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POST-TRANSCRIPTIONAL CONTROL OF HUMAN CELLULAR SENESENCE

A Dissertation Presented
By

David M. Burns

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY
July 15th, 2010

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POST-TRANSCRIPTIONAL CONTROL OF HUMAN CELLULAR SENESCENCE

A Dissertation Presented By

David M. Burns

Approved as to style and content by:

Timothy F. Kowalik, Ph.D., Chair of Committee

Roger J. Davis, Ph.D., Member of Committee

Victor R. Ambros, Ph.D., Member of Committee

Joan A. Steitz, Ph.D., Member of Committee

Joel D. Richter, Ph.D., Dissertation Mentor

Anthony Carruthers, Ph.D.
Dean of the Graduate School of Biomedical Sciences

July 15th, 2010

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ABSTRACT

The central dogma of biology asserts that DNA is transcribed into RNA and RNA is translated into protein. However, this overtly simplistic assertion fails to portray the highly orchestrated and regulated mechanisms of transcription and translation. During the process of transcription, RNA provides the template for translation and protein synthesis as well as the structural and sequence specificity of many RNA and protein-based machines. While only 1-5% of the genome will escape the nucleus to be translated as mRNAs, complex, parallel, highly-conserved mechanisms have evolved to regulate specific mRNAs. Trans-acting factors bind cis-elements in both the 5' and 3' untranslated regions of mRNA to regulate their stability, localization, and translation. While a few salient examples have been elucidated over the last few decades, mRNA translation can be reversibly regulated by the shortening and lengthening of the 3' polyadenylate tail of mRNA. CPEB, an important factor that nucleates a complex of proteins to regulate the polyadenylate tail of mRNA, exemplifies a major paradigm of translational control during oocyte maturation and early development. CPEB function is also conserved in neurons and somatic foreskin fibroblasts where it plays an important role in protein synthesis dependent synaptic plasticity and senescence respectively. Focusing on the function of CPEB and its role in mRNA polyadenylation during human cellular senescence, the following dissertation documents the important finding that CPEB is required for the normal polyadenylation of p53 mRNA necessary for its normal translation and onset of senescence. Cells that lack CPEB have abnormal levels of mitochondria and ROS production, which are demonstrated to arise from the direct result of hypomorphic p53 levels. Finally, in an attempt to

recapitulate the model of CPEB complex polyadenylation in human somatic cells, I unexpectedly find that Gld-2, a poly(A) polymerase required for CPEB-mediated polyadenylation in *Xenopus laevis* oocytes, is not required for p53 polyadenylation, but instead regulates the stability of a microRNA that in turn regulates CPEB mRNA translation. Furthermore, I demonstrate that CPEB requires Gld-4 for the normal polyadenylation and translation of p53 mRNA.

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LIST OF ABBREVIATIONS

APA	Alternative polyadenylation site selection
CAMKII	Calmodulin Kinase II
cAMP	Cyclic Adenosine Monophosphate
Cdk	Cyclin dependent kinase
CPE	Cytoplasmic Polyadenylation Element
CPEB	Cytoplasmic Element Binding Protein
CPSF	Cleavage and Polyadenylation Specificity Factor
CSF	Cytostatic Factor
DNA	Deoxyribonucleic acid
eIF	Eukaryotic initiation factor
ELAVL1	Embryonic Lethal Abnormal Vision-Like
ePAB	Embryonic poly(A) binding protein
ESTs	Expressed sequence tags
FISH	Fluorescence in situ hybridization
G2	Growth-phase 2
GLD	Germline Deficient
GPCR	G-coupled Protein receptor
hnRNP	Heterogeneous nuclear ribonucleic proteins
HuR	Hu Antigen R
iPS	Induced pluripotent stem cells
Kd	Knockdown
KH	K Homology
KO	Knockout

LTP	Long-term potentiation
MEFs	Murine embryonic fibroblasts
MBT	Mid-blastula transition
mRNA	Messenger RNA
mRNP	mRNA-protein complex
NGDN	Neuroguidin
PABP	Poly(A) binding proteins
PBE	Pumilio Binding Element
Poly(A)	Polyadenosine
PRE	Pumilio Response Element
Rb	Retinoblastoma
RINGO	Rapid Inducer of G2-M transitions in oocytes
RIS	Reprogramming Induced Senescence
RNP	Ribonucleic Protein
ROS	Reactive Oxygen Species
RRM	RNA recognition motif
SELEX	systematic evolution of ligands by exponential enrichment
snRNP	Small nuclear ribonucleic protein machines
3'-UTR	3'-untranslated region

CHAPTER I

INTRODUCTION

The lifecycle of a messenger RNA (mRNA) begins in the nucleus of all eukaryotic cells where newly synthesized RNAs are rapidly transcribed from a deoxyribonucleic acid (DNA) template. The nascent RNA will co-transcriptionally receive a methylated-guanosine cap to protect the 5' end and will shed intronic sequences during the process of RNA splicing, culminating with a 3' cleavage and addition of a polyadenylate (poly(A)) tail (Danckwardt et al. 2008). Even before the process of transcription has terminated, the nascent RNA is refashioned by precipitant small nuclear ribonucleic protein machines (snRNPs), heterogeneous nuclear ribonucleic proteins (hnRNPs) and serine/arginine (SR) proteins (Dreyfuss et al. 2002). This highly orchestrated birthing of the mRNA is just the beginning as proteins continue to be deposited along its entire ribonucleic sequence, some of which will stay stably bound for the remainder of the journey, while other proteinacious factors will come and go (Dreyfuss et al. 2002; Moore 2005). Remarkably, only 10% of all transcribed RNA will leave the nucleus and only as little as 1-5% will leave as mRNA (Mattick and Makunin 2005). This transcriptional intermediate has transformed from a simple stretch of ribonucleic acids into a highly modified and now seemingly mature amalgamation of both proteins and mature messenger RNA known as the mRNA-protein complex (mRNP). Importantly, the RNA-binding proteins associated with the mRNP will dictate the fate of the mRNA.

The mRNP is now ready for export from the nucleus to the cytoplasm in procession 5' to 3', where it will be translated into protein by the ribosome, a complex >3 mega-dalton machine of roughly 60% RNA and 40% protein (Kohler and Hurt 2007). mRNA translation, a vastly complex process involving many factors whereby the mRNA is decoded by the ribosome from trinucleotide sequences called codons can simply be divided into three steps; initiation, elongation and termination (Sonenberg and Hinnebusch 2009; Jackson et al. 2010). Translation initiation constitutes the assembly of the elongation-competent 80S ribosome on the mRNA, where the initiator methionine anti-codon is base-paired with the start AUG codon (Kozak 1983). Next, the elongation phase is a cycle of aminoacyl-tRNA delivery and peptide bond formation until the process terminates with the nascent peptide release and ribosome dissociation (Mathews et al. 2007). Some mRNAs will be translated immediately, while others will be stored and shipped off to various cellular depots until they are needed, such as the leading edge of lamellipodia, mitotic spindles, and neuronal synapses (Besse and Ephrussi 2008; Holt and Bullock 2009); these stored mRNAs are shipped and translationally repressed by RNA-binding proteins until they are locally translated by the ribosome.

During the process of mRNA translation, initiation is the most rate limiting step (Lodish and Jacobsen 1972; Palmiter 1975). It is reasonable for translational initiation to be both rate limiting and the point at which general translation is arrested in an effort to conserve energy during times of cellular stress (Sonenberg and Hinnebusch 2009). But what about the opposite scenario: Do specific mRNAs have translational precedence over one another and if so, how does this occur? Because a given mRNA must be translated at the expense of a different mRNA, it seems highly plausible for cells to actively control not only general translation, but specific mRNA translation as well. Often, it is the complement of mRNA-associated RNA-binding

proteins that can promote or repress the translational capacity of a given mRNA though the mechanisms by which this occurs are not fully understood.

The mRNA's lifecycle would not be complete without the death or decay of the mRNA. mRNA decay is initiated first by decapping, then 5' degradation or 3' degradation by RNA exonucleases, and ultimately complete nucleolytic destruction (Garneau et al. 2007; Goldstrohm and Wickens 2008).

RNA-binding proteins: Master regulators of mRNA metabolism.

mRNAs are constantly bound by a dynamic panel of RNA-binding proteins in the form of an mRNP. Surprisingly, of the ~800 putative RNA binding proteins in the human genome predicted by the identification of either an RNA recognition motif (RRM) or K Homology (KH) domain, little is known about the mechanisms of how this class of proteins may regulate mRNA localization, translation and/or mRNA stability (Sanchez-Diaz and Penalva 2006; Mathews et al. 2007). However, it is generally acknowledged that translational control by RNA-binding proteins affords the cell an evolutionarily novel repertoire of exquisite control mechanisms; this allows for extensive innovation both spatially, as in the example of body patterning by Orb and Bruno during *Drosophila* development (Webster et al. 1997), and temporally, as in the example from oocyte maturation and translational control at the synapse for synaptic plasticity by the Cytoplasmic Element Binding Protein (CPEB) (Richter 2007). Translational control of specific mRNAs affords the cell additional control mechanisms including: i) enables cells to quickly respond to extracellular cues as exemplified by the *Xenopus* oocyte, ii) a degree of flexibility by

allowing the reversible control of protein synthesis and associated high energy demands, exemplified by HuR (also known as Embryonic Lethal Abnormal Vision-like or ELAVL1) antagonism of microRNA-122 translation repression (Bhattacharyya et al. 2006) , iii) coordinates and organizes mRNAs into ontologically relevant mRNP units post-transcriptionally (Hieronymus and Silver 2004), and finally iv) preferential movement of mRNAs in and out polyribosomes (Proweller and Butler 1996).

In the cytosolic compartment, the translation of one mRNA must come at the cost of translating another. RNA-binding proteins can influence this translational balance for specific mRNAs in many different ways. One such mechanism that enhances movement of specific mRNAs into translating polysomes, is the cytoplasmic polyadenylation of mRNAs. The poly(A) tail of an mRNA acts as a scaffold for RNA-binding proteins, such as the poly(A) binding protein PABP which promotes translation. Although the poly(A) tail is initially added in the nucleus, the length of this tail can be altered in the cytoplasm to affect the translational efficiency of specific mRNAs. A cis-element found in the 3'-UTR of some mRNAs which stimulates polyadenylation is known as the cytoplasmic polyadenylation element (CPE) (McGrew et al. 1989). The CPE is a binding platform for the CPE binding protein (CPEB) which nucleates factors that can both remove and add poly(A) tails; experiments utilizing the *Xenopus laevis* oocyte detail mechanistically how CPEB can accomplish this feat (Barnard et al. 2004; Kim and Richter 2006).

CPEB function in *Xenopus laevis* oocytes.

Most metazoans undergo some sort of sexual reproduction. Specialized cells termed gametes arise from germ cells via meiosis. The gametes will fuse to form the zygote that quickly develops into the blastula. After blastulation, the embryo continues to differentiate into various organs and tissues and ultimately a mature individual. The two gametes, the sperm and ova, not only contribute genetic material, but a centrosome from the sperm and cytoplasm from the oocyte. The oocyte's cytoplasm supports early embryogenesis by supplying many factors, including some 10^{12} ribosomes and maternal mitochondria, as well as maternally derived mRNAs synthesized during oocyte development; these dormant mRNAs are required for both maturation of the oocyte and for early embryonic divisions, as these events proceed in the absence of transcription (Ferrell 1999). Transcription remains silenced for many cleavage cell divisions, until the mid-blastula transition (MBT), as in the African clawed frog *Xenopus laevis* and fruit fly *Drosophila*, but only two cell divisions in mice and up to eight in human. Whereas the cleavage divisions in mammals take two to three days, roughly one-tenth of the total gestation period in mice, eleven cleavage divisions will occur in seven hours in *Xenopus* (Gilbert 1997). Thus, the maternally synthesized sets of transcripts are targets for multiple, most likely conserved, translational regulatory mechanisms responsible for generating the early embryonic proteome.

Oocytes from *Xenopus laevis* represent an ideal system amenable to biochemical dissection and experimentation to test paradigms of translational control and cell cycle regulation. Early oocyte maturation in *Xenopus laevis* proceeds up to the dictyate stage of pachytene in prophase I where it then arrests; this arrest is comparable to a Growth-phase 2 (G2) arrest observed in somatic mammalian cells. Oocyte maturation is thus the process of hormonally-stimulated cell cycle resumption and meiotic-phase progression where the oocyte

reduces its genome from tetraploid to haploid in preparation for fertilization. Early experiments demonstrated that stores of select maternally derived mRNAs become translationally active upon progesterone stimulation; these mRNA were shown to enter the translating polyribosomal fractions by sucrose gradient sedimentation (Dworkin et al. 1985; McGrew et al. 1989). The entrance of mRNAs into the polysomal fraction is due to an increase in the mRNAs 3' poly(A) tail length demonstrated by removing this tail by oligo(dT) + RNase H (Richter 1991; Gray and Wickens 1998). Subsequent experiments by McGrew and Richter (1991) using serial deletion analysis of a reporter RNA sequence identified an AU-rich element required for progesterone induced polyadenylation and named it the Cytoplasmic Polyadenylation Element (CPE). The CPE, whose general consensus sequence is UUUUUAU, was also demonstrated to require an additional hexanucleotide sequence AAUAAA that had previously been shown to bind the CPSF complex (Shatkin and Manley 2000). Together, the CPE and hexanucleotide sequence are required for the increase in the reporter mRNA G10, previously identified to shift in size after progesterone treatment (McGrew et al. 1989). UV-crosslinking experiments led to the identification of the protein that binds the CPE: the CPE binding protein (CPEB) (Hake and Richter 1994). From the many experiments, mostly conducted in *Xenopus laevis* oocytes, four models of CPEB-mediated translational control emerge: i) the masking model (Figure 1), ii) the opposing deadenylation-polyadenylation model (Figure 2), iii) poly(A) tail stabilization model through ePAB (Figure 3), and iv) the CPE combinatorial code. The four models are historically distinct, but mechanistically overlapping and inter-dependent.

The models of CPEB function.

After identifying the cis-element and trans-acting factor required for cytoplasmic polyadenylation in frog oocytes, rigorous experimentation led to the identification of many other components of the CPEB-polyadenylation complex (Stebbins-Boaz et al. 1999; Mendez and Richter 2001; Barnard et al. 2004; Kim and Richter 2006; Kim and Richter 2007; Richter 2007). The underlying theme of how CPEB regulates translation is via polyadenylation-induced translation, but over the last twenty years four models of CPEB mediated translational control have emerged. Because, CPEB itself has no catalytic activity and is thus simply an RNA-binding protein, CPEB is merely the core of a complex of auxiliary enzymes that can reversibly inhibit and/or promote translation by adding or removing adenosine ribonucleotides to the 3' end of mRNA. CPEB-associated factors can also block eukaryotic initiation factor 4G (eIF4G) entry to prevent translational initiation (Richter 2007).

As previously stated, translation is primarily limited by the rate of the initiating ribosome. Thus, by increasing the polyadenylate tail of mRNA, the poly(A) binding proteins (PABPs) are recruited and specifically bind polyadenylate stretches. These PABPs (of which there are at least four cytoplasmic isoforms in human cells) then form a protein-protein-protein bridge via PABP, eIF4G and eIF4E to promote the recruitment of the small ribosomal subunit and the ternary complex containing initiator methionine (Sonenberg and Hinnebusch 2009; Jackson et al. 2010) – creating the so called „cap to tail synergy“ summarized by the closed loop model of translation (Sachs and Wahle 1993; Tarun et al. 1997). Initial co-immunoprecipitations using antibody against CPEB followed by mass-spectrometry identified the eIF4E-binding protein Maskin, α -helical triple coiled-coil protein, as a component of the CPEB-complex (Stebbins-Boaz et al. 1999). One of the first models to emerge from these studies was the Masking-Model of

translational control (Figure 1). However, the mouse and human orthologue of Maskin TACC3 does not contain any obvious eIF4E binding domain. Interestingly, another CPEB and eIF4E binding protein Neuroguidin (Ngdn), could possibly substitute in function for Maskin in mammals (Jung et al. 2006).

