cell or recovered from the medium was also examined. Erk-1 was used as a loading control.

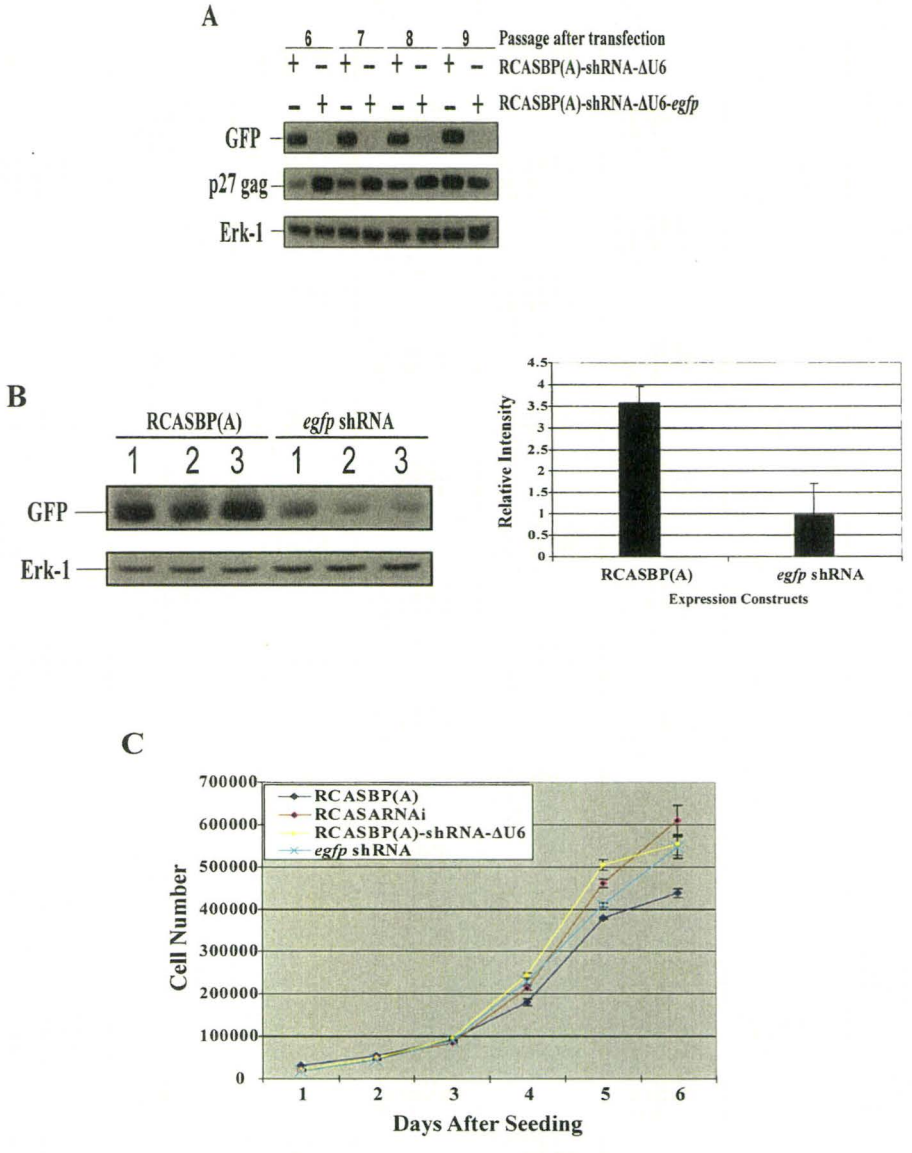


Figure 9. Sustained inhibition of GFP expression by shRNA. **A)** GFP expression in control and e*gfp* shRNA expressing CEF. Protein lysates were prepared from the 6th passage after transfection of the control and retroviral vector expressing the e*gfp* shRNA. Levels of GFP and p27 gag, recovered from the tissue culture medium, were analyzed by Western blotting analysis. Erk-1 was used as a loading control; **B)** A GFP expression plasmid (pGFP-N1) was transfected with pRSV-βGal at the 5th passage into CEF infected with the RCASBP(A) control vector or e*gfp*-shRNA expression virus. pRSV-βGal was used to control for transfection efficiency. The results of triplicate samples are shown and quantitated. The inhibition of transiently expressed GFP was over 70% in CEF expressing the e*gfp* shRNA. **C)** The proliferation of CEF infected with the control vectors RCASBP(A), RCASBP(A)RNAi {Das, 2006 #6360} and RCASBP(A)-shRNA-ΔU6 is compared to that of CEF expressing the e*gfp* shRNA. Error bars represent the standard error from the mean.

