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Role of CPEB in Senescence and Inflammation: A Dissertation

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ROLE OF CPEB IN SENESCENCE AND INFLAMMATION

A Dissertation Presented

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DEDICATION

With my deep respect and gratitude I dedicate this work to my scientific mentors:

Lev Ovchinnikov, Jeffrey Nickerson and Joel Richter

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CHAPTER I

INTRODUCTION

Cellular mRNAs are synthesized in the nucleus and are exported to the cytoplasm where they can be actively translated, stored in a dormant form, or degraded. From birth to death, each mRNA is bound by an intricate combination of RNA binding proteins that regulate mRNA export, transport, localization, stability, and translation. Some of the RNA binding proteins, such as the cold-shock proteins, bind mRNAs with a low degree of sequence-specificity while other proteins bind with high specificity to cis-elements in untranslated regions (5'UTR or 3'UTR) (Macdonald, 2001; Andreassi and Riccio, 2009).

mRNA-specific regulatory factors control mRNA fate via recruitment of different multi-protein complexes that assemble in a dynamic process regulated by different signaling events. In many cases, non-coding RNAs (siRNA or miRNAs) can be also recruited to mRNA binding multi-protein complexes (known as mRNPs) where they control the mRNA expression. In turn, these complexes influence mRNA translation by using different mechanisms including interfering with binding of translational factor eIF4E or ribosomal subunits, mediating miRNA repression, or controlling cytoplasmic polyadenylation and/or deadenylation (Macdonald 2001; Fabian et al., 2010; Groppo and Richter, 2009).

Modulating of the length of poly(A) tail is one of the well-described mechanism for controlling mRNA translation and stability. Many studies show that the shortening of poly(A) correlates with mRNA repression and/or mRNA degradation whereas the addition of poly(A) is coincident with active translation and protection of mRNA from ribonuclease activity (Richter, 1999; Pique et al., 2006). There are several known cis-elements in the 3'UTR of mRNA such as the Nanos response element (NRE), Pumilio binding element (PBE), embryonic deadenylation element (EDEN), AU-rich element (ARE), and Cytoplasmic Polyadenylation Element (CPE), all of which were shown to be important for shortening of poly(A) tails of its target mRNAs (Macdonald 2001). In addition, multiple miRNA-binding sites in 3'UTRs can also mediate inhibition of translation via deadenylation. On the other hand, CPE sequences, in concert with the polyadenylation hexanucleotide (HEX), were shown to be important for the extension of poly(A) tails (Mendez and Richter, 2001). Moreover, the CPE element can perform a dual function through a signal-dependent switch from shortening to elongating of mRNA poly(A) tails and therefore from translational repression to activation. In some cases, there is a certain degree of synergy (e.g., among the CPE, PBE, and HEX) or competition (e.g., between the CPE and ARE, or the ARE and miRNAs) between two or more cis-elements, which adds an additional level of regulation to mRNA translation (Pique et all., 2008).

The Cytoplasmic Polyadenylation element (CPE) is a specific U-rich sequence (UUUUAAUU or UUUUAU) that is bound by an evolutionarily

conserved mRNA binding protein, the Cytoplasmic Polyadenylation Binding Protein (CPEB). CPEB possesses two RNA-recognition motifs (RRMs) and two zinc-finger motifs that are required for CPE-binding activity (Hake et al., 1998). It was shown that phosphorylation of CPEB by the kinase Aurora A shifts CPEB-dependent shortening of mRNA tails toward their polyadenylation (Mendez et al., 2000). CPEB was originally discovered and extensively studied in early development in *Xenopus laevis* oocytes where it was found to be a critical factor for meiotic progression and oocyte maturation through the regulation of cyclin B1 and mos mRNAs (Stebbins-Boaz et al., 1996; Mendez and Richter, 2001).

The observation of CPEB-dependent translational control of these cell cycle-related mRNAs in frog oocytes lead to the idea that CPEB could also play a role in the mitotic cell cycle in frog embryos and, perhaps, in mammalian cells. In fact, there is growing evidence of CPE- and CPEB-dependent translation, mRNA stability, and mRNP transport in rodent cells. CPEB was shown to be involved in the cell cycle, synaptic plasticity in neurons (Richter 2007) and, as presented in this dissertation, in cellular senescence and LPS-induced inflammation.

The mechanism of CPEB-dependent translation in *Xenopus* oocytes

CPEB was originally discovered in the early 1980s in the oocytes of the African frog *Xenopus laevis*. The absence of active transcription in oocytes is advantageous for studying translational control, especially via the regulation of

polyadenylation, which is the dominant mechanism of gene expression control in early oocytes (Vassalli et al., 1989; Paris et al., 1988) . Thus, unmatured oocytes stay quiescent for many days in G2-like state and contain a huge pool of maternal mRNA being stored in a dormant form with a shortened poly(A) tails (about 20-40 adenosines).This supply of dormant mRNAs is important material for the future oocyte cell division and promotion of embryonic polarity (Tadros et al., 2005).

At this stage, CPEB binds to CPE-elements (UUUUUAAU) located in close proximity to the “nuclear” polyadenylation hexanucleotide sequence AAUAAA (HEX), a cis-element that is bound by Cleavage and polyadenylation specificity factor (CPSF), and promotes deadenylation of several mRNAs, including CyclinB1 and Mos. Mechanistically, CPEB binding to CPE leads to the assembly of the large protein complex containing a scaffold protein Symplekin, the poly(A) polymerase Gld-2, poly(A) ribonuclease PARN and eIF4E-binding protein Maskin. Simultaneous binding of Maskin to CPEB and eIF4E results in disruption of eIF4E-eIF4G interaction and in distortion of the “closed loop” between 5’-3’ UTRs of mRNA that is necessary for effective 40S ribosomal subunit recruitment and initiation of translation. Disruption of the mRNA end-to-end interaction by Maskin helps PARN to override the activity of GLD2 leading to the shortening of the poly(A) tail (Figure 1A) (reviewed in Richter 2007).

To reinitiate meiosis and trigger cell cycle progression toward maturation into a fertilizable egg, oocytes require stimulation with the hormone

progesterone, which boosts polyadenylation of many CPE-containing mRNAs. The progesterone signaling cascade activates Aurora A, a kinase that phosphorylates CPEB at serine 174, leading to CPEB protein complex remodeling (Mendez et al., 2000).The phosphorylation event results in a stronger interaction between CPEB, bound to CPE, and CPSF, bound to the HEX. In this scenario, PARN gets excluded from the complex, giving the advantage to Gld2 catalyzed polyadenylation. The newly elongated poly(A) tail (that is about 150 adenosines long) recruits embryonic polyadenylation binding protein, ePAB that stabilizes poly(A) and, at the same time, interacts with eIF4G by competing Maskin out from the binding to eIF4E. Establishing the strong ePAB-eIF4G interaction leads to the “closed loop” formation and resumption of efficient mRNA translation (Figure 1B) (reviewed in Richter 2007).

In addition to progesterone, oocyte maturation and CPE-mediated mRNA translation can be induced by the insulin signaling pathway via activation of PI3K and PKC zeta kinases. Despite different upstream signaling components, both progesterone and insulin signaling pathways elicit the activation of GSK-3 kinase, which in turn, triggers dissociation and activation of Aurora A from the GSK-3/Axin/ Aurora A inhibitory complex. Phosphorylation of CPEB by activated Aurora A stimulates CPE-dependent mRNA translation of proteins required for oocyte maturation, such as Mos and CyclinB1 (Sarkissian et al., 2004).

During further cell divisions and progression of oocytes to Meiosis II, CPEB level is dramatically reduced via phosphorylation, subsequent ubiquitination and degradation by proteosomal machinery in a SCF (beta-TrCP) dependent manner. Degradation of CPEB helps to release a multiple CPE-containing mRNAs, such as Cyclin B1, from CPEB-mediated control and promotes entry into meiosis II (Mendez et al., 2002; Setoyama et al., 2007).

Importantly, CPEB not only regulates the translational status of CPE-containing mRNAs, but also localizes its target mRNAs to specific locations such as the animal pole of oocytes, embryonic spindles and centrosomes. Moreover, interfering with this "local" embryonic CPEB-dependent translational control by depletion of CPEB leads to multiple defects in chromosome segregation, spindle formation and cell cycle arrest (Groisman et al., 2000; Eliscovich et al.,2008).

The number of CPEs in the 3'UTR of an mRNA, their location and relationship with the other cis-elements, such as HEX or PBE, were shown to influence the CPEB-depenent protein synthesis and differentially regulate mRNA translation at different times and locations. Moreover, since many mRNAs contain CPEs in their 3'UTR, the combination of additional cis-elements and their distance between each other and interacting proteins ensures precise timing and specific location of translation for each particular mRNA (Pique et al., 2008).

Besides factors described above, additional RNA-binding proteins were shown to be associated with the CPEB-protein complex, such as frog germ cell-specific Y-box protein 2 (FRGY2), Xp54 RNA helicase, Pat1, RNA-associated

protein 55 (RAP55), CstF77, APLP2, xGEF suggesting existence of a variety CPEB complexes whose function still has to be determined (Standart and Minshall, 2008). Some of these proteins are known components of mammalian mRNP granules such as P-bodies and miRNA complexes. CPEB was also found to be localized in mRNA-dependent way to the transcriptionally active lampbrush chromosome (Lin et al., 2010). However the function of CPEB1 in this location is still unclear.

The Roles of CPEB in Mammalian Cells

Recently, CPEB was shown to regulate multiple CPE-containing targets in mammalian cells where it is involved in different physiological functions (Richter 2007). Mice lacking CPEB are not only viable but have a normal lifespan, which provides a good model for studying CPEB function. However, CPEB knockout mouse has impacted fertility due to disruption of synaptonemal complex and chromatin fragmentation at the pachytene stage of meiosis (Tay et al., 2001). CPEB mRNA and protein are abundant in many mammalian cell types and tissues with a highest degree of expression in brain, testis and ovary. More detailed investigations have lead to a growing list of CPEB KO mouse defects including changes in learning, memory, senescence and inflammation.

Most of the CPEB protein localizes to the cytoplasm, where it can be also found as a component of mRNA granules such as processing bodies (P-bodies) and stress granules (SG) (Wilczynska et al., 2005) that are involved in control of

mRNA translation and stability (Buchan and Parker, 2009). Both types of granules contain dormant mRNA in a form of mRNPs and different proteins. Some of these proteins are specific structural components (G3BP in SGs and DCP1in P-bodies), where others are transient proteins that have a high exchange rate with the cytoplasm (Kedersha and Anderson, 2007).

Briefly, P-bodies are the cytoplasmic foci enriched in proteins known to be involved in mRNA repression, decaping and degradation (such as decapping proteins Dcp1/Dcp2, deadenylase complex CCR4-Not, exonuclease Xrn-1 , RNA helicase Dhh1p/rck/p54 protein, and components of miRNA machinery including GW182, Ago2 and miRNAs) (Coller and Parker, 2005; Anderson and Kedersha, 2009). Recently, it was shown that the miRNA pathway leads to mRNA repression followed by increased in the P-bodies formation (Jackson and Standart, 2007; Fabian et al., 2010). CPEB was shown to co-localize with P-bodies components, however its function in this foci is not fully described (Wilczynska et al., 2005).

SG granules are larger cytoplasmic granules that are induced by environmental stress or by overexpression of some SG proteins. In general, SG are considered to be a stalled initiation complexes and they are highly enriched in translation initiation factors eIF4E, eIF4G, eIF3 , small ribosomal subunits and multiple mRNA binding proteins (FMRP,TIAR,G3BP, CPEB, Y-box protein) (Anderson and Kedersha, 2009).

Both SG and P-bodies are highly dynamic structures (Balagopal and Parker, 2009). First, they are known to interact with each other, as and some protein components can be found in both types of granules and can shuttle between the two granules (Wilczynska et al., 2005; Buchan and Parker, 2009). Second, mRNA repression in these granules was shown to be reversible (Bhattacharayaa et al., 2006) and recruitment of some proteins and their mRNA targets to the granules is dependent on stimulation (Kedersha and Anderson, 2007). Finally, all mRNA granules are highly mobile structures that utilize the microtubule dependent transport. Moreover, they were found to be attached to microtubules and display different types of movements starting from the most common oscillatory and retrograde to the less abundant anterograde movements (Loschi et al., 2009). Thus, SG and P-bodies can provide a more dynamic and versatile directed translational control (Aizer et al., 2008; Anderson and Kedersha, 2009).

The localization of CPEB in cytoplasmic foci seems to be conserved among different organisms. Thus, *C.elegans* and fruit fly CPEB resides in a complex with p54 helicase and RAP55 protein as a component of germinal granules which consists from some known P-bodies markers and RNPs involved in germ cell development (Standart and Minshall, 2008; Arkov and Ramos, 2010). In addition, most of the components of CPEB complex in Xenopus oocytes also were found to be components of these cytoplasmic structures.

Although the function of CPEB in P-bodies and SG is still unknown, it is appealing to speculate that CPEB can repress mRNA in these granules (in concert with other proteins or miRNAs) until its delivered by microtubule network and released to the site of mRNA translation in a certain time point.

As was discussed above, in *Xenopus* oocytes and in the variety of mammalian cells, CPEB mainly localizes to the cytoplasm. However, upon treatment with a chemical inhibitor of the Crm-1-dependent nuclear export (Leptomycin B) CPEB accumulates in the nucleus indicating that CPEB is a nuclear-cytoplasmic shuttling protein (Ernoult-Lange et al., 2009). The full function of CPEB in the nucleus is still under investigation. However, it was proposed that CPEB can repress mRNA during nuclear export and also control pre-mRNA alternative splicing. In fact, in the nucleus CPEB associates with some factors involved in mRNA processing such as Symplekin, Pol II, eIF4AIII, Cstf-64 and affects splicing of mouse Col91a gene (Lin et al., 2010).

The Role of CPEB in Neurons

The accumulated evidence suggests that translation is required for long term memory formation (LTD) and for local protein synthesis in stimulated synapses. CPEB is involved in translational control and expressed in the different brain regions, with a higher abundance in hippocampus and was shown to be involved in regulation of memory formation and synaptic plasticity (Richter and

Klan, 2009). Mice lacking CPEB have defects in memory extinction, which is likely due to defects in long term potentiation (LTP) and long term depression (LTD)(Alarcon et al., 2004; Berger-Sweeney et al., 2006). In addition, CPEB was shown to affect dendrite branching, which is known to be important for establishing neuronal communications (Bestman and Cline, 2009). Interestingly, neurons in the mouse brain contain all important mediators of CPEB-dependent translation and respond to multiple signaling pathways, which can potentially lead to CPEB-dependent translation in the brain. First, there are many CPE-containing mRNAs that are activated upon activation of receptors in the brain areas with a high abundance of CPEB (Du and Richter, 2005). Second, symplekin, poly(A) polymerase, eIF4E and Maskin can also be detected in neurons and synapses. Third, the progesterone signaling pathway including GSK-3 and Aurora A kinases, is highly active in some areas of the brain such as purkinje cells and hippocampus (Yao et al., 2002; Foy et al., 2010; Tsutsui 2008). Moreover, neurons in the brain have very active insulin signaling, which was also shown to trigger CPEB-dependent translation (Chiu et al., 2010)

To ensure the local protein synthesis at synapses, mRNA must be delivered in a dormant state packed in the form of an mRNP complex (Zukin et al., 2009). In neurons there are different types of mRNPs involved in mRNA localization, sorting, storing and degradation (transport mRNPs, stress granules and P-bodies). All of these granules are highly dynamic structures which can possibly exchange some factors between each other. In neuronal dendrites the

mRNA granules are localized in both axon and dendrites in a microtubule-dependent manner and also can be seen at synapses and in synaptoneurosomes. These mRNP granules get dramatically reduced in a number after electrical depolarization of cultured neurons, which correlates with a increased turnover rate of structural components of P-bodies (Zeitelhofer et al., 2008). Recently published study in Aplysia sensory neuron, found CPEB in mRNA granules similar to SG which increases in size and shows the decreased mobility upon stimulation (Si et al., 2010; Chae et al., 2010).

In neurons, CPEB binds CamKII 3'UTR within the transport granules and delivers mRNA in a microtubule dependent manner. The regulation of CaMKII mRNA by CPEB was shown to be CPE-dependent and activated by the NMDA signaling cascade (Wu et al., 1998; Huang et al., 2003). Additional studies show that deletion of CPEB affects the transport of mRNA granules and leads to defects in dendrite branching. Besides CamKII, many more potential neuronal CPE-containing targets of CPEB were identified (BDNF and c-jun, tPA, engrailed1) and more mRNAs were proposed as potential CPEB targets (Oe and Yuneda, 2010; Du and Richter, 2005).

Taking into account the abundance of CPEB in the brain together with the fact that multiple neuronal mRNAs contain CPE-elements, CPEB might have a potential role in many neurological functions.

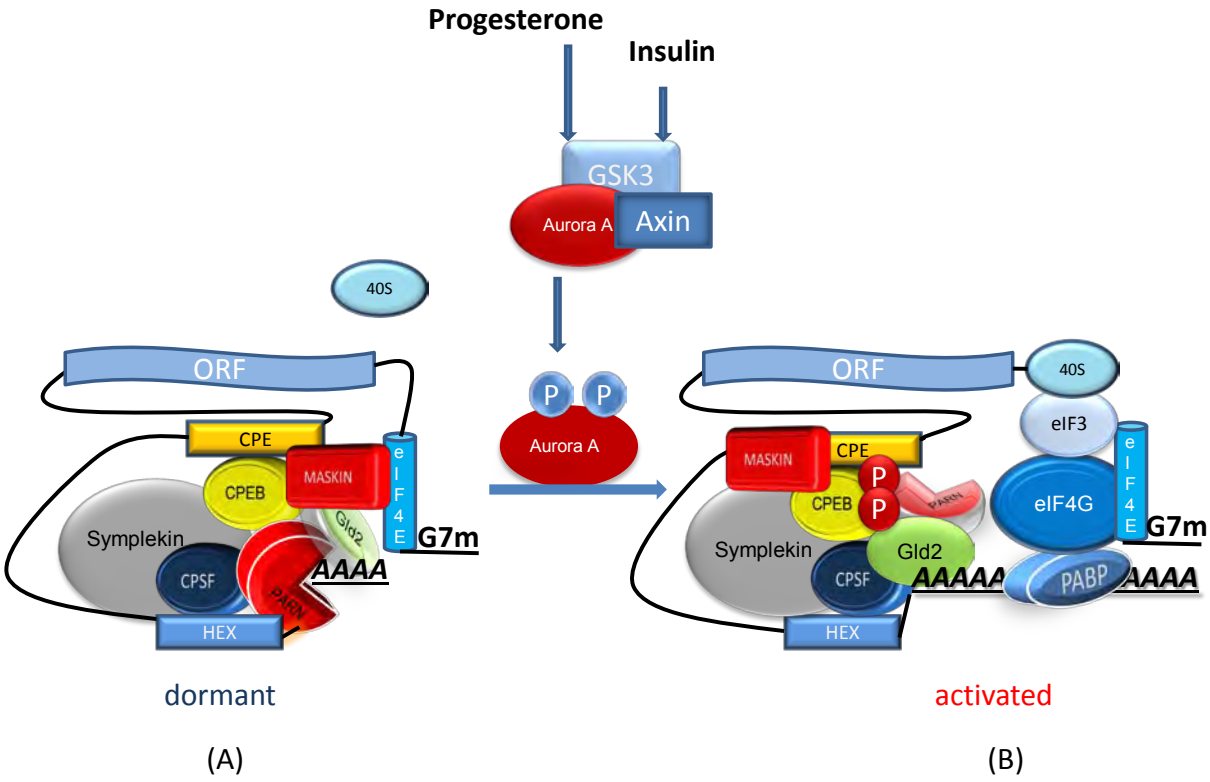
Progesterone
Insulin
GSK3
Aurora A
Axin
40S
ORF
CPE
CPEB
MASKIN
eIF4E
Symplekin
CPSF
PARN
Gld2
AAAA
G7m
HEX
P
P
Aurora A
ORF
40S
eIF3
MASKIN
CPE
P
P
CPEB
PARN
eIF4G
eIF4E
G7m
Symplekin
Gld2
CPSF
AAAAA
PABP
AAAA
HEX
dormant
activated
(A)
(B)

Figure 1

Figure 1. Translational control by CPEB in early development.

CPEB bound to the CPE element recruits multiple factors resulting in translational arrest of mRNA with a short polyA tale (A). Stimulation with progesterone or insulin activates Aurora A which phosphorylates CPEB and triggers CPEB protein complex remodeling resulting in resumed polyadenylation and translation (B) (See detailed description in the text (p.4-5)).

Senescence

Mouse Embryonic Fibroblasts (MEFs) are the classical model for characterization of senescence associated signal transduction, oxidative stress and tumorogenesis. Newly established fibroblast cell cultures can replicate normally for a several passages, slowly progressing toward the abrupt irreversible growth arrest, known as senescence. Senescent cells display flattened cell morphology, senescence-associated heterochromatin foci, and express a high level of senescence-associated acidic β-galactosidase and stay arrested for many days (Sharpless et al., 2001). Finally, they may acquire multiple genetic alterations and can reinitiate replication, a transition point known as the senescence bypass or immortalization. Therefore, cellular senescence is considered to be a protective mechanism from uncontrolled proliferation and received considerable attention due to its antitumorigenic effect. From the other hand, bypass of senescence and immortalization leads to unlimited cell divisions and higher susceptibility to transformation and cancerogenesis (Braig et al., 2005;Chen et al., 2005;Collado et al., 2005).

In regular cell culture conditions, naturally accruing oxidative stress is the major cause of cellular senescence since the cells that cultured in oxygen restricted environment proliferate for an indefinite amount of passages. Senescence can be also stimulated by the application of excessive DNA

damaging stress such as UV, X-ray, and Hydroperoxide or by overexpression of some oncogenes and tumor suppressors (Ben-Porath and Weinberg, 2005).

Despite the fact that mouse cell senescence is telomere-independent and has an early onset (about 10 passages in cell culture), when the human cell senescence depends on telomere erosion and has a later onset (about 70 passages in cell culture), both human and mouse cells have two major senescence pathways, $p16^{INK4a}$/Rb and $p19^{ARF}$/p53/ (Fig.2).

$p16^{INK4a}$/Rb pathway

RB protein regulates the G1/S transition stage of cell cycle by controlling the E2F transcription factor. Briefly, the cell-cycle controlled regulation of cyclin-dependent kinases (CDKs) such as CDK4, CDK6, and CDK2 lead to phosphorylation of Rb, thus controlling its activity. In turn, phosphorylated Rb is not able to repress the E2F transcription factors, which mediates the expression of multiple genes involved in G1/S progression of cell cycle such as cyclin E and cyclin A. The p16 protein is the cyclin dependent kinase inhibitor that prevents Rb phosphorylation via inhibition of CDKs. Elevated level of p16, overexpression of RB, or inhibition of E2F are strongly associated with growth arrest and cellular senescence (Campissi, 2003; Sherr 2004).

$p19^{ARF}$/p53/p21 pathway

This pathway is activated by $p19^{ARF}$, which triggers inactivation of MDM2, a protein that is involved in degradation of the tumor suppressor gene p53. Activated p53 triggers expression of many genes involved in cell proliferation arrest and apoptosis. The cell cycle inhibitory function of p53 is mediated via regulation of protein p21, the cyclin dependent kinase inhibitor that alters CDK2/cyclin-E and CDK4/cyclin-D activity. p53 is a very strong tumor suppressor and its inactivation leads to the development of multiple cancers. Conversely, overexpression of p53 leads to growth arrest and prevents tumorigenesis (Sherr 2004).