In the *Xenopus* oocyte, upstream signaling events initiated by progesterone binding to an as yet unidentified receptor generate a signal transduction cascade along with an increase in cyclic adenosine monophosphate (cAMP) cellular concentration that promotes the activity of Eg2 (Andresson and Ruderman 1998), the human orthologue of Aurora-A kinase. In its active state, the kinase Eg2 phosphorylates CPEB at serine residue 174 within the amino acid sequence motif LDSR (Mendez et al. 2000b). The phosphorylation of CPEB by Eg2 initiates the polyadenylation of stored dormant maternal mRNAs that have a short 3' tail of approximately twenty adenosines. CPEB itself cannot polyadenylate mRNA, thus predicting that CPEB must then either recruit additional factors, weaken its affinity for inhibitory factors (such as Maskin), or change the activity of a previously bound factor (for example, stimulate CPSF activity). Furthermore, the observation that almost all mRNAs receive a poly(A) tail before leaving the nucleus also predicts that a deadenylase must at some point interact with CPEB-bound mRNAs prior to CPEB phosphorylation, polyadenylation, and ensuing translation. Separate co-immunoprecipitation experiments led to the identification of three additional factors residing in the CPEB complex: symplekin, a protein scaffold and tight junction protein (Barnard et al. 2004), xGld-2 (Barnard et al. 2004), a non-canonical poly(A) polymerase required for CPEB mediated polyadenylation in *Xenopus*, and PARN (Kim and Richter 2006), an enzyme possessing 3' RNA exonuclease activity. Thus, a model is suggested by the work of Kim and Richter (2006), whereby mRNAs transcribed in the nucleus of *Xenopus* oocytes are spliced and

polyadenylated, after which CPEB binds the mRNA and nucleates a complex containing: symplekin, maskin, CPSF complex, Gld-2 and PARN (Figure 2). CPEB then binds its target mRNA in the nucleus as experiments demonstrate CPEB can shuttle between cytoplasm and nucleus (Lin et al. 2010). CPEB-bound PARN then deadenylates CPE-containing mRNAs and they lay dormant until activated by progesterone (Kim and Richter 2006). Because PARN presumably has overriding or greater deadenylating activity, PARN activity opposes the polyadenylation activity of Gld-2 in the cytoplasm until meiotic resumption and CPEB phosphorylation by Eg2. The phosphorylation of CPEB at the critical S174 residue then reduces the affinity of CPEB for PARN, thereby expelling PARN from the complex and ensuing default polyadenylation by Gld-2 (Kim and Richter 2006; Richter 2007). This model exemplifies one of the themes of translational control: translational control affords the cell with a poised and immediate response to extracellular cues (Mathews et al. 2007). Additionally, the presence of the deadenylase PARN in the CPEB-complex may guard against the precocious polyadenylation and translation of repressed mRNAs (Kim and Richter 2006).

After the initial events of Eg2 activation and phosphorylation of CPEB, Cdk1 complexes with cyclin B, an mRNA target of CPEB, to initiate the cell's embryonic cell cycle. Additional translational control mechanisms exist upstream or parallel to CPEB, namely derepression of RINGO/SPY mRNA by the RNA-binding protein Pumilio-2, to ensure rapid and robust cell cycle activation (Padmanabhan and Richter 2006; Vasudevan et al. 2006). However, stemming from the observation that CPEB becomes hyperphosphorylated in later stages of oocyte maturation (Mendez et al. 2002), Kim and Richter (2007) demonstrated that embryonic poly(A) binding protein (ePAB) is also bound to CPEB. ePAB, identified by both the Moon and Steitz groups (Zelus et al. 1989; Voeltz et al. 2001; Seli et al. 2005) further stabilizes the poly(A) tail

by disassociating from CPEB after CPEB hyperphosphorylation, presumably from proline directed Cdk1 phosphorylation sites (Mendez et al. 2002; Kim and Richter 2007). This release of ePAB from the complex may also concentrate ePAB and eIF4G, weakening Maskin affinity for eIF4E, and thus promoting eIF4F complex formation and translational initiation (Figure 3).

The final model of CPEB-regulated cytoplasmic polyadenylation involves an additional factor that binds another common cis-element in the 3'-UTR of CPE containing mRNAs, the Pumilio Response Element (PRE) or Pumilio Binding Element (PBE). As previously discussed, the composition of the complex of proteins required for cytoplasmic polyadenylation is well characterized; however, the context of the CPEB complexes positioning on the 3'-UTR of an mRNA is poorly defined (Charlesworth et al. 2004; Pique et al. 2008). Also, the observation that not all CPE-containing mRNAs are polyadenylated at the same time during oocyte development (Ballantyne et al. 1997; de Moor and Richter 1997; Mendez et al. 2002) suggests other cis-acting elements such as the Pumilio Binding Element (PBE) (Nakahata et al. 2003) may operate in parallel and organize mRNAs into functionally relevant sets (Hieronymus and Silver 2004; Licatalosi and Darnell 2010). Finally, very little is known about the identity of specific individual CPE containing mRNAs, but because many maternal CPE-containing mRNAs can be differentially polyadenylated temporally, additional sequence specific instructions residing in the 3'-UTR are predicted to dictate when, and perhaps where, CPE mRNAs are polyadenylated. Of course this control is beyond the scope of the CPEB-complex and is inextricably linked to the sequence context of the non-coding regions (5' and 3' UTRs) of specific mRNAs.

CPEB family proteins.

Most invertebrate and vertebrate metazoans have been shown to possess a CPEB or CPEB-like orthologue (Richter 2007). CPEB is most similar to Orb2 in the fly *Drosophila melanogaster* and Cbp-3 in the nematode *Caenorhabditis elegans*, maintaining a high degree of amino acid conservation, thus predicting a similar degree of functional conservation as well (Figure 5). Higher vertebrate metazoans have been shown to also contain three additional CPEB-like proteins. Huang et al. (2006) used systematic evolution of ligands by exponential enrichment (SELEX) and structural probing experiments to demonstrate that CPEB4 has a preference for a structural cis-element and not a CPE-like sequence. Therefore, the CPEB4 binding sequence is different from CPEB1 and most likely binds functionally distinct target mRNAs (Huang et al. 2006). Very little is known about either the target mRNAs for CPEB2 and CPEB3 family member proteins or how CPEB2 or CPEB3 regulates these mRNAs. Further mechanistic elucidation of the function of CPEB family members CPEB2, CPEB3, and CPEB4 await experimentation. Interestingly, the Mendez Group recently demonstrated that CPEB4 can co-immunoprecipitate xGld-2 poly(A) polymerase, and thus may be functionally similar to CPEB1 polyadenylation complex (Igea and Mendez 2010) .

Conservation of CPEB function in distant metazoans.

After a plethora of work done in the *Xenopus* oocyte, a CPEB-knockout mouse was made nearly a decade after the identification of the CPE (McGrew et al. 1989; Tay and Richter 2001). The initial impetus for the creation of the knockout (KO) mouse was to test whether CPEB was required for both meiotic and mitotic cell cycles. The prediction was that CPEB loss-of-function through complete gene ablation would result in a phenotype similar to that observed in mos-

deficient mice: a parthenogenetic activation of oocytes (Hashimoto et al. 1994). *Mos*, a serine/threonine kinase, is a cytosolic factor (CSF) that importantly initiates a MAP-kinase cascade that liberates *Xenopus* oocytes from meiotic arrest, but is also required for the arrest of meiosis at the end of metaphase II (Sagata 1997). Oocytes of *mos* null animals have a high rate of spontaneous parthenogenetic activation and later develop ovarian cysts and tumor due to a failed ovulation (Colledge et al. 1994; Hashimoto et al. 1994; Choi et al. 1996). Importantly, *mos* is under control of CPEB in *Xenopus* oocytes (Stebbins-Boaz et al. 1996). Unexpectedly, female CPEB knockout mice, otherwise normal in appearance, have undetectable or vestigial ovaries (Tay and Richter 2001). Male CPEB knockout mice have disrupted spermatogenesis and suffer from hypogonadism (Tay and Richter 2001). Thus, both male and female CPEB knockout mice are sterile (Tay and Richter 2001).

CPEB mRNA is also highly expressed in distinct regions of the mammalian brain (Theis et al. 2003). Experiments in the last decade in the field of learning and memory have shown a requirement of protein synthesis for strengthening synaptic efficacy during the early phase of learning (Schuman 1997). Fluorescence in situ hybridization (FISH) experiments demonstrated that CPEB mRNA was present in the dendritic layer of the hippocampus (Wu et al. 1998) and, intriguingly, Calmodulin Kinase II (CamKII), an important player essential for long-term potentiation (LTP) and neuronal differentiation, has CPEs in its 3'-UTR (Wu et al. 1998). Importantly, the necessary components for local translation are also present in dendritic shafts and spines, such as ribosomes, initiation factors, and mRNAs (Eberwine et al. 2001; Job and Eberwine 2001). Thus, Wu et al. (1998) surmised neurons could recapitulate what had been previously shown in frog oocytes, that polyadenylation by CPEB could stimulate translation required for local protein synthesis and synaptic efficacy. Wu et al. (1998) demonstrated by

purifying synaptosomes from dark-reared animals, with and without visual stimulation from light, not only exhibit an increase of 1.7 fold in CamKII protein, but have an increase in poly(A) tail length. These findings are significant not only because they demonstrate that the CPEB-mediated translational control mechanism found in frog oocytes is conserved between distant metazoans, but also because they show that other types of cells, such as polarized post-mitotic hippocampal neurons, can employ CPEB-mediated translational control under quite distinct circumstances.

As significant and interesting as the findings are that CPEB is involved in neurons and germ cell differentiation, CPEB KO mice are surprisingly normal (Richter 2007). One explanation for the lack of additional aberrant phenotypes in the CPEB KO mouse is that in the absence of CPEB1, the organism can compensate the loss-of-function with that of another CPEB family member protein. Compensation by other family member proteins is a common theme as demonstrated by the D-type cyclin family knockout mice (Ciemerych et al. 2002; Kozar et al. 2004; Ciemerych and Sicinski 2005). Recent experiments from the Mendez group demonstrate that CPEB4 can bind the CPE, albeit with one-tenth the affinity. Igea and Mendez (2010) also demonstrate that important polyadenylation enzymes, such as the poly(A) polymerase Gld-2, can co-immunoprecipitate with CPEB4 (Igea and Mendez 2010). Regardless, because CPEB KO mice are sterile, a viable alternative to breeding the mice was to culture murine embryonic fibroblasts (MEFs) to further investigate the role of CPEB in somatic cell cycle (Groisman et al. 2006).

Cellular Senescence.

Primary murine embryonic fibroblasts (MEFs) proliferate normally in cell culture for up to five passages when they then begin to experience a cell cycle arrest between passages five through eight. This proliferative cell cycle arrest has been termed cellular senescence in mice and is generally thought to occur as the result of cell culture stress from abnormally high levels of 20% atmospheric oxygen in comparison to the 4% normally found in most tissues (Parrinello et al. 2003). However, senescence is now generally accepted as *bona fide* tumor-suppressor mechanism important for prevention of malignant tumors (Collado et al. 2005; Collado and Serrano 2005). Senescence has a wide range of triggers from Reactive Oxygen Species (ROS) and DNA damage – presumably due to high oxygen levels and UV-damage – to the aberrant expression of oncogenes, improper cellular contacts, lack of nutrients, inflammatory cytokines, and telomere decapping (Ben-Porath and Weinberg 2004).

The initial master regulator of senescence onset is p53, then the retinoblastoma protein (Rb) (Dimri 2005). When cultured by the standard 3T3 protocol (300,000 cells, trypsinize, 3 days), CPEB KO MEFs surprisingly did not senesce but instead continued to proliferate and were immortal (Groisman et al. 2006). CPEB lacking a zinc finger, and thus deficient in the ability to bind RNA, cannot rescue senescence. However, CPEB reintroduction by way of viral transduction at early, but not late passages, can rescue senescence. These experiments thus demonstrate that CPEB is required for senescence onset. Groisman et al. (2006) further demonstrated that in cells lacking CPEB, the oncogene c-MYC is abnormally expressed at high levels, by both western-blot and by measuring the movement of c-MYC mRNA from monoribosomes to polyribosomes in CPEB WT versus KO MEFs. Groisman et al. (2006) further showed that the aberrant expression of c-MYC is sufficient for senescence bypass in MEFs. As

noted by the Mendez laboratory, the possibility of CPEB complex acting as a repressor of translation is consistent with the sequence context of c-MYC mRNA in that it has PBE sequences in close proximity to CPE's (Pique et al. 2008). However, it is open to experimentation to determine if this possibility is in fact true.

Senescence in murine cells is quite different than that of human cells. Credited as one of the first to rigorously investigate the finite lifespan of cultured cells, Leonard Hayflick in the 1960's proposed that cells possess some sort of counting mechanism that prevents them from proliferating indefinitely and that this in turn is somehow related to organismal aging (Shay and Wright 2000). Later, it was shown that telomere erosion was one such counting mechanism (Stewart et al. 2003; Stewart and Weinberg 2006). Because MEFs generated from laboratory mice have constitutively active telomerase, the RNP responsible for maintaining and lengthening telomeres, this excludes the possibility that telomere erosion is important in the senescence of MEFs, or rather the lack of senescence in CPEB KO MEFs. Also different in mice and human cells is that human cells are more resistant to transformation. As compared to human cells, fewer genetic lesions are required to transform mouse cells (Hahn 2002; Itahana et al. 2004). Therefore, important questions remained as to the requirement of CPEB for senescence in human cells.

The next two chapters of this dissertation are thus concerned with the investigation of CPEB function in human somatic cells. In Chapter 2, CPEB loss-of-function studies were undertaken by knocking down CPEB mRNA in primary foreskin fibroblasts (hFS), in order to assess the importance of CPEB in human cellular senescence. Here we show that human cells lacking CPEB have impaired senescence resulting in a lifespan extension of nearly five-fold, and have maintained telomeres (Burns and Richter 2008). Similar to the findings in mouse cells,

CPEB can rescue senescence at early, but not late passages. Where perhaps different from MEFs, however, was the finding that human cells lacking CPEB had decreased mitochondrial numbers and corresponding ROS production and a compensatory increase in lactate production.

Importantly, a CPEB-dependent translational defect of the p53 tumor suppressor protein mRNA was also found. Starting with the finding that CPEB is required for both normal senescence onset and polyadenylation of p53 mRNA, Chapter 3 then continues to describe initial attempts to recapitulate the findings from *Xenopus* oocytes by confirming the requirement for the predicted non-canonical poly(A) polymerase Gld-2 for the normal polyadenylation of the CPEB targeted mRNA of p53. What follows details the unexpected findings that Gld-2 is surprisingly not required for p53 mRNA, but is instead an upstream regulator of CPEB mRNA translation by microRNA mediated translational repression.

Because roughly one-third to one-fourth of human genes contain at least one putative CPE motif, understanding the molecular function of the CPEB cytoplasmic polyadenylation complex in normal somatic cells is as crucially important as it has been demonstrated for oocyte maturation, embryonic development, learning and memory. The mRNA binding protein CPEB seems to be less conserved in its regulatory amino-terminal end, but is highly conserved in its RNA binding domains, suggesting that CPEB target genes are most likely also conserved (Figure 4). By identifying a novel CPEB-complex member and demonstrating regulatory features of CPEB mRNA translation within the biologically relevant context of cellular senescence, the findings presented in this dissertation have advanced our understanding of the critical functions of CPEB in human cells.

CHAPTER 2

CPEB Regulation of Human Cellular Senescence, Energy Metabolism, and p53 mRNA Translation

David Burns¹ and Joel D. Richter¹

¹Program in Molecular Medicine University of Massachusetts Medical School Worcester, MA
01605

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Correspondence to:

Dr. Joel D. Richter

Program in Molecular Medicine

University of Massachusetts Medical School

373 Plantation Street, Suite 204

Worcester, MA 01605

Tel: (508) 856-8615; fax: (508) 856-4289; email: joel.richter@umassmed.edu

SUMMARY

Cytoplasmic Polyadenylation Element Binding Protein (CPEB) stimulates polyadenylation and translation in germ cells and neurons. Here, we show that CPEB-regulated translation is essential for the senescence of human diploid fibroblasts. Knockdown of CPEB causes skin and lung cells to bypass the M1 crisis stage of senescence; re-introduction of CPEB into the knockdown cells restores a senescence-like phenotype. Knockdown cells that have bypassed senescence undergo little telomere erosion. Surprisingly, knockdown of exogenous CPEB that induced a senescence-like phenotype results in the resumption of cell growth. CPEB knockdown cells have fewer mitochondria than wild type cells and resemble transformed cells by having reduced respiration and reactive oxygen species (ROS), normal ATP levels, and enhanced rates of glycolysis. p53 mRNA contains cytoplasmic polyadenylation elements (CPEs) in its 3' UTR, which promote polyadenylation. In CPEB knockdown cells, p53 mRNA has an abnormally short poly(A) tail and a reduced translational efficiency, resulting in about a 50% decrease in p53 protein levels. An shRNA-directed reduction in p53 protein by about 50% also results in extended cellular lifespan, reduced respiration and ROS, and increased glycolysis. Together, these results suggest that CPEB controls senescence and bioenergetics in human cells at least in part by modulating p53 mRNA polyadenylation-induced translation.