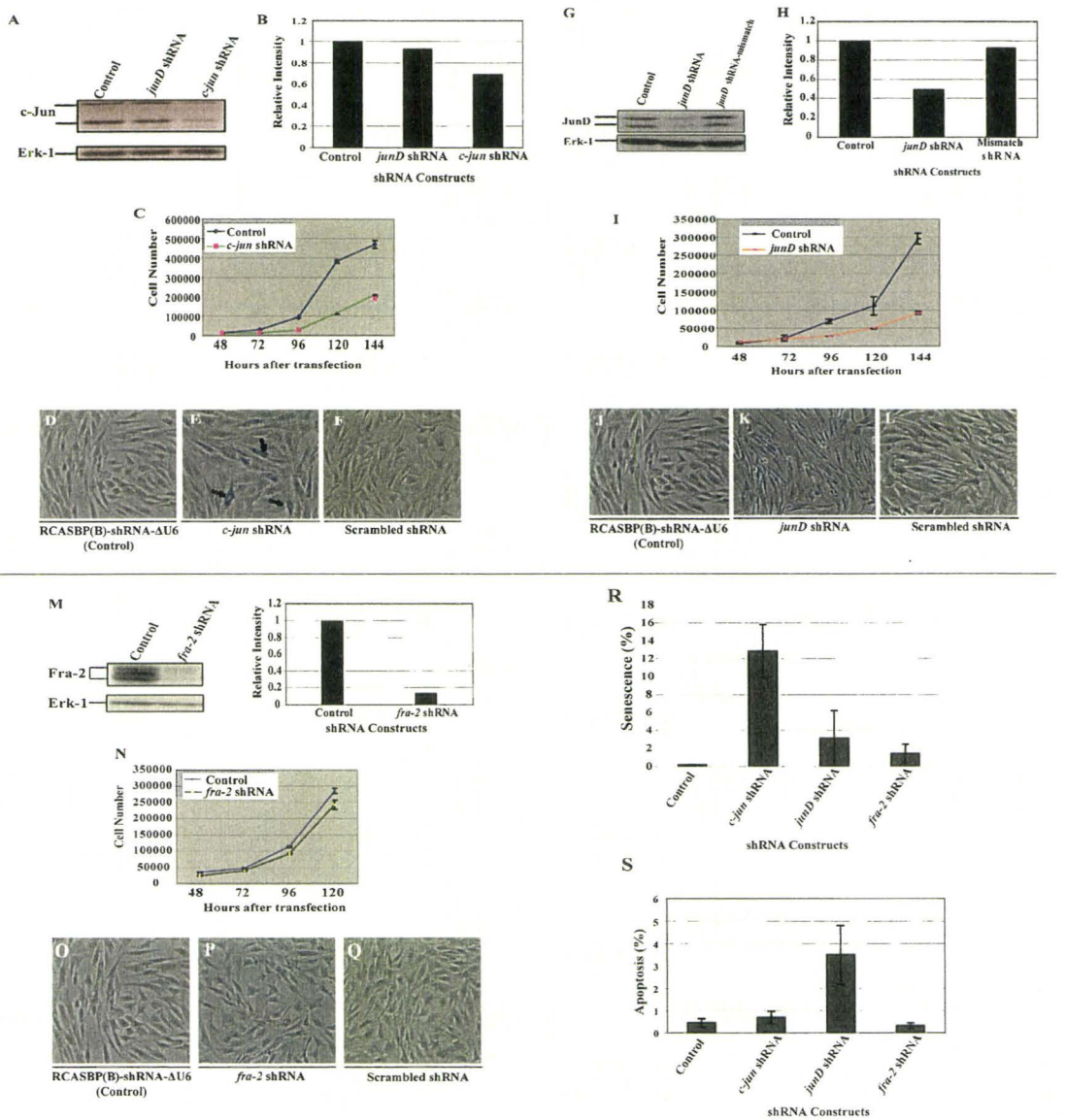


Figure 10. Effect of c-Jun, JunD, Fra-2 down-regulation by shRNA in normal CEF. Down-regulation of c-Jun expression by shRNA was examined by Western blotting analysis (panel A). The level of c-Jun inhibition was determined by densitometry (panel B). The reduction of c-Jun expression impaired cell proliferation (panel C) and induced premature entry into senescence while the control vector or expression of the mismatch form of *c-jun* shRNA did not have any significant effects (SAβG assays; panels D-F). The *junD*-821 shRNA reduced the expression of JunD and inhibited cell proliferation (panels G-I). The levels of JunD