RAS oncogene

RAS proteins belong to the family of small GTPases that includes three family members such as H-RAS, K-RAS and N-RAS. RAS protein is a very potent oncogene and its hyperactivation ultimately lead to many cancers. Transfection of immortalized cells with RAS also leads to the tumorigenic phenotype. However, if introduced to the primary cells in the early passages RAS induces premature senescence triggering the accumulation of tumor suppressors p53 and $p16^{INK}$, providing a classical example of oncogene induced senescence (OIS). RAS-induced senescence was shown to require the activation of

RAF/MEK/ERK and/or PI3K/AKT pathways, which components are known play a critical role in cell proliferation and differentiation (Serrano et.al., 1997).

Myc oncogene

In addition to the RAS oncogene, overexpression of the transcriptional factor c-Myc, also block proliferation of primary cells due to its pro-apoptotic effect mediated by enhanced transcription of $p19^{ARF}$ and a subsequent stabilization of p53 (Zindy et al., 1998). However, the normal function of c-Myc oncogene is to enhance cell proliferation and its overexpression can promote immortalization and tumorigenesis in cells with altered $p19^{ARF}$/p53 pathway. In fact, the increased level of c-Myc was found in many types of tumors (Nesbit et al., 1999). From the other hand, c-Myc deficiency leads to a dramatic reduction in proliferation rate due to very low levels of CDK4–6/D and CDK2/E activity (Mateyak et al., 1999). Many cell cycle related genes such as CDK2-activating phosphatases Cdc25A and B, E2F-2 and D-type cyclins are known transcriptional targets of c-Myc (Dang et al., 1999; Cole et al., 1999)

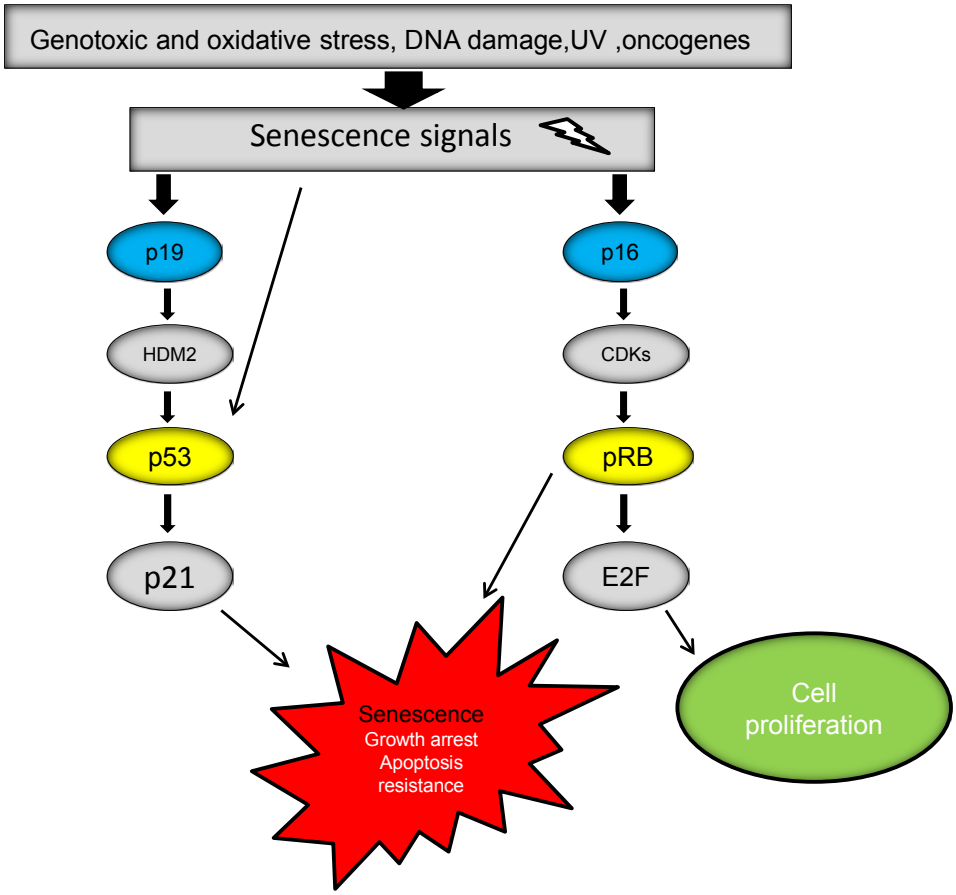
Genotoxic and oxidative stress, DNA damage,UV ,oncogenes
Senescence signals
p19
HDM2
p53
p21
p16
CDKs
pRB
E2F
Senescence
Growth arrest
Apoptosis
resistance
Cell
proliferation

Figure 2

Figure 2. Molecular pathways of cellular senescence. Senescence signals triggered by genotoxic or oxidative stress, UV, DNA damage or by oncogene overexpression activate $p19^{ARF}$/p53/p21 or $p16^{INK4a}$/Rb pathways (see pathway description in the text (p.16-17)) leading to the growth arrest, apoptosis resistance and senescence.

Inflammation

Microbial and viral infections, as well as different types of tissue injuries including cardiac infarction and ischemia, are counteracted by an acute immune response that protects the organism from harmful stimuli and initiates the healing process (Medzhitov 2008; Akira et al., 2006; Gueler et al., 2004). Despite its protective role, not properly terminated or excessive inflammation can cause endotoxic shock, a life threatening condition that leads to detrimental tissue damage of multiple organs (Aggarwal et al., 2003; Gerber and Nau, 2010).

The inflammatory response is regulated by cytokines and chemokines, small secreted proteins that act on immune system pathways in picomolar concentrations by binding to specific cell-surface receptors (Dinarello et al., 2004; Conti et al., 2004). The expression of these signaling molecules is a time-dependent event that is tightly regulated at the level of transcriptional activation and repression, as well as at the level of mRNA stability and translational control (Hao and Baltimore,2009; Anderson, 2009). High level of cytokines, or their prolong expression, strongly correlates with increased mortality due to the septic shock (Gullo et al., 2010; Krakauer et al., 2010; Brunialty et al., 2006). In addition to the acute inflammatory response, unregulated production of proinflammatory cytokines and chemokines can lead to chronic inflammation, a hallmark of many diseases such as diabetes, cancer, asthma, obesity, arthritis, atherosclerosis and

neurodegeneration (Tracey et al.,1993; Pickup, 2004; Dandona et al., 2004; Philip et al., 2004, Phillips et al., 2004).

LPS signaling pathway

In bacterial infection, an inflammatory signaling cascade can be triggered by liposaccharide (LPS), an endotoxin derived from bacterial walls. Injection of LPS into mice dramatically induces the level of proinflammatory IL-6 in serum, which is one of the first response cytokines whose level is highly correlated with the severity of the endotoxic stress. Inflammation is characterized by increased blood flow and capillary permeability and the recruitment of polymorphonuclear granulocytes (neutrophils), cells with high phagocyte activity, blood monocytes, which can differentiate into macrophages, and dendritic cells. Cytokines are secreted by all three cell types (Janeway et al., 2005). Macrophages also display phagocytic activity and because of their long life span, play important roles during the immune response (Gordon and Taylor, 2005). At the cell surface, LPS interacts with LPS binding protein, which together bind CD14 and myeloid differentiation factor 2 (MD2). This protein complex engages the TLR4 receptor and facilitates the activation and subsequent nuclear translocation of the first response transcription factors including AP-1, NFκB, C/EBPb, c-Jun, STATs, and IRFs (Akira et al., 2001; Kawasaki et al., 2003). These transcription factors act in combinatorial fashion to insure a fast immune response.

Activated TLR4 receptor elicits a downstream signaling cascade through MyD88-dependent pathway. This pathway starts when MyD88 (an adaptor protein) engaged by TLR4 recruits multiple additional adaptors and activates a number of kinases including IKK (leading to NFκB activation), MKK3/6 (leading to activation of p38), and MKK4/7 (which activates JNK).

NFκB transcription complex

NFκB (nuclear factor kappa-light chain-enhancer of activated B cells) is an evolutionarily conserved transcription factor that is activated via different pathways by multiple stimuli. It contributes to diverse cellular processes such as cellular proliferation and stress response, cell death, and inflammation (Barnes et al., 1997; Pachl et al., 1999; Lappas et al., 2002). NFκB can activate or repress gene expression through its binding to the specific DNA sequences within promoters and enhancers known as κB elements. Activation of NFκB leads to the increased transcription of different inflammation mediators including IL-1, TNFα, IL-6, IL-8, and enzymes such as cyclooxygenase-2 (COX2) and iNOS (Hoffmann et al., 2006; Pahl et al., 1999), which, in turn, can further propagate NFκB activation (Barnes and Karin, 1997).

Uncontrolled activation of NFκB ultimately leads to multiple inflammatory disorders including autoimmune diseases and septic shock (Tak et al., 2001). In addition, sustained NFκB activation is found in many types of cancers (Karin et

al., 2006). Conversely, the inhibition of NFκB during bacterial induced inflammation leads to the reduced production of proinflammatory proteins and decreased neutrophil infiltration. There are a growing number of NFκB inhibitors that can reduce NFκB activation and cytokine production (Ivanenkov et al., 2008).

NFκB is a family of proteins containing similar N-terminal Rel homology domains (RHD) that are important for dimerization and DNA binding. The family has five members: RelA (p65), RelB, c-Rel, NFκB1 (p50/p105) and NFκB2 (p52/p100). Based on their structure, the first three proteins can be grouped together because they all posses a common inhibitory c-terminal ankyrin motif. In contrast, p100 (precursor of p52) and p105 (precursor of p50) has to be processed by limited proteolysis (Nolan and Baltimore, 1992; Hoffmann et al., 2002; Karin,2006) or by translational inhibition (Moore et al., 1999) to generate active shorter proteins.

All NF-kb family members act as dimers (homo- or hetero-) that have different DNA binding specificities, transcriptional activities, and mechanisms of activation (Ghosh et al., 2005). The RelA, RelB, and c-Rel transactivation domains (TAD), which are responsible for engaging co-activators and displacing repressors, function as transcriptional activators. In contrast, both p50 and p52 proteins, which lack transactivation domains and are present as homodimers, can only act as repressors. However, they can activate transcription when

heterodimerized with TAD-containing proteins such as Bcl-3 or other members of the NFκB family. (Baeuerle and Henkel,1994).

Mechanism of the NFκB activation

At steady state in unstimulated cells, the NFκB heterodimer RelA(p65)/p50 localizes in the cytoplasm via interaction with inhibitory proteins from the IκB family. This family includes seven members: IκBα, IκBβ, IκBγ, BCL-3, IκBε, and the precursor proteins p100 and p105, all of which contain a conserved c-terminal ankyrin repeats sequence. These ankyrin repeats mask the nuclear localization sequence (NLS) of RelA, thus preventing its nuclear translocation (Stancovski and Baltimore, 1997; Xiao and Ghosh, 2005). Upon LPS signaling, IκBα is hyperphosphorylated by activated inhibitor of κB kinase (IKK), and is rapidly degraded by the proteasome in a ubiquitin-dependent fashion (DiDonato et al., 1997). The exposed NLS domain of NFκB directs its nuclear translocation followed by DNA-binding to promoter regions. Therefore, the balance between synthesis and degradation of IκBα is critical for NFκB activation. Newly synthesized IκBα then binds NFκB and brings it back to the cytoplasm. These events are classified as the canonical NFκB activation pathway, which is the major pathway induced by LPS. Alternatively, IKK activity can trigger a noncanonical pathway via enhanced p100 processing to p52 (Scheidereit et al., 2006)

IKK complex

The IKK complex consists of three major kinase subunits: IKKα (IKK1), IKKβ (IKK2), and NEMO or IKKγ. The IKKα and IKKβ subunits, when in heterodimer form, directly phosphorylate S32 and S36 residues of IκBα. IKKγ has no enzymatic activity but coordinates upstream signaling pathways. In the canonical pathway, the IKK complex is activated by kinases such as TAK1, which phosphorylates IKKβ on residues Ser177 and Ser181 (Mercurio et al., 1997; Hoffmann et al., 2002). This posttranslational modification is not only necessary but sufficient for phosphorylation of IκBα on Ser32/36 (Zandi et al., 1997). Upon phosphorylation, the IκBα become ubiquitinated (by Skp1–Culin Roc1/Rbx1/Hrt-1–F-box, which belongs to the SCF family of ubiquitin ligases) followed by proteosomal degradation (Ben-Neriah, 2002). In contrast, activated IKKα triggers an alternative noncanonical pathway whereby proteosomal processing of p100 elicits p52-RelB heterodimer formation and nuclear translocation (Karin, 2006).

Posttranscriptional regulation of inflammation

In addition to transcriptional control, mammalian cells display a variety of post-transcriptional mechanisms that mediate the inflammatory response. This additional level of regulation occurs by way of regulatory elements in the 3'UTRs of particular mRNAs. These 3'UTR elements control the timing and extent of

mRNA destruction and/or translation, thereby providing strong temporal control of the immune response. Based on the time at which they are activated, the genes can be subdivided into three groups, the first of which consist early response genes, which are induced within an hour after stimulation. This group encodes a number of mRNAs with cis-elements (AU-rich elements, or AREs), which are involved in mRNA stability. The second and third groups represent intermediate (at 2 hours after stimulation) and late (by 12 hours) response genes (Hao et al., 2009).

The ARE-binding proteins (TTP, TIA-1, AUF1, HuR) are essential cis-factors involved in the stability and translational repression of ARE-containing mRNAs Deficiency in ARE binding proteins usually leads to the multiple inflammatory conditions, most often, due to increased stability of cytokine TNFα mRNA The cytokine production is also regulated via miRNAs which act in concert with RNA binding proteins (Zhang et al., 2002; Anderson, 2010).

CHAPTER II

Control of cellular senescence by CPEB

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Abstract

Cytoplasmic polyadenylation element-binding protein (CPEB) is a sequence-specific RNA-binding protein that promotes polyadenylation-induced translation. While a CPEB knockout (KO) mouse is sterile but overtly normal, embryo fibroblasts derived from this mouse (MEFs) do not enter senescence in culture as do wild-type MEFs, but instead are immortal. Exogenous CPEB restores senescence in the KO MEFs and also induces precocious senescence in wild-type MEFs. CPEB cannot stimulate senescence in MEFs lacking the tumor suppressors p53, $p19^{ARF}$, or $p16^{INK4A}$; however, the mRNAs encoding these proteins are unlikely targets of CPEB since their expression is the same in wild-type and KO MEFs. Conversely, Ras cannot induce senescence in MEFs lacking CPEB, suggesting that it may lie upstream of CPEB. One target of CPEB regulation is myc mRNA, whose unregulated translation in the KO MEFs may cause them to bypass senescence. Thus, CPEB appears to act as a translational repressor protein to control myc translation and resulting cellular senescence.

Introduction

Regulated mRNA translation plays an important role in early animal development and in the central nervous system. In oocytes, embryos, and neurons, several dormant mRNAs have short poly(A) tails; when the poly(A) tails are elongated in response to external stimuli, translation ensues (Wu et al. 1998; Groisman et al. 2002). The cytoplasmic polyadenylation element-binding protein (CPEB) is the key factor that controls this process; it binds the cytoplasmic polyadenylation element (CPE) in the 3′ untranslated regions (UTRs) of responding mRNAs. When phosphorylated on S174 or T171 (species-dependent), CPEB promotes polyadenylation by stimulating the activity of Gld-2 (Barnard et al. 2004), an atypical poly(A) polymerase (Kwak et al. 2004). The newly elongated tail then is bound by poly(A)-binding protein (PABP), which promotes general translation by augmenting the assembly of the eIF4F initiation complex (Kahvejian et al. 2005). How the poly(A) tail and PABP stimulate translation of CPE-containing RNAs was revealed when a CPEB coimmunoprecipitating protein was identified. This protein, Maskin, binds not only CPEB, but also the cap-binding factor eIF4E (Stebbins-Boaz et al. 1999; Richter and Sonenberg 2005). The Maskin–eIF4E association competitively inhibits the eIF4E–eIF4G association; thus, there is no eIF4G-assisted recruitment of the

40S ribosomal subunit to the 5′ end of the mRNA. The poly(A) tail and PABP help eIF4G out-compete Maskin for binding to eIF4E, which results in eIF4F initiation complex assembly and translation (Cao and Richter 2002). Maskin phosphorylation also helps this protein dissociate from eIF4E (Barnard et al. 2005).

In early-stage embryos of *Xenopus laevis*, CPEB promotes polyadenylation-induced translation of cyclin B1 mRNA, which helps drive these atypical mitotic cell cycles that lack G1 and G2 phases (Groisman et al. 2002). Indeed, the injection of neutralizing CPEB antibody or dominant-negative mutant forms of this protein inhibits cell division (Groisman et al. 2000; Mendez et al. 2002). In contrast to these results in *Xenopus*, CPEB knockout (KO) mice are viable and appear overtly normal, although they have defects in germ cell development (Tay and Richter 2001; Tay et al. 2003) and neuronal synaptic plasticity (Alarcon et al. 2004) and exhibit some behavioral anomalies (Berger-Sweeney et al. 2006). The observations that CPEB is essential for mitotic progression in *Xenopus* embryos and that mice lacking this gene display no phenotype that is obviously related to aberrant cell cycle progression might seem contradictory. However, some genes that are important for cell cycle progression in cultured cells have little effect in KO mice (Sherr and Roberts 2004;Malumbres and Barbacid 2005). To determine whether CPEB is important for cell cycle progression in mice, embryo fibroblasts (MEFs) from wild-type and CPEB KO animals were cultured according to a standard 3T3 protocol; there were no

detectable differences in cell division. However, several passages after the initial cell cycle experiments were performed, the wild-type cells ceased to divide and entered a senescent stage, as expected (Sharpless et al. 2001). Amazingly, the KO MEFs did not senesce, but instead bypassed this process and were immortal up to at least 40 passages.

Senescence is a process that limits the number of times a cell divides in vitro; in mouse cells, it can be induced by DNA damage, the activation of certain oncogenes, or the stress of certain culture conditions (Ben-Porath and Weinberg, 2005). In vivo, senescence may be a tumor-suppression mechanism to prevent malignant transformation (Braig et al. 2005;Chen et al. 2005;Collado et al. 2005;Michaloglou et al. 2005) and contribute to organismal aging (Patil et al. 2005). While several genes influence senescence, perhaps the most central are the tumor suppressors p53, Rb, $p19^{ARF}$, and $p16^{INK4A}$. While $p19^{ARF}$ and $p16^{INK4A}$ are derived from the same locus, $p16^{INK4A}$ is a cyclin-dependent kinase (CDK) inhibitor and thus an activator of Rb, a transcriptional corepressor. $p19^{ARF}$ activates the transcription activator p53 by antagonizing Mdm2, the ubiquitin ligase that mediates its destruction (Sherr 2004). Primary mouse cells that lack either of the abovementioned four tumor suppressors are immortal (Campisi 2003; Sherr 2004).

While exogenous CPEB restored senescence when expressed in CPEB KO MEFs and even induced precocious senescence in wild-type MEFs, it could not

induce senescence in p53, $p19^{ARF}$, or $p16^{INK4A}$/$p19^{ARF}$ double-KO MEFs. Conversely, activated Ras, which induces senescence in wild-type MEFs, could not do so in CPEB KO MEFs. The CPEB KO MEFs yielded a few small foci when grown in soft agar, suggesting that they may be partially transformed; however, they did not yield tumors when injected into athymic (nude) mice. p53, Rb, $p19^{ARF}$, and $p16^{INK4A}$ were all expressed in CPEB KO MEFs at levels similar to those observed in wild-type MEFs, although the Rb pocket proteins showed some changes. On the other hand, myc levels were aberrantly high in the KO MEFs, indicating that the mRNA encoding this protein might be misregulated in the KO MEFs. Indeed, myc RNA coimmunoprecipitates with CPEB from wild-type MEFs, and the CPE-containing myc 3′ UTR, when appended to a reporter RNA, is responsible for elevated levels of translation in KO versus wild-type MEFs. Moreover, the elevated expression of myc in the wild-type MEFs may cause them to bypass senescence. These and other data suggest that CPEB-mediated down-regulation of translation is necessary for mouse cells to become senescent.

Results

CPEB regulates cell senescence

To determine whether CPEB is important for the cell cycle, mouse embryo fibroblasts (MEFs) were prepared from embryonic day 14.5 (E14.5) embryos. At passage 2, the cells were cultured in medium lacking serum for 24 h and then cultured a subsequent 29 h in medium containing 10% serum. At several times, the cells were subjected to FACS analysis; both wild-type and KO MEFs recovered from quiescence induced by serum deprivation in an identical manner (Supplementary Fig. 1).

Wild-type and KO MEFs were further cultured according to a standard 3T3 protocol. A Western blot of the KO MEFs confirms that they do not express detectable levels of CPEB (Fig. 1A, left). Growth curves of four KO MEF lines show that they all bypassed senescence, while a wild-type line senesced after passage 6 (Fig. 1A, right). In other experiments, 14 of 17 KO MEF lines were found to be immortal, while seven of seven wild-type lines became senescent (data not shown). Although only 12 passages are depicted in the figure, the KO MEFs have been cultured up to ~40 passages, demonstrating that they are immortal. A heterozygous MEF line also was immortal, indicating that the amount of CPEB is important for senescence (Fig. 1A). In other experiments, five of five additional heterozygous MEF lines also were immortal (data not shown).

To assess whether the KO MEFs were immortal because they lacked CPEB and not because of a mutation elsewhere in the genome, the KO MEFs were infected with a retrovirus expressing CPEB or, as a control, an empty virus. CPEB caused the KO MEFs to stop dividing, express senescence-associated β-galactosidase, and assume a flattened morphology typical of senescent cells (Ben-Porath and Weinberg 2005). Moreover, CPEB caused wild-type MEFs to undergo senescence prematurely (passage 4 instead of passage 7) and to express β-galactosidase (Fig. 1B; at right, a Western blot shows that similar amounts of heterologous CPEB were synthesized in wild-type and KO MEFs). In other experiments, late (more than ~25) passage KO MEFs or immortalized cells derived from wild-type MEFs infected with the same CPEB-expressing retrovirus elicited no discernible effect on cell division or morphology (data not shown). Therefore, this rescue experiment demonstrates that CPEB is essential for cell senescence and eliminates the possibility that the senescence bypass in the KO MEFs was caused by mutations in other genes.

To gain insight into other genes that might act in concert with CPEB, we considered three that are known to be necessary for senescence: p53, $p19^{ARF}$, and $p16^{INK4A}$. We obtained MEFs that lack p53, $p19^{ARF}$, or $p19^{ARF}$ and $p16^{INK4A}$, which when cultured according to our 3T3 protocol, were, indeed, immortal (Fig. 1C). While CPEB induced the premature senescence of wild-type MEFs at passage 4 (as noted in Fig. 1B), it could not rescue senescence in MEFs that

lack these three genes (cells infected at passage 4 and examined at passage 11). Thus, CPEB requires these tumor suppressors to induce senescence.