INTRODUCTION

Initiation is the rate-limiting step for translation of most eukaryotic mRNAs and requires both a 5' methylated guanosine cap ($m^7G[5']ppp[5']N$) and a 3' poly(A) tail. The ends of the RNA are brought into close proximity by a protein-protein-protein bridge composed of eIF4E (the cap binding factor), eIF4G, and poly(A) binding protein (PABP) (Tarun and Sachs 1996; Tarun et al. 1997; Wells et al. 1998). PABP may facilitate the interaction of eIF4G with eIF4E, which is necessary for initiation since eIF4G, via the multisubunit eIF3, positions the 40S ribosomal subunit on the 5' end of the mRNA (Sonenberg and Hinnebusch 2007). One mode of translational control that is particularly important for germ cell development is the abrogation of this 5'-3' protein bridge by the near absence of the poly(A) tail (and hence PABP) and by the association of a specialized eIF4E binding protein with some mRNAs. These inert (masked) mRNAs are activated by subsequent poly(A) tail growth the binding of PABP to poly(A), and the replacement of the eIF4E binding protein with eIF4G. The cytoplasmic polyadenylation element (CPE) controls poly(A) tail length; it resides in mRNA 3' UTRs and serves as the binding site for CPEB, a factor that associates with Gld2, a poly(A) polymerase (Barnard et al. 2004), PARN, a deadenylating enzyme (Kim and Richter 2006), ePAB, a poly(A) binding protein (Kim and Richter 2007), Maskin, an eIF4E binding protein (Stebbins-Boaz et al. 1999; Cao et al. 2006), CPSF, a multi-subunit RNA binding complex (Mendez et al. 2000b; Dickson et al. 2001), and symplekin, a probable scaffold or assembly protein (Barnard et al. 2004). In response to developmental cues, CPEB becomes phosphorylated, causing the expulsion of PARN from the RNP complex and results in Gld2-catalyzed polyadenylation (Mendez et al. 2000b; Kim and Richter 2006). The newly elongated poly(A) tail is bound by ePAB that in turn

binds eIF4G; this complex displaces Maskin from eIF4E, thus circularizing the RNA and promoting initiation (Barnard et al. 2005; Cao et al. 2006; Kim and Richter 2006).

CPEB-controlled translation has also been found to modulate neuronal synaptic plasticity (Klann and Richter 2007; Richter 2007) and cellular senescence in murine embryonic fibroblasts (MEFs) (Groisman et al. 2006). Like apoptosis, senescence is a mechanism that prohibits unrestricted cell proliferation. DNA damage, nutrient deprivation, improper cell contacts, and oncogenic signaling all converge on the p53 and/or retinoblastoma (Rb) tumor suppressor pathways to initiate cell cycle arrest and entry into senescence (Lowe et al. 2004; Campisi and d'Adda di Fagagna 2007). While senescence is usually examined in cultured cells, recent studies in animals have demonstrated that it is an important barrier to malignant transformation (Braig et al. 2005; Chen et al. 2005; Collado et al. 2005; Michaloglou et al. 2005).

The bypass of senescence, or immortalization, is required for but does not necessarily lead to cellular transformation. Although both phenomena are often studied in mouse and human cells, there are a number of differences between the two organisms, indicating the complexity of molecules that influence these processes. For example, cultured mouse cells are relatively easy to transform, requiring the addition of only one or two oncogenes (Drayton and Peters 2002). Normal diploid human cells, however, are more refractory and require the inactivation of the p53 and Rb proteins as well as the activation of the catalytic subunit of the telomerase, hTERT. In addition, mouse telomeres are typically 25-60 kb in length while those in humans are much shorter, about 10-15 kb; thus, telomere erosion is not necessarily essential for senescence in mouse cells while it is in human cells (Chin et al. 1999). Moreover, murine cells senesce abruptly in culture 5-10 times faster than human cells. These and other differences between mouse and human cells notwithstanding, it is increasingly evident that in both species, cellular

and replicative senescence are not only triggered by cellular perturbations that generate a DNA damage response, such as oncogene expression and cell culture stress (Lee et al. 1999; Wu et al. 2004), but that senescence onset and maintenance requires a sustained DNA damage response (Hemann and Narita 2007).

While comparing rates of mitosis between MEFs derived from wild type (WT) and CPEB knockout (KO) mice, we made the startling observation that the KO MEFs did not senesce as did WT MEFs, but instead were immortal (Groisman et al. 2006). Reintroduction of CPEB into early passage KO MEFs induced them to senesce; reintroduction of CPEB into late passage KO MEFs (i.e., those that had gone through several passages beyond when WT MEFs senesce) did not. CPEB-mediated senescence required the tumor suppressors p53 and p19^{ARF}; conversely, Ras-induced senescence required CPEB. Myc protein levels were elevated in the KO MEFs and more myc mRNA was found on polysomes compared to WT MEFs. Most importantly, shRNA knockdown of myc in the KO MEFs caused them to become senescent. These data indicated that in mouse cells, the removal of CPEB-inhibited myc RNA translation led to immortalization.

Here, we investigate the importance of CPEB for senescence of normal human diploid fibroblasts. While WT and mock-infected human foreskin fibroblasts senesced after about 70 population doublings, those infected with a lentivirus harboring a shRNA against CPEB did not; the lifespan of these cells was extended by nearly five-fold. A second infection of these knockdown cells with a retrovirus expressing murine CPEB restored a senescence-like phenotype in early but not late passage cells. Human WI-38 lung fibroblasts also bypassed senescence when CPEB levels were reduced. As expected, CPEB knockdown cells that bypassed senescence retained long telomeres. Interestingly, promotion of the senescence-like phenotype by overexpressed CPEB could be reversed when the levels of this protein were

reduced. CPEB knockdown cells had abnormally low levels of mitochondria; they also resemble transformed cells in that they had reduced respiration but an elevated rate of glycolysis presumably to maintain homeostatic ATP levels. CPEB knockdown cells contained ~50% reduction of p53, and p53 mRNA had a shorter poly(A) tail and a reduced translational efficiency compared to p53 mRNA in wild type cells. A ~50% reduction of p53 levels in cells containing normal levels of CPEB also bypassed senescence and had reduced mitochondrial mass and respiration. We propose that the senescence bypass and change in energy metabolism observed in CPEB knockdown cells is due at least in part to dysfunctional p53 mRNA polyadenylation and translation.

RESULTS

CPEB is required for senescence in primary human cells

To determine whether primary human cells require CPEB to become senescent, human diploid foreskin fibroblasts were, in two separate occasions, infected at passage eight with a lentivirus expressing one of two different shRNAs against CPEB RNA (shCPEB) as a control for possible off-target effects. As an additional control, the cells were also infected with an empty lentivirus or one expressing shRNA against mRNA encoding the tetracycline resistance gene (shTETR). The lentivirus vector also expressed GFP, which indicated that ~70% of cells were infected with the virus. The cells were analyzed for growth and morphology without drug selection.

The efficiency of the CPEB knockdown was monitored by both RT-PCR of RNA (data not shown) and by western analysis (Fig. 1A). CPEB was reduced by greater than 80% compared to the control shTETR knockdown; the upper band in the panel might represent phosphorylated CPEB. After ~68 population doublings, the mock-infected and shTETR-infected cells stopped dividing and assumed a flat senescent-like morphology. These cells also stained for β -galactosidase activity at acid pH, a common marker for senescence (Itahana et al. 2007) (Fig. 1D, and data not shown). The shCPEB-infected cells, however, continued to grow, did not undergo a morphology change, and did not stain for β -galactosidase activity (Fig. 1A and D). Moreover, while mock-infected and shTETR-infected cells expressed high levels of p21^{CIP1} and

p16^{INK4A}, which is consistent with entry into senescence, the cells infected with shCPEB did not (data not shown and Fig. 5).

Cells expressing shCPEB were infected with a retrovirus expressing mouse CPEB, which while >95% identical to human CPEB, would not be a target of the human shCPEB. When the cells were infected with the retrovirus after 20 or 68 doublings, they quickly entered a senescence-like state as indicated by the cessation of cell division, assumption of flattened morphology, and staining for β -galactosidase activity. However, when the cells were infected with the retrovirus after 87 population doublings, none of these events took place. Thus, the reintroduction of CPEB into knockdown cells induces a senescence-like state at early and middle, but not late, passages.

We also assessed whether CPEB is required for senescence in WI-38 human lung fibroblasts. These cells were infected with the same lentiviruses used for the foreskin fibroblasts; while the mock infected or control infected cells entered senescence after an additional ten population doublings, those with reduced levels of CPEB did not and indeed reached at least 38 doublings (Fig. 1B). Thus, at least two human cell types require CPEB to enter senescence.

Finally, the zinc finger of CPEB was deleted, which in addition to containing two RNA recognition motifs, is required for CPE binding (Hake et al. 1998). This mutant protein was unable to restore senescence (a mock infection also did not rescue senescence) (Fig. 1C-E). Therefore, CPEB must bind RNA to induce the senescence-like phenotype.

CPEB is required for the suppression of telomere maintenance

Because senescence in human cells is generally accompanied by telomere loss, we measured telomere length in cells infected with shCPEB by a telomere oligonucleotide ligation assay (T-OLA) (Stewart et al. 2003) and by fluorescence in situ hybridization (FISH) (Henegariu et al. 2001). The T-OLA assay (Fig. 2A, B) shows that as expected, the telomeres of WT or shTETR infected cells eroded as the cells entered senescence. While the shCPEB-infected cells also underwent some telomere shortening (e.g., at 90 days post-infection), the erosion was not as severe as with the control cells. This maintenance of telomere length was also evident by FISH for the telomeric region (Fig. 2C). That is, at 90 days post-infection with shCPEB, there was clearly hybridization to the telomeric region. In contrast, cells infected with shTETR underwent extensive telomere erosion as evidenced by the lack of a FISH signal to this same region. As expected, WT cells also lacked a FISH signal due to telomere shortening. These data indicate that CPEB is required, directly or indirectly, for telomere erosion.

The CPEB-induced senescence-like arrest is reversible

Two approaches were used to determine whether the CPEB-induced senescence-like phenotype is reversible. First, WT fibroblasts were infected with lentivirus expressing human HA-CPEB on day one, which was followed two days later by the infection of another lentivirus expressing shRNA against this same CPEB; the cells were analyzed four days later (Fig. 3A).

Western analysis shows that the shRNA efficiently knocked down HA-CPEB (Fig. 3B). As expected, shCPEB, shGFP (another control), or mock lentivirus infections had no effect on cell growth when the cells were also infected with a retrovirus expressing CPEB lacking a zinc finger (hCPEB Δ ZF), which is unable to bind RNA, or with a retrovirus that expressed no heterologous protein (mock) (Fig. 3C). However, while retrovirally transduced CPEB efficiently attenuated cell growth as shown previously (see Fig. 1), the cells returned to prolific cell division once CPEB was knocked down by the lentivirus-expressed shCPEB (Fig. 3C).

In a second approach to address reversibility, sequences encoding a CPEB-GFP fusion protein were cloned into a vector containing the tetracycline response element. Following infection and antibiotic selection, the cells were infected with a lentivirus encoding the tetracycline repressor (Tet^R); several days later, the cells were incubated with doxycyclin (DOX), which will repress the expression of CPEB. The cells were then split; some were further incubated with DOX while others were transferred to DOX-free medium (-DOX). The cells were analyzed 4 and 8 days later (Fig. 3D). Fig. 3E demonstrates that CPEB (-DOX) inhibited cell division; conversely, the subsequent down-regulation of CPEB expression (+DOX) allowed the cells to continue to divide. Finally, CPEB-GFP was readily detected in cells incubated in the presence, but not the absence, of DOX (Fig. 3F). These results demonstrate that the CPEB-induced senescence-like phenotype is reversible.

CPEB is necessary for stress-induced cellular senescence

Cellular senescence is generally thought to be a response to stresses that induce telomere shortening, oncogene activation, reactive oxygen species (ROS), etc. (Ben-Porath and Weinberg 2005). In MEFs, at least one inducer of senescence, constitutively activate Ras, requires CPEB (Groisman et al. 2006). This is also the case with human fibroblasts that have reduced CPEB levels (Supplementary Fig. 1). Also, as in CPEB KO MEFs, Ras failed to induce senescence in human fibroblasts with reduced CPEB (Supplementary Fig. 1). Because Ras is involved in modulating levels of reactive oxygen species (ROS) (Irani et al. 1997; Archer and Bar-Sagi 2002), we used two approaches to examine whether ROS was also involved in the CPEB-induced senescence-like phenotype. First, WT fibroblasts were treated with 200 μ M N-acetylcysteine (NAC), an oxygen free radical scavenging agent, followed by infection of a retrovirus expressing CPEB. While CPEB strongly induced senescence in untreated cells, its ability to do so in NAC-treated cells was substantially reduced (Supplementary Fig. 2A). Moreover, fibroblasts treated with hydrogen peroxide to increase the ROS concentration readily senesced, but did not do so when they had reduced CPEB levels (Supplementary Fig. 2B).

While indirect, these results suggest some connection between CPEB and ROS, and possible changes in cellular bioenergetics. To examine bioenergetics directly, we measured mitochondrial respiration in two CPEB knockdown cell lines; Fig. 4A shows that relative to WT or shTETR control cells, mitochondrial respiration in these cells was reduced by about 50%. To investigate the origin of this reduced respiration, we stained WT, shTETR, and shCPEB cells with Mitotracker, which reflects mitochondrial mass, followed by stacking of confocal images. These images indicate that shCPEB caused a reduction of mitochondrial number (Fig. 4B); the quantification of the Mitotracker fluorescent signal indicates that there was nearly eight fold

fewer mitochondria (Fig. 4C). This decrease was confirmed by a western blot for cytochrome C, a mitochondrial marker (Fig. 4D).

Because mitochondrial oxidative phosphorylation generates most of the cell's ATP, it might be inferred that the CPEB knockdown fibroblasts contain less ATP than WT cells. However, the level of ATP was nearly identical between WT and shCPEB cells (Fig. 4E). Transformed cells also have reduced respiration but maintain relatively normal levels of ATP by increasing glycolysis (Bensaad and Vousden 2007). To determine whether this is also the case with CPEB knockdown cells, the amount of lactate, an indicator of glycolysis, was determined. Indeed, the CPEB knockdown cells produced about five times more lactate than did WT cells (Fig. 4F), indicating a substantial up-regulation of glycolysis. Finally, CPEB knockdown cells were also found to have a decrease in ROS levels (Fig. 4G), as might be expected from the reduction in mitochondrial respiration.

CPEB-induced senescence requires p53

In MEFs, CPEB requires p53 to induce senescence (Groisman et al. 2006), 2006). To determine whether this is also the case with human cells, fibroblasts were infected with a retrovirus expressing GSE-22, a p53 dominant negative peptide that inhibits p53 activity (Beausejour et al. 2003). Two days later, the cells were infected with a virus expressing CPEB; the cells were then analyzed for growth and p21, a target gene of p53 (Figs. 5A-C). While CPEB induced senescence in cells lacking GSE-22, they were unable to do so if they contained the inhibitory peptide. Moreover, GSE-22 prevented p21 expression, thus demonstrating that it

indeed inhibited p53 activity. These results indicate that CPEB-induced senescence requires p53 in human cells.

CPEB control of p53 mRNA translation

Several senescence-related proteins were analyzed in extracts derived from WT, shTETR, and shCPEB cells. p53 as well as K382-acetylated p53 were reduced by about 50-60% in shCPEB-containing cells; the cell cycle inhibitory proteins p21^{CIP1} and p16^{INK4A} were also reduced, consistent with the bypass in senescence (Fig. 6A). Because p53 mRNA levels were not commensurately lower with the p53 protein levels (Fig. 6B), we inferred that CPEB might control, directly or indirectly, the post-transcriptional regulation of p53. One indication that this regulation might be direct is the fact that two conserved CPEs are found in the 3'UTRs of p53 mRNA from several mammalian species (Fig. 6B). To examine whether these CPEs are functional, radiolabeled RNA corresponding to the p53 3'UTR was injected into *Xenopus* oocytes, followed by incubation with progesterone, which stimulates oocyte maturation and CPE-dependent polyadenylation. Indeed, the p53 3'UTR underwent robust polyadenylation. Moreover, a p53 3'UTR with mutated CPEs exhibited little polyadenylation (Fig. 6C), demonstrating that the p53 3'UTR has the proper sequences to promote cytoplasmic polyadenylation. In transfected fibroblasts, HA-tagged wild type CPEB, but not a CPEB with deleted zinc fingers, efficiently co-precipitated p53 mRNA; GAPDH mRNA, a negative control was not precipitated under any condition (Fig. 6E). Finally, we investigated whether a knockdown of CPEB would alter the poly(A) tail length of p53 mRNA. Fig. 6F shows a PCR-

based PAT assay (Salles and Strickland 1995) for poly(A); while the poly(A) tail of p53 RNA reached nearly 200 nucleotides in WT cells, it was only about ~50 nucleotides in shCPEB knockdown cells. Thus, CPEB controls p53 RNA poly(A) tail length in human fibroblasts.

Because p53 protein levels are maintained by a balance of synthesis and destruction, we sought to determine which of these processes was controlled by CPEB. WT and shCPEB-infected fibroblasts were first starved of methionine and cysteine, then pulsed with ³⁵S-methionine and ³⁵S-cysteine, followed by a chase with radio-inert methionine and cysteine. p53 was then immunoprecipitated and the decay of this protein was monitored by SDS-PAGE and phosphorimaging. While shCPEB had no effect on the decay rate of general cellular proteins, there was a stabilizing effect on p53 (Fig. 7A,B). This result, however, is complicated by the fact that p53 is a positive regulator of the human homologue of mdm2, the E3 ligase that controls ubiquitin-mediated p53 destruction (Fig. 7C). In other words, elevated p53 levels induce mdm2 transcription, which in turn leads to p53 destruction. Consequently, we have used alternative approaches to determine why p53 levels are reduced in CPEB knockdown cells. First, WT and shCPEB infected cells were pulsed with ³⁵S-methionine and ³⁵S-cysteine in the presence of the proteasome inhibitor MG132, followed by p53 immunoprecipitation. Compared to total protein, the rate of p53 synthesis was ~50% lower in the shCPEB-infected cells compared to WT (Figs. 7D, E).