were determined by densitometry (panel H). SAβG assays revealed no induction of senescence in conditions of JunD inhibition (panels J-L). Fra-2 expression was abolished by the corresponding shRNA (panel M). Loss of Fra-2 expression did not impair proliferation or induce any visible phenotype in normal CEF (panels N-Q). **R)** Quantitation of SAβG-positive cells in the presence of various shRNA as described above. **S)** Quantitation of apoptosis in CEF expressing a shRNA for *c-jun*, *junD* or *fra-2* or the control vector RCASBP(B)-shRNA-ΔU6 as determined by the Tunel assay.

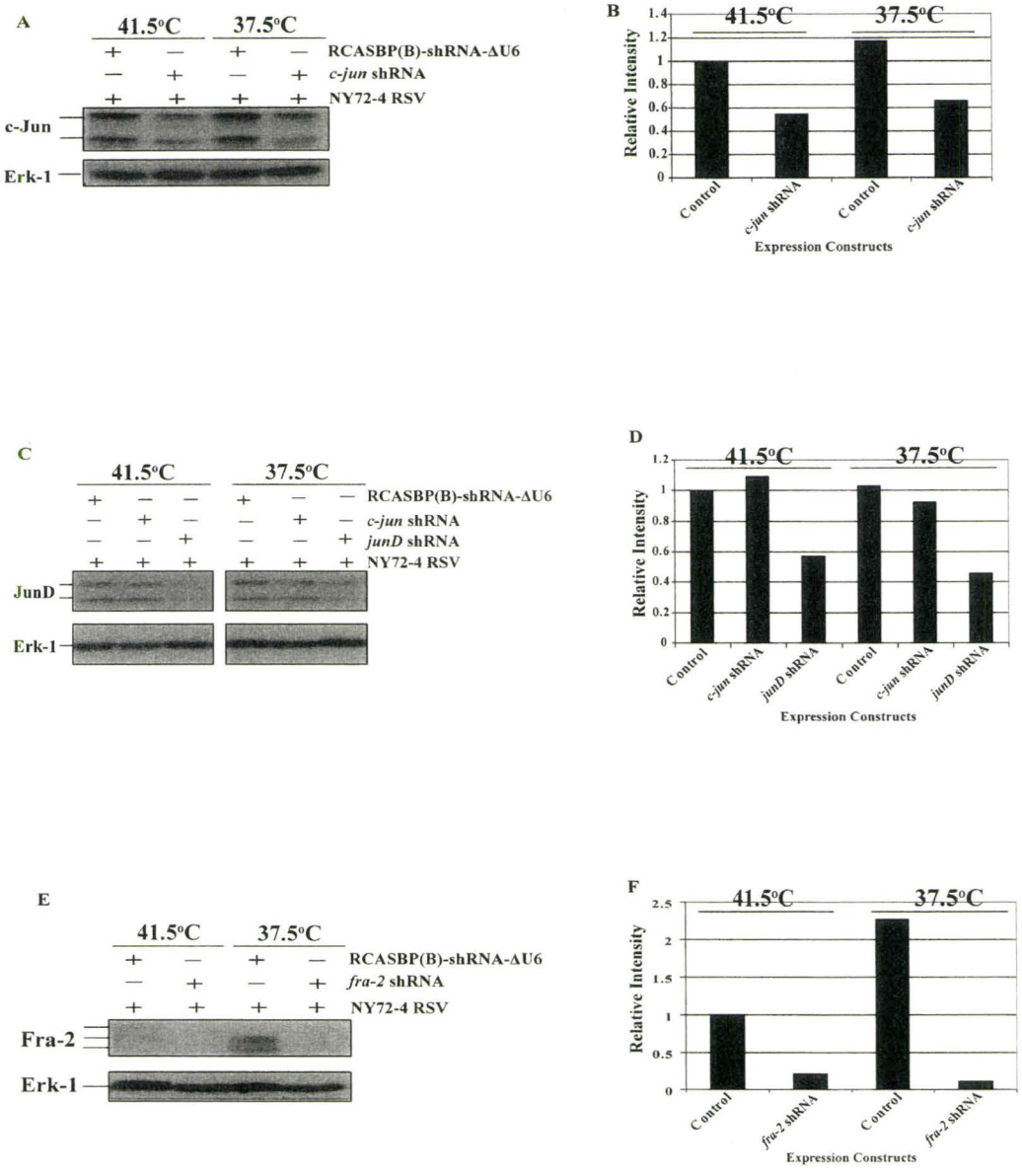