Finally, we determined whether oncogenic Ras, which induces premature senescence in mouse primary cells (Serrano et al. 1997), could also do so in cells that lack CPEB. CPEB KO and wild-type MEFs, as well as $p19^{ARF}$ KO MEFs, were infected with a retrovirus expressing Ki-Ras at passage 2, subjected to antibiotic selection at passage 3, and examined at passage 5. While this protein induced senescence of wild-type cells, it did not do so with the $p19^{ARF}$ or CPEB KO MEFs (Fig. 1D). Thus, Ras-induced senescence cannot overcome the loss of these proteins.

Transformation potential of CPEB KO MEFs

The observation that CPEB KO MEFs are immortal suggests that they might display some features of a transformed phenotype. We examined four parameters to assess whether the KO MEFs might be at least partially transformed. First, transformed cells often display an elongated morphology; however, in contrast to immortalized cells derived from wild-type MEFs or early-passage wild-type and KO MEFs, late-passage KO MEFs had a distinctive round shape (Fig. 2A) that is not obviously related to partial transformation. Second, another assay to identify transformed cells is their ability to grow in medium containing reduced serum, typically 1%. Under this culture condition, neither the

KO nor the wild-type MEFs grew irrespective of whether they were infected with a Ras-bearing retrovirus (Fig. 2B). However, when the medium contained the normal 10% serum, the KO MEFs grew at a faster rate than immortalized cells derived from wild-type MEFs with or without exogenous Ras expression. These results suggest that the KO MEFs might have a tendency toward transformation. Third, we examined the ability of the KO MEFs to grow in soft agar, which is a widely used indicator of transformation. While immortalized cells derived from wild-type MEFs did not form any colonies in this medium, the KO MEFs did, although the colonies were small and not abundant (Fig. 2C). When transformed with Ras, the immortalized cells derived from wild-type MEFs and the KO MEFs both formed large and numerous colonies, although the latter were larger and more numerous. These data also suggest a tendency of KO MEFs toward transformation. Fourth, we determined whether late-passage (immortal) KO MEFs formed tumors in nude (athymic) mice. For comparison, immortalized cells derived from wild-type MEFs as well as both cell types transformed with Ras were also injected into nude mice. No tumors were observed in animals injected with immortalized KO or wild-type cells (data not shown), but as expected, tumors were evident in animals injected with cells (wild-type and KO) that were transformed with Ras (Fig. 3A). Curiously, the 10 tumors derived from the KO MEFs all had large hematomas, while none of the 10 tumors derived from wild-type cells were so distinguished (Fig. 3A, arrows). Although the tumors appeared to grow at the same rate (Fig. 3B), the final weights (after 36 d of growth) of the

wild-type tumors were usually greater than the KO tumors (Fig. 3C) (p = 0.01, Student's t-test). Moreover, the morphologies of the tumors were different; the cells containing CPEB were densely packed and slightly elongated, while those lacking CPEB were larger, more irregularly shaped, and with nuclei usually displaced to the periphery (Fig. 3D). While the significance of these morphology differences remains to be elucidated, the aggregate data indicate that the CPEB KO MEFs are probably not transformed unless they are challenged with an oncogene.

Altered levels of senescence-associated proteins in CPEB KO MEFs

To examine whether key proteins that are thought to influence senescence are aberrantly expressed in the KO MEFs, a series of Western blots was performed (Fig. 4). The levels of p53, $p19^{ARF}$, $p16^{INK}$, and Rb (unphosphorylated and phosphorylated forms) were nearly the same in wild-type and KO MEFs, indicating that they are unlikely to be substrates of CPEB regulation. Note that the p53 antibody reacted with two proteins from wild-type and KO MEFs; to firmly identify p53, protein from p53 KO MEFs was also analyzed in parallel lanes, which demonstrated that the indicated band is, indeed, p53 and that it was present at similar levels in wild-type and KO MEFs. Myc as well as the Rb pocket proteins p130 and 107 were present at higher levels in the KO MEFs and all have putative CPEs (Fig. 4), suggesting that aberrant translation of one or more of them in the KO MEFs might be responsible for senescence bypass.

The overexpression, not underexpression, of these proteins in the KO MEFs was a surprising finding. For example, the ablation of CPEB activity in *Xenopus* oocytes or embryos prevents translation (Groisman et al. 2000; Mendez et al. 2002) as does the removal of CPEB from oocytes of KO mice (Tay and Richter 2001). Thus, in these cases, CPEB is an activator of translation. Moreover, CPEB-stimulated translation requires phosphorylation of S174 or T171 (*Xenopus* and mouse, respectively) (Mendez et al. 2000; Tay et al. 2003). To assess whether CPEB-controlled senescence requires RNA binding and/ or T171 phosphorylation, the KO MEFs were infected with retroviruses expressing wild-type and deletion mutant CPEB proteins. Figure 5 shows that while wild-type CPEB rescued senescence in KO MEFs, CPEB proteins lacking either the two RNA-binding domains or the zinc finger, all of which are necessary for RNA binding (Hake et al. 1998), failed to do so. Conversely, CPEB with a T171A mutation did rescue senescence (this construct also contained an S177A; while there is no evidence that this residue is phosphorylated, the motif in which this residue lies strongly resembles that surrounding T171 and thus could be a secondary phosphorylation site). Therefore, while RNA binding is necessary for CPEB-mediated senescence, T171 phosphorylation is not. Because this modification is required for polyadenylation (at least in oocytes and probably neurons as well), it may be that in MEFs, CPEB-regulated translation might not require polyadenylation.

CPEB inhibits myc RNA translation

To identify possible mRNA substrates of CPEB in MEFs, we performed a series of ribonucleoprotein (RNP) coimmunoprecipitation experiments. MEFs were infected with viruses expressing HA-CPEB (HA antibody precipitation is more efficient than CPEB antibody precipitation), followed by immunoprecipitation, RNA extraction, and RT–PCR detection of RNAs selected on the basis of their expression in MEFs and whether they have putative CPEs (Fig. 6A). RNAs encoding the Rb pocket proteins p107 and p130 and myc were immunoprecipitated with HA-CPEB (Fig. 6A, lane 2, cf. lane 1, where HA was immunoprecipitated from uninfected cells.) RT– PCR of a serial dilution of total RNA indicates the relative amount of the precipitated RNA (Fig. 6A, lanes 3–6). In addition, the RNA levels were the same in wild-type and KO MEFs and in KO MEFs infected with the CPEB-containing virus (Fig. 6A, lanes 7–9). Controls in which the reverse transcription step was omitted did not yield amplification products (data not shown). These data show that some, but not all, CPE-containing RNAs can be immunoprecipitated with CPEB.

Because Rb protein levels were the same in wild-type and KO MEFs, the RNA encoding this protein is unlikely to be regulated by CPEB. On the other hand, the levels of p107, p130, and myc were all elevated in the KO MEFs (Fig. 4). Any of these proteins could contribute to senescence bypass; however, p107 and p130 have been knocked out in mice with no reported precocious MEF senescence

(Sage et al. 2000), which would be expected if they were the direct downstream effectors of CPEB. (Note, however, that while oncogenic Ras induces senescence in Rb KO MEFs, it cannot induce senescence in Rb/p107 or Rb/p107/p130 KO cells, thereby demonstrating the involvement of the pocket proteins in senescence [Sage et al. 2000; Pepper et al. 2001].) Because myc has been reported to be involved in senescence (Lutz et al. 2002) and because high levels of myc are detected in the CPEB KO MEFs that bypass senescence, we have focused our initial attention on this mRNA.

A UV cross-linking experiment was used to determine whether myc RNA is a direct target of CPEB. Recombinant CPEB was mixed with the myc 3′ UTR that was synthesized in vitro in the presence of [^{32}P]UTP. In some cases radioinert competitor RNA lacking or containing the CPE was also added to the mix, which was followed by UV irradiation, RNase digestion, and analysis by SDS-PAGE and PhosphorImaging (Fig. 6B). CPEB was efficiently cross-linked to the RNA, which was reduced only when the competitor RNA contained a CPE. Thus, CPEB directly interacts with the myc 3′ UTR CPEs.

Next, we repeated the CPEB-myc RNA coimmunoprecipitation experiment from MEFs as in Figure 6A, but in this case, excess competitor RNA lacking or containing the CPE was added to the extract prior to and during the immunoprecipitation procedure (Fig. 6C). The CPE-containing RNA effectively competed away the myc RNA from binding CPEB, while the CPE-lacking RNA

had no effect. These data indicate that CPEB interacts with the myc 3′ UTR CPEs in MEFs.

To confirm that the translation of myc RNA is regulated by CPEB, as suggested by the data in Figure 4, extracts from wild-type and KO MEFs were centrifuged through sucrose gradients to resolve the polysomes and the nontranslating RNPs, which generally sediment less than 80S. The RNA was extracted from each fraction and subjected to real-time quantitative RT–PCR for actin and myc RNAs. When compared with actin RNA (whose sedimentation did not change in the wild-type vs. KO MEFs) (data not shown), the results in Figure 6D show that in wild-type MEFs, a substantial portion of myc RNA was not translated, but that much of this material was recruited into polysomes in the KO MEFs. Thus, CPEB appears to repress the translation of myc RNA.

Finally, a reporter *Renilla* luciferase RNA was appended with either this sequence or a control vector sequence; plasmid DNAs encoding these RNAs, together with one encoding firefly luciferase to serve as an internal standard, were transfected into wild-type and CPEB KO MEFs followed by an analysis of luciferase activity. While both MEF types translated the RNA with the vector 3′ UTR to about the same extent, the KO MEFs translated the RNA with the myc 3′ UTR with greater efficiency than wild-type MEFs (Fig. 6E). Taken together, the results in Figure 6 demonstrate that CPEB inhibits myc mRNA translation.

The regulated translation of Myc RNA by CPEB controls cell senescence

Plasmid DNA encoding myc RNA with its own 3′ UTR or one composed of vector sequence was transfected into wild-type and CPEB KO MEFs; DNA encoding vector sequence was also transfected. While none of the RNAs had an effect on growth of the CPEB KO MEFs, myc RNA with the vector 3′ UTR caused the wild-type cells to bypass senescence when compared with the RNA encoding myc with its own 3′ UTR or the noncoding RNA (Fig. 7A). Moreover, a Western analysis for Myc shows that the levels of this protein were high in wild-type MEFs transfected with myc RNA with the vector 3′ UTR, but lower in wild-type MEFs transfected with the other RNAs; Myc was also high in KO MEFs under all conditions (Fig. 7A). These results, together with those showing that similar amounts of heterologous myc RNA were present in the transfected cells (Fig. 7A; the lower panel depicts quantitative real-time PCR for the myc 3′ UTR), suggest that CPEB represses the translation of myc RNA when it has its own CPE-containing 3′ UTR, and that the levels of myc are important for regulating cell senescence.

These results imply that reduced myc levels in KO MEFs would induce senescence. To assess this possibility, a short hairpin RNA (shRNA) was used to knock down myc RNA; shRNA for GFP served as a control. Figure 7B (top) shows that relative to the control shRNA, the myc shRNA knocked down myc to

>95%. A growth curve demonstrates that with reduced myc levels, the cells ceased to divide (Fig. 7B, middle panel; the cells are shown in the bottom panel).

Finally, we also assessed possible apoptosis in MEFs overexpressing myc. Although $p19^{ARF}$ and p53 levels were somewhat elevated in these cells, we detected no significant apoptosis as assessed by TUNEL labeling or Western blotting for several apoptotic markers such as caspase 3, BAX, or BCL-2 (Supplementary Fig. 2). These data indicate that CPEB-mediated senescence occurs via translational control of myc RNA.

Discussion

This report demonstrates the necessity of CPEB for cellular senescence in MEFs. CPEB requires p53, $p19^{ARF}$, and $p16^{INK4A}$ to induce senescence; conversely, activated Ras requires CPEB to stimulate this process. The CPEB KO MEFs are not transformed, and while they, like immortalized cells derived from wild-type MEFs, are transformed by Ras, their responses to this oncogene are not identical. That is, Ras causes the KO MEFs to form more and larger foci in soft agar compared with wild-type MEFs, and induces distinct shape changes in the KO MEFs in tumors formed in nude mice. While the significance of these results remains to be elucidated, they do indicate that immortalized wild-type and CPEB KO MEFs are not identical. Indeed, p53, whose disruption is usually associated with immortalization, is expressed at normal levels in the KO MEFs.

Finally, CPEB interacts with several CPE-containing mRNAs whose encoded products are present at aberrantly high levels in the KO MEFs (p130, p107, myc) and the translation of one of them, myc, appears to regulate senescence.

The observation that CPEB is essential for cellular senescence is surprising. Several other proteins have been implicated in this process including eIF4E (Ruggero et al. 2004), a general factor that regulates all cap-dependent translation. Thus, the number of mRNAs whose translation could be affected by eIF4E overexpression is extremely large. CPEB, on the other hand, interacts only with CPE-containing RNAs, thus vastly restricting the potential mRNA substrates on which it could act to induce senescence. Even so, the identification of myc RNA as one substrate involved in this process was unexpected because its translation is reported to be under internal ribosome entry site (IRES) control, while CPEB mediates translation through eIF4E (see introduction and below). On the other hand, myc has long been known to regulate cell proliferation; it is low in nondividing cells and high in rapidly dividing cells (Campisi et al. 1984). Its overexpression can enhance the cell cycle, especially by shortening G1 (Karn et al. 1989), but can induce apoptosis as well (Thompson 1998). MEFs and other cells lacking myc have a reduced rate of proliferation (Mateyak et al. 1999). Our results show that a two-to threefold elevation in myc levels caused by the aberrant translation of its mRNA when the CPEs are deleted can lead to senescence bypass. Conversely, we show that a knockdown of myc causes MEFs to stop dividing.

We also examined the effects of myc overexpression on apoptosis in MEFs. In wild-type and KO MEFs, over-expressed myc induced a mild up-regulation of p53 and $p19^{ARF}$, but we could detect no evidence of apoptosis (Supplementary Fig. 1). It is known that myc overexpression can induce apoptosis, as noted above, but in our case, myc may not have been at a sufficiently high level to induce apoptosis. Moreover, in the CPEB KO MEFs, perhaps other factors whose translation is controlled by CPEB are reduced or enhanced, which could offer some protection against apoptosis.

Translational control by CPEB

How CPEB might repress myc translation may be gleaned from its activity in other cells. In *Xenopus* oocytes, an RNP complex includes CPE-containing RNA, CPEB, Maskin, and eIF4E, among other proteins. Through its association with CPEB, Maskin prevents translation of CPE-containing RNAs by preventing the interaction between eIF4E and eIF4G (Cao and Richter 2002). This repression is reversed when CPEB is phosphorylated by Aurora A on S174 (frog) or T171 (mouse) (Mendez et al. 2000; Tay et al. 2003). Because a T171A CPEB mutation rescues senescence, the phosphorylation of this residue, if it occurs in MEFs, cannot be necessary for the translational regulatory event by CPEB that mediates senescence. CPEB could still use Maskin-like molecules to repress translation. In this regard, a new CPEB and eIF4E-binding factor has been identified in *Xenopus*, mouse, and human cells. This protein, neuroguidin,

represses translation in a CPE-dependent manner (Jung et al. 2006), and thus could mediate the activity of CPEB that is necessary for senescence.

CPEB also interacts with the RNA helicase p54 (Minshall et al. 2001). This protein inhibits translation by an unknown mechanism, but its activity may be related to its residence in p-bodies (Coller and Parker 2005). p-bodies, or processing bodies, were first shown to be centers for RNA destruction (Sheth and Parker 2003), but are now known to harbor translationally dormant mRNAs as well (Brengues et al. 2005). Recent evidence indicates that in HeLa cells, CPEB is present in p-bodies (Wilczynska et al. 2005). We have confirmed that in MEFs, CPEB is also present in p-bodies, among other regions in the cell (data not shown). While we do not know if myc RNA is also present in p-bodies, we speculate that it might be. Conversely, myc RNA might not be p-body associated in CPEB KO MEFs, thereby resulting in elevated levels of translation. It should also be noted that myc RNA contains an IRES (Stoneley et al. 1998), and thus its translation would require neither eIF4E nor eIF4G. However, myc RNA is also translated by cap-dependent translation, and the mode of translation it uses, cap versus IRES, is cell type dependent (Stoneley et al. 2000).

Is CPEB a tumor suppressor?

As determined by the absence of growth without serum, in soft agar, or in nude mice, the CPEB KO MEFs are not transformed. On the other hand, the KO MEFs

transformed with Ras grow faster in medium containing serum, form larger foci in soft agar, and yield unusually shaped cells in nude mice compared with wild-type cells expressing Ras. These results suggest that MEFs lacking CPEB are slightly more susceptible to Ras-mediated transformation than CPEB-containing MEFs. If this is the case, then the CPEB KO mice might contract cancer at a higher rate than wild-type mice, especially if they express activated Ras or perhaps are exposed to a chemical mutagen. While neither wild-type nor CPEB KO animals aged nearly a year show signs of cancer (data not shown), experiments will be initiated to assess whether oncogenes or other carcinogens elicit an elevated rate of malignancy in the CPEB KO mice.

Material and Methods

Reagents

Mouse CPEB (Gebauer and Richter 1996) was cloned into the XhoI–NotI sites of C-POZ, a retrovirus vector (Nakatani and Ogryzko 2003), or into the XhoI sites of pMSCVhyg (Clontech Laboratories, Inc.). The c-myc ORF, with or without its own 3′ UTR (a gift from Michael Cole, Dartmouth Medical School, Hanover, NH), was cloned into the BamHI–EcoRI sites of pBabe, a retrovirus vector (Morgenstern and Land 1990). The myc 3′ UTR was cloned into the XbaI–NotI sites of *Renilla* luciferase (pRL-TK; Promega).

Antibodies for cyclin E (C19), cyclin A (H432), cyclin D1 (DCS-6), Rb (IF8), p107 (C18), p130 (C20), and p21 (C19) were a gift from Andrew Koff (Memorial Sloan-Kettering, New York) and are designated according to Santa Cruz Biotechnology. Other antibodies were purchased from commercial suppliers (c-myc, sc764, $p16^{INK4A}$, M-156 from Santa Cruz Biotechnology; $p19^{ARF}$, ab80–100, Abcam; the hemagglutinin epitope, PRB-101P, Covance; phospho-Rb, Ser 887/811, Ser 795, Ser 780, Cell Signaling; tubulin, Sigma).

Cell culture and immunostaining

Wild-type, $CPEB^{+/-}$, and $CPEB^{-/-}$ MEFs were derived from E12.5–E14.5 embryos and cultured in Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum using a 3T3 protocol. The cells were infected (Danos and Mulligan 1988) with retroviral vectors C-POZ mCPEB, pBabe-c-Myc-Puro, pMSCV-mCPEB-Hyg, and pMSCV-Ki-Ras-Puro (gift from Valentina Evdokimova, University of British Columbia, Vancouver, BC, Canada), which were packaged using Bosch and Phoenix cells (Pear et al. 1993). To knock down myc, cells were infected with retrovirus pMSCV-puro into which the myc shRNA (or as a control, GFP shRNA) was cloned (Open Biosystems, clone SM2166 H-3). The infected cells were selected with 2 μg/mL puromycin or 400 μg/mL hygromycin, or by using magnetic beads containing antibody against the IL3 receptor, which is expressed on the surface of cells infected with C-POZ retrovirus vector (Nakatani and Ogryzko

2003). CPEB in pMSCV-Hyg was also introduced to $p19^{ARF}$ KO, $p16^{INK4A}/p19^{ARF}$ KO (gifts from Norman E. Sharpless, University of North Carolina, Chapel Hill, NC), and p53 KO (gift from Stephen Jones, University of Massachusetts Medical School, Worcester, MA) MEFs at passage 4. Senescence-associated β-galactosidase activity was determined at pH 6.0 (Dimri et al. 1995). Soft agar assays were performed according to Clark et al. (1995).

To visualize the microtubules, wild-type and CPEB KO MEFs at passages 3 and 25 were immunostained with primary antibody against α-tubulin and secondary antibody conjugated with green Alexa Fluor 488 (Molecular Probes). DNA was stained with DAPI. The coverslips were mounted with Prolong (Molecular Probes), and images were obtained with a Nikon Eclipse (E600) microscope at 100×.

The TUNEL reaction was performed using an In Situ Death Detection POD kit (Roche). DNase I-treated cells were used as a positive control.

Biochemical assays

Wild-type MEFs were infected with a retrovirus encoding Flag-and HA-tagged CPEB. Cells obtained from 150 mM plates were lysed in buffer containing 20 mM Tris-HCl (pH 8), 0.2 mM EDTA, 0.1% Tween 20, 0.1 M KCl, 10% glycerol, 10 mM β-mercaptoethanol, and protease inhibitors and subsequently subjected to RNP

coimmunoprecipitation sequentially with antibody directed against each epitope (Nakatani and Ogryzko 2003). The RNA was extracted from the final precipitate and subjected to an RT reaction with SuperScript II (Invitrogen) with an oligo(dT) primer.

Renilla luciferase was cloned into pRL-TK vector (Promega) mRNA and appended with either the myc 3′ UTR (the myc 3′ UTR sequence is found in Ensembl ENSMUST22971) or a 3′ UTR derived from vector sequence; the RNA was synthesized in vitro with T7 RNA polymerase (mMassage mMachine kit; Ambion). Each of these RNAs was mixed with RNA encoding firefly luciferase appended with a 3′ UTR vector sequence and transfected into wild-type and CPEB KO MEFs with Trans-Messenger Transfection Reagent (Qiagen). The amount of *Renilla* luciferase in each of the MEFs was then determined 4 h after transfection with the Dual-Luciferase Reporter Assay System (Promega) and expressed as a fold change in CPEB KO versus wild-type MEFs.

Wild-type and CPEB KO MEFs were infected at passage 2 with pBabe-c-Myc-Puro (myc ORF with its own 3′ UTR or one with a vector 3′ UTRone) or pBabe-Puro (vector alone). The levels of myc mRNA and protein were assessed by the RT–PCR reaction and Western blotting, respectively. Total RNA or protein was prepared from 100 mM plates of MEFs cultured to passage 4. One microgram of total RNA was used for RT–PCR or quantitative real-time one-step RT–PCR with the QuantiTect SYBR RE-PCR Qiagen kit. The relative fold changes were

calculated using the $2^{-\Delta\Delta Ct}$ method. Western blot analyses were performed on one-fourth of the material from cells grown on 100 mM plates.

Polysome fractionation was performed according to Ruan et al. (1997). MEFs at passage 4 were obtained from three 10-cm plates and were centrifuged through 15%–50% sucrose and fractionated, and the RNA from each fraction was extracted and used for oligo(dT)-primed quantitative real-time PCR for myc and actin RNAs. The amount of myc RNA was normalized to the amount of actin RNA in each fraction. Relative fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method.

For UV cross-linking (Hake and Richter 1994), [^{32}P]UTP-labeled myc 3′ UTR (20 fmol) was mixed with recombinant CPEB (1 µmol) plus 0, 3, 6, and 30 pmol of competitor RNA (cyclin B1 3′ UTR containing or lacking the CPE) and irradiated with UV light. The RNA was then digested with RNase A and T1, and the products were resolved by SDS-PAGE and PhosphorImaging.