The translational efficiency of p53 mRNA was determined by polysome sucrose density centrifugation of extracts from WT and shCPEB-infected cells. The gradients were fractionated and following RNA extraction, p53 mRNA was assayed by Q-RT-PCR using GAPDH mRNA as an internal standard. Fig. 7F demonstrates that in shCPEB-infected cells, there was a shift in the

sedimentation profile of p53 mRNA from heavy to lighter polysomes, consistent with a reduction in translational efficiency.

Senescence bypass and alteration in bioenergetics in p53 knockdown cells

We next investigated whether the ~50% reduction in p53 in CPEB knockdown cells is sufficient for the senescence bypass and change in bioenergetics (Fig. 8A). To do so, we stably expressed a p53 shRNA via lentiviral gene transfer, which reduced p53 protein levels by about 50% (Fig. 8B). As expected, expression of p21, a p53 target gene, was also inhibited (Fig. 8B). The 50% reduction of p53 induced senescence bypass, as did expression of the GSE-22 dominant negative p53 peptide (Fig. 8C). The 50% reduction of p53 also resulted in reduced mitochondrial respiration (Fig. 8D) and ROS (Fig. 8E), and stimulated a greater than 6 fold increase in lactate production, indicating substantial up-regulation of glycolysis (Fig. 8F). To assess what factors might be downstream of CPEB and p53 that influences the change in bioenergetics, we determined the levels of synthesis of cytochrome oxidase 2 (SCO2), which has been reported to modulate the Warburg effect in a p53-dependent fashion (Matoba et al. 2006). Fig. 8F demonstrates that SCO2 levels were reduced in cells in which either CPEB or p53 were knocked down, suggesting that the influence of CPEB on bioenergetics occurs via p53 mRNA translation and SCO2.

Response to chemical carcinogen in CPEB knockout mice

Based on the results of Groisman et al (2006) and this study that describe the importance of CPEB in cell growth and metabolism *in vitro*, we have initiated studies to examine the relative importance of CPEB in ageing and malignant transformation in mice. CPEB knockout mice have ~2-2.3 year lifespans that are virtually indistinguishable from those of wild type animals; they also display no unusual proclivity for tumor formation (data not shown). However, CPEB KO mice do form papillomas at a significantly faster rate than wild type animals in a two-step DMBA-TPA carcinogenesis assay (Supplementary Fig. 3). Thus, CPEB does appear to offer some protection against at least one type induced tumor formation and perhaps other induced stresses as well.

DISCUSSION

We demonstrate that reduced levels of CPEB cause a bypass of senescence in primary human cells. While these cells have a nearly five-fold extended lifespan, their rate of cell division eventually begins to slow and they cease to divide after about 93 population doublings. While mouse cells (MEFs) that lack CPEB also bypass senescence, they do not begin to slow even after >40 passages and are immortal (Groisman et al. 2006). In both mouse and human cells lacking (or with reduced) CPEB, the re-introduction of CPEB at early passages restores a senescence-like phenotype; a similar re-introduction of CPEB into late passage cells, however, has little effect on cell division. While it is unclear why only early passage cells respond to exogenous CPEB, such experiments do demonstrate that it is CPEB and not another factor that is responsible for the senescence bypass. In both MEFs and human fibroblasts, p53 is required for CPEB-induced senescence while CPEB is required for Ras-induced senescence ((Groisman et al. 2006); Fig. 5; Supplementary Fig. 1). These similarities between mouse and human cells notwithstanding, CPEB-controlled senescence in MEFs is mediated at least in part by myc while in human cells, one key factor is p53. In MEFs containing or lacking CPEB, there is no detectable change in the amount of p53 over many cell passages while myc protein is elevated in CPEB KO MEFs (Groisman et al. 2006). Myc mRNA is also translated more efficiently in the KO MEFs and is at least one factor that mediates immortalization when CPEB is absent since a knockdown of myc in CPEB KO MEFs causes a cessation of cell division (Groisman et al. 2006). In human skin fibroblasts, there is no evidence for CPEB control of myc translation that contributes to senescence. On the other hand, CPEB mediates poly(A) tail length and translational control of p53 mRNA. Without the translational enhancement of p53 mRNA by

CPEB, p53 levels are reduced to less than half their normal levels, which leads to senescence bypass (Fig. 8; see also (Rogan et al. 1995; Wei et al. 2003; Lynch and Milner 2006) and changes in bioenergetics (see below).

Our results indicate that CPEB controls p53 mRNA polyadenylation and translation, possibly in a manner similar to that which occurs in vertebrate germ cells (Tay and Richter 2001; Tay et al. 2003; Barnard et al. 2004; Kim and Richter 2006; Richter 2007). If this is the case, then other factors such as Gld2 and PARN, which mediate CPEB-directed polyadenylation in oocytes, may have the same function in human fibroblasts, and a reduction in their steady state levels might modulate senescence.

In response to DNA damage, ribosomal protein L26 (rpL26) has been reported to stimulate p53 mRNA translation while nucleolin inhibits it (Takagi et al. 2005). p53 mRNA translation is also enhanced by the RNA binding protein HuR in response to UV irradiation (Mazan-Mamczarz et al. 2003). While we have no evidence that CPEB regulates p53 mRNA translation in response to DNA damage, it appears to regulate steady state translation by insuring that the p53 poly(A) tail is the proper length. Polyadenylation is a complex process regulated in both the nucleus and cytoplasm. For example, both nuclear pre-mRNA and cytoplasmic mRNA polyadenylation can be regulated during the cell cycle (Colgan et al. 1996; Groisman et al. 2006) and in response to certain signaling events (Wu et al. 1998; Mellman et al. 2008). Moreover, poly(A) dynamics are also regulated by environmental stress (Hilgers et al. 2006) and by miRNAs (Wu et al. 2006). We speculate that CPEB activity may be necessary for maintaining steady state p53 levels under normal conditions where it has important functions in cellular ageing and energy metabolism (Bensaad and Vousden 2007).

Reversible CPEB-induced senescence-like phenotype

The observation that the CPEB-induced senescence-like phenotype was reversible was surprising because senescence is generally considered to be an irreversible process. On the other hand, there have been reports of senescence reversibility under particular circumstances. For example, (Macip et al. 2006) have shown that in p53 null cells, a reactive oxygen species-induced senescence-like phenotype is reversible. This type of senescence reversibility may not be directly related to that described here since oxidative stress of CPEB knockdown cells does not readily induce senescence (Supplementary Fig. 2). Moreover, Beausejour et al. (2003) have shown that senescence accompanying telomere shortening can also be reversed. While the nature of the CPEB reversibility of senescence requires further investigation, it is clear that cells' entry into senescence is particularly sensitive to the amount of this protein. That is, in human cells, a knockdown of CPEB, either endogenous or exogenous, to ~20% of normal levels results in senescence bypass. In MEFs, cells heterozygous for CPEB also bypass senescence (Groisman et al. 2006). Conversely, cells containing relatively low amounts of exogenous CPEB require several passages before they senesce whereas cells containing high levels senesce much faster. We speculate that the amount of CPEB is important for the relative translational efficiency of p53 mRNA, which in turn could be responsible for the rate, or timely onset, of senescence.

CPEB, p53, and energy metabolism

CPEB knockdown fibroblasts have nearly eight fold fewer mitochondria compared to WT cells and overall, respire about half as well. This startling observation suggests that these cells have reduced ATP levels and thus would probably divide more slowly than wild type cells. Such is not the case, however, because knockdown cells have a very high rate of glycolysis; normal levels of ATP are therefore generated and the cell division rate at early passages is indistinguishable from wild type. The so-called „Warburg effect“ of reduced oxygen consumption and elevated glycolysis was recognized many years ago as a hallmark of cancer cells (Shay and Wright 2000; Gatenby and Gillies 2004; Bensaad and Vousden 2007). Based on the Warburg effect, it might be inferred that CPEB knockdown skin fibroblasts are transformed; this is unlikely to be the case, however, because they are contact inhibited (data not shown). Moreover, the CPEB KO MEFs do not grow in reduced serum, do not show significant anchorage independent growth, and do not form tumors when injected into nude mice (Groisman et al. 2006). In the human CPEB knockdown fibroblasts, it seems likely that the Warburg effect is due to reduced levels of p53 (Fig. 8; (Matoba et al. 2006). In p53 null or even hypomorphic cells, aerobic respiration is reduced and glycolysis is elevated. One downstream gene whose expression was recently reported to be regulated by p53, synthesis of cytochrome oxidase 2 (SCO2), may be responsible for several of these changes in energy metabolism (Matoba et al. 2006). This protein is necessary for assembly of the multi-protein cytochrome C oxidase (COX) complex, which forms the molecular foundation for oxidative phosphorylation. Indeed, SCO2 is reduced in CPEB as well as p53 knockdown cells (Fig. 8), which probably is responsible for the lowered respiration and elevated glycolysis. However, there are likely to be a number of mRNAs whose translation is mis-regulated in cells that lack CPEB, some of which are likely to contribute

to the senescence bypass and/or the Warburg effect. In this vein, (Wajapeyee et al. 2008) recently identified a growth factor, IGFBP7, and 16 additional factors that control BRAF-induced senescence. In MEFs, CPEB is necessary for Ras and probably BRAF-induced senescence, suggesting that mRNAs encoding some of these factors might be under the translational control of CPEB. We are presently investigating whether the translational regulation of other mRNAs modulates senescence.

Materials and Methods

Cells and culture conditions

Primary human foreskin fibroblasts were obtained from the Cell Culture Core Facility of the Yale University Skin Disease Research Center and cultured as described (Rangarajan et al. 2004) in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum. About 10^6 cells, counted with a hemocytometer following treatment with trypan blue, were passaged every three days. Human lung WI-38 fibroblasts were cultured in a similar manner.

Virus production, infection, and cell lines

Amphotropic retroviruses and lentiviruses were produced by transient transfection of 293T cells with a transfer vector and amphotropic packaging plasmids encoding VSV-G and gag-pol using Lipofectamine 2000 (Invitrogen). Human cells at 50% confluency were infected for 8-12 hr with viral supernatants containing 7 μ g/ml polybrene. Typically 70-90% infection efficiency was achieved as assessed by using a GFP-encoding viral gene or by immunostaining cells using anti-HA (Covance). After infection, fresh media was added to the infected fibroblasts.

Some cells were analyzed by western blotting for p16 (BD Biosciences), p21 (Santa Cruz Biotechnology), p53 (DO-1, Neomarkers), CPEB (Affinity BioReagents), cytochrome C (BD

Pharminggen), SCO2 (Dr. Paul M. Hwang, NHLBI-NIH), and b-actin (Abcam). Other cells were fixed with 0.2% glutaraldehyde and stained for b-galactosidase activity at acidic pH according to Dimri et al (1995).

Telomere Analysis

A telomere oligonucleotide ligation (TOLA) assay was performed as previously described (Stewart et al. 2003). Briefly, DNA extracted from WT, shTETR, and shCPEB infected cells (5 mg each) was added to a 20 ml reaction volume containing 0.5 pmol ³²P-end-labeled (CCCTAA)₄ oligonucleotide and hybridized for 12-15 h at 50 °C followed by addition of 20 units Taq DNA ligase (New England Biolabs) at 50 °C for 5 h. After ligation, the DNA was precipitated, dried, resuspended and applied to a 5% polyacrylamide-6 M urea gel. To ensure equal DNA loading of TOLA gels, 10 ng of each TOLA sample was analyzed by Q-PCR for the GAPDH gene. Primer sequences are presented in the Supplemental material.

Metaphase chromosomes from WT, shTETR, and shCPEB infected cells were prepared as described (Henegariu et al. 2001) and blocked with COT1 DNA and hybridized with 50 ng of a locked nucleic acid (LNA) probe (TTAGGGTTAGGGTTAGGG; locked nucleotides are underlined) that was 3'' end conjugated with Cy3.

RNA analysis

To examine the p53 mRNA poly(A) tail, total RNA (300 ng) was pre-annealed with 5-prime phosphorylated oligo d(T)₁₈ (20 ng/ml) followed by reverse transcription (RT) with an oligo d(T)₁₈ anchor primer (5'-GCTTCAGATCAAGGTGACC-d(T)₁₈). Subsequent PCR used 2 ml from the previous RT with primers specific for p53 RNA and the linker in the presence of dNTPs plus ³²P-ATP. Details can be found in the Supplementary material.

Immunoprecipitation of mRNA-protein complexes was performed on early passage fibroblasts infected with virus encoding HA-CPEB (Peritz et al. 2006), followed by RT-PCR for p53 and GAPDH mRNAs.

Analysis of p53

Control and shCPEB infected fibroblasts were cultured in methionine and cysteine-free media (Invitrogen) for 45 min and then cultured in media containing 140 mCi ³⁵S methionine and ³⁵S cysteine (ProMix, Amersham) for 30 min. The cells were then washed and cultured in fresh DMEM supplemented with 2 mM each of methionine and cysteine for the times indicated. The cells were then frozen and stored until they were lysed and used to immunoprecipitate p53 (DO-1 antibody, Neomarkers), which was analyzed by SDS-PAGE and phosphorimaging. Details of the procedure may be found in the Supplementary material.

Some cells were cultured in methionine/cysteine free media as noted above in the presence of MG132, a proteasome inhibitor, for 1 hr, followed by a 15 min culture in 100 mCi ³⁵S methionine and cysteine; p53 was then immunoprecipitated and analyzed as noted above.

Bioenergetics

To measure oxygen consumption, $\sim 4 \times 10^5$ cells were washed and resuspended in 200 ml Krebs-Ringers solution plus HEPES (125 mM NaCl, 1.4 mM KCl, 20 mM HEPES, pH 7.4, 5 mM NaHCO₃, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 1 mM CaCl₂) containing 1% BSA. Cells from each condition were aliquoted into a BD Oxygen Biosensor System plate (BD Biosciences) in triplicate. Plates were assayed on a SAFIRE multimode microplate spectrophotometer (Tecan) at 1-minute intervals for 60 minutes at an excitation wavelength of 485 nm and emission wavelength of 630 nm.

Lactate assay

The concentration of lactate in 40,000 cells was measured as described by Chang et al (1992); the NADH produced by the conversion of lactate to pyruvate by lactate dehydrogenase was measured at 340nm using a SAFIRE multimode spectrophotometer (TECAN).

ATP

ATP concentrations were determined using a CellTiter-Glo Luminescent Cell viability assay kit (Promega) by first plating approximately 40,000 cells in 96 well format plate and following the manufacturer's instructions.

Mitochondria

Images were obtained from live cells plated on coverslips after incubating them with 500nM Mitotracker-Red (Molecular Probes). Cells were stained cells for 20 min. in 10% FBS, DMEM, 5% CO₂ using a Zeiss AxioVert 200M Confocal with a PerkinElmer UltraView Spinning Disc. Images were analyzed with Metamorph and Imarus software, similar to (Kang et al. 2007).

ROS

ROS levels in 80,000 cells were determined by using 10 mM CM-H₂DCFDA (dichlorodihydrofluorescein diacetate) (Invitrogen) in Krebs-Ringer bicarbonate buffer (Sigma-Aldrich). Using a Tecan plate reader, CM-H₂DCFDA was excited at 485 nm and detected at 530 nm with a 4x4 pattern reader.

Detailed procedures

Details of all procedures can be found in the supplementary material.

Supplemental Experimental Procedures

Virus production, infection, and cell lines

Amphotropic retroviruses and lentiviruses were produced by transient transfection of 293T cells with the appropriate transfer vector and amphotropic packaging plasmids encoding VSV-G and gag-pol using Lipofectamine 2000 (Invitrogen). Both mouse and human cells at ~60% confluency were infected for 8-12 hr with viral supernatants containing 7 $\mu\text{g/ml}$ polybrene. Typically, 70-90% infection efficiency was achieved using this protocol as assessed by GFP fluorescence with a GFP-encoding virus or HA staining with a virus expressing HA-CPEB. After infection, fresh media was added to the cells.

SA- β -galactosidase assay

Cells were fixed with 0.2% glutaraldehyde (Sigma) and incubated 12 hr at 37°C in a filtered solution containing 1 mg/ml X-Gal (Sigma) in 150 mM NaCl, 2 mM MgCl₂, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, and 40 mM sodium phosphate buffer (pH 6.0).

Ligation mediated poly(A) test (LM-PAT), Xenopus oocyte injection and poly(A) test (PAT)

For the analysis of poly(A) of p53 mRNA, 5 µg of total RNA was extracted using Trizol reagent and annealed to oligo d(T)₁₈-linker (500 µg/ml; 5'-GCGAGCTCCGCGGCCGCGT₁₈-3'); dNTPs were added, followed by a further incubation at 65°C for 5 min. After quick chilling on ice, reverse transcription was performed with Superscript II (Invitrogen) according to the manufacturer's instructions. p53-specific oligomers and oligo d(T)₁₈-linker, and 1 µl of cDNA template were used in a 25 µl PCR reaction with PlatinumTaq (Invitrogen). Trace amounts of ³²P-dATP was added to the PCR reactions. The PCR products were digested with a p53-specific exonuclease BstXI (New England Biolabs) to ensure p53 specific amplification and analyzed on polyacrylamide gels.