Figure 11. Repression of c-Jun, Jun D and Fra-2 expression by shRNA in v-Src transformed CEF. CEF were co-infected with the temperature sensitive mutant NY72-4 RSV and a group virus B expressing a shRNA for *c-jun*, *junD* or *fra-2* or the control virus RCASBP(B)-shRNA-ΔU6 (Control). The repression was determined at the non-permissive (NP) and permissive temperature (P) of 41.5˚C and 37.5˚C, respectively, by Western blotting analysis (panels A, C, E) followed by densitometry (panels B, D, F). Erk-1 was used as loading control.

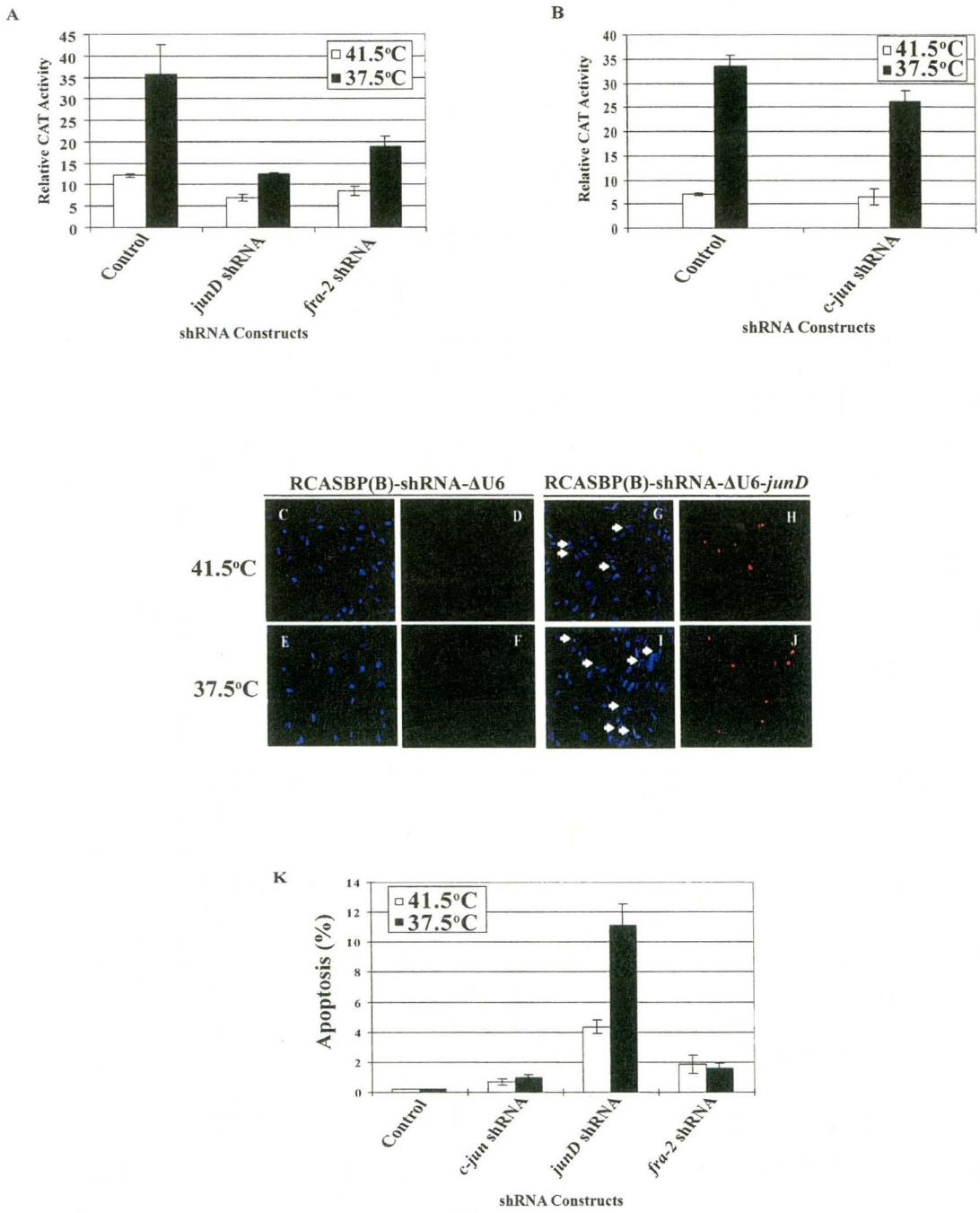


Figure 12. Apoptosis induced by v-Src in response to JunD down-regulation. **A-B)** Effects of c-Jun, JunD and Fra-2 down-regulation by shRNA on the activity of AP-1 in 72-4 RSV-infected CEF. **C-J)** Tunel assays in NY72-4 RSV infected CEF expressing a shRNA for *junD*. **K)** Quantitation of apoptosis induced by the control virus or a virus expressing shRNA for individual AP-1 members. Error bars indicate the standard errors.

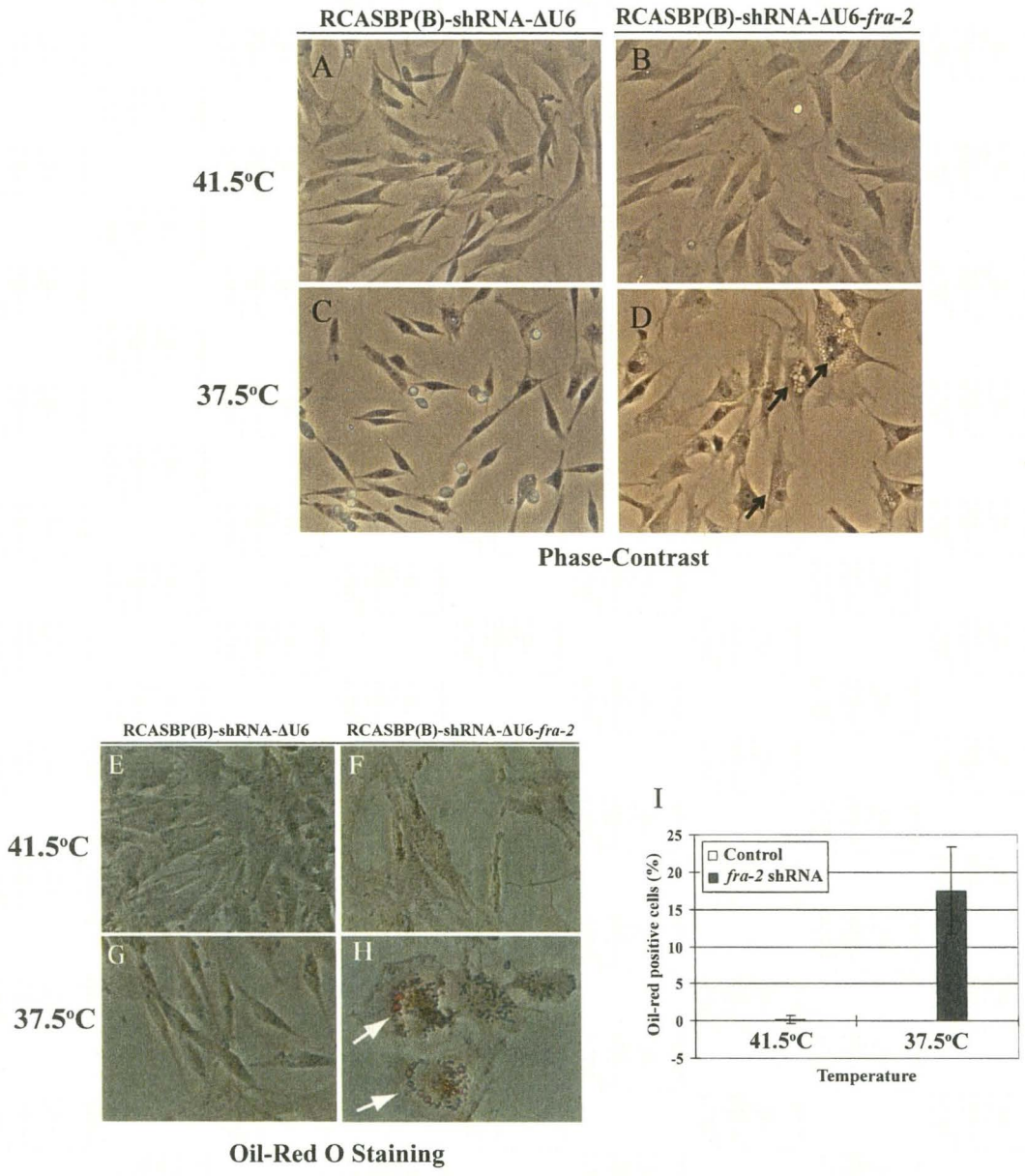


Figure 13. Formation of lipid vesicles in NY72-4 RSV infected CEF expressing the *fra-2* shRNA. Vesicles (arrows) appear following the activation of the temperature sensitive v-Src kinase in NY72-4 RSV infected CEF expressing the *fra-2* shRNA (A-D: Phase contrast microscopy; E-H: Oil-Red O staining). **I)** Quantitation of Oil-Red O positive cells. Error bars indicate the standard errors.

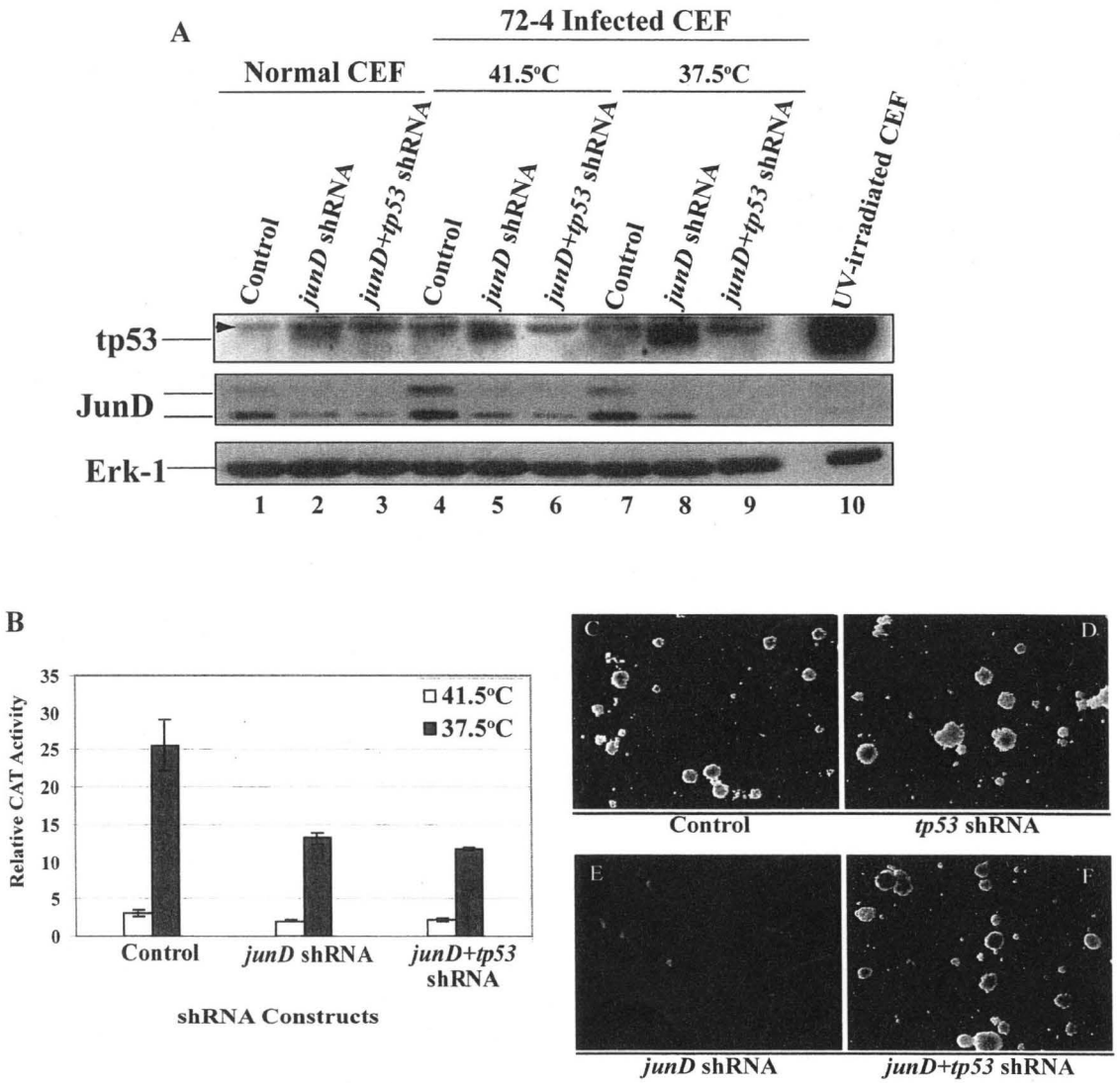


Figure 14. Induction of tp53 in CEF expressing the jun D shRNA. **A)** Western blotting analysis of tp53 level in CEF infected with a control virus (RCASBP(A)-shRNA-ΔU6), a virus expressing the *junD* shRNA or separate shRNA for *junD* and *tp53* (*junD* + *tp53*). The induction of tp53 in UV-irradiated CEF was used as a positive control (lane 10). Expression of the *tp53* shRNA abrogates the accumulation of tp53 in CEF co-infected with NY72-4 RSV and a virus expressing the *junD* shRNA (lanes 5-6 and lanes 8-9). The arrowhead points to a protein of slower mobility whose expression is not induced by UV irradiation or the expression of the *tp53* shRNA and therefore appears to be unrelated to tp53. **B)** Transient expression assays for AP-1 activity in NY72-4 RSV infected CEF expressing a single shRNA for *junD* or two shRNA for *junD* and *tp53*. **C-F)** Soft agar assays of CEF co-infected with NY72-4 RSV and a control virus (RCASBP(A)-shRNA-ΔU6), a virus encoding a single shRNA for *junD* or *tp53*, or two shRNA species for *junD* and *tp53* (*junD+tp53*); magnification: 10X. The down-regulation of tp53 re-established the anchorage-independent proliferation of v-Src transformed CEF with Jun D/AP-1 inhibition.