Mice and histology

Immortalized wild-type and CPEB KO MEFs, some of which were infected with a Ki-Ras-expressing retrovirus, were injected subcutaneously into 12-wk-old male athymic (nude) mice (Charles River) (~10^6 cells per injection, one injection near each hind limb of each of 10 mice). The mice were euthanized 6 wk post-

injection, and the tumors were dissected, weighed, fixed, and processed for histological analysis. Paraffin-embedded sections were stained with hematoxylin and eosin.

Acknowledgments

We thank David Garlick, Nic Jones, Michelle Kelleher, and Jackie Lees for advice and Mike Cole, Valentina Evdokimova, Regina Groisman, Steve Jones, Andy Koff, Martine Roussel, and Norman Sharpless for reagents. V.M. was supported by an Institutional Post-doctoral Training Grant (HD07312). This work was supported by grants from the NIH (HD37267 and GM46779) and the G. Harold and Leila Y. Mathers Charitable Foundation. Additional core support from the Diabetes and Endocrinology Research Center Program Project (DK32520) is gratefully acknowledged.

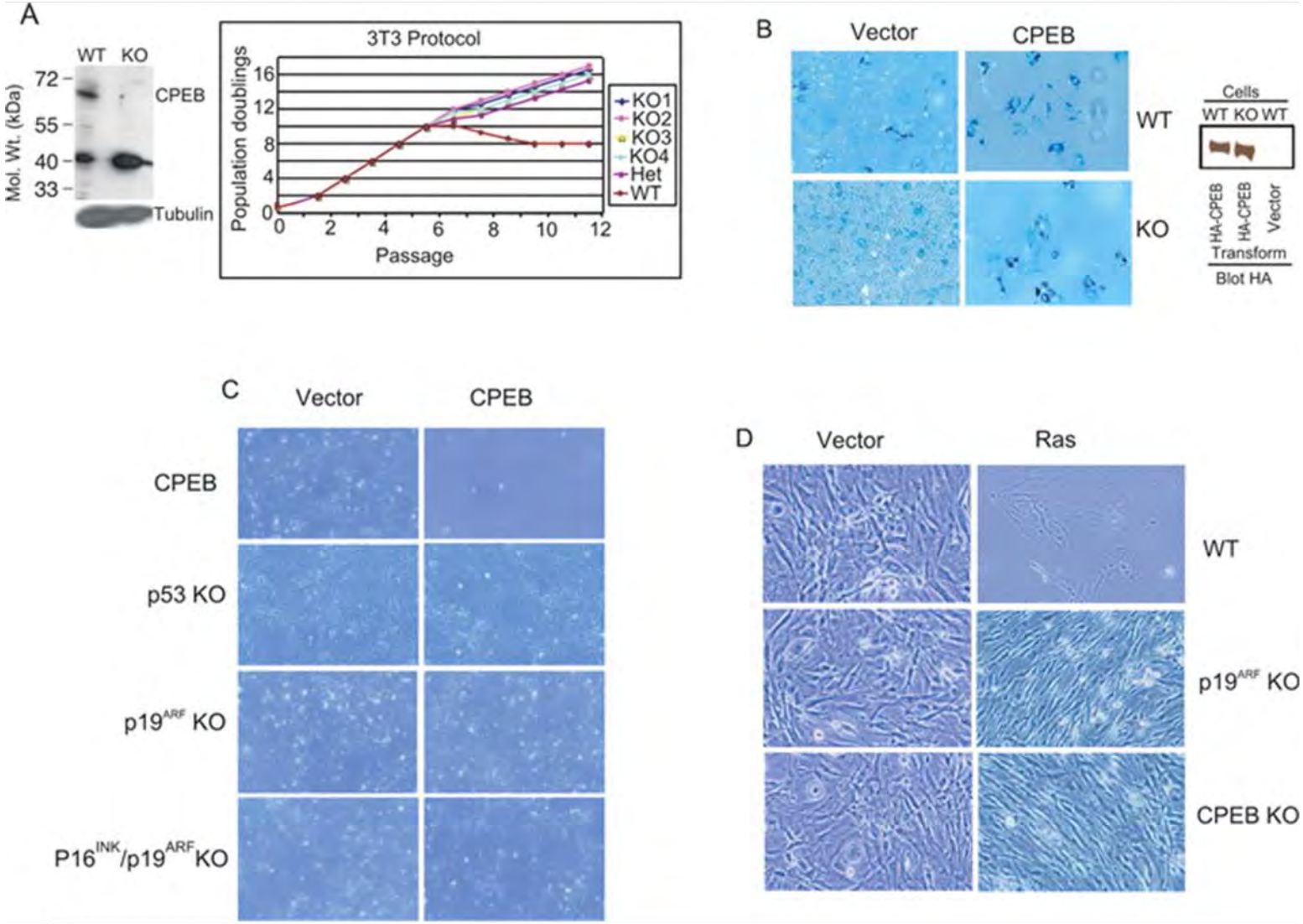
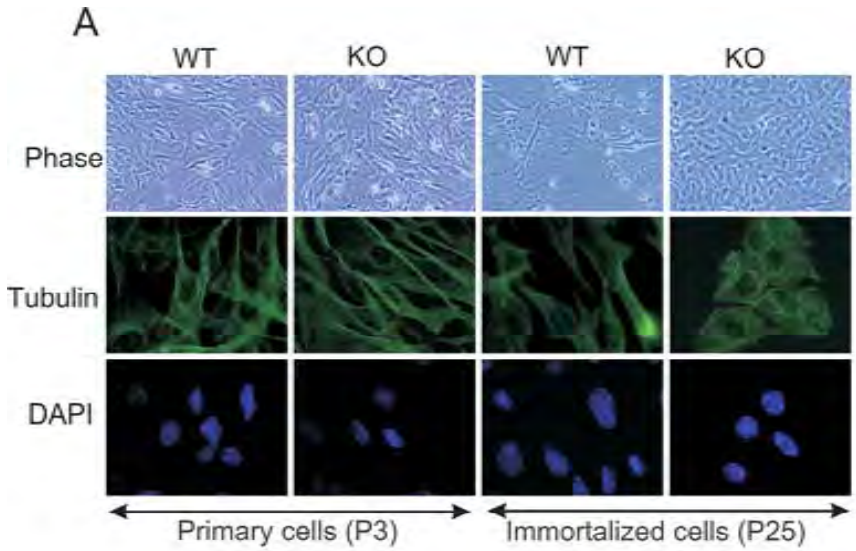
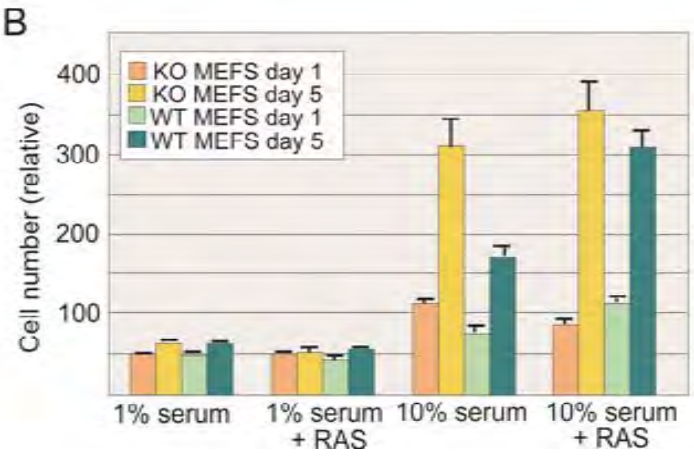
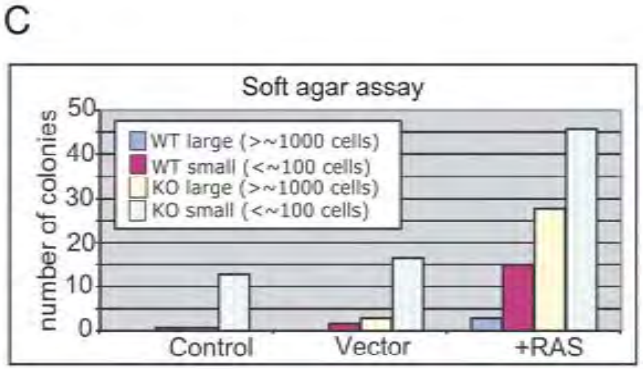

A
WT KO
72
55
40
33
Mol. Wt. (kDa)
CPEB
Tubulin
3T3 Protocol
Population doublings
16
12
8
4
0
0 2 4 6 8 10 12
Passage
KO1
KO2
KO3
KO4
Het
WT
B
Vector
CPEB
WT
KO
Cells
WT KO WT
HA-CPEB
HA-CPEB
Vector
Transform
Blot HA
C
Vector
CPEB
CPEB
p53 KO
p19ARF KO
P16INK/p19ARF KO
D
Vector
Ras
WT
p19ARF KO
CPEB KO

Figure 1

Figure 1. CPEB controls senescence in MEFs. (*A*, *left*) A Western blot of wild-type (WT) and CPEB KO MEFs probed for CPEB and tubulin. (*Right*) The rate of population doublings of four CPEB KO MEF lines and, for comparison, one wild-type line and one CPEB heterozygous line. The MEFs were cultured according to a 3T3 protocol. (*B*) Wild-type and CPEB KO MEFs were infected with a retrovirus expressing HA-tagged CPEB, or an empty control vector, at passage 2 and then fixed at passage 4 (wild type) or passage 11 (KO) and stained for β-galactosidase activity. Also shown is a Western blot demonstrating the expression of HA-tagged CPEB in wild-type and KO MEFs. (*C*) MEFs lacking CPEB, $p19^{ARF}$, $p16^{INK}$/$p19^{ARF}$, or p53 were infected with the CPEB-encoding virus or an empty vector at passage 4 and examined by phase contrast microscopy at passage 11. (*D*) MEFs derived from wild-type, CPEB KO, and $p19^{ARF}$ KO animals were infected at passage 2 with a retrovirus expressing Ki-Ras or an empty vector, selected by puromycin at passage 3, and examined by phase contrast microscopy at passage 5.


A
WT
KO
WT
KO
Phase
Tubulin
DAPI
Primary cells (P3)
Immortalized cells (P25)


B
KO MEFS day 1
KO MEFS day 5
WT MEFS day 1
WT MEFS day 5
Cell number (relative)
400
300
200
100
1% serum
1% serum + RAS
10% serum
10% serum + RAS


C
Soft agar assay
number of colonies
50
40
30
20
10
0
WT large (>~1000 cells)
WT small (<~100 cells)
KO large (>~1000 cells)
KO small (<~100 cells)
Control
Vector
+RAS

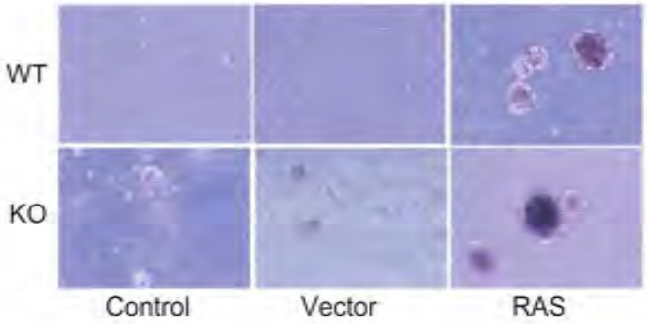

WT
KO
Control
Vector
RAS

Figure 2

Figure 2. CPEB KO MEFs have features consistent with partial transformation. (*A*) Wild-type (WT) and CPEB KO MEFs were examined by phase contrast microscopy and immunostained for α-tubulin at passages 3 and 25 (P3 and P25) to assess cell morphology. The cells were also stained with DAPI to visualize nuclei. (*B*) Wild-type and CPEB KO MEFs were cultured for up to 5 d in medium containing 1% or 10% serum and analyzed for cell number by staining with crystal violet (average ± SD, $n = 3$). Some of the MEFs were also infected with a retrovirus expressing Ki-Ras and were similarly examined. (*C*) Wild-type and CPEB KO MEFs, some of which were infected with a Ki-Ras-containing virus or an empty virus vector as before, were grown on soft agar for 2 wk. Small foci were estimated to have ~100 cells, while large foci were estimated to have ~500–1000 cells.

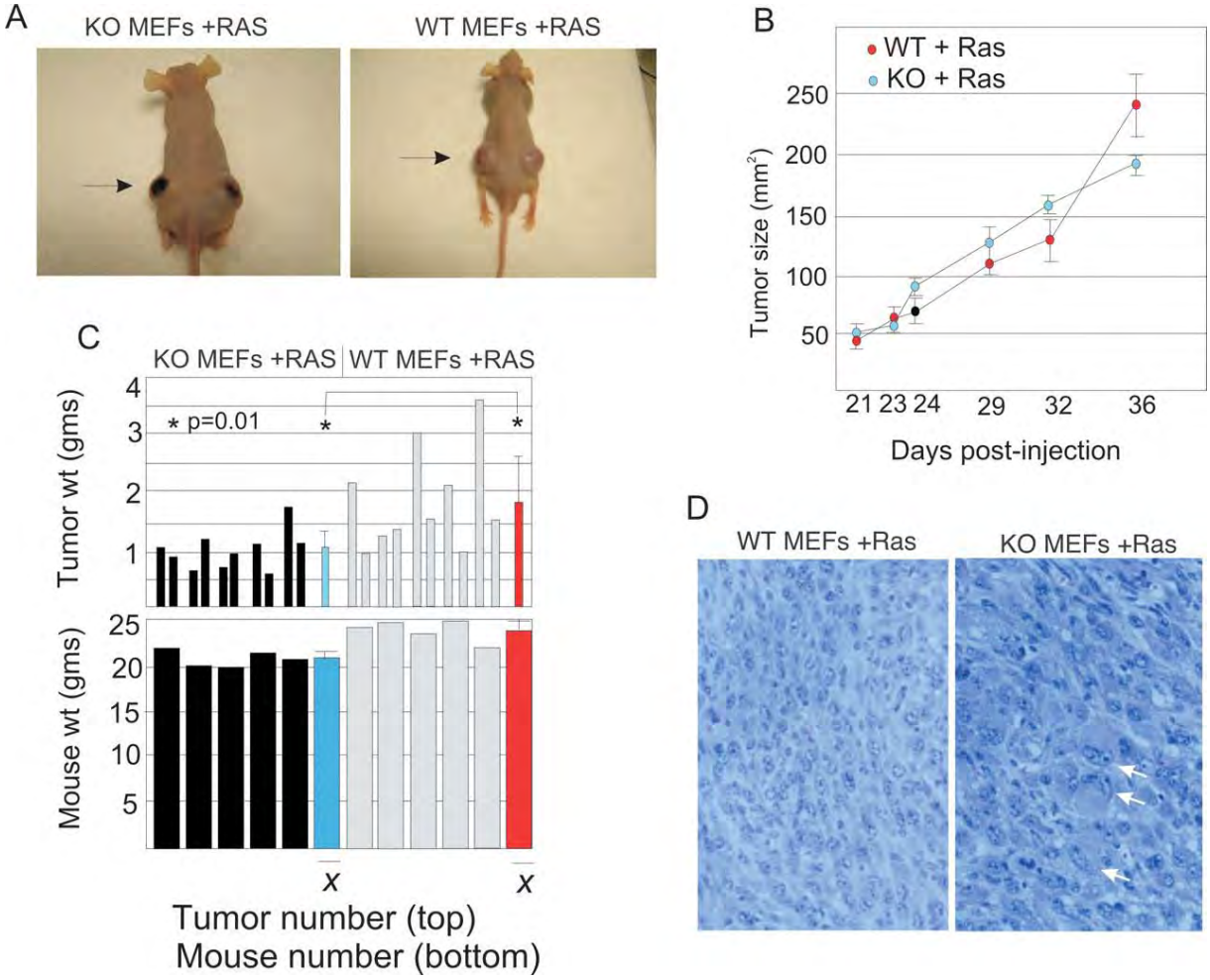

A
KO MEFs +RAS
WT MEFs +RAS
B
WT + Ras
KO + Ras
Tumor size (mm²)
250
200
150
100
50
21 23 24 29 32 36
Days post-injection
C
KO MEFs +RAS
WT MEFs +RAS
* p=0.01
Tumor wt (gms)
4
3
2
1
Mouse wt (gms)
25
20
15
10
5
x
x
Tumor number (top)
Mouse number (bottom)
D
WT MEFs +Ras
KO MEFs +Ras

Figure 3

Figure 3. Tumors derived from wild-type (WT) and CPEB KO MEFs transformed with Ki-Ras. Immortalized cells derived from wild-type MEFs grown according to a 3T3 protocol as well as CPEB KO MEFs were infected with a retrovirus expressing Ki-Ras. The cells were injected subcutaneously into athymic (nude) mice (five mice each, two injections per mouse near each hind limb). All Ki-Ras-containing cells formed tumors irrespective of genotype (10/10 WT, 10/10 CPEB KO). (*A*) Representative pictures of the tumors at the sixth week after injection; note the hematoma on the tumors derived from the CPEB KO MEFs (cf. those denoted by arrows). All 10 tumors derived from the Ras-transformed KO cells had hematomas. (*B*) Tumor growth. (*C*) Final tumor and mouse weight 6 wk after injection. (*D*) The tumors shown in *A* were excised, sectioned, and stained with hematoxylin and eosin. Representative sections are shown. The arrows denote cells with enlarged cytoplasm and displaced nuclei.

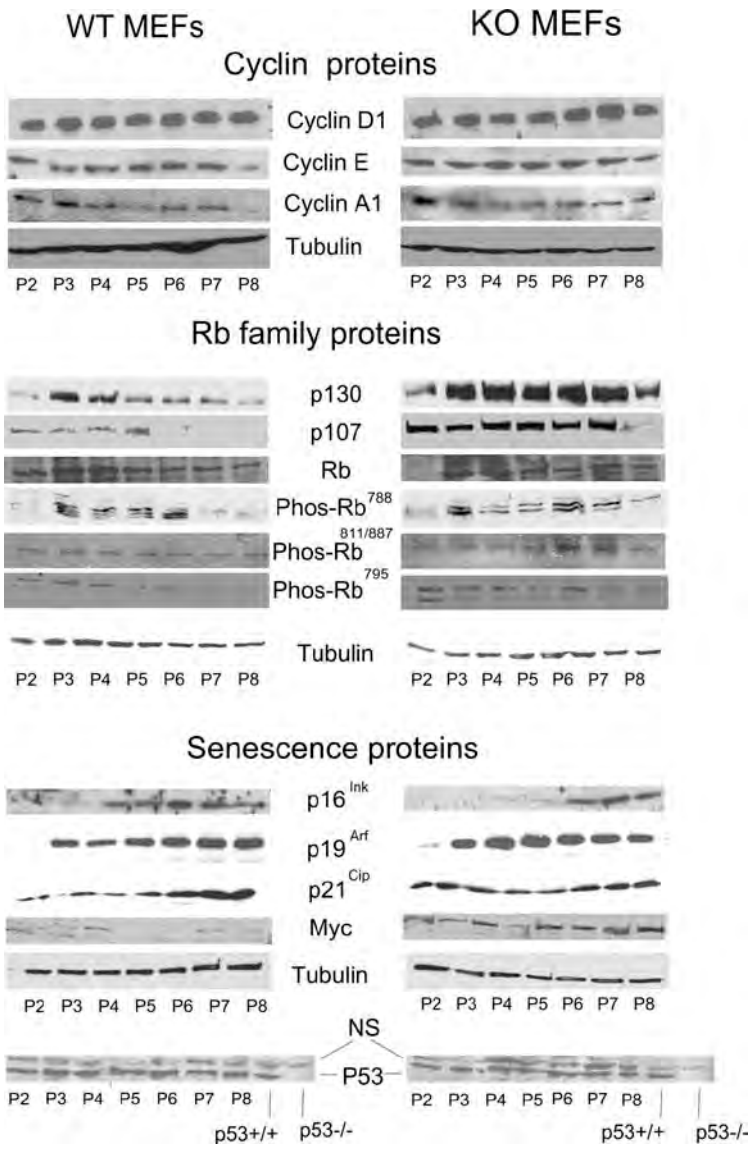

WT MEFs
KO MEFs
Cyclin proteins
Cyclin D1
Cyclin E
Cyclin A1
Tubulin
P2 P3 P4 P5 P6 P7 P8
Rb family proteins
p130
p107
Rb
Phos-Rb788
Phos-Rb811/887
Phos-Rb795
Tubulin
P2 P3 P4 P5 P6 P7 P8
Senescence proteins
p16Ink
p19Arf
p21Cip
Myc
Tubulin
P2 P3 P4 P5 P6 P7 P8
NS
P53
P2 P3 P4 P5 P6 P7 P8
p53+/+
p53-/-
P2 P3 P4 P5 P6 P7 P8
p53+/+
p53-/-

Figure 4

Figure 4. Expression levels of proteins involved in the cell cycle and cell senescence. Wild-type (WT) and CPEB KO MEFs were collected over passages 2–8 and examined by Western blot ting for the indicated proteins. Tubulin served as the loading control and as a standard for film exposures among blots. Be cause the p53 antibody reacted strongly with two proteins from wild-type and CPEB KO MEFs, extracts from p53 KO and parallel wild-type MEFs were also analyzed. The faster migrating protein was absent from the p53 KO MEFs, demonstrating that it is p53. (NS) Nonspecific band.

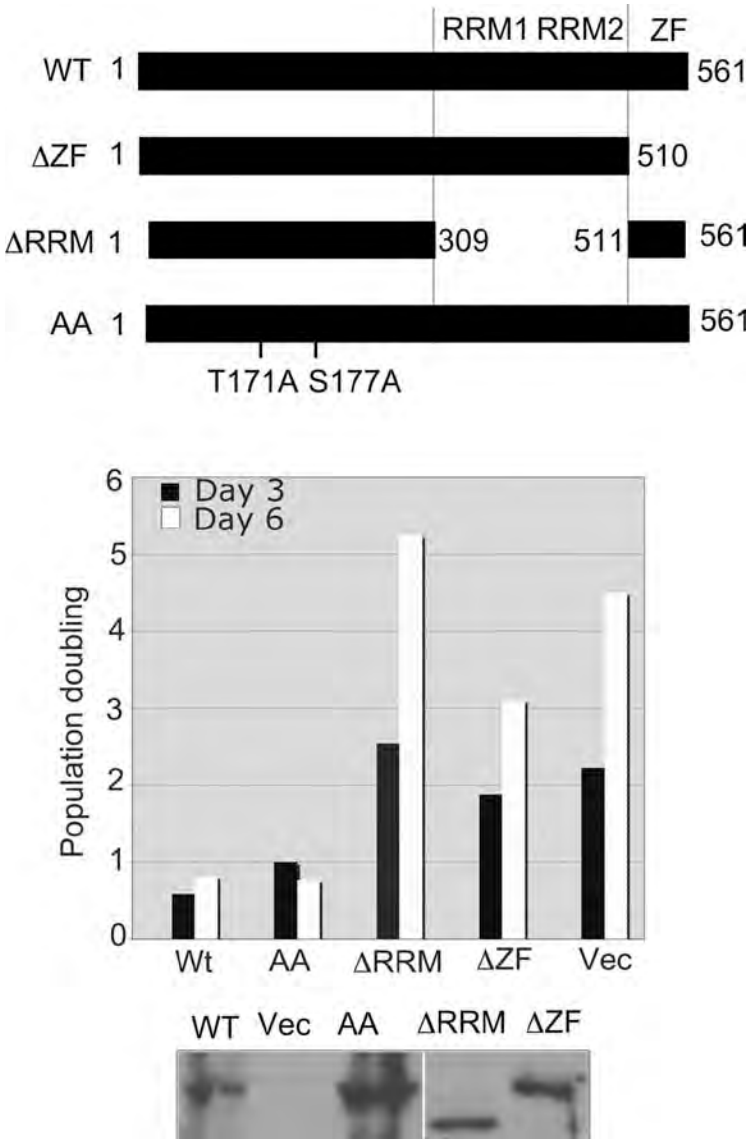

RRM1 RRM2 ZF
WT 1
561
ΔZF 1
510
ΔRRM 1
309
511
561
AA 1
561
T171A S177A
Day 3
Day 6
Population doubling
0
1
2
3
4
5
6
Wt
AA
ΔRRM
ΔZF
Vec
WT Vec AA ΔRRM ΔZF

Figure 5

Figure 5. RNA binding but not phosphorylation is necessary for CPEB rescue of senescence. The CPEB KO MEFs were infected with virus expressing no CPEB (Vec), wild-type CPEB (WT), CPEB lacking the RRMs (ΔRRM), CPEB lacking the zinc finger (ΔZF), or CPEB with T171A/S177A mutations that prevent cytoplasmic polyadenylation. The cells were selected and counted on days 3 and 6. The Western blot shows the relative levels of the virally expressed proteins

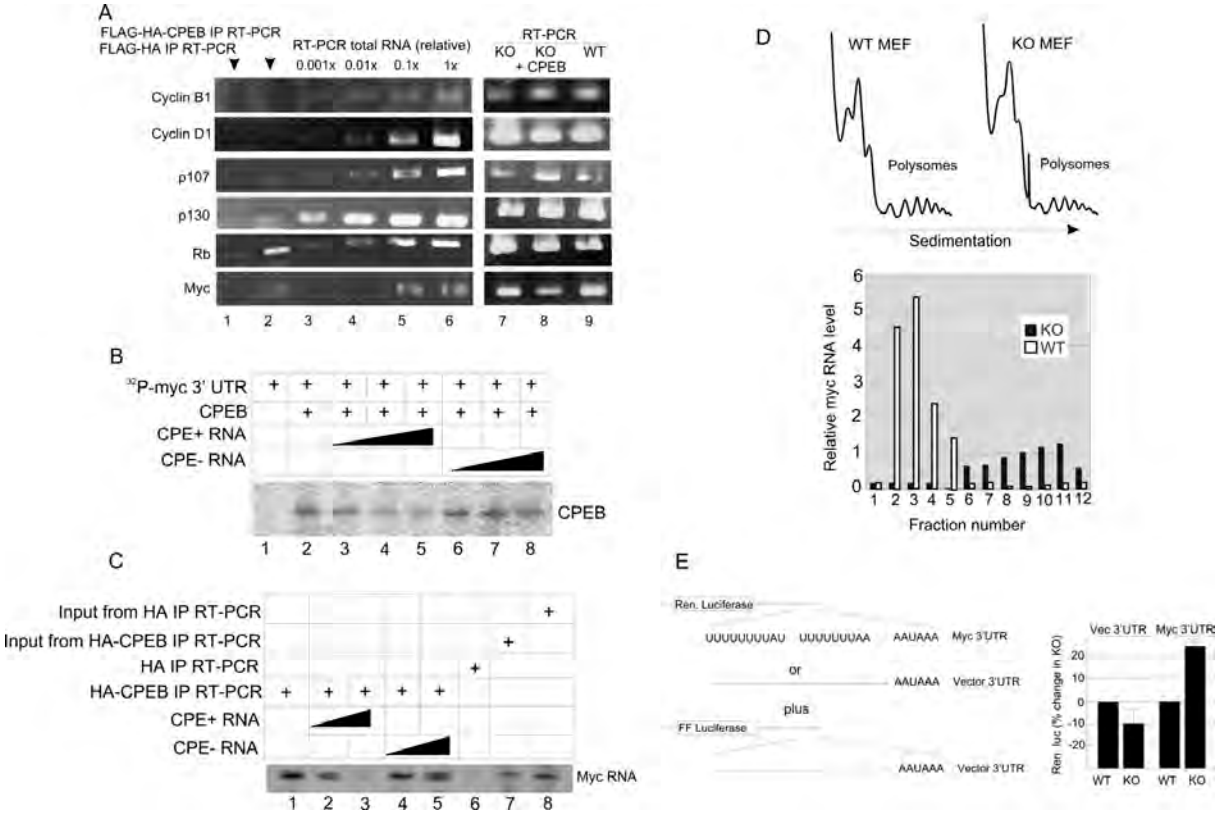

A
FLAG-HA-CPEB IP RT-PCR
FLAG-HA IP RT-PCR
RT-PCR total RNA (relative)
0.001x 0.01x 0.1x 1x
RT-PCR
KO KO WT
+ CPEB
Cyclin B1
Cyclin D1
p107
p130
Rb
Myc
1 2 3 4 5 6 7 8 9
B
32P-myc 3' UTR
CPEB
CPE+ RNA
CPE- RNA
CPEB
1 2 3 4 5 6 7 8
C
Input from HA IP RT-PCR
Input from HA-CPEB IP RT-PCR
HA IP RT-PCR
HA-CPEB IP RT-PCR
CPE+ RNA
CPE- RNA
Myc RNA
1 2 3 4 5 6 7 8
D
WT MEF
KO MEF
Polysomes
Polysomes
Sedimentation
Relative myc RNA level
KO
WT
0 1 2 3 4 5 6
1 2 3 4 5 6 7 8 9 10 11 12
Fraction number
E
Ren. Luciferase
UUUUUUUUAU UUUUUUUAA AAUAAA Myc 3'UTR
or
AAUAAA Vector 3'UTR
plus
FF Luciferase
AAUAAA Vector 3'UTR
Vec 3'UTR Myc 3'UTR
Ren. luc (% change in KO)
20 10 0 -10 -20
WT KO WT KO

Figure 6

Figure 6. Translational regulation of myc mRNA by CPEB. (*A*) MEFs were infected with Flag-and HA-tagged CPEB and subsequently subjected to RNP coimmunoprecipitation sequentially with antibody directed against each epitope. The RNA was extracted from the final precipitation and subjected to RT–PCR for the denoted RNAs. In addition to the immunoprecipitated RNP complexes, total RNA from wild-type (WT) and KO MEFs was serially diluted and analyzed for RNA levels by RT–PCR. (*Right*) The relative input levels of the RNAs by RT–PCR from wild-type, CPEB KO, and CPEB KO MEFs that were infected with virus expressing CPEB. (*B*) ^{32}P myc 3′ UTR was mixed with recombinant CPEB and, in some cases, with CPE-containing (CPE+) or CPE-lacking (CPE−) RNA. The mixture was UV-irradiated, RNase-digested, and analyzed by SDS-PAGE and PhosphorImaging. (*C*) MEFs infected with the same plasmids as in *A* were mixed with CPE-containing (CPE+) or CPE-lacking (CPE−) RNAs prior to HA immunoprecipitation and RT–PCR for myc RNA. (*D*) Wild-type and CPEB KO MEFs at passage 4 were centrifuged through sucrose gradients and fractionated, and the myc and actin RNAs in each fraction were determined by quantitative real-time PCR. The histogram depicts the amount of myc RNA relative to actin RNA in each fraction. The UV scans of the gradients (absorbance at 254 nm) are depicted at the *top*. (*E*) Schematic diagram of *Renilla* luciferase RNA appended with either the myc 3′ UTR (the CPEs and the polyadenylation hexanucleotide AAUAAA are shown) or a 3′ UTR derived from vector sequences but containing the AAUAAA. Each of these in vitro synthesized RNAs was mixed with RNA

encoding firefly (FF) luciferase appended with a 3′ UTR vector sequence, and transfected into wild-type and CPEB KO MEFs. The FF luciferase construct served as an internal control for transfection efficiency. The amount of *Renilla* luciferase in each of the MEFs was then determined 5 h after transfection and expressed as the fold change in CPEB KO versus wild-type MEFs. The average of three experiments (±SD) is shown.

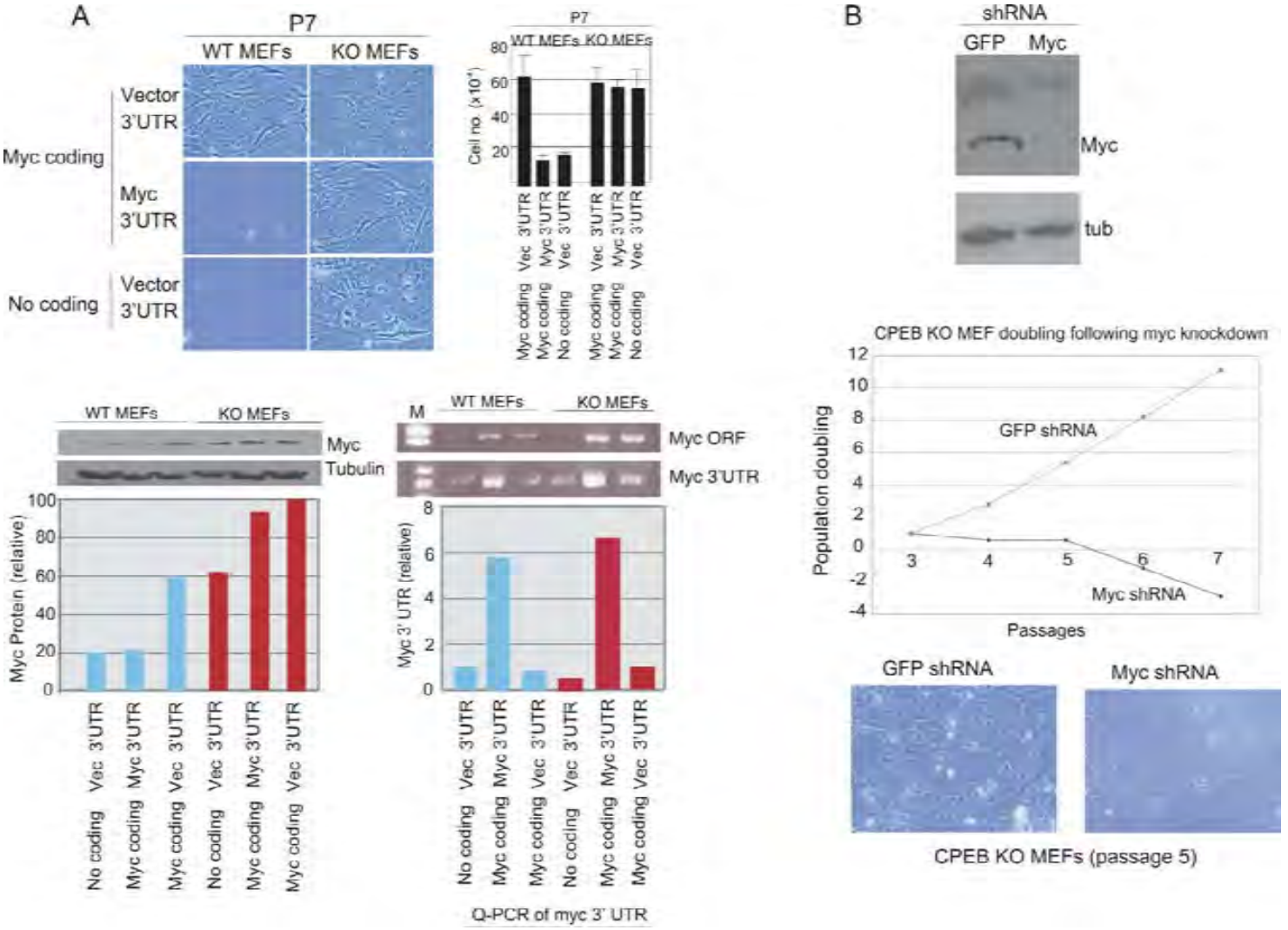
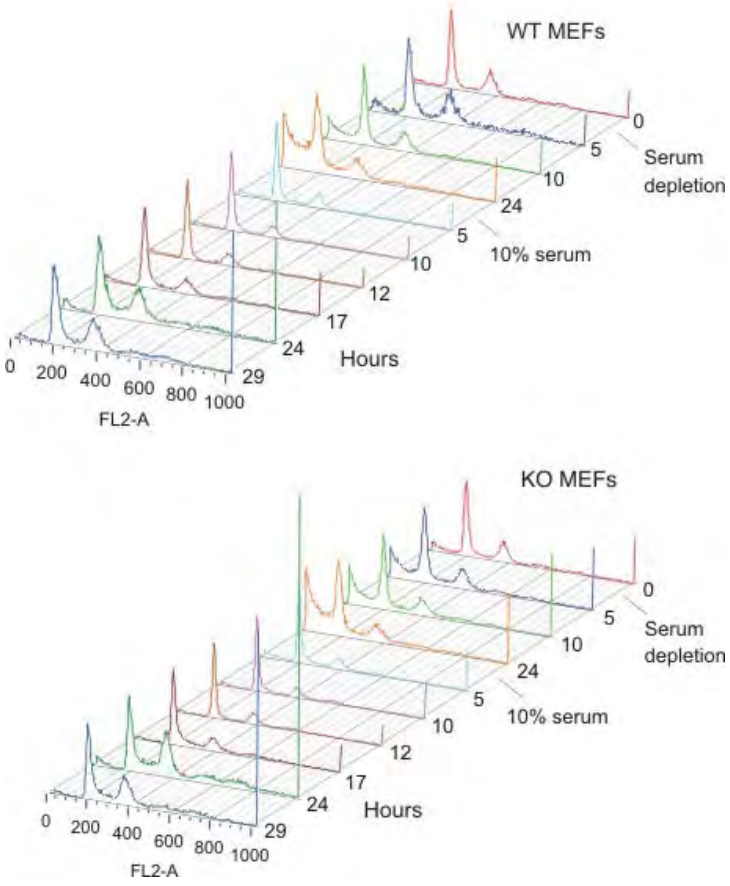

A
P7
WT MEFs
KO MEFs
Vector 3'UTR
Myc coding
Myc 3'UTR
No coding
Vector 3'UTR
Cell no. (x10⁴)
Vec 3'UTR
Myc 3'UTR
Vec 3'UTR
Myc coding
Myc coding
No coding
WT MEFs
KO MEFs
Myc
Tubulin
Myc Protein (relative)
M
Myc ORF
Myc 3'UTR
Myc 3' UTR (relative)
Q-PCR of myc 3' UTR
B
shRNA
GFP
Myc
Myc
tub
CPEB KO MEF doubling following myc knockdown
Population doubling
GFP shRNA
Myc shRNA
Passages
GFP shRNA
Myc shRNA
CPEB KO MEFs (passage 5)

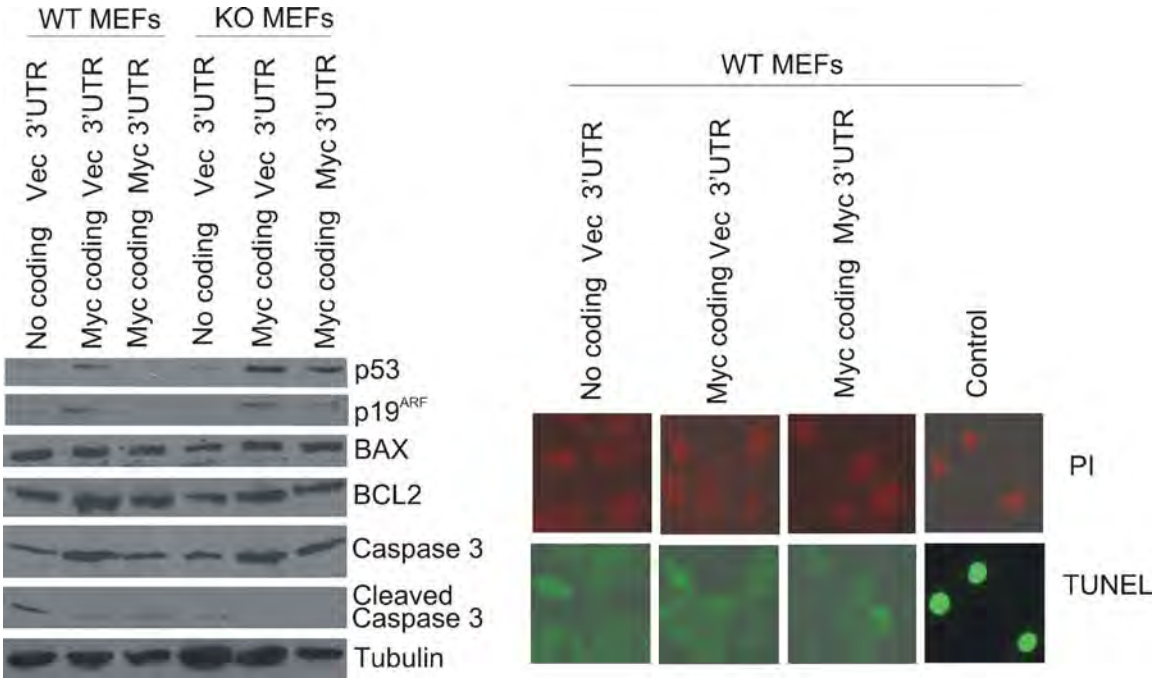
Figure 7

Figure 7. Myc RNA translation by CPEB controls senescence. (*A*) At passage 2, wild-type (WT) and CPEB KO MEFs were transfected with a retrovirus expressing the myc-coding region appended with its own 3′ UTR or a 3′ UTR derived from vector sequences. Some MEFs were infected with a virus containing only the vector. At passage 7, cell number was assessed by microscopy and by counting with a hemocytometer. Some of the cells were also used for Western blotting for myc and tubulin, and for RT–PCR of the myc ORF and 3′ UTR. In addition, the myc 3′ UTR was quantified by real-time PCR (histogram). The levels of this RNA were made relative to actin RNA, which was also quantified by real-time PCR. (*B*, *top* panel) KO MEFs were infected with shRNAs for myc or GFP at passage 2 and puromycin-selected, and a Western analysis for myc protein was performed at passage 5. The cells were also examined for population doubling (*middle* panel) and phase contrast microscopy (*bottom* panel).



Supplementary Figure 1

Supplementary Figure 1. Cell cycle rates of WT and CPEB KO MEFs. Cells of each genotype at passage 2 were cultured in medium lacking serum for 24 hours and then cultured a subsequent 29 hours in medium containing 10% serum. At several times, the cells were stained with propidium iodide and subjected to FACS analysis.



Supplementary Figure 2

Supplementary Figure 2. Immunoblots of p53, $p19^{ARF}$, Bax, BCl2, Caspase 3, cleaved Caspase 3 and tubulin were performed on WT and KO MEFs that were infected with retroviruses harboring the sequences noted above. The right panel shows TUNEL reactions for the cells infected with the sequences noted above. PI, propidium iodide.

Author's contributions

Maria Ivshina performed the experiments and assays and fully contributed the Fig.1, Fig. 2A, Fig. 3 (except western blots for p53 and c-Myc) and participated in cloning of myc-3'UTR contracts described in Fig.7. All experiments described in Fig. 2B, Fig. 2C, Fig. 3, Fig. 1S were done in equal contribution with Irina Groisman.

CHAPTER III

CPEB-regulation of NFκB Nuclear Localization Mediates the Inflammatory Immune Response

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Abstract

CPEB is a sequence-specific RNA binding protein that regulates cytoplasmic polyadenylation-induced translation. We report here that CPEB KO mice are hypersensitive to LPS-induced endotoxic shock, which correlates with elevated serum levels of the proinflammatory cytokines IL-6, IL-8 and IL-12. Peritoneal macrophages from the KO mice, as well as a CPEB-depleted macrophage cell line, not only secrete more IL-6 than control cells in response to LPS, but also have prolonged retention of NFκB in the nucleus, which is responsible for elevated IL-6 transcription. The amount of nuclear NFκB correlates with reduced levels of IκBα, which is hyperphosphorylated and rapidly degraded. Collectively, these data suggest that CPEB deficiency enhances the inflammatory response via delayed resolution of NFκB signaling.

Introduction

Inflammation is triggered by bacterial pathogens and lipopolysaccharide (LPS), a component of their cell walls, which activates the transcription of multiple inflammatory response genes including cytokines and chemokines. Despite the importance of these inflammation mediators for the host defense against infection, their uncontrolled excessive production can elicit multiple organ failure and lethality resulting in septic shock. Therefore, the limitation of cytokine production provides an essential step in the termination of inflammation and the prevention of endotoxic tissue damage (Aggawat et al., 2003; Medzitov et al., 2008).

Production of the inflammatory mediators is controlled at multiple levels including transcription, translation, and protein stability (Anderson, 2009). In the presence of LPS, the immune response is triggered through a Toll-like receptor (TLR) signaling pathway (Kawasaki et al., 2003) resulting in the activation of several transcription factors including NFκB (Xiao and Ghosh, 2005). Many signaling events that converge on NFκB lead to the activation of the IκB kinase (IKK) complex (Mercurio et al., 1997; Zandi et al., 1997), which in turn phosphorylates IκBα, a factor that normally retains NFκB in the cytoplasm (Stancovski and Baltimore, 1997). Phosphorylated IκBα is rapidly destroyed, thus releasing NFκB to translocate to the nucleus and activate transcription of target genes (Hoffmann et al., 2006). In addition to the activation of transcription, LPS

triggers the stabilization of multiple mRNAs, an event that is dependent upon the interplay of several 3'UTR-binding proteins such as those that associate with the AU-rich element (ARE) stabilization/destruction sequence (Zhang et al., 2002; Akira et al., 2006; Hao and Baltimore, 2009).

The Cytoplasmic Element Binding Protein, CPEB, is an mRNA binding protein that interacts with the CPE, a U-rich sequence in mRNA 3'UTRs to control poly(A) tail length and translation (Hake et al.1994; Richter 2007). As a consequence of its control of polyadenylation and translation, CPEB regulates germ cell development (Tay et al. 2001), neuronal synaptic plasticity (Alarcon et al. 2004, Klann and Richter 2007), and cellular senescence (Groisman et al.2006, Burns et al.2008). Two observations suggested that CPEB might also be involved in the immune response. First, CPEB activation by phosphorylation is a result of extra-cellular signaling events including those induced by stress such as that which occurs during cellular senescence. Second, the ARE, a 3'-UTR cis-element in cytokine mRNAs resembles the CPE, indicating that CPEB might influence their stability and/or translation. In this report, we have investigated CPEB involvement in the immune response. We find that CPEB KO mice are hypersensitive to endotoxic shock and that they secrete high levels of cytokines. Peritoneal macrophages derived from the KO animals, as well as a macrophage cell line depleted of CPEB produce excessive amounts of the cytokines. We find that cytokine production is due to an aberrantly long nuclear retention of NFκB, a transcription factor that induces cytokine mRNA synthesis. IκBα, a protein

required for cytoplasmic NFκB retention, is hyperphosphorylated and destroyed at an abnormally high rate upon CPEB depletion, thereby liberating NFκB for nuclear translocation and transcriptional activation.

Results and Discussion

LPS induction of high levels of serum cytokines results in septic shock (Krakauer et al.,2010). To determine whether CPEB might be involved in this inflammatory response, the peritoneal cavities of wild type (WT) and CPEB KO mice were injected with LPS LD_{50}. The rate of LPS-induced lethality of the KO mice was substantially elevated relative to that of WT animals, and reached 100% by day 6 (Figure 1A). Both WT and KO animals had normal levels of white blood cells and platelets prior to LPS injection (data not shown), indicating no obvious predisposition to inflammation. Histological analysis of liver and lung tissue samples taken 24 h after LPS injection revealed an increased number of neutrophils in both WT and CPEB KO animals. However, the levels of neutrophils in the KO samples were significantly higher, indicating an especially strong inflammatory response (Figure 1B). Next, we analyzed the levels of blood serum cytokines after control PBS or LPS injection by performing ELISA for 96 different mediators of inflammation. Only 6 of the 96 showed a statistically significant change; of these 6, IGF-bp3, IGF-1, IL-6, IL-12, and IL-8 (KC) were little altered

upon PBS injection irrespective of genotype. L-selectin, was elevated in the serum in both PBS and LPS injected KO mice. LPS injection elicited significantly higher levels of IL-6, IL-8, IL-12p40, IGF-1, and IGF-BP3 in the KO animals (Figure 2). Interestingly, the proinflammatory cytokines IL-6, IL-8 and IL-12p40 are major markers of lethal sepsis, which correlate with the increased lethality on the LPS injected CPEB KO mice. To begin to determine why the cytokine levels were abnormally elevated in the KO mice, we analyzed immune cell lineages from thymus, lymph node, and spleen by FACS. In no case did we observe any significant difference in immune cell progenitors or mature immune cells (data not shown). Because macrophages are one major source of cytokine production, we analyzed IL-6 from wild-type and CPEB KO mice-derived peritoneal macrophages treated with LPS in vitro. As shown in Figure 3A, LPS-induction of IL-6 in the KO macrophages was elevated by ~2 fold, which is consistent with the results observed in the KO animals. We also assessed the level of IL-6 after shRNA-mediated depletion of CPEB in a macrophage cell line (RAW264.7), which has an inflammatory response to LPS similar to that of primary macrophages. As shown in Figure 2B, the level of CPEB RNA in cells infected with lentivirus expressing shRNA for CPEB was strongly reduced compared to a scrambled control shRNA. Although LPS induced IL-6 in control and lentivirus-shCPEB infected cells, depletion of CPEB resulted in ~3 fold higher levels of this cytokine. Because LPS induces a transcriptional program culminating in interleukin production, we used RT-PCR to determine whether CPEB depletion

had an effect on LPS induced IL-6 transcription (Figure 3D). LPS elicited elevated levels of IL-6 RNA in both control and CPEB depleted cells. However, the CPEB deficient cells had a higher level of IL-6 RNA following LPS treatment, suggesting that the level or activity of a transcription factor may be controlled by CPEB.

NFκB is one of the major LPS-activated transcription factors implicated in IL-6, IL-8 and IL-12 cytokine production (Pahl et al., 1999) Moreover, NFκB mRNA encoding the 65 kDa subunit (p65, RelA) contains CPEs in its 3' UTR, suggesting that it may be a direct target of CPEB. Consequently, we assessed whether NFκB inhibitors could prevent the accumulation of the high LPS-induced IL-6 levels in CPEB knockdown cells (Figure 4A). Although LPS induced >3 fold increase in IL-6 in CPEB depleted cells, this increase was abrogated by two different inhibitors of NFκB activity, JSH-23 and 5HPP-33. Thus, NFκB is a major contributor to IL-6 production in CPEB deficient cells in response to LPS.

Next, because NFκB transcriptional activity is tightly controlled by its nuclear localization, we examined the levels of IκBα, a cytoplasmic NFκB anchoring protein. In WT cells, LPS induces a transient decrease in IκBα levels (Xiao and Ghnosh, 2005; Mercurio et al., 1997); in Figure 4B, IκBα was reduced at 0.5 hrs post LPS and returned pre-LPS levels by 4 hours. Unexpectedly, CPEB depletion resulted in a dramatic decrease in the levels of this protein at 0.5 and 4 hr in response to LPS (Figure 4B). These results imply that NFκB nuclear localization, directly or indirectly, would be regulated by CPEB. To assess this

possibility, we immunostained control and CPEB-depleted macrophages for NFκB at 0-4 hours post-LPS treatment. Figure 4C demonstrates that prior to LPS administration, NFκB was cytoplasmic in cells containing or lacking CPEB; after 30 min however, when IκBα was destroyed, NFκB relocated to the nucleus in both cell types. As expected, in control cells after 4 hr in LPS, IκBα levels were restored and NFκB relocated to the cytoplasm. In contrast, LPS-treated CPEB knockdown cells in which IκBα levels were low, NFκB remained nuclear. We observed similar results in LPS-treated peritoneal macrophages derived from WT and CPEB KO mice (Figure 5). These results suggest that CPEB control of IL-6 production in response to LPS is mediated by NFκB subcellular localization.

Because IκBα mRNA contains 3' UTR CPEs, we suspected that it might be under the translational control of CPEB. To address this possibility, control and CPEB-depleted macrophages were treated with LPS for 4 hours, followed by pulse labeling for 15 min with ^{35}S-methoinine and immunoprecipitation of IκBα. Knockdown of CPEB had no discernable effect on the synthesis of this protein (Figure 6A). This result suggests that CPEB controls IκBα at the level of protein degradation.

To assess this possibility, control and CPEB deficient cells were incubated with LPS and with or without MG132, a proteosomal inhibitor. Figure 6B shows that, as in Figure 4B, there were low levels of IκBα when CPEB depleted cells were treated with LPS. In contrast, IκBα in CPEB depleted cells incubated with

the proteosome inhibitor was restored to control levels. We further examined the phosphorylation of IκBα at Ser 32/36, which is required for ubiquitination and subsequent degradation (Mercurio et al. 1997). Indeed, in depleted cells treated with LPS and MG132, which maintains IκBα stability, ser 32/36 were hyper-phosphorylated (Figure 6B). Based on these data, we propose that CPEB knockdown elicits IκBα instability, possibly by activating IKKalpha/beta complex or other unknown kinases, resulting in NFκB nuclear retention and IL-6 transcription (Figure 6C).

The enhanced degradation of IκBα can be a direct result of either CPEB-dependent regulation of proteases that control IκBα degradation (such as Skp1–Culin Roc1/Rbx1/Hrt-1–F-box) (Ben-Neriah, 2002), or CPEB-dependent regulation of IκBα or NFκB modifications (such as acetylation, phosphorylation, or glycosylation) (Xiao and Ghosh, 2004) that, in turn, lead to IκBα instability. In both cases, we envision that CPEB enhances upstream signaling events that culminate in prolonged NFκB nuclear retention. For example, CPEB could control the level or activation of IKKα/β, one of the main kinases that phosphorylates IκBα ser32/36, which in turn would mediate the amount of NFκB in the nucleus (see above). In addition, a defect NFκB signal termination can lead to the prolong activation of this transcription factor. Some possibilities include expression of suppressor cytokine signaling proteins (SOCS1 and

SOCS3) (Ilangumaran et al., 2004) and de-ubiquitinating enzymes (A20 and Cezanne) (Verstrepen et al., 2010;Enesa et al., 2008).

In addition to transcription, the inflammatory response is regulated by the stability and/or translatability of cytokine mRNAs, which can feedback and modulate NFκB activity (Hoffmann and Baltimore, 2006). A number of RNA binding proteins control cytokine RNA expression. For example, mice lacking AUF1, an ARE-binding protein, suffer from dermatitis and chronic inflammation (Sadri et al., 2009); when challenged with LPS, the animals have elevated serum cytokines, particularly TNFα, and a high rate of mortality (Lu et al., 2006). Another ARE binding protein that mediates the inflammatory response is TTP, which when deleted in mice, causes an autoimmune response (cachexia, arthritis, dermatitis, autoantibody production) and elevated TNFα. Mice lacking TIA-1, another ARE binding protein, exhibit elevated TNFα and, depending on the genetic background, arthritis. Surprisingly, TTP/TIA-1 double knockout mice develop severe arthritis and have high levels of TNFα mRNA but reduced amounts of TNFα protein (Phillips et al., 2004) The mRNA encoding AUF1 has several CPEs in its 3' UTR, consequently, we considered the possibility that CPEB might mediate the inflammatory response through translational control of AUF1 mRNA. However, AUF1 levels are elevated, not reduced, in CPEB KO mice (data not shown), suggesting that CPEB-mediated inflammation does involve AUF1. Moreover, TNFα levels are not significantly altered in CPEB KO animals (data not shown). Thus, while there may be interplay among the various

ARE binding proteins for controlling the stability and/or translation of cytokine mRNAs, CPEB does not appear to among them.

Finally, it is interesting to note that only upon LPS treatment, in either injected animals or stimulated cells, is CPEB deficiency manifest by elevated cytokine production. This observation suggests that in the non-stressed state, CPEB depletion has little detectable effect on inflammation, and that only upon the application of stress (i.e., LPS) does CPEB deficiency allow certain signaling events to occur without normal constraints, thus leading to dramatic phenotypes. In this sense, CPEB might be considered a stress-response protein. Indeed, CPEB deficient primary mouse or human cells do not senesce as do WT cells (senescence is a stress response), but are immortal (Groisman et al., 2006; Burns and Richter, 2008). In the brain, CPEB deficiency results in a deficit in synaptic plasticity, which is induced by another type of stress, electrical stimulation (Alarcon et al., 2004; Zearfoss et al., 2008). Various signaling events may converge on CPEB, or perhaps more properly, CPEB-bound mRNAs, which are normally tightly controlled; when CPEB is absent, unrestricted signaling may produce a range of phenotypes depending upon the cell or tissue being examined.

Materials and Methods

Animal studies

All experiments were conducted in accordance with approved NIH and institutional protocols for the treatment and handling of vertebrate animals. Three month old agouti CPEB WT and KO males (13 each with matching littermates) were injected IP with LPS (20 mg/kg). The general condition and survival of animals were monitored every 12 hours for 6 days. Animal survival rates were plotted using Kaplan-Meier method and significance was scored by Log-Rank test.

Histology

For histological analysis, 4 WT and 4 CPEB KO mice were sacrificed 24 hrs post-LPS injection. Liver and lung tissues were fixed overnight at 4°C in 10% buffered formaldehyde, paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E).

Measurements of cytokine levels

100 ml of blood were collected from the tail of each animal (4 animals per group) following PBS or LPS injection. Blood serum cytokine levels were determined with a cytokine ELISA array (RayBiotech) containing 96 different cytokines. The

membranes were probed according the manufacturer's protocol. The blots were quantified by scanning densitometry.

IL-6 secretion from primary macrophages or RAW 264.7 cell line was measured by mouse anti-IL-6 ELISA (BD Biosience). Equal numbers of macrophages were plated in triplicate (5×10^5 cells/well) and were treated with 100 mg/ml LPS (Sigma) for 0 to 4 h. The media samples were collected and used to assess IL-6 levels by ELISA according to the manufacturer's protocol

Antibody

Immunoblot analysis were performed with purified polyclonal antibodies against NFκB (p65) (Santa Cruz Biotechnology), IκBα and pIκBα (Cell Signaling), β-actin (Sigma).

Immunofluorescence

Macrophages were grown in 12- well tissue culture plates on glass coverslips and fixed with 4%formaldehyde, permeabilized with Triton X-100, blocked with normal goat serum and stained with NFκB antibody (p65).

Lentiviral production and infections

Viral stocks were prepared as described (Morgenstern and Land 1990; Kinsella and Nolan 1996), using the 293Tcells and the retroviral plasmid vectors containing CPEB-shRNA or scrambled-ShRNA in PLL.3 vector. The

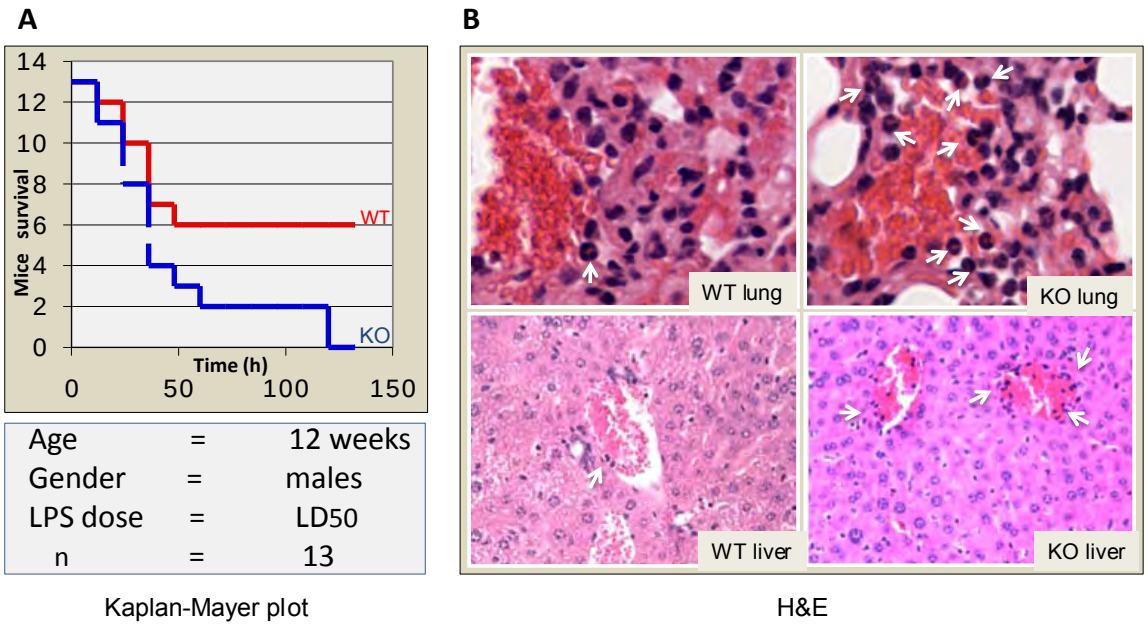
macrophages were infected for 5 days and the CPEB knockdown was confirmed by RT-PCR.

Metabolic labeling of cells

Cells were incubated in starvation medium (without methionine, 2 mM thymidine, no serum) for 15 min and then labeled (150 µCi/mL Redivue ^{35}S-methionine, Amersham Pharmacia) for 15 min. Cells were collected, washed 3 times in cold PBS and lysed in RIPA buffer. Immunoprecipitations were performed with anti-IκBα polyclonal antibody (Cell Signaling) overnight on ice. For some experiments, the LPS (1mg/ml) was added to the cells for 4 hours before starvation.

Cell cultures

RAW 264.7 cells were purchased from TACC and were cultured in high glucose DMEM containing 10% FBS, and antibiotic/animycotic solution (GIBCO, Grand Island, NY) at 37°C in 5% CO_2. To isolate peritoneal cells, mice were injected with thioglyconate (3%) for 4 days, after which time the animals were sacrificed and the peritoneal cavities were washed twice with 5ml of cold DMEM. The peritoneal cells were collected, centrifuged for 10 min at 1,500rpm at 4°C. The cells were resuspended and plated in culture dishes and allowed to adhere for 3 h. The cells were then washed twice with PBS to remove debris and contaminating cells and used for ELISA and immunofluorescence analysis.



Kaplan-Mayer plot

H&E

Figure 1

Figure 1. LPS-induced inflammatory response. A. WT and CPEB KO mice (males, 12 weeks of age), were injected IP with LPS LD50 and a Kaplan-Mayer survival curve was determined. The KO mice exhibited statistically significant decreased survival ($p=0.03$). B. Histological analysis (H&E staining) of liver and lung tissue obtained from control and LPS injected WT and KO mice. White arrows indicate increased neutrophil infiltration in KO animals following LPS injection.

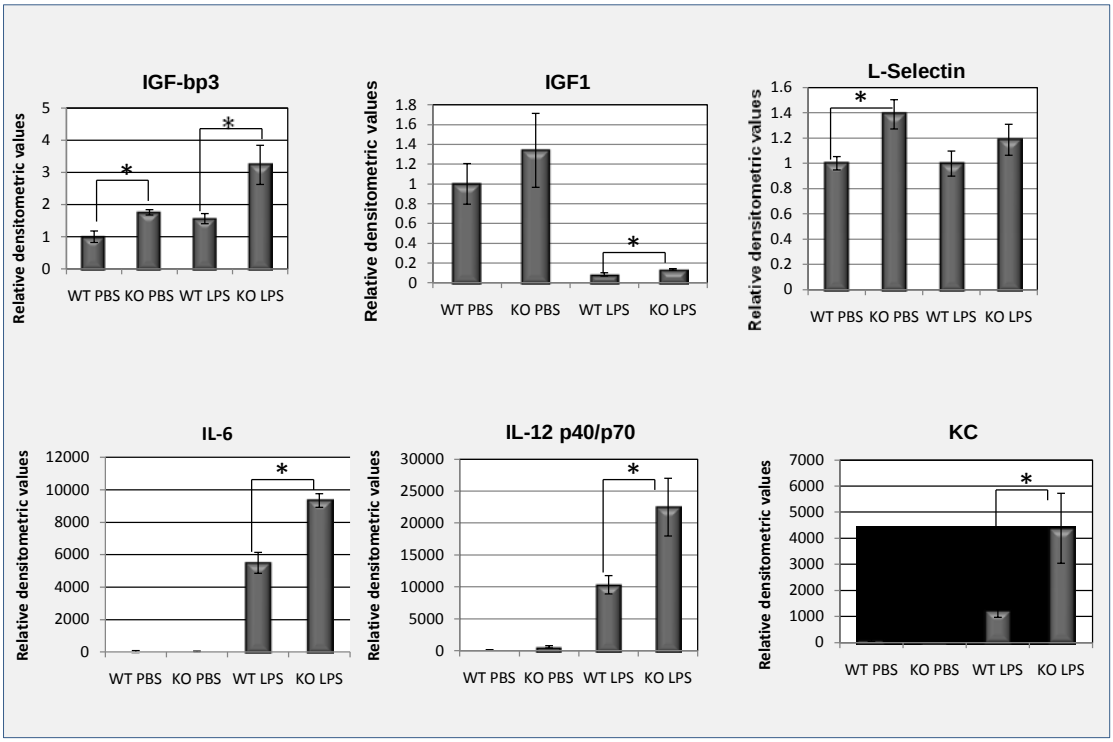

IGF-bp3
Relative densitometric values
5
4
3
2
1
0
*
*
WT PBS KO PBS WT LPS KO LPS
IGF1
Relative densitometric values
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
WT PBS KO PBS WT LPS KO LPS
L-Selectin
Relative densitometric values
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
WT PBS KO PBS WT LPS KO LPS
IL-6
Relative densitometric values
12000
10000
8000
6000
4000
2000
0
*
WT PBS KO PBS WT LPS KO LPS
IL-12 p40/p70
Relative densitometric values
30000
25000
20000
15000
10000
5000
0
*
WT PBS KO PBS WT LPS KO LPS
KC
Relative densitometric values
7000
6000
5000
4000
3000
2000
1000
0
*
WT PBS KO PBS WT LPS KO LPS

Figure 2

Figure 2. The blood serum cytokine levels in CPEB KO and WT mice. Blood serum was collected from 4 animals per group 2h of post-PBS or LPS injection and the levels of 96 secreted proteins were measured by ELISA. Six of the proteins (IGF-bp3, IGF1, L-selectin, IL-6, Il-12, and KC) were consistently different between the 4 groups. The histograms represent the relative densitometric values of the proteins that were significantly different among each group. (p-value < 0.05 marked with *; n=4)

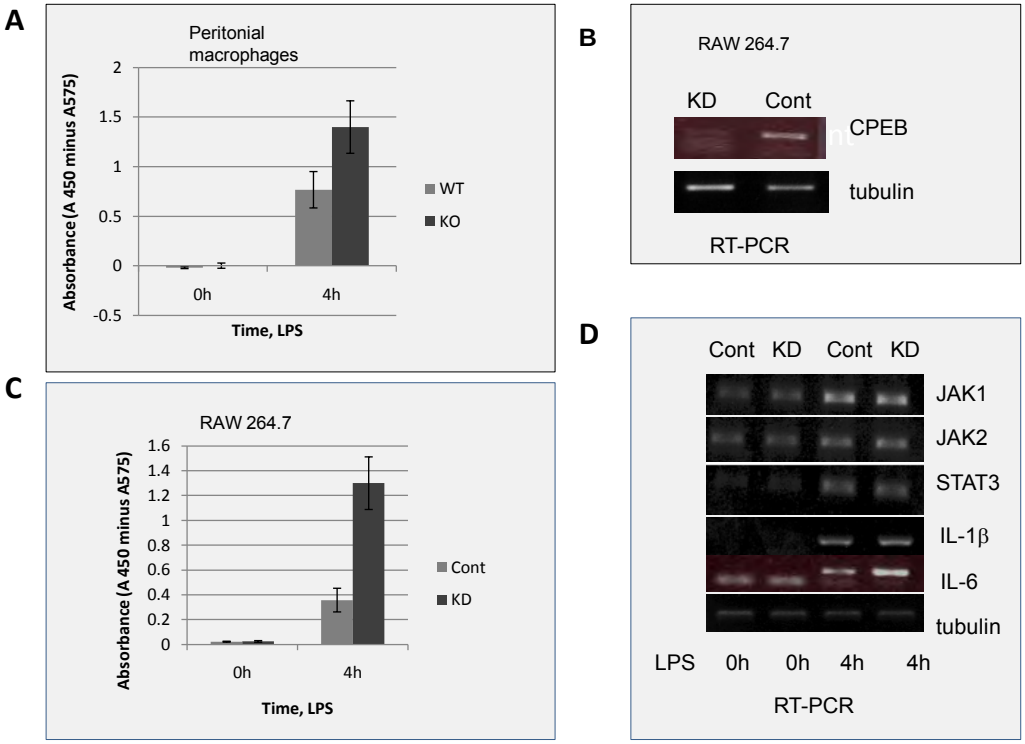

A
Peritonial macrophages
Absorbance (A 450 minus A575)
2
1.5
1
0.5
0
-0.5
0h
4h
Time, LPS
WT
KO
B
RAW 264.7
KD
Cont
CPEB
tubulin
RT-PCR
C
RAW 264.7
Absorbance (A 450 minus A575)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0h
4h
Time, LPS
Cont
KD
D
Cont KD Cont KD
JAK1
JAK2
STAT3
IL-1β
IL-6
tubulin
LPS 0h 0h 4h 4h
RT-PCR

Figure 3

Figure 3. Knockdown of CPEB in RAW 264.7 macrophage cell line causes increased IL-6 secretion. A. Peretonial macrophages from CPEB KO or WT mice were treated with LPS (100 mg/ml) for 0 or 4h, after which time the media were collected and processed for IL-6 ELISA. (n=3) B. RAW 264.7 macrophages were infected with lentiviruses expressing either a scrambled shRNA (control) or one targeting CPEB (KD). Five days post-infection, the cells were treated with LPS (100 mg/ml) for 0 or 4h, after which time the media were collected and processed for IL-6 ELISA. (n=3) C. RNA was extracted from the cells noted in panel B and used to examine the level of *cpeb* mRNA, by semi-quantitative RT-PCR. D. RNA was extracted from the cells noted in panel C and used to examine the level of *Il-6* mRNA, by semi-quantitative RT-PCR. The mRNA level of the other inflammatory proteins (JAK1, JAK2, STAT3, IL-1b) and tubulin were used as a control.

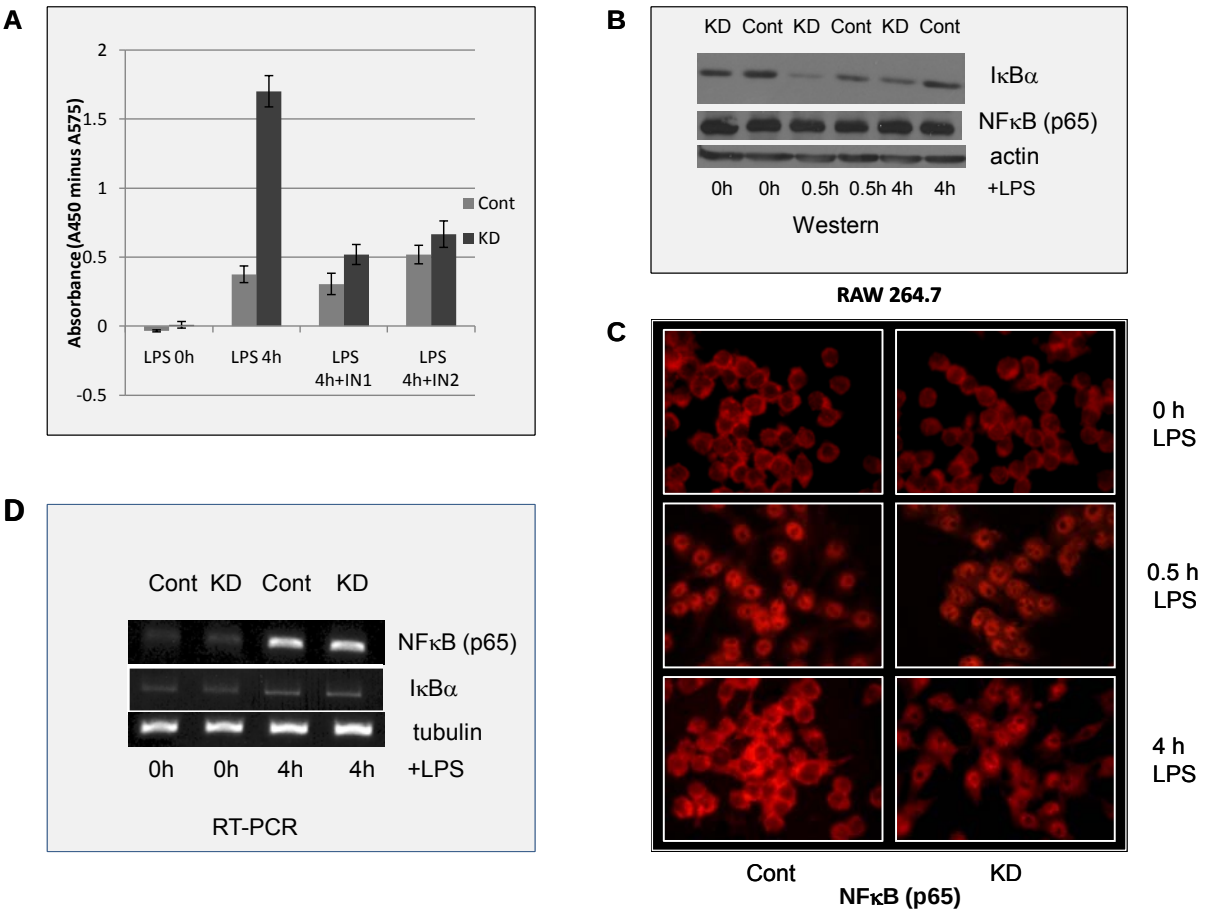

A
Absorbance (A450 minus A575)
2
1.5
1
0.5
0
-0.5
LPS 0h
LPS 4h
LPS 4h+IN1
LPS 4h+IN2
Cont
KD
B
KD Cont KD Cont KD Cont
IκBα
NFκB (p65)
actin
0h 0h 0.5h 0.5h 4h 4h +LPS
Western
RAW 264.7
C
0 h LPS
0.5 h LPS
4 h LPS
Cont
KD
NFκB (p65)
D
Cont KD Cont KD
NFκB (p65)
IκBα
tubulin
0h 0h 4h 4h +LPS
RT-PCR

Figure 4

Figure 4. Prolong nuclear retention of NFκB in CPEB-depleted macrophages treated with LPS. A. Macrophages infected with lentiviruses expressing scrambled (control) or CPEB targeting (KD) shRNAs were incubated with two different inhibitors of NFκB (JSH-23 and 5HPP-33) for 2 hours followed by addition of LPS for an additional 4 hours. The media were then collected and examined for IL-6 by ELISA. B. Control and CPEB-depleted macrophages were treated with LPS for 0, 0.5, or 4 hrs and then process for western blot analysis of NFκB (p65), IκBα and tubulin as a loading control. C. Cells treated as described above were fixed at the corresponding time points and processed for immuno-fluorescence for NFκB. In contrast to control cells, the CPEB-depleted cells treated with LPS for 4 hours maintained NFκB in the nucleus. D. RNA was extracted from cells treated as above and used for RT-PCR analysis of NFκB, IκBα, and tubulin. (n=3)

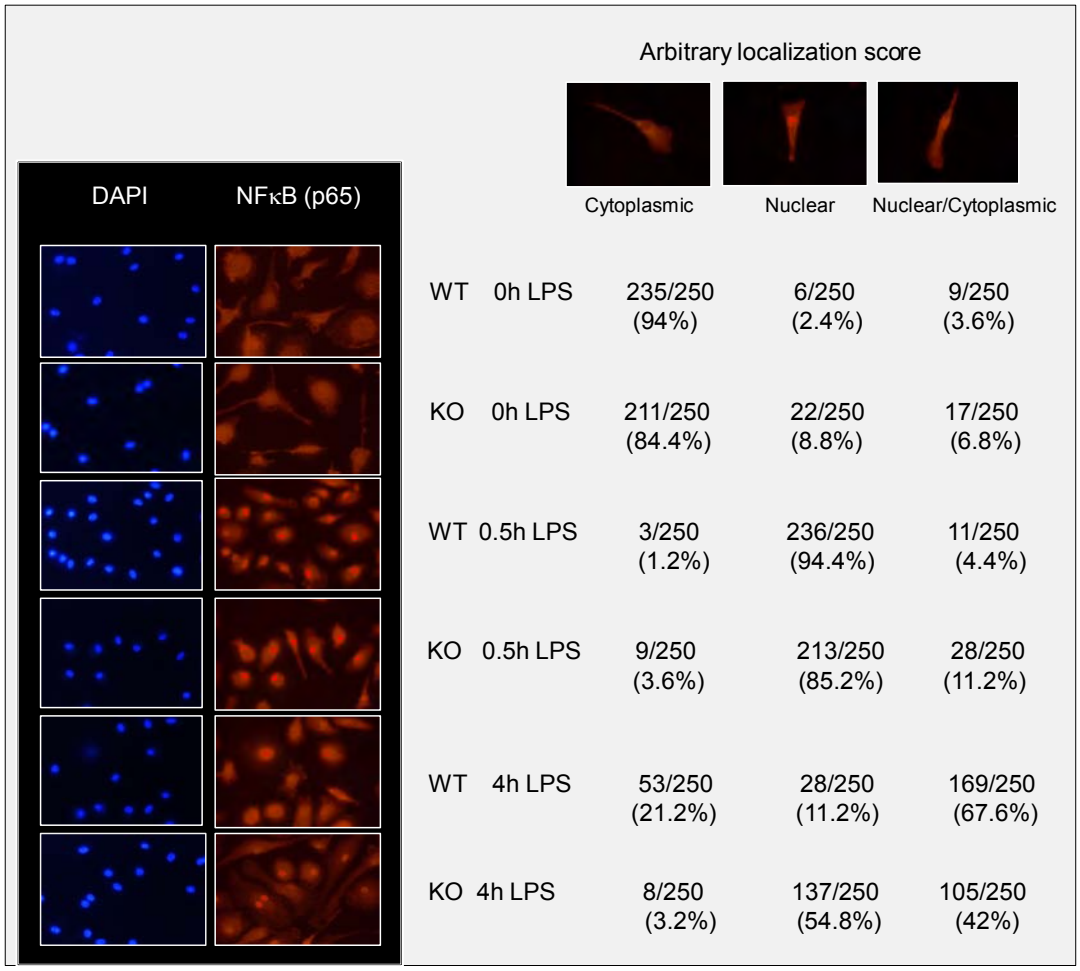

Arbitrary localization score
DAPI
NFκB (p65)
Cytoplasmic
Nuclear
Nuclear/Cytoplasmic
WT 0h LPS 235/250 (94%) 6/250 (2.4%) 9/250 (3.6%)
KO 0h LPS 211/250 (84.4%) 22/250 (8.8%) 17/250 (6.8%)
WT 0.5h LPS 3/250 (1.2%) 236/250 (94.4%) 11/250 (4.4%)
KO 0.5h LPS 9/250 (3.6%) 213/250 (85.2%) 28/250 (11.2%)
WT 4h LPS 53/250 (21.2%) 28/250 (11.2%) 169/250 (67.6%)
KO 4h LPS 8/250 (3.2%) 137/250 (54.8%) 105/250 (42%)

Figure 5

Figure 5. Nuclear retention of NFκB in peritoneal macrophages treated with LPS derived from CPEB KO and WT mice. Peritoneal macrophages were treated with LPS for the times indicated, then fixed and immunostained for NFκB (p65). 250 cells were randomly chosen and scored for nuclear versus cytoplasmic NFκB. The localization of NFκB was determined by comparing with cells showing mostly cytoplasmic, mostly nuclear, or even distributed between the two compartments.

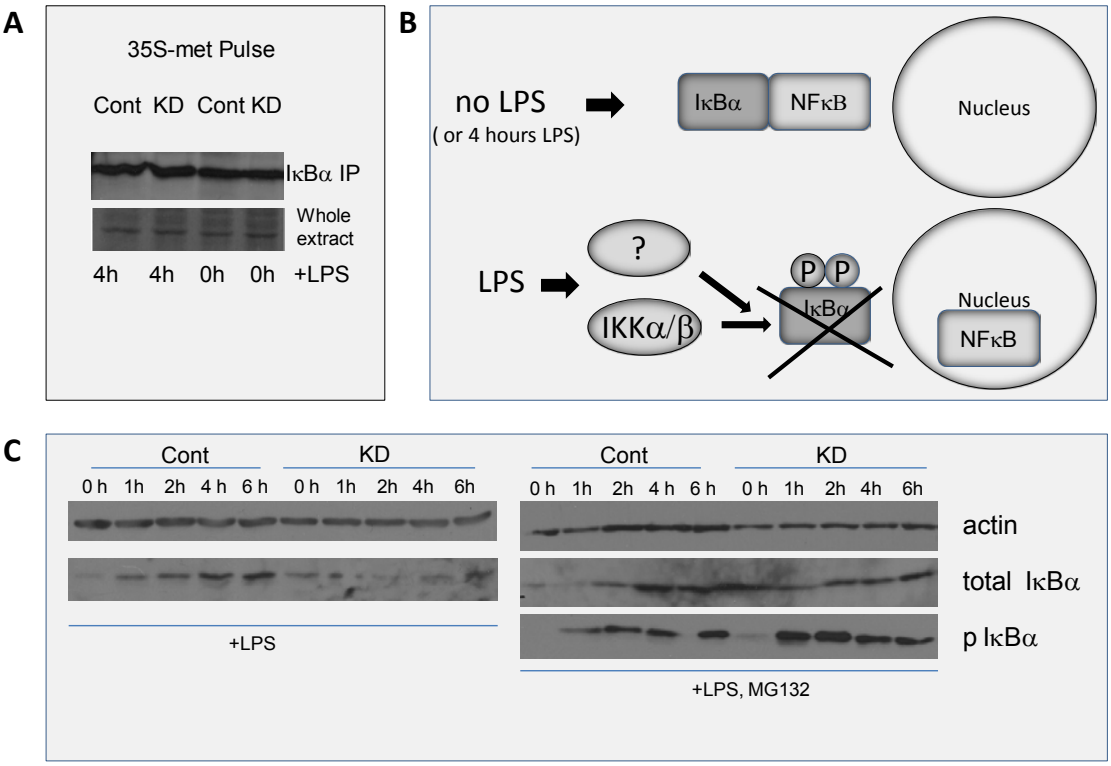

A
35S-met Pulse
Cont KD Cont KD
IκBα IP
Whole extract
4h 4h 0h 0h +LPS
B
no LPS
(or 4 hours LPS)
IκBα
NFκB
Nucleus
LPS
?
IKKα/β
P P
IκBα
Nucleus
NFκB
C
Cont
KD
0 h 1h 2h 4 h 6 h 0 h 1h 2h 4h 6h
+LPS
Cont
KD
0 h 1h 2h 4 h 6 h 0 h 1h 2h 4h 6h
+LPS, MG132
actin
total IκBα
p IκBα

Figure 6

Figure 6. IκBα is hyper-phosphorylated in CPEB-deficient cells following LPS treatment. A. Control and CPEB-depleted cells were treated with LPS for 0 or 4 hours followed by 15 min incubation with ^{35}S-methionine and subsequent immunorecipitation of IκBα. The panel shows that the rate of synthesis of IκBα is unaffected by CPEB depletion or LPS. Also show is labeled protein from the whole cell extract. B. A schematic view of the mechanism of NFκB nuclear translocation.Without LPS NFκB retained in the cytoplasm through the interaction with IκBα protein. Upon LPS stimulation IκBα is phosphorylated and subsequently degraded thus, releasing NFκB that translocates to the nucleus. C. Control or CPEB-deficient macrophages were treated LPS and in some cases, with the proteosomal inhibitor MG132 for 0-6 hours. Proteins extracts were then western blotted and probed for IκBα, pIκBα, or actin as a loading control. Note that IκBα is hyper-phosphorylated in CPEB knockdown cells treated with LPS and MG132.

Authors' contributions

Maria Ivshina conceived and coordinated the study, designed and performed the majority of the experiments and assays and drafted the manuscript.

Ilya Alexandrov participated in the statistical analysis and in the coordination of the study, mice breeding and handling, mice survival experiments and cytokine arrays.

Joel Richter participated in the design of the study, its coordination and helped draft the manuscript.

Tsioshi Udagawa participated in the design and cloning of CPEB-shRNA

I acknowledge DERC facility and Dr. David Garlick for the help with the histological analysis.

CHAPTER IV

Discussion and Conclusions

In the Chapter II of this dissertation I presented the data that CPEB deficient mouse embryonic fibroblasts have an abnormal life span (when cultured *in vitro*) and proliferate without entering the irreversible cell cycle arrest known as a senescence.

This study was initiated from a simple observation that in early development in frog oocytes CPEB regulates several cell cycle-related genes, including Cyclin B1 and Mos during meiotic and mitotic divisions. Moreover, depletion of CPEB results in cell cycle arrest and defects in the mitotic apparatus (Groisman et al., 2000). Next, we hypothesized that CPEB may play a role in the mammalian cell cycle. As a model for our study we decided to use the CPEB knockout mice, which provides a convenient source of CPEB KO cells. With the many examples in the literature describing viable knockouts of different cell cycle related genes, the fact that CPEB KO mice does not display any obvious abnormality (except a defect in fertility) and has a normal lifespan was not that surprising. First, we decided to compare the cell cycle progression of the embryonic fibroblasts obtained from CPEB KO and WT mice. To our

disappointment, no significant difference in the initial cell divisions between both genotypes was found. Surprisingly, after several passages in cell culture we observed a reduction of WT cell number and unusual flat cell morphology. At the same time we could still culture the CPEB KO cells for many more passages without noticing a reduction in proliferation. In fact, what we observed in case of WT cells was a well described and studied phenomena named senescence (Sharpless et al., 2001). In our case, CPEB KO cells bypassed senescence and displayed unusual round cell phenotype at later passages. To confirm that this effect is due to CPEB deficiency, we attempted to rescue senescence in CPEB KO cells by CPEB overexpression. In fact, reintroduction of CPEB could restore the senescence phenotype (reduced proliferation, flat morphology and expression of b-Gal) after a few rounds of proliferation, showing that bypass of senescence is CPEB-specific. Senescence is considered to be a protective mechanism to prevent uncontrolled cell division and tumorogenesis. Thus, the next question was if CPEB KO cells display any characteristics of cell transformation.

In addition, we observed that CPEB KO MEFs are resistant to the premature RAS-induced senescence, the effect which is common among the cells with altered tumor suppressor pathways. Notably, CPEB KO MEFs infected with the Ras oncogene at later passages can form some colonies in soft agar, suggesting a possible change toward tranformation. However, injection of these cells to the athymic mice leads to the formation of tumors indistinguishable from the

matching WT. We were unable to find any significant changes in the level of many cell-cycle and senescence related proteins. However, in CPEB KO cells we observed an increased amount of c-Myc, an oncogene which possesses a few potential CPE-elements in its 3'UTR. In fact, we found that CPEB can regulate level of c-Myc via repression of its mRNA. We also found increased levels of the RB-related p107 and p130 proteins in CPEB KO cells, however additional experiments need to be performed to clarify the significance of these changes.

Later studies of CPEB function during the human cell senescence (Burns et al., 2006) confirmed our initial observations. First, despite the difference in size of the telomeres and a life span between mouse and human cells, knockdown of CPEB in primary human cells also resulted in bypass of senescence. Second, this phenotype can be rescued by CPEB overexpression. Third, human cells were also resistant to Ras-induced premature senescence. However, levels of c-Myc in the human cells were unchanged and CPEB was found rather to regulate p53 polyadenylation.

Collectively this data confirm the role of CPEB in both mouse and human senescence and extends our knowledge of CPEB function in mammalian cells. Considering the amount of protein containing the putative CPE-elements it is likely that CPEB involved in regulation of other senescence related proteins.

In Chapter III of this dissertation, I investigated the role of CPEB in LPS-induced inflammation. As suggested by multiple studies, all stages of the inflammatory response including onset, progression and resolution, greatly rely on translational control and mRNA stability. Notably, regulated mRNA translation provides a rapid and, in most cases, reversible way to regulate protein levels that results in acute activation and fast termination of inflammation. Thus, this project began with the hypothesis that CPEB, an RNA-binding protein that regulates translation, could be involved in the control of protein synthesis during the inflammatory response.

Interestingly, many potential activators of CPEB were shown to have a crucial role in immune response. For example, progesterone, the hormone that triggers CPEB-dependent polyadenylation in *Xenopus* oocytes, has a role in the alleviation of inflammation. It was shown that pretreatment of macrophages with progesterone inhibits LPS-induced expression of inflammatory proteins such as IL-6, TLR4, and iNOS and induces the expression of proteins involved in the resolution of inflammation such as SOCS1. In addition, GSK-3 activation (a kinase that inhibits Aurora A and leads to the formation of inhibitory CPEB complex) was shown to promote the IL-6 production in macrophages and in the central nervous system where it regulates microglia migration and inflammation (Yuskaitis and Jope, 2009). Conversely, inhibiting GSK-3 reduces the inflammatory response and has an important clinical application in the treatment

of septic shock, chronic inflammatory and neurodegenerative diseases (Ko et al., 2010).

In contrast to CPEB, the role of other 3'UTR binding proteins such as AUF1, TTP, TIA-1, and HuR in inflammation is well described in the literature. All of these proteins bind AU-rich sequences, which in part resembles the CPE element (UUUUUAU) and elicit post-transcriptional regulation via the control of mRNA stability. Most of ARE-binding protein deficient mice show altered cytokine stability (TNFα is the most affected) and have changes in the inflammatory response (Anderson, 2010). Consequently, we proposed that CPEB might have a similar function in controlling cytokine production. We also considered the possibility of CPEB either working in synergy or competing with ARE-binding proteins. Finally, because the 3'UTRs of some ARE-binding proteins (for example, AUF1 and HuR) have a multiple CPE-elements we did not exclude the possibility of CPEB-dependent control of their expression.

In our study, CPEB KO mice were found to be less tolerant to IP injections of LPS and have an increased influx of neutrophils in the lung and liver indicating the presence of a hyperactivated inflammatory response. Currently, there are no obvious indications of increased inflammation in CPEB KO mice when raised under normal conditions. The CPEB KO mice have a lifespan comparable to WT, a total blood count in a normal range, and a similar of immune cell progenitors compared to WT. In contrast, LPS, which is a highly potent stimulator of

inflammation, elicits the dramatic changes in CPEB KO mice compared to WT. This suggest that perhaps an additional strong signal, similar to the one that activates CPEB by progesterone in *Xenopus* oocytes is required to activate the CPEB and thus CPEB-dependent control of protein expression. For example, the activation of the NMDA receptor is required to activate CPEB-dependent translation in neurons.

Because LPS elicits the inflammatory response via an increase of multiple proinflammatory cytokine production, we compared the cytokine level in CPEB KO and WT mice. By performing the ELISA test for 96 inflammatory cytokines, we found an LPS-stimulated increase in blood serum level of IL-6, IL-8, IL-12 in of CPEB KO mice. The elevated levels of these cytokines were previously described as a “signature” of endotoxic shock and were shown to correlate with the increased mortality. Out of 96 cytokines, we could only detect significant changes in 6 of them, perhaps due to the limited expression of cytokines at the time point we used.

We also observed that LPS promoted induction of IL-6 the CPEB-deficient RAW 267.4 macrophages, a cell line with a fully functioning LPS signaling pathway. In addition, we detected an increased amount of IL-6 mRNA in CPEB-deficient cells, suggesting the increase an IL-6 transcription or mRNA stability.

LPS stimulated signaling pathways trigger the activation of the NFκB transcription factor that has a central role in inflammation. To investigate whether

the increased production of IL-6 was NFκB-dependent, we tested whether the NFκB inhibitors can reduce the amount of secreted IL-6. In fact, we observed a great reduction of IL-6 by the NFκB inhibitors. We proposed that the increased in cytokine production is triggered via activation of the common inflammatory transcriptional factor NFκB. NFκB activity is tightly controlled by its nuclear translocation and is inhibited via binding to IκBα in the cytoplasm. We confirmed the NFκB localization and observed its prolonged nuclear retention in CPEB-deficient macrophages, which correlated with a reduced level of IκBα in CPEB KO macrophages.

After the ruling out the possibility of CPEB-controlled translation of IκBα (the rate of biosynthesis of IκBα was unaffected) we conclude that it is likely that CPEB regulates proteasomal degradation of IκBα. Moreover, the level of IκBα is restored by incubating cells with the proteasomal inhibitor MG132, and we also observed a higher level of IκBα phosphorylation at Ser32/36, which was shown to be directly linked to the stability of the protein.

Collectively, we show that deletion of CPEB can lead to hypersensitivity to the LPS, increased cytokine production, and prolonged nuclear retention of NFκB due to CPEB-dependent control of IκBα protein stability. In this study we focused on the activation of a single transcription factor, NFκB, and its regulators, however we do not exclude the possibility that CPEB may affect other transcription factors known to be involved in cytokine production. For example,

transcription factors such as c-Jun and STAT3 have been shown to have synergistic effects with NFκB.

Also the exact mechanism of CPEB-dependent IκBα destabilization is still unknown. One possibility includes CPEB-dependent regulation of proteases known to control IκBα degradation such as Skp1-Culin Roc1/RBx1/Hrt-1-F-box. Another possibility includes enhanced posttranslational modifications of both IκBα and NFκB (such as acetylation and phosphorylation) by activated upstream signaling molecules, which in turn lead to higher NFκB activity and IκBα degradation. The enhanced phosphorylation of IκBα at S32 and S36 residues suggests that, perhaps, IKK kinase complex could be the one of the upstream signaling molecules.

Improperly terminated cytokine signaling signals back to NFκB could provide a constant input for its activation. Thus, it remains possible that CPEB-dependent control of cytokine mRNA stability feeds back to NFκB. In addition, the lack of terminating signals such as SOCS 1 and SOCS3, histone deacetylating enzymes (HDACs), phosphatases (for example PTPs) or deubiquitinases (such as A20 and Cezanne), were shown to lead to the increased NFκB activation.

It was recently shown that in human monocytes, reduction in cytokines correlates with miRNA inhibition and increased P-bodies formation (after 8 hours). Because CEPB is also a component of p-bodies the question of whether

CPEB represses cytokine mRNA expression in these cytoplasmic structures and its relationship to miRNAs is still open. The abundance of CPE-elements in the 3'UTRs of many mRNAs suggests the complexity of the CPEB signaling network. Thus it will be interesting to analyze the CPE content in the early response group of inflammatory mRNAs in comparison to the inflammatory genes in the late response group. Knowing when and how CPEB is activated in response to the LPS can provide us with a greater understanding of CPEB-dependent signaling.

Finally, the inflammatory pathways overlap with the diabetes and cancer pathways, leading to the question of whether CPEB functions under these inflammatory conditions. There are some literature reports describing a variety of cancers with a significant change in the level of the CPEB interacting protein, Symplekin, as well as upstream signaling components (progesterone and estrogen receptors, Akt1, GSK-3, Aurora A, parafibromin).

Transformation of CPEB deficient mouse embryonic fibroblast with the RAS oncogene can promote the growth large colonies (foci) in soft agar assays, indicating that in this system CPEB may function as a tumor-suppressor. However, injection of such fibroblasts into the athymic mice yielded tumors indistinguishable from the control cells, showing that this function is not sufficient to increase the rate of cancer growth in animals. In contrast, CPEB KO mice are more susceptible to formation of papillomas when the chemical mutagens DMBA-TPA are applied, suggesting some tissue specificity in cancer

progression. In addition, a few reports show that some ovarian cancers have a significant change in CPEB mRNA levels, however it is not clear if this difference is a cause or consequence of cell transformation.

At this time, it is difficult to predict the function of CPEB in cancer development due to its potential involvement in a bidirectional control of CPE-containing mRNAs (including oncogenes and tumor suppressors as well as their regulators). For example, simultaneous regulation by CPEB of cell cycle proteins (cyclin B1), oncogenes (c-Myc) and tumor suppressors (p53) can create a fragile balance between normal cell division and cancer progression.

The normal life span of CPEB KO mice suggests that it is likely that additional external DNA-damaging stimuli, leading to tissue specific signaling events and changes in multiple genes, will be required for shifting this balance toward CPEB-driven tumorigenesis. Elucidating the timing of translational control for all specific CPEB-targets can also help us to understand the complexity of CPEB-dependent cancer progression.

APPENDIX I.

Centrosomal localization of CPEB4

CPEB4 is an RNA-binding protein that belongs to the CPEB family of proteins; it shares strong sequence similarity to CPEB and to the other family members, CPEB2 and CPEB3 (Theis et al., 2003). Similar to CPEB (described in early in this dissertation), CPEB4 possesses 2 RRMs and 2 Zn-finger domains that are critical for its mRNA binding. However, CPEB4 binds to the CPE with low affinity and, instead, has a high specificity to a distinct mRNA sequence with a secondary structure resembling a poly(U) loop (Huang et al., 2006). Unlike CPEB1, CPEB4 does not have an Aurora A phosphorylation sites, but has recognition motifs for some other kinases such as CamKII, PKA and p70S6. This suggests that despite its similarity to CPEB1, CPEB4 has a different mechanism of regulation and, perhaps, binds to a different subset of mRNA targets.

Among all CPEB proteins, CPEB4 was shown to be the most abundant in hippocampal area of the brain (as detected by mRNA in situ hybridization and protein immunostaining) and Purkinje cells of the cerebellum. In hippocampal cultured neurons, CPEB4 was associated with mRNA in the form of RNP dendritic granules located in the post synaptic density. The mRNA encoding CPEB4 in the brain was rapidly induced by kainite, a very potent inducer of seizures. CPEB4 is also expressed in immune cells such as macrophages (Raw 267.4 cell line) (data not shown). In other types of immune cells, e.g.,

thymocytes, CPEB4 was shown to be a transcription target of ROR gamma, a nuclear receptor involved in different physiological aspects including the regulation of thymocytes differentiation and circadian rhythms (Xi et al., 2006).

As suggested from recent work in *Xenopus* oocytes CPEB4, similar to CPEB1, is involved in translational control via polyadenylation (Novoa et al., 2010; Igea et al., 2010). In fact both proteins are differentially regulated in early development (as shown by a correlation between increased level of CPEB4 and a decreased level of CPEB1) with CPEB1 being a prevalent translational regulator during early oocyte development and CPEB4 acting as a translational regulator later in development (i.e., meiotic arrest) (Igea et al., 2010). In *Xenopus* oocytes CPEB1, which is important for cell cycle progression via regulation of cell cycle related proteins such as Mos and Cyclin B1, was shown to mediate the localization of these mRNAs to the centrosomes and spindles (Groisman et al.,2000; Eliscovich et al., 2008). Moreover the depletion of CPEB1 leads to the multiple centrosomal and spindle abnormalities and results in a cell cycle arrest (Groisman et al., 2000). In addition, exogenous CPEB1 can be co-localized with centrosomes in Hela cells (data not shown). Here, we describe centrosomal localization of CPEB4 in mammalian cells and begin to characterize the significance of this observation.

This work began from the simple observation that in mammalian cells stained with affinity purified CPEB4 antibody, in addition to being dispersed in the

cytoplasm, CPEB4 displays a very distinct dot-like cytoplasmic staining pattern, resembling the centrosome. In fact, when co-stained with the centrosomal marker gamma-tubulin, CPEB4 was shown to be highly co-localized with the centrosomes in many different cell types including Hela and RPE cells (Fig.1), and HFS or MCF7 cells (data not shown). By using cell lines stably expressing GFP-centrin B, a centrosomal marker that unequally localizes to the mother centriole, we noticed that the majority CPEB4 co-localizes in an opposite manner to CentrinB, i.e, showing specificity to the daughter centriole in a cell cycle stage independent manner (Fig.2). Because the centrosome is the major microtubule organizing center, staining of some proteins in the centrosome can be a result of aggregation of proteins attached to the microtubules. However, CPEB4 localization to centrosomes was not dependent on microtubules since their disruption by nocadozole did not affect CPEB4 localization (Fig 3, upper panel), except a modest increase in the number of “dots” observed in some cells. However this result does not exclude the possibility that CPEB4 was delivered to the centrosome via microtubules. To confirm the specificity of CPEB4 centrosomal localization, we assessed CPEB4 distribution in Hela cells transfected with CPEB4-shRNA. As shown in Fig.4, we observed CPEB4 co-staining with the centrosomal marker gamma tubulin only in nontransfected cells. In contrast, centrosomal staining of CPEB4 was barely detectable in the cells transfected with CPEB4-shRNA. To further confirm the specificity of CPEB4 localization to the centrosome, we biochemically purified centrosomes from Hela

cells by using discontinuous sucrose gradients after preincubation with 1 µg/ml cytochalasin D and 0.2 µM nocodazole for 1 h according to the method of Moudjou and Bornens (1998) (Wigley et al.,1999).

As shown in Fig 3A. CPEB4 was enriched in the both cytoplasmic (confirmed with alpha tubulin antibody) and centrosomal (confirmed by gamma-tubuling staining and HSP proteins 70 and 27) fractions of the gradient.

Next, we investigated whether CPEB4 depletion has an impact on cell cycle progression or the centrosome replication cycle. Compared to cells infected with a lentivirus expressed a scrambled shRNA, RPE cells containing a virus expressing shRNA for CPEB4 (Fig 5B), we observed a mild arrest in a S-phase of the cell cycle, suggesting a possible role of CPEB4 at this stage. However we did not observed any cell cycle abnormality when a similar experiment was performed in Hela cells (data not shown). In addition we did not find any centrosomal cycle defects as both cell lines, irrespective of CPEB4 depletion, showed equal centrosomal amplification induced by hydroxyurea (data not shown).

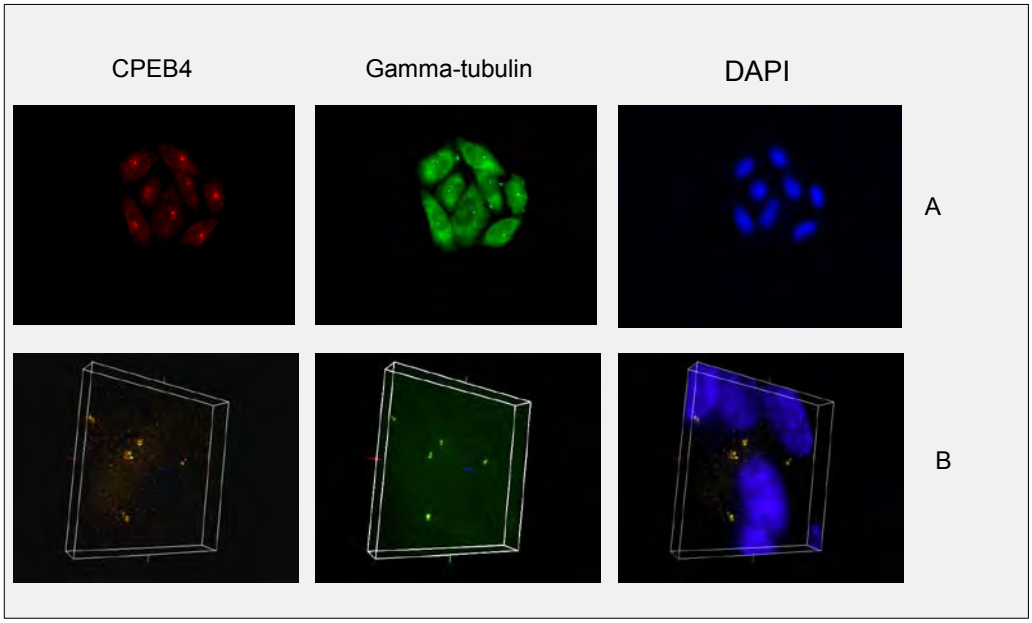


Figure 1

Figure1. CPEB4 on centrosomes. A. Asynchronously dividing Hela cells were methanol-fixed and immunostained with affinity purified antibodies to CPEB4 (red), the centrosomal marker gamma tubulin (green), and DAPI (blue). Localization of the CPEB4 and gamma tubulin were detected by indirect immunofluorescence. B. RPE cells were immunostained as in panel A and visualized by spinning disk confocal microscopy followed by 3D image reconstruction.

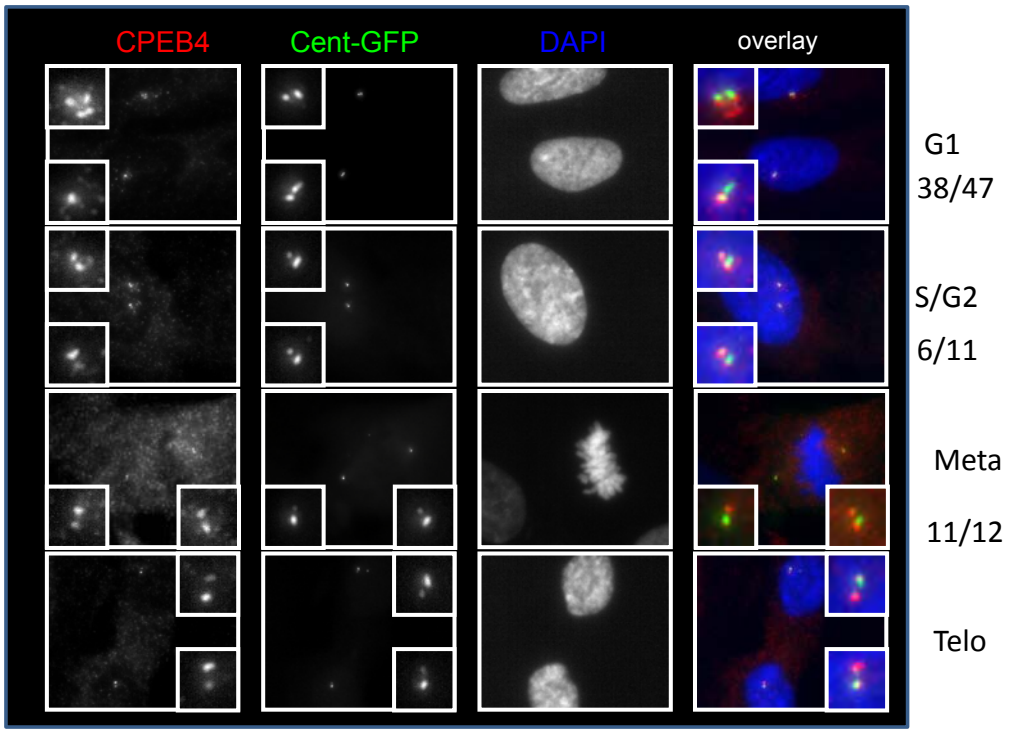


Figure 3

Figure 2. CPEB4 preferentially localizes to the daughter centriole throughout the cell cycle. Asynchronously dividing Hela cells were methanol-fixed and immunostained with affinity purified CPEB4 antibody and co-localized with stably transformed GFP-centrin, a centrosomal marker. Images were collected by confocal microscopy at different stages of the cell cycle. The strongly fluorescent dots of GFP-centrin are indicative of mother centrioles. The numbers indicates the cells counted.

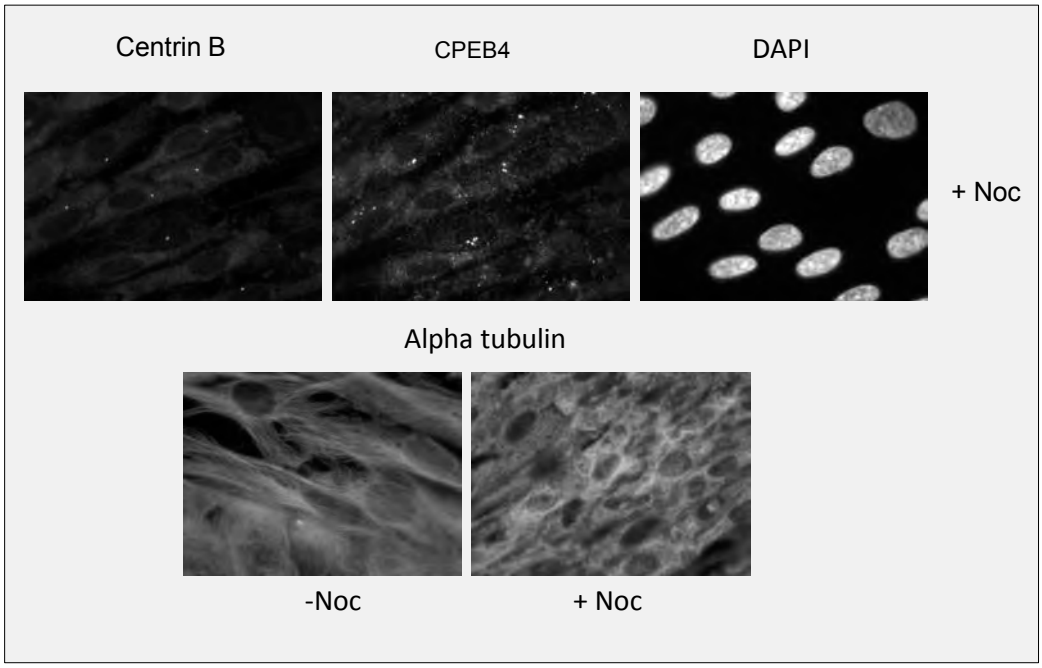


Figure 3

Figure 3. CPEB4 localizes to the centrosome in nocadazole treated Hela cells. Hela cells treated with 5μg/ml nocadazole for 3 hours were methanol-fixed and immunostained with affinity purified antibodies to CPEB4 and gamma tubulin; there were also stained with DAPI (upper panel). To confirm microtubule de-polymerization by nocadozole, the cells were stained with antibody against alpha tubulin (Lower panel). The images were obtained by indirect fluorescence.

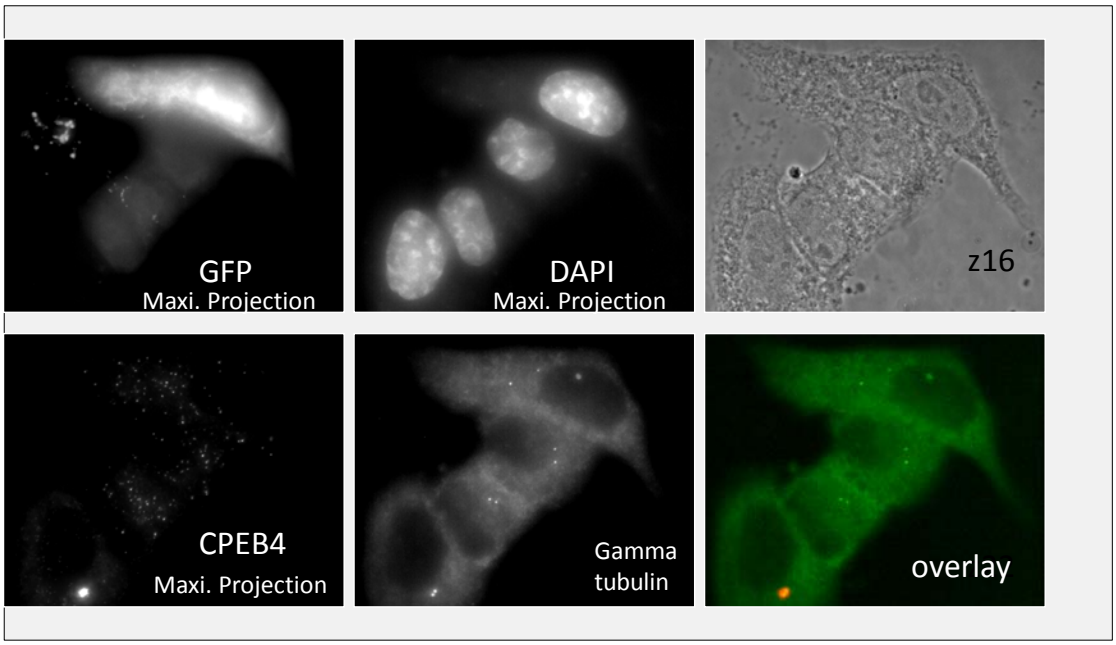

GFP
Maxi. Projection
DAPI
Maxi. Projection
z16
CPEB4
Maxi. Projection
Gamma
tubulin
overlay

Figure 4

Figure 4. shRNA mediated depletion of CPEB4 eliminates its detection on centrosomes. Hela cells transfected with lentivirus expressing GFP and shRNA were cultured for CPEB4 for 48h, fixed by methanol and stained for CPEB4 (red) and gamma tubulin (green). The images were obtained by using confocal z-section analysis. Note that CPEB4 only localizes to the centrosome in cells lacking GFP (and hence the shRNA for CPEB4). In cells expressing GFP (and hence the shRNA for CPEB4), there is no CPEB4 detection on centrosomes.

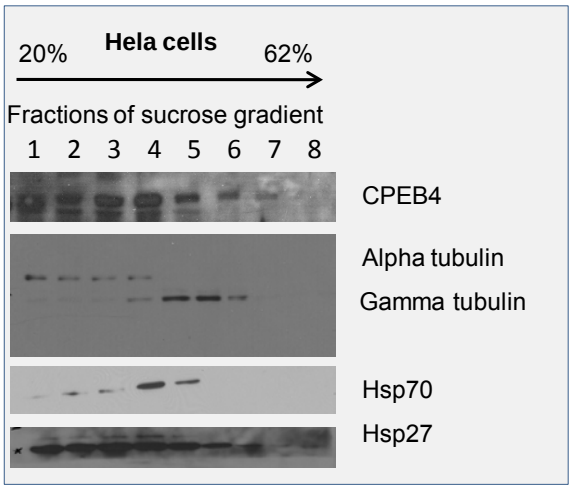
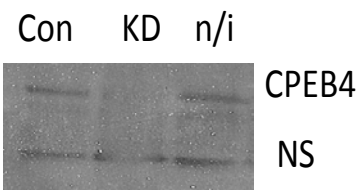

20%
Hela cells
62%
Fractions of sucrose gradient
1 2 3 4 5 6 7 8
CPEB4
Alpha tubulin
Gamma tubulin
Hsp70
Hsp27

Figure 5

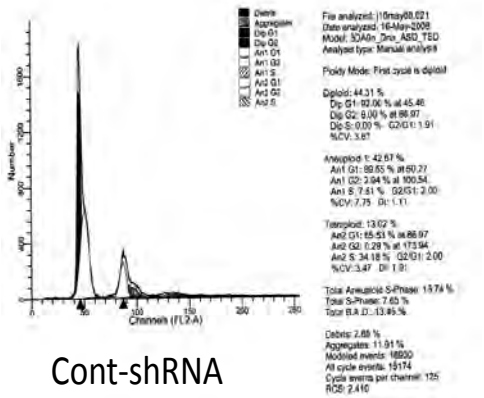
Figure 5. CPEB4 protein co-sediments with the centrosomes. Centrosomes were prepared by 20-62% discontinuous sucrose gradient centrifugation as described in the text. Equal amounts of fractions 1-8 were collected from the top of the gradient and subjected to western blot analysis. The purity of the centrosomal preparation was confirmed by alpha-tubulin exclusion from the centrosomal fractions. Note that CPEB4 and heat shock proteins Hsp70 and Hsp27 (as a controls) co-sediment with the centrosomal protein gamma tubulin (fractions 5-7).

A

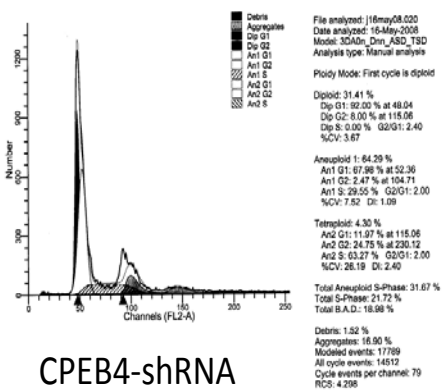


Western

B



Cont-shRNA



CPEB4-shRNA

Figure 6

Figure 6. Cell cycle analysis of CPEB4 deficient RPE cells. A. RPE cells were infected with lentivirus expressing shRNA for CPEB4 or scrambled shRNA as a control. The cells were exponentially grown for 5 days after infection; CPEB4 knockdown was confirmed by western blot with CPEB4 antibody. NS denotes a nonspecific band, KD refers to shRNA for CPEB4, “cont” refers to a scrambled shRNA, “n.i” refers to control cells not infected with a virus**.** B. Asynchronously dividing RPE cells infected with virus expressing shRNA for CPEB4 or scrambled shRNA as a control were stained with propidium iodide and analyzed by flow cytometry. Note the slight block of the cell cycle in S-phase in CPEB4 deficient cells.

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