Oocyte injections and progesterone induced polyadenylation assay was performed as previously described (McGrew et al. 1989).

IP-RT-PCR

Immunoprecipitation of mRNA-protein complexes was performed on early passage fibroblasts infected with the amphotropic virus expressing c-pOZ CPEB-HA. The procedure was performed according to Peritz et al (2006). The following gene specific primers were used for RT reactions and subsequent PCR. 125ng DNase treated total RNA was used for RT reactions. One µl of RT mix was used for 25 PCR cycles. The following primers were used; GAPDH forward primer 5''-

CGCTCTCTGCTCCTCCTGTT; GAPDH reverse primer 5'-CCATGGTGTCTGAGCGATGT;
P53 forward primer 5'-TCTTGCATTCTGGGACAGCC; p53 reverse primer 5'-
AGCTCGTGGTGAGGCTCCCCT.

Polyribosome gradients

Extracts derived from infected cells were used for polysome gradient centrifugation as prepared as described by Ruan et al (1997). Briefly, fibroblasts cells cultured in 10-mm culture dishes were harvested 24 h after transfection by replacing the culture media with fresh media containing cycloheximide (Sigma) at a final concentration of 100 µg/ml for 5–10 min. The cells were washed with PBS, trypsinized, pelleted and resuspended in low-salt buffer (20 mM Tris-HCl (pH 7.5), 10 mM NaCl, 3 mM MgCl₂). Triton-X 100 was added to the cell suspension to a final concentration of 0.3% (v/v) and the cells were lysed on ice using a 1-ml Dounce homogenizer. The solution was centrifuged for 1 min at 10,000xg at 4 °C and the supernatants were layered on top of linear 15%–50% (w/v) sucrose gradients (11 ml). Centrifugation was carried out in a Beckmann SW41Ti Rotor at 36,000 r.p.m. for 2 h at 4°C. Polysome profiles were monitored by absorbance of light with a wavelength of 254 nm (A₂₅₄).

Quantitative PCR

Trizol reagent (Invitrogen) was used to extract RNA from 400 μ l polysome fractions from fibroblasts processed as described above. The RT reactions were performed with Superscript II (Invitrogen) using 1 or 5 μ l RT mix,. Quantitative PCR reactions were conducted using Sybr Green PCR MasterMix (Qiagen) on an ABI 7700 real time thermocycler. The primers used for PCR and RT are described above.

Lactate assay

40,000 cells were plated in a 6-well tissue culture plate (BD Biosciences); one day later, they were washed and suspended in 250 μ l PBS before addition of HCl to a final concentration of 0.03 N HCl. The cells were then frozen at -20 C until use.. Thawed cells were suspended in 50 μ l of 400 mM 2-amino-2-methylpropanol (Sigma), pH 9.9. A 4 ml assay cocktail containing 1 ml 400mM glutamate, pH 9 in 10mM PBS, 1 ml 15mM β -nicotinamide adenine dinucleotide (Sigma), pH6.5 in 10 mM PBS, 2 ml 10mM PBS. A typical assay included 40 μ l assay cocktail, 100 μ l sample or lactate standards (to a generate standard curve). Using a Tecan plate reader, absorbance of 340nm light prior to incubation with enzymes was performed. Twenty microliters of enzyme cocktail (200 U/ml L-lactate dehydrogenase and 80 U/ml glutamic-pyruvic transaminase in 10mM phosphate buffer, pH 6.5) was then added and the mixture was incubated 20 min at room temperature; absorbance at 340nm light was then measured.

Metabolic p53 labeling and immunoprecipitation

350,000 cells per 6mm plate were cultured for 30-45min in 5% dialyzed FCS in methionine and cysteine free media (Invitrogen); 140 μCi ^{35}S Met/Cys (ProMix, Amersham) was then added and the cells were cultured for 30 min. The radioactive media was removed and the cells cultured in DMEM with 10% FBS, DMEM and 2mM each of cysteine and methionine. The cells were then washed with 2 ml ice-cold PBS, centrifuged, and stored frozen until use. The cells were suspended in 100 μl 50 mM Tris, pH 7.5, 1% SDS, 5 mM DTT, boiled for 10 min and the insoluble material pelleted. To the supernatant was added 1.2ml 50 mM Tris, pH7.5, 250 mM NaCl, 5 mM EDTA, 0.5 NP-40, and protease inhibitors (complete protease inhibitor, BoehringerMannheim). A 50 μl slurry of protein A Sepharose was then added and rotated in cold room for 1 hour. The beads from this pre-clearing step were then removed and 1 μl p53 DO-1 antibody was added and the mixture rotated for 1 hr. in a cold room. A 50 μl slurry of protein A Sepharose was added to samples and rotated for 1 hour. The samples were then centrifuged briefly and to the supernatant was added additional p53 antibody and protein A Sepharose. The immunoprecipitation procedure was repeated two more times; the beads were then combined and resuspended with 100 μl SDS gel loading buffer for analysis by electrophoresis and phosphorimaging.

In other experiments, 3.5×10^6 cells were cultured in methionine and cysteine-free media as above, but were also treated with 50 μM MG132 for 1 hr. The cells were then labeled in ^{35}S methionine and cysteine-containing media for 15 min prior to p53 immunoprecipitation.

Acknowledgements

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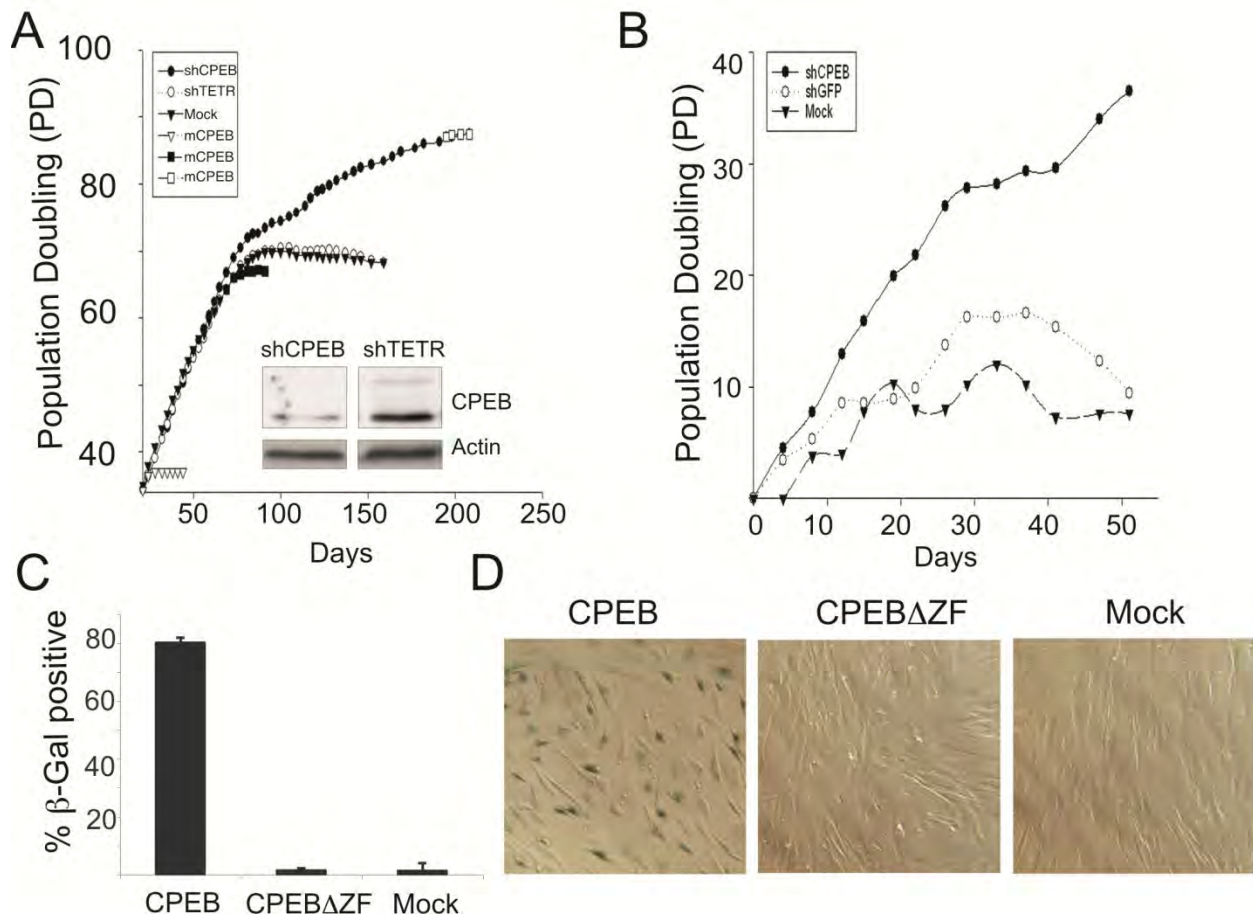


Figure 1. CPEB is necessary for cellular senescence.

Figure 1. CPEB is necessary for cellular senescence. (A) Human foreskin fibroblasts were infected with a lentiviruses encoding shRNA targeting CPEB mRNA (shCPEB), or the tetracycline resistance mRNA (shTETR), as a control. Additional cells were mock infected with an empty lentivirus. Population doublings were then determined by counting cells with a hemocytometer. Some cells were also infected with a retrovirus expressing mouse CPEB (because of differences in the primary sequence, mouse CPEB mRNA is not a target of the shCPEB), followed by cell counting. The inset shows a western blot probed for CPEB and actin; these extracts were prepared from cells 40 days post-lentivirus infection. (B) Human lung fibroblasts (WI-38) were infected with lentiviruses targeting CPEB or, as a control GFP; mock refers to infection with an empty virus. The cells were counted as in part A. (C and D) Foreskin fibroblasts previously infected with shCPEB were also infected with retroviruses expressing wild type CPEB or a CPEB lacking a zinc finger, which renders the protein incapable of RNA binding. Some cells were also mock infected. The cells were then stained for b-galactosidase at acid pH and were counted (panel C), and visualized by bright field microscopy three days after retrovirus infection (D).

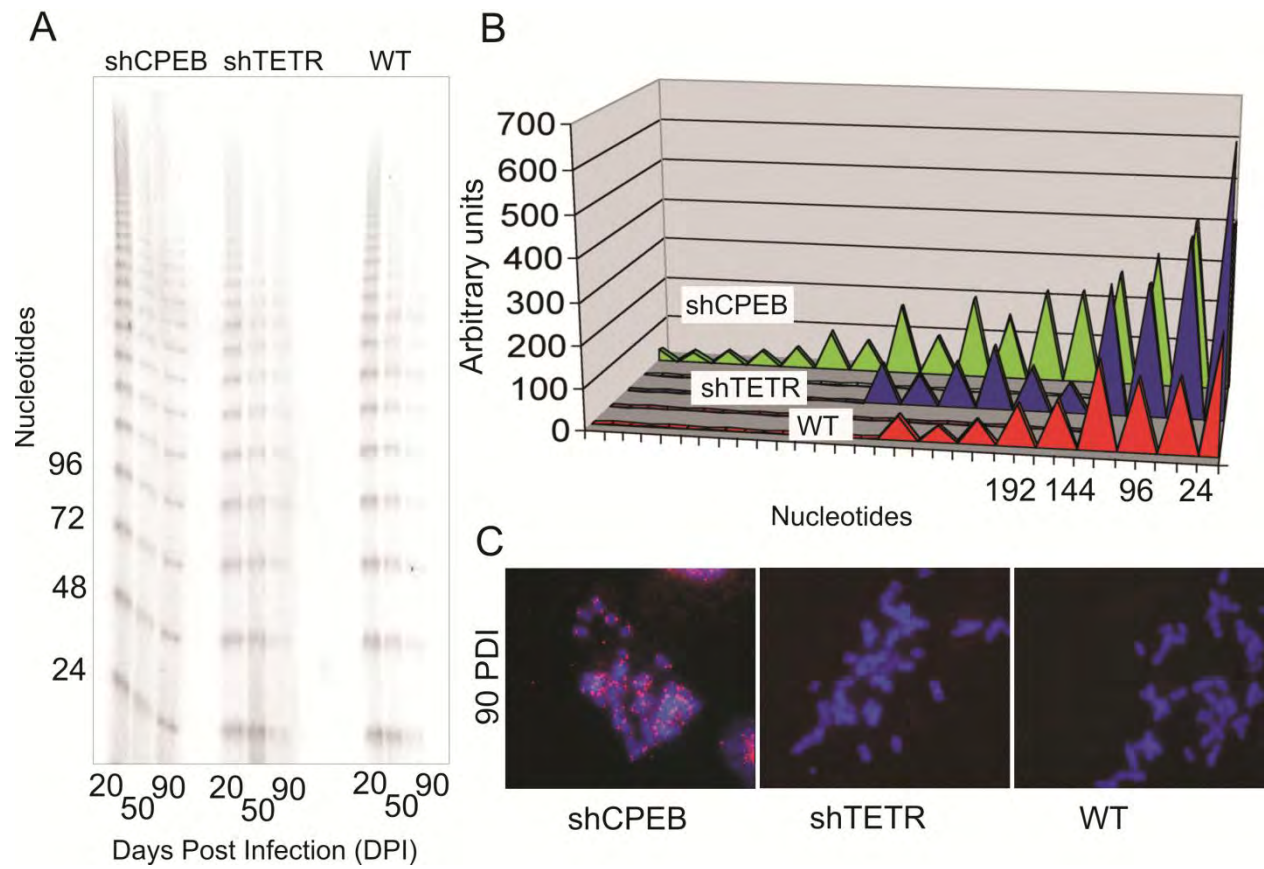


Figure 2. CPEB knockdown cells retain long telomeres

Figure 2. CPEB knockdown cells retain long telomeres. (A) DNA was extracted from skin fibroblasts infected with shCPEB and shTETR-containing lentiviruses 20, 50, or 90 days post-infection and used to determine telomere length by the telomere oligonucleotide ligation assay (TOLA). (B) The relative telomere lengths derived from the analysis in panel A were quantified by scanning desitometry. (C) shCPEB, shTETR, and noninfected wild type (WT) cells were fixed at 90 days post-infection and used for fluorescence in situ hybridization to telomeric regions (telo-FISH) using a Cy3-conjugated locked nucleic acid oligonucleotide (LNA). The DNA was stained with DAPI.

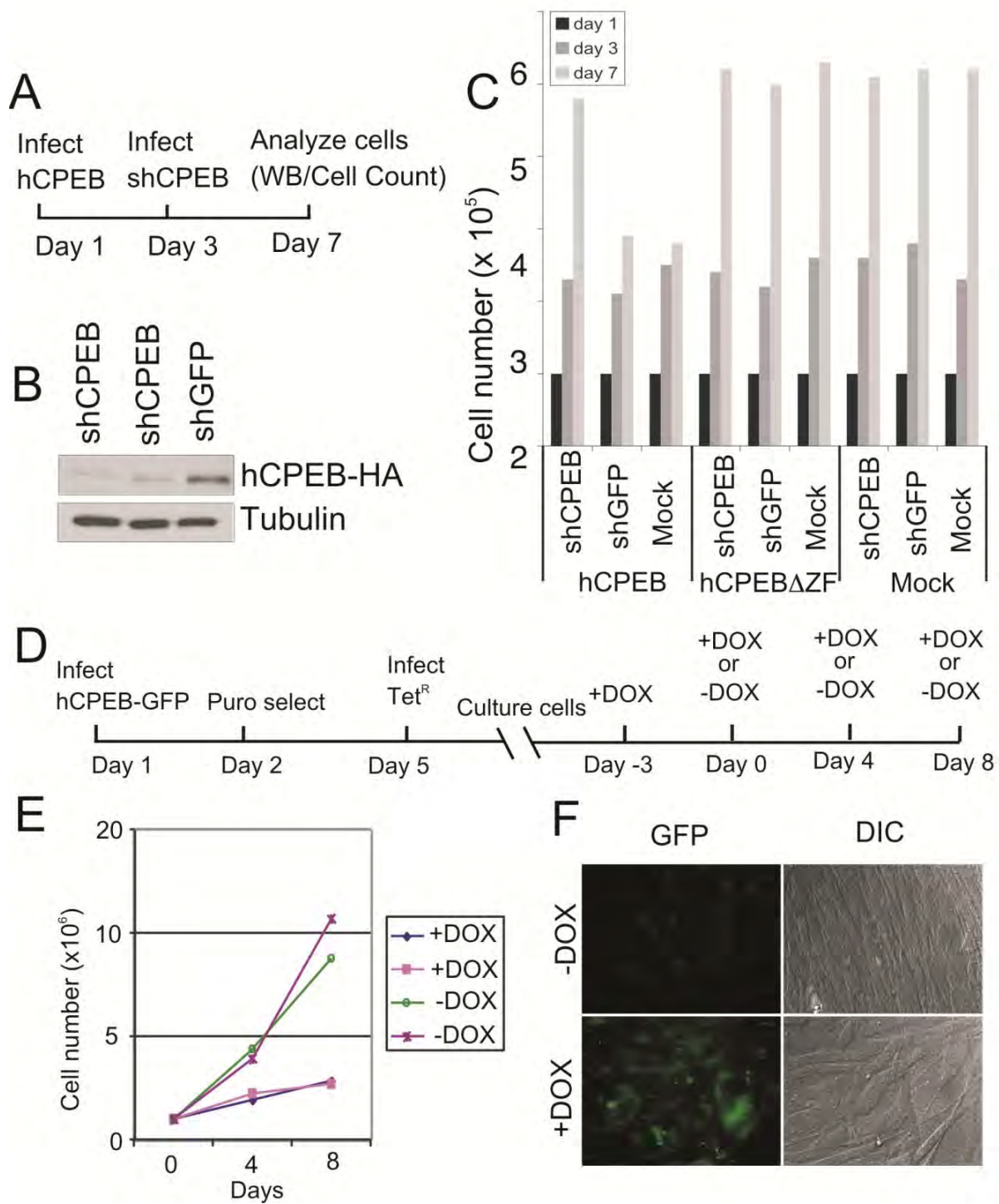


Figure 3. The CPEB-induced senescence-like phenotype is reversible.

Figure 3. The CPEB-induced senescence-like phenotype is reversible. (A) Schematic representation of one sequence of events used to examine the reversibility of the CPEB-induced senescence-like phenotype. (B) Western blot for CPEB and tubulin derived from fibroblasts that were sequentially infected with a retrovirus encoding HA-tagged human CPEB (hCPEB-HA) followed by a lentivirus encoding shCPEB (two different sequences) or shGFP. (C) Cell numbers were determined following sequential infection of the viruses noted in panel A. (D) Schematic representation of the experimental timeline when cells were infected with a retrovirus expressing CPEB under the control of the tetracycline repressor. Skin fibroblasts were infected with a virus expressing a CPEB-GFP fusion protein under the control of the Tetracycline Response Element (TET-ON), followed the next day by puromycin selection. Three days later, the cells were infected with a virus encoding the tetracycline repressor (TET^R); the cells were then cultured for several days in the presence of doxycyclin (DOX), which allows GFP-CPEB expression to remain high. Some cells were then cultured in medium lacking DOX, which will repress GFP-CPEB transcription. (E) Growth chart of 2 cell lines containing exogenous CPEB-GFP under control of the TET^r cultured in the absence or presence of DOX. (F) Live cell images of GFP-CPEB containing cells cultured in the absence or presence of DOX.

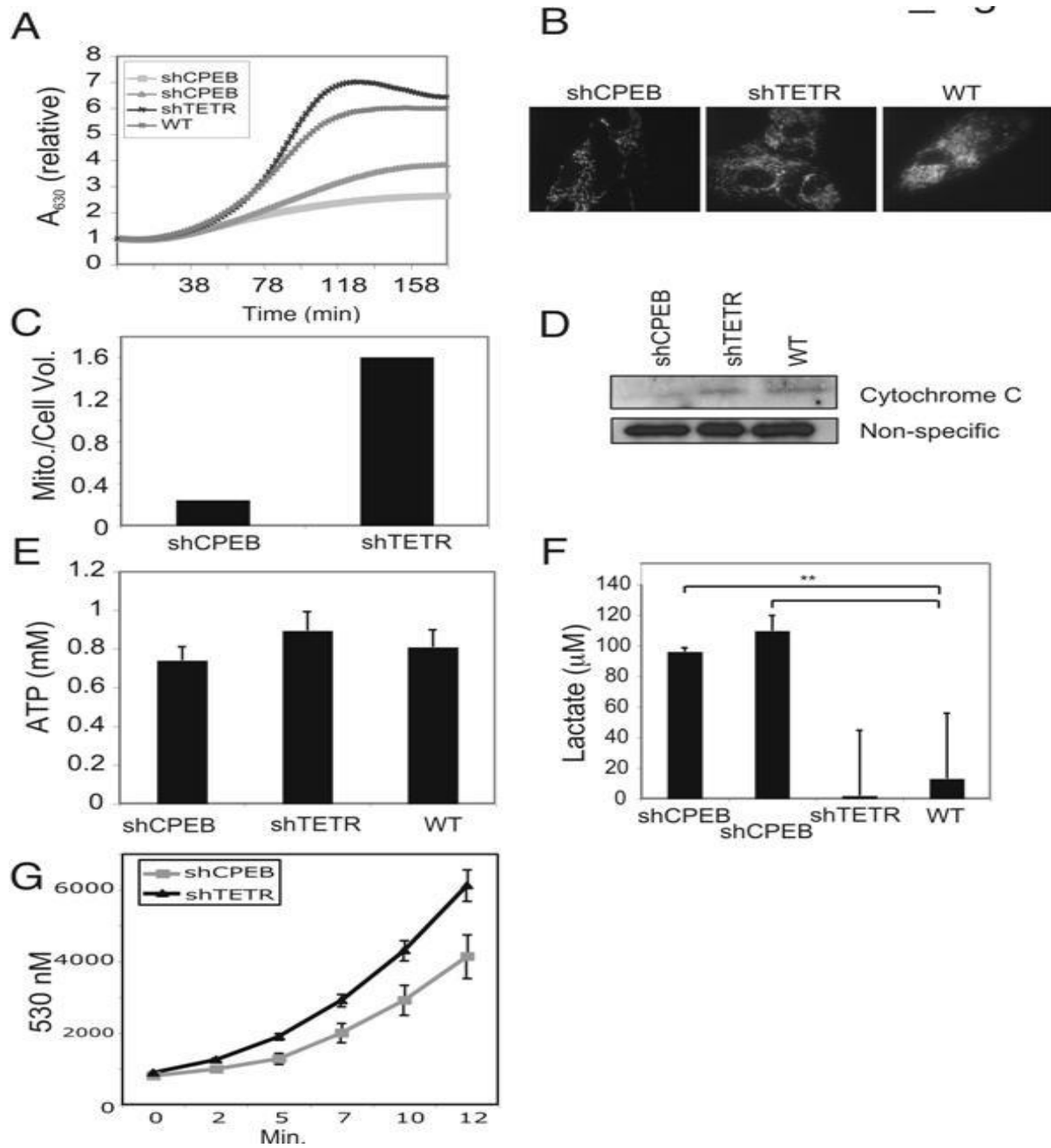


Figure 4. Reduced respiration and mitochondrial number in CPEB knockdown cells.

Figure 4. Reduced respiration and mitochondrial number in CPEB knockdown cells. (A) Fibroblasts infected with shCPEB (targeted to two different sequences), shTETR, or empty virus (mock) were cultured for approximately 47 days when they were used to measure oxygen consumption. (B) Z-plane stacks of confocal images obtained from live cells, some of which were infected with shCPEB or shTETR, stained with MitotrackerRed to visualize mitochondria. (C) Quantification of MitotrackerRed fluorescence from panel B. (D) Immunoblot of cytochrome C from WT, shTETR, and shCPEB infected cells. A nonspecific immuno-reactive band served as a loading control. (E) WT, shCPEB, and shTETR cells were used to measure ATP concentration. (F) Lactate, a indicator of glycolysis, was determined in shCPEB (targeting two different sequences) and control cells. (G) Determination of relative ROS levels in cells expressing shCPEB or shTETR.

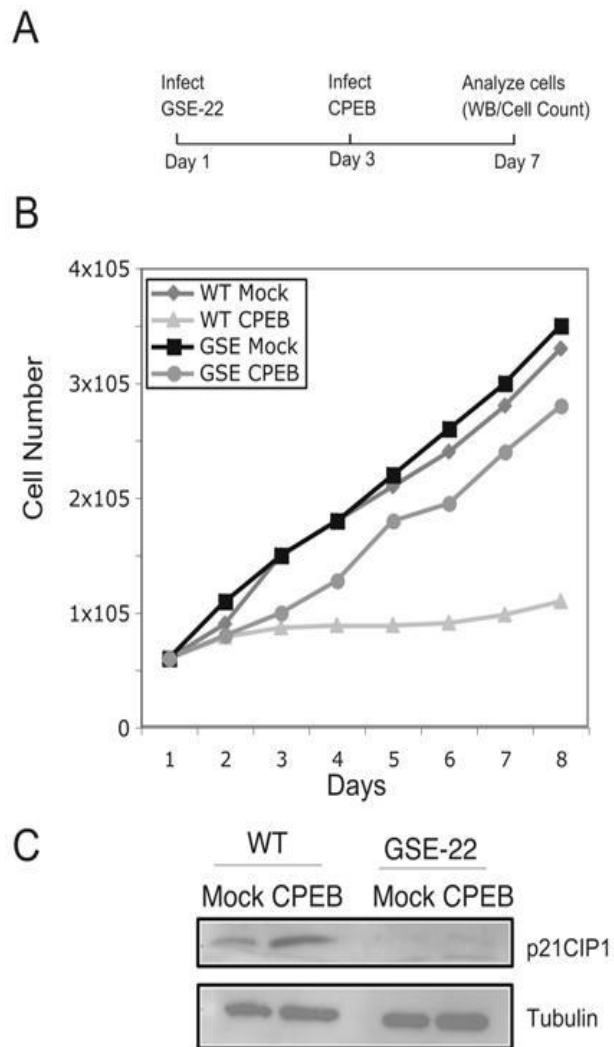


Figure 5. CPEB induced senescence requires p53.

Figure 5. CPEB induced senescence requires p53. (A) Fibroblasts were infected with a retrovirus expressing GSE-22, a p53 dominant negative peptide. Two days later, the cells were infected with a virus harboring CPEB; examination of the cells began on day 7. (B) Growth curves of GSE-22 or CPEB-infected or mock infected cells. (C) Western blot analysis of p21 and tubulin in infected or mock infected cells.

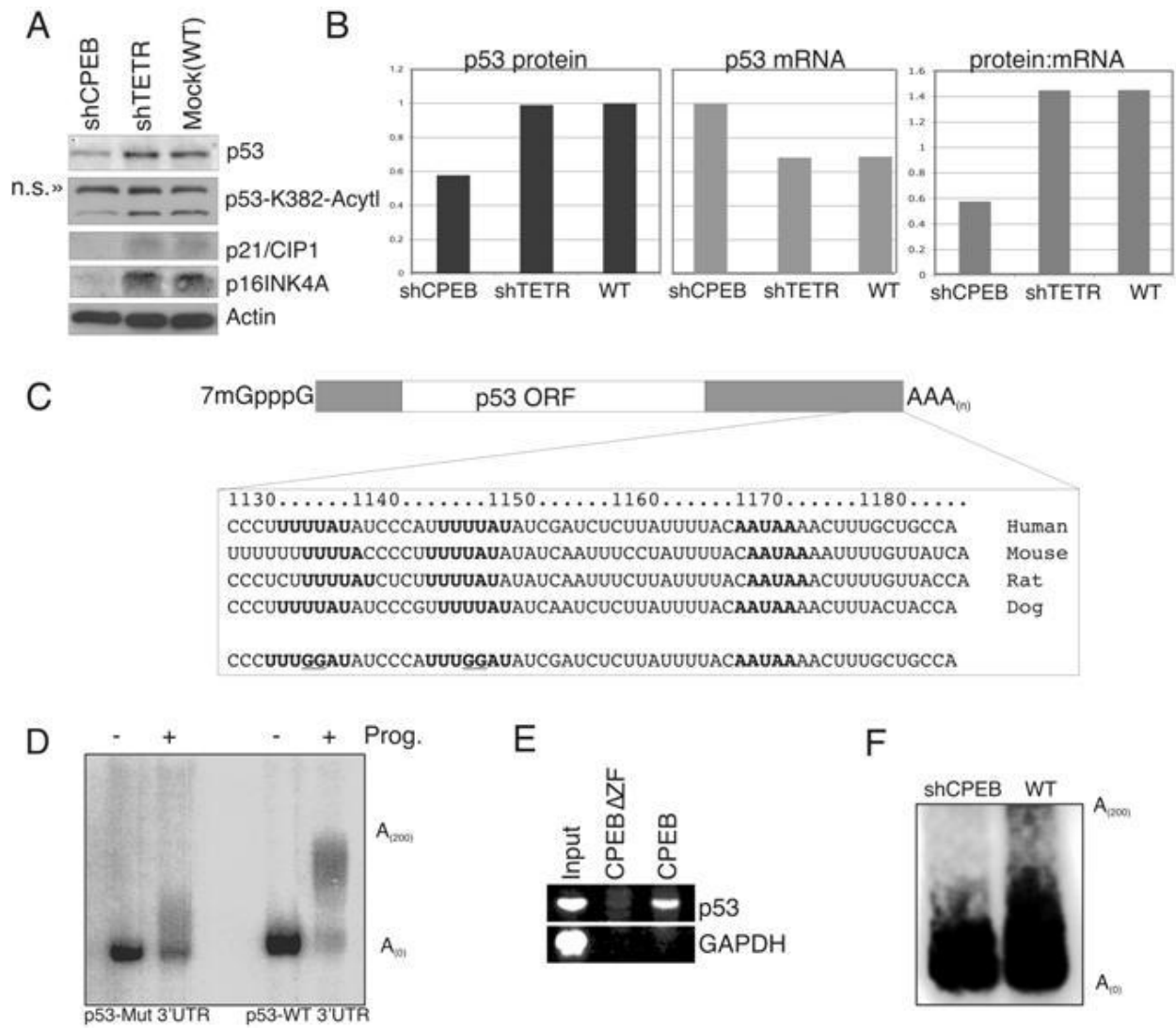


Figure 6. CPEB promotes cytoplasmic polyadenylation of p53 mRNA.

Figure 6. CPEB promotes cytoplasmic polyadenylation of p53 mRNA. (A) Extracts from shCPEB shTETR, or mock infected cells were probed with antibodies specific for p53, K382 acetylated p53, p21^{CIP1}, p16^{INK4A}, and actin. (B) The amount of p53 protein in panel A was quantified by densitometry, and the amount of p53 RNA in other infected cells was quantified by Q-RT-PCR; the ratios of these values was then plotted. (C) Diagram of the salient features of the p53 mRNA 3' UTRs from several mammals; the CPEs and AAUAAA hexanucleotide are in bold. The bottom sequence denotes dinucleotide substitutions in each of the two CPEs. (D) The human WT and mutated p53 3' UTRs were radiolabeled and injected into *Xenopus* oocytes, which were then treated with progesterone to induce meiotic maturation and cytoplasmic polyadenylation. (E) Fibroblasts infected with a retrovirus encoding HA-CPEB or HA-CPEB lacking the zinc finger were used for HA antibody co-immunoprecipitation and RT-PCR detection of p53 mRNA and the non-CPE-containing GAPDH mRNA. (F) Ligation-mediated polyadenylation test (LM-PAT) assay was used to estimate the poly(A) tail length of p53 mRNA in WT and shCPEB knockdown cells.

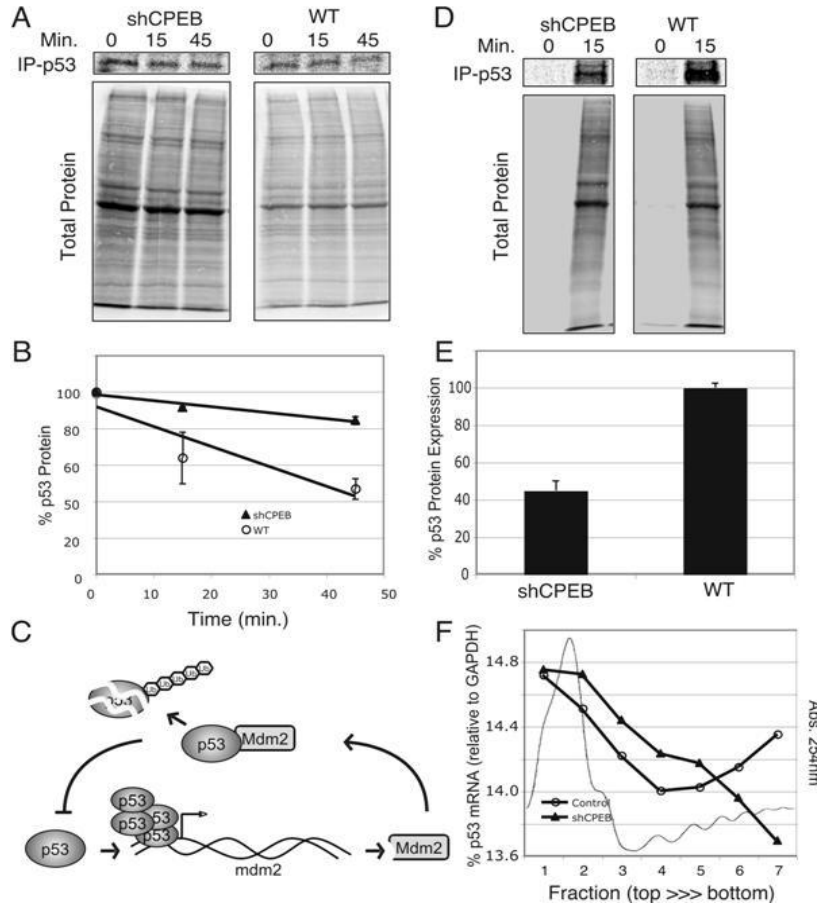


Figure 7. CPEB controls p53 mRNA translation.

Figure 7. CPEB controls p53 mRNA translation. (A and B) WT and shCPEB infected cells were incubated in methionine and cysteine-free medium for 30 minutes, followed by an incubation with ^{35}S -methionine and ^{35}S -cysteine for 30 minutes and then an incubation of up to 45 minutes with excess radioinert methionine and cysteine. p53 was immunoprecipitated from the extracts at 0, 15, and 45 minutes of the amino acid chase and resolved by SDS-PAGE, as was total cell protein. (C) Diagram illustrating a feedback loop where high levels of p53 induce transcription of the E3 ligase mdm2, which in turn induces p53 destruction. Ub refers to ubiquitin. (D and E) WT and shCPEB infected cells were incubated in methionine and cysteine free media for 1 hour followed by incubation in ^{35}S -methionine and ^{35}S -cysteine for 15 minutes. p53 was then immunoprecipitated and analyzed by SDS-PAGE and phosphorimaging. The quantification of p53 levels in three different experiments is shown. (F) Extracts from WT and shCPEB-infected fibroblasts were centrifuged through 15-50% sucrose gradients, fractionated, and scanned with 254 nm light. The relative amounts of p53 and GAPDH mRNAs were quantified by Q-RT-PCR. A representative polysome profile (absorbance at 254 nm light) from wild type cells is shown.

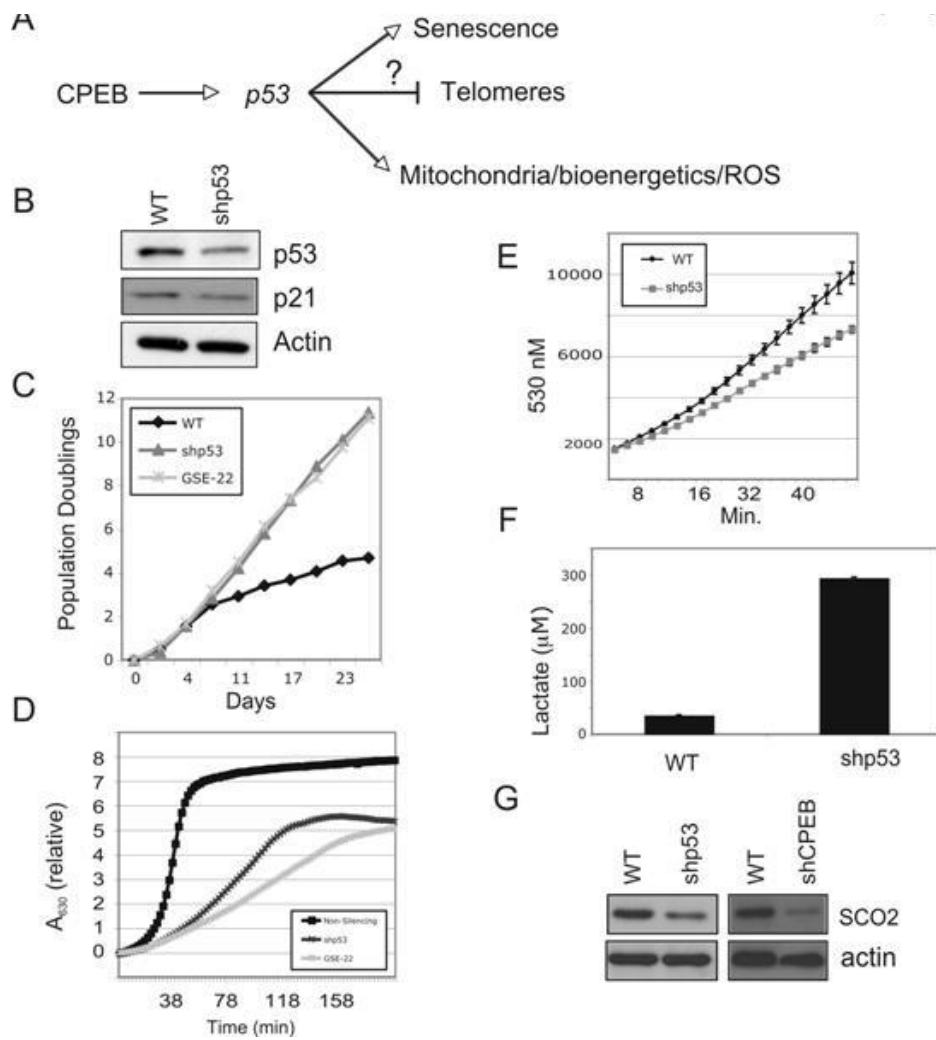
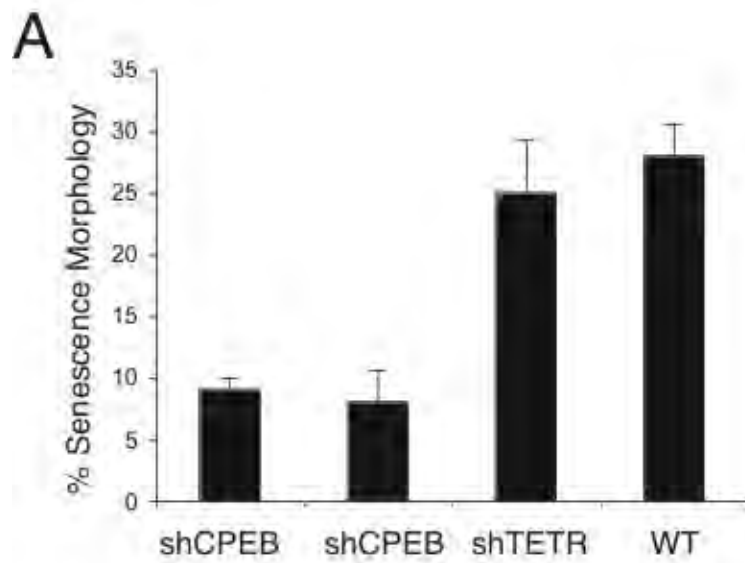
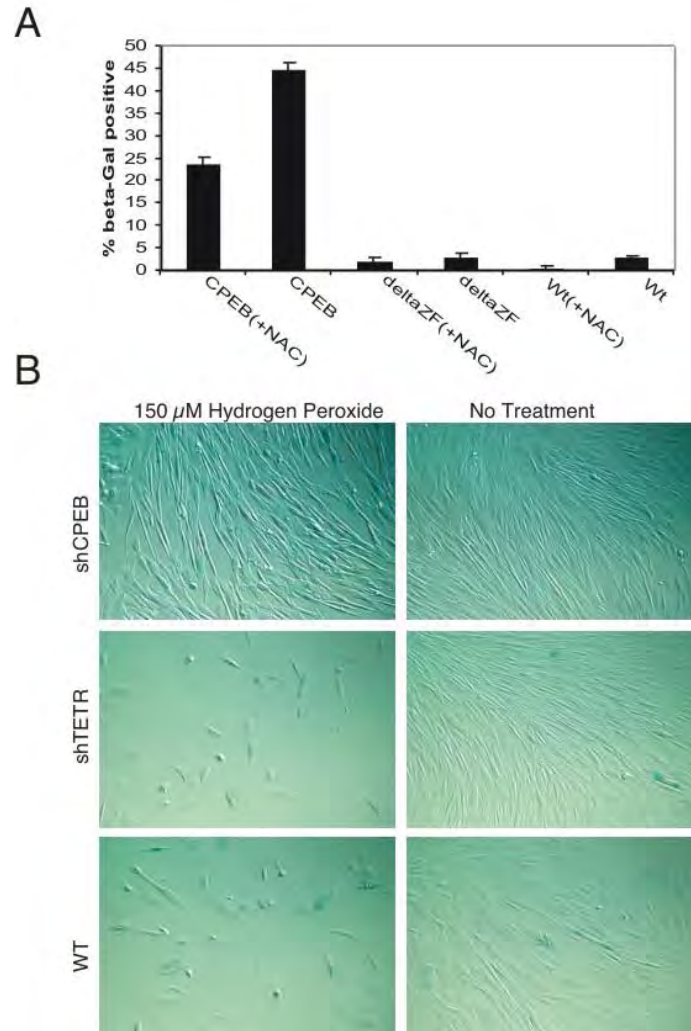


Figure 8. p53 regulation of senescence and bioenergetics.

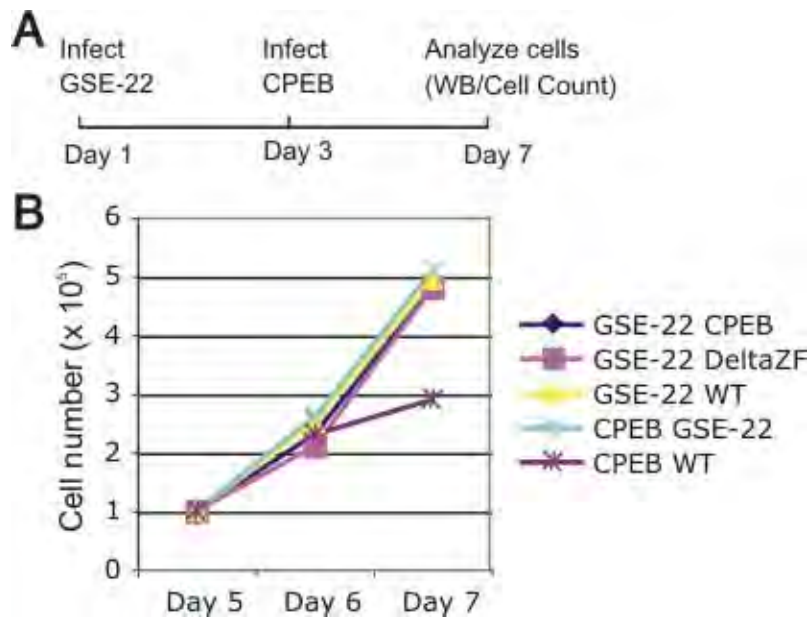
Figure 8. p53 regulation of senescence and bioenergetics. (A) Proposed pathway in which CPEB, at least in part, influences senescence, telomere maintenance, and bioenergetics. (B) Western blot showing a shRNA-directed~50% knockdown of p53, which inhibits the expression of p21. (C) Growth curves of wild type cells or cells infected with shp53 or GSE-22. (D) Oxygen consumption in cells infected with a non-silencing shRNA, shp53 RNA, or GSE-22, a p53 inhibitory peptide. (E) Relative ROS levels in wild type or shp53 infected cells. (F) Levels of lactate in wild type and shp53 knockdown cells. (G) Western blot of SCO2 in CPEB and p53 knockdown cells.



Supplementary Figure 1. Ras requires CPEB to induce senescence. (A) Cells passaged for 32 days were infected with a retrovirus containing RASV12; two days post-infection, GFP-positive cells were scored for flat senescent-like morphology.



Supplementary Figure 2. Oxidative stress-induced premature senescence requires CPEB. (A) primary human foreskin fibroblasts were mock infected or infected with retroviruses expressing CPEB or CPEB Δ ZF. The resulting cultures were then split and sister cultures were treated with 200 μ M N-acetyl cysteine (NAC). The cells were then stained for SA- β -galactosidase activity and counted. (B) WT and shCPEB and shTETR infected cells were treated with 150 μ M H₂O₂ for two hours after which they were washed with PBS and grown in normal growth media overnight. The cells were then stained for SA- β -galactosidase activity.



Supplementary Figure 3. CPEB requires p53 activity to induce senescence. (A) Schematic diagram to describe the order in which cells were infected with different viruses. Human fibroblasts were infected with a lentivirus encoding GSE-22, a dominant negative form of p53, or CPEB. The cells were then infected two days later with other viruses encoding CPEB or GSE-22. (B) Growth chart demonstrating that two cell lines continue to proliferate irrespective of when they express CPEB.

CHAPTER 3

Gld2 Regulation of miR-122 Stability Mediates CPEB/Gld4 Control of p53 mRNA

Polyadenylation-Induced Translation

David Burns, Andrea D'Ambrogio, Stephanie Nottrott, and Joel D. Richter

Program in Molecular Medicine University of Massachusetts Medical School Worcester, MA
01605

[*Running head:* polyadenylation regulatory cascade]

[*Key words:* CPEB; senescence; polyadenylation; translation; p53; Gld4]

Correspondence to:

Dr. Joel D. Richter

Program in Molecular Medicine

University of Massachusetts Medical School

373 Plantation Street, Suite 204

Worcester, MA 01605

Tel: (508) 856-8615; fax: (508) 856-4289; email: joel.richter@umassmed.edu

Author Contributions

All experiments performed by D.M.B except otherwise noted. A.D. performed co-immunoprecipitations of CPEB and Gld-4. S.N. designed and constructed luciferase reporter constructs. D.M.B and J.D. R. designed experiments and wrote the manuscript.

SUMMARY

The cytoplasmic polyadenylation element binding protein (CPEB) nucleates a complex of factors on specific mRNA 3' untranslated regions to regulate polyadenylation-induced translation. Gld2 is a non-canonical poly(A) polymerase that interacts with CPEB to catalyze polyadenylation. Because p53 mRNA polyadenylation/translation is controlled by CPEB in primary human diploid fibroblasts, we surmised that Gld2 would be the enzyme responsible for poly(A) addition. Surprisingly, depletion of Gld2 promoted p53 mRNA polyadenylation/translation, as well as enhanced the stability of CPEB mRNA. Using mRNA reporters containing the CPEB 3' UTR, two sequences, which correspond to miR-122 binding sites, were found to regulate translation, as did an antagomir of miR-122. Although miR-122 is thought to be liver-specific, it is present in human primary fibroblasts but destabilized by Gld2 knockdown. Further experiments demonstrate that Gld4 is the CPEB-associated poly(A) polymerase that regulates p53 mRNA polyadenylation/translation. Thus, p53 mRNA translational homeostasis is maintained by a regulatory hierarchy composed of Gld2/miR-122/CPEB/Gld4.

INTRODUCTION/RESULTS

Cytoplasmic polyadenylation-induced translation controls germ cell development (Mendez et al. 2000a; Tay and Richter 2001), neuronal synaptic plasticity (Wu et al. 1998; Alarcon et al. 2004; Zearfoss et al. 2008), and cellular senescence (Groisman et al. 2006; Burns and Richter 2008), a tumor-suppressor mechanism that limits the replicative lifespan of cells (Stewart and Weinberg 2006; Campisi and d'Adda di Fagagna 2007). Although polyadenylation is mediated by several factors three core ones are CPEB, a sequence-specific RNA binding protein that specifies which mRNAs undergo polyadenylation; Gld2, a poly(A) polymerase, and PARN, a deadenylating enzyme. Poly(A) tail length is governed by the interaction of PARN with the ribonucleoprotein (RNP) complex: when PARN is associated with the RNP, the poly(A) tail is short because its deadenylating activity overrides the polyadenylating activity of Gld2. When PARN dissociates from the RNP complex following signal-induced CPEB phosphorylation, the poly(A) tail is lengthened by constitutively-active Gld2 (Barnard et al. 2004; Kim and Richter 2006; Kim and Richter 2007).

Mouse embryo fibroblasts (MEFs) derived from CPEB knockout (KO) mice do not senesce as do MEFs derived from wild type (WT) mice, but instead are immortal. Senescence is rescued when ectopic CPEB is expressed in the KO MEFs and potentiated when expressed in WT MEFs (Groisman et al. 2006). Human foreskin fibroblasts depleted of CPEB bypass senescence and divide ~270 days compared to WT cells that senesce after about 90 days. As with the mouse cells, ectopic expression of CPEB rescues senescence in knockdown cells and potentiates senescence in WT cells. CPEB controls the polyadenylation-induced translation of p53 mRNA, and indeed CPEB-induced senescence requires p53. Depletion of CPEB also

induces the “Warburg Effect” where mitochondrial respiration is reduced and cells produce ATP primarily through glycolysis (Burns and Richter 2008).

To investigate the possibility that CPEB control of p53 polyadenylation would require Gld2, human primary foreskin fibroblasts were stably transduced with lentiviruses expressing two different shRNAs against the Gld2 coding sequence. Surprisingly, Gld2 depletion (Fig. 1a, 1b) elicited an increase in both p53 protein levels (Fig. 1c) and p53 mRNA polyadenylation (Fig. 1d). Also unexpectedly, depletion of Gld2 resulted in increased oxygen consumption (Fig. 1e) and entry into a senescence-like cell cycle arrest as evidenced by β -galactosidase staining at acid pH (Fig. 1f). In comparison, CPEB depleted cells had decreased oxygen consumption, fewer cells staining with β -galactosidase, increased lifespan, and most importantly, reduced poly(A) tail size on p53 mRNA and a 50% reduction in p53 protein levels (Burns and Richter 2008).

These paradoxical results prompted us to examine CPEB levels in Gld2 depleted cells because CPEB is required for normal p53 mRNA translation (Burns and Richter, 2008). After comparing the levels of CPEB nuclear pre-mRNA by intron-specific qPCR and mostly cytoplasmic mRNA by exon-specific qPCR, we found that although the pre-mRNA levels, which generally reflect transcription, were nearly unchanged, cytoplasmic mRNA levels increased by about two-fold (Fig. 2a). Thus, in the absence of Gld2, CPEB mRNA unexpectedly was more stable.

Surmising that Gld2 might control p53 protein levels via CPEB, we next used a Renilla luciferase (RLuc) and firefly luciferase (Fluc) reporter system to investigate post-transcriptional regulation of CPEB by Gld2. As shown in Figs. 2b and 2c, a knockdown of Gld2 resulted in nearly an 80% increase in RLuc expression as well as when the 3′ most 455 nucleotides of the CPEB 3′ UTR were deleted. By comparison, expression of the reporter harboring either the full

length CPEB 3' UTR or a 3' deletion was unaffected in Gld2 knockdown cells (Fig. 2c).

Additional serial deletions of the distal CPEB 3' UTR demonstrated that there were two regions, encompassing nucleotides 300-480 and 480-560, which elicited increases in reporter translation following Gld2 knockdown; moreover, the repression mediated by these two regions was additive (Fig. 2d).

Analysis of the two regions of the CPEB 3' UTR that mediated translational repression by Gld2 revealed the presence of two potential miR-122 binding sites (Supplemental Figure 1). Although miR-122 is thought to be liver-specific and account for ~70% of the total population of microRNAs in that tissue (Lagos-Quintana et al. 2002), deletion of these specific sites, either individually or combined, nonetheless alleviated translational repression in a dose-dependent manner in Gld2 depleted cells (Fig. 3a), and were nearly identical to that observed with the large deletions (Fig. 2d). These results suggest that miR-122 might repress CPEB mRNA translation in human skin fibroblasts and indicate that this miRNA might be more widely distributed than originally thought. Indeed recent evidence shows that miR-122 is present in human skin (Holst et al. 2010) and even HEK293 cells (Liao et al. 2010).

To assess directly whether miR-122 might repress CPEB mRNA expression, cells were electroporated with a locked nucleic acid (LNA) antagomir for miR-122, or as a control, a scrambled LNA. The miR-122 antagomir enhanced reporter expression by nearly 4.5 fold relative to control (Fig. 3b). Importantly, when miR-122 LNA antagomir-transduced cells were pulsed labeled with ³⁵S-methionine for 15 min followed by p53 immunoprecipitation and analysis by SDS-PAGE and phosphorimaging, there was a two-fold increase in the synthesis of p53 protein (Fig. 3e). These data indicate that human primary skin fibroblasts contain miR-122 and that Gld2 controls its steady state levels or activity. Fig. 3c indeed demonstrates that these

cells do contain miR-122 and that Gld2 knockdown reduces the level of this miRNA by nearly 40 fold. These results are consonant with those of Katoh et al (2008), who demonstrated that in liver, Gld2 is essential for miR-122 stability.

While consistent with the hypothesis that miR-122 mediates p53 mRNA translation via CPEB, these data do not eliminate the possibility that miR-122 could act via another molecule to regulate p53 synthesis (note that p53 mRNA has no miR-122 site according to Targetscan.org or Microna.org). Consequently, we infected cells with a lentivirus expressing shRNA for CPEB as well as the miR-122 antagomir followed by a 15 minute pulse of ³⁵S-methionine pulse and p53 immunoprecipitation. Fig. 3f shows that although miR-122 antagomir alone induced an increase in p53 synthesis, the antagomir plus shRNA for CPEB elicited to increase. Taken together, these data demonstrate that Gld2 activity stabilizes miR-122, which in turn reduces CPEB expression; CPEB then acts directly on p53 mRNA to mediate poly(A) tail length and translation.

If not Gld2, what poly(A) polymerase works with CPEB to modify p53 mRNA polyadenylation and translation? We thought that a non-canonical poly(A) polymerase, i.e., one that lacks an RNA binding domain and thus would require another factor such as CPEB to be tethered to the RNA, would most likely be involved. Two cytoplasmic enzymes have this characteristic, Gld4 (PAPD4) (Schmid et al., 2008) and MitoPAP (PAPD1) (Mullen and Marzluff, 2008). Both polymerases were depleted with shRNAs (data not shown), but only the loss of Gld4 reduced p53 translation (Fig. 4a) and polyadenylation (Fig. 4d). Moreover, ectopically-expressed Gld4-Flag and CPEB-HA co-immunoprecipitate together (Fig. 4c). Importantly, Gld-4-flag immunoprecipitates p53 mRNA in knockdown control cells (shTETR) but not in CPEB knockdown cells (Fig. 4b).

DISCUSSION

The polyadenylation of almost all mRNAs occurs after 3' poly(A) site selection and cleavage in the nucleus (Mathews et al. 2007). However, as exemplified by the *Xenopus* oocyte maturation, the poly(A) tail shortening and lengthening is a crucial strategy to regulate translation of specific mRNAs (Mendez and Richter 2001). One such mechanism that affords post-transcriptional regulation through poly(A) tail shortening and lengthening is mediated through a complex of proteins that, at its core, has the CPEB RNA-binding protein (Richter 2007). Much is known about how CPEB and the associated factors deadenylate and polyadenylate specific mRNAs in the *Xenopus* oocyte (Richter 2007). However, little is known about how this feat is accomplished in higher vertebrate metazoans. Because our laboratory recently identified a biologically relevant and important target of the CPEB-polyadenylation complex during senescence onset (Burns and Richter 2008), we sought to further characterize the mechanism of how CPEB might add adenylates to mRNAs. Starting by knocking down the previously identified poly(A) polymerase xGld-2 (Barnard et al. 2004), we unexpectedly find a complex translational control cascade involving miR-122 control of CPEB mRNA translation and identify a compensatory or alternative non-canonical poly(A) polymerase hGld-4 (Schmid et al. 2009) required for p53 mRNA polyadenylation.

After the unexpected finding that hGld-2 knockdown increased p53 polyadenylation and translation, we wondered why hGld-2, which is co-expressed with CPEB mRNA, is not in complex with CPEB? There are at least three possibilities. Firstly, Gld-2 could be unavailable, found in alternative complexes with other RNA-binding proteins or retained in the nucleus (data not shown) to function in alternative complexes. Consistent with this idea, is the finding that

CPEB4 can co-immunoprecipitate xGld-2 in *Xenopus* extracts (Igea and Mendez 2010). Secondly, perhaps xGld-2 is not as conserved in the way we might have predicted (Igea and Mendez 2010). Consistent with this possibility is that xGld-2 shares only ~40% amino acid identity with its human orthologue. Thirdly, perhaps Gld-2 is post-translationally modified in ways that exclude it from CPEB-complex in somatic cells versus early embryonic cells. The elucidation of Gld-2 function in human somatic cells awaits further experimentation.

Why would miR-122 regulate CPEB mRNA translation? Translational control cascades are commonplace when rapid and coordinated cytoplasmic events occur as illustrated the process of meiotic resumption in the *Xenopus* oocyte (Vasudevan et al. 2006). Perhaps coordinated p53 mRNA translation ensures a rapid response by increasing p53 protein levels sufficient to enact cell cycle arrest during cell division when ongoing transcription is absent. Also, post-transcriptional regulation by various RNA-binding proteins could combinatorially organize and regulate p53 mRNA specifically to promote translation in opposition to other CPEB target mRNAs. Along these lines, the Filipowicz group has demonstrated that ectopic Hu antigen R (HuR), an AU-rich RNA binding protein, is able to antagonize miR-122 function causing the derepression of CAT-1 reporter mRNA (Bhattacharyya et al. 2006). Interestingly, HuR has been linked to p53 mRNA stability (Gorospe 2003; Mazan-Mamczarz et al. 2003). Perhaps the 3'-UTR is more intricately involved in the translation of p53 mRNA than has been fully realized.

As the Suzuki laboratory first demonstrated, and we confirm here, Gld-2 is required for the stabilization of miR-122 and its normal expression (Katoh et al. 2009). We focus on miR-122 as being Gld-2 regulated, but are struck by the lack of data on other microRNAs that are similarly regulated and wonder if the adenylation of small non-coding RNAs is more common and widespread than we think? Recent deep sequencing results of small RNAs demonstrate that

approximately 20% of all RNA-seq reads from cloned neuroblastoma miRNAs have a non-templated adenylate in the first position, suggests it is more than probable (Schulte et al. 2009). While stabilizing microRNAs by non-template adenylate addition is a feasible strategy to regulate mRNA translation and/or mRNA stability, how and why Gld-2 polymerase activity is limited to add only one adylate to the 3-prime end of microRNAs is unknown. The mechanism of Gld-2 adenylate addition awaits further experimentation.

METHODS

Cells and culture conditions

Primary human foreskin fibroblasts were obtained from the Cell Culture Core Facility of the Yale University Skin Disease Research Center and cultured as described (Rangarajan et al. 2004) in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum. About 10^6 cells, counted with a hemocytometer following treatment with trypan blue, were passaged every three days. Human lung WI-38 fibroblasts were cultured in a similar manner.

Virus production, infection, and cell lines

Amphotropic retroviruses and lentiviruses were produced by transient transfection of 293T cells with a transfer vector and amphotropic packaging plasmids encoding VSV-G and gag-pol using Lipofectamine 2000 (Invitrogen). Human cells at 50% confluency were infected for 8-12 hr with viral supernatants containing $7\mu\text{g/ml}$ polybrene. Typically 70-90% infection efficiency was achieved as assessed by using a GFP-encoding viral gene or by immunostaining cells using anti-HA (Covance). After infection, fresh media was added to the infected fibroblasts.

Some cells were analyzed by western blotting for, p53 (DO-1, Neomarkers), and b-actin (Abcam). Other cells were fixed with 0.2% glutaraldehyde and stained for b-galactosidase activity at acidic pH according to Dimri et al (1995).

Oxygen Consumption

To measure oxygen consumption, $\sim 4 \times 10^5$ cells were washed and resuspended in 200 ml Krebs-Ringers solution plus HEPES (125 mM NaCl, 1.4 mM KCl, 20 mM HEPES, pH 7.4, 5 mM NaHCO₃, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 1 mM CaCl₂) containing 1% BSA. Cells from each condition were aliquoted into a BD Oxygen Biosensor System plate (BD Biosciences) in triplicate. Plates were assayed on a SAFIRE multimode microplate spectrophotometer (Tecan) at 1-minute intervals for 60 minutes at an excitation wavelength of 485 nm and emission wavelength of 630 nm.

Analysis of p53

Control and shCPEB infected fibroblasts were cultured in methionine and cysteine-free media (Invitrogen) for 45 min and then cultured in media containing 140 mCi ³⁵S methionine and ³⁵S cysteine (ProMix, Amersham) for 30 min. The cells were then washed and cultured in fresh DMEM supplemented with 2 mM each of methionine and cysteine for the times indicated. The cells were then frozen and stored until they were lysed and used to immunoprecipitate p53 (DO-1 antibody, Neomarkers), which was analyzed by SDS-PAGE and phosphorimaging.

Additionally, Cells were cultured in methionine/cysteine free media as noted above in the presence of MG132, a proteasome inhibitor, for 30 min., followed by a 15 min culture in 100

$\mu\text{Ci } ^{35}\text{S}$ methionine and cysteine; p53 was then immunoprecipitated and analyzed as noted above.

FIGURES

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Figure 1. Depletion of Gld2 enhances p53 expression.

Figure 1. Depletion of Gld2 enhances p53 expression. (a) RT-PCR of Gld2 and tubulin RNAs following infection of human foreskin fibroblasts with a lentivirus containing an shRNA against Gld2 (b) Knockdown of Gld-2-HA in cells infected with a retrovirus expressing Gld2-HA and a lentivirus expressing shRNA for Gld2. Tubulin served as a loading control. (c) Western blot showing enhanced expression of p53 following knockdown of Gld2. (d) Ligation-mediated polyadenylation test (LM-PAT) assay was used to estimate the poly(A) tail length of p53 mRNA in WT cells, shGld-2 knockdown cells (two shRNAs targeting different regions of Gld2 were used), cells expressing ectopic CPEB, and cells expressing ectopic that lacks a zinc finger and hence is unable to bind RNA (CPEB Δ ZF) (Burns and Richter, 2008). (e) Oxygen consumption in cells infected with shCPEB, shGld-2, or empty vector. (f) Mock or shGld2 infected cells were stained for β -galactosidase at acidic pH, which denotes cellular senescence. Cell number was also determined with a haemocytometer, population doublings were plotted as growth curves of wild type cells or cells infected with shGld2.

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Figure 2. Gld2 knockdown increases CPEB mRNA and translation by a post-transcriptional mechanism.

Figure 2. Gld2 knockdown increases CPEB mRNA and translation by a post-transcriptional mechanism. (a) Fold change of nuclear (intron-containing) or predominantly cytoplasmic (exon-containing) CPEB following Gld2 depletion. Actin RNA was used to normalize qRT-PCR data. (b) A schematic of Rluc-CPEB-3'-UTR reporter constructs used in the following experiments. (c and d) Cells were infected with plasmids encoding firefly luciferase as a control and the Renilla luciferase plasmids noted in panel B. The cells were then depleted of Gld2 and the amount of Renilla luciferase, relative to firefly luciferase, was determined. The amount of Renilla luciferase activity derived from RNA containing the entire CPEB 3'-UTR was arbitrarily set at 100.

Figure 3. miR-122 activates p53 mRNA translation by repressing CPEB mRNA expression.

Figure 3. miR-122 activates p53 mRNA translation by repressing CPEB mRNA expression. (a) Human skin fibroblasts were transduced with firefly luciferase as an internal standard and Renilla luciferase appended with the full length CPEB 3' UTR or UTRs containing deletions in putative miR-122 binding sites. The data are expressed as described in Figure 2. (b) Skin fibroblasts were transduced with plasmids encoding firefly and Renilla luciferase containing the full length CPEB 3' UTR and then electroporated with miR-122 LNA antagomir, an LNA with a scrambled sequence, or no LNA. The luciferase data are expressed as described in Figure 2. (c) mir-122 expression levels were measured using qPCR for the mature mir-122 and normalized to actin mRNA expression levels. (d and e) Skin fibroblasts transduced with Mock-GFP, miR-122 LNA, or scrambled LNA were incubated in methionine and cysteine-free medium containing the proteasome inhibitor MG132 for 30 minutes, followed by a pulse incubation with ³⁵S-methionine for 15 min. p53 protein was then immunoprecipitated from these cells and resolved by SDS-PAGE, as was total cell protein. Gels were exposed to phosphor-imaging screens and quantified using ImageQuant software. (f) Skin fibroblasts were treated as in panels c and d after first being infected with lentiviruses expressing either shRNAs against the TET repressor as a control or CPEB.



Figure 4. Gld-4 is an alternative poly(a) polymerase required for normal p53 polyadenylation.

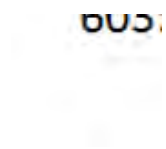
Figure 4. Gld-4 is an alternative poly(a) polymerase required for normal p53 polyadenylation.

(a) Western blot of p53 from normal hFS cells that express either shGld-4 or shMitoPAP. (b) p53 mRNA is immunoprecipitated by Gld-4-flag from cells which have been infected with a lentivirus expressing either knockdown control (shTETR) or CPEB knockdown. GAPDH is used as a negative control as it lacks CPEs. (c) Co-immunoprecipitations from (hFS or HeLa) cells transfected with Gld-4-Flag and CPEB-HA constructs. (d) LM-PAT from either Mock or shGld-4 infected cells. The poly(a) tail length is quantitated for Mock and shGld-4 treated cells. (e) semi-quantitative RT-PCR amplification of p53-exon specific or p53-intron specific in Mock, shGld-4 or shMitoPap cells.

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Figure 5. Proposed model of Gld-2 stabilization of mir-122, CPEB mRNA repression, and reduced p53 polyadenylation

Figure 5. Our findings suggest a model where Gld-2 adenylates miR-122 thereby stabilizing miR-122 and repressing the translation of CPEB mRNA. If miR-122's stability is affected by the loss of Gld-2, CPEB mRNA is derepressed and translated where it can then bind p53 mRNA to polyadenylate p53 mRNA by Gld-4.



Supplemental Figure 1. MiR-122 binding sites on the 3' UTR of CPEB mRNA

CHAPTER 4

GENERAL DISCUSSION, CONCLUSION AND PREDICTIONS

In the previous chapters, I have presented evidence for the requirement of CPEB and cytoplasmic polyadenylation for the onset of senescence and p53 mRNA polyadenylation. The finding that CPEB is required for the normal polyadenylation of p53 mRNA is significant for two major reasons; i) p53 is a tumor suppressor protein commonly mutated in ~50% of all human cancers, and ii) p53 is a crucial regulator of the response to DNA damage and other genotoxic stresses such as reactive oxygen species (ROS). I have also demonstrated that CPEB, surprisingly, does not require the non-canonical Gld-2 polyadenylation enzyme, but instead requires Gld-4 for p53 mRNA polyadenylation and translation. Interestingly, Gld-2 regulates the stability of microRNA-122 that in turn regulates the translation and stability of CPEB mRNA.

The findings presented here exemplify two major themes in the field of translation control. First, translational control mechanisms are often conserved in cells and tissues as distant as oocytes, neurons, and now somatic skin fibroblasts (Mathews et al. 2007; Richter 2007). Second, translational control cascades can rapidly regulate important cellular processes like hormone-induced meiotic cell cycle resumption and cell cycle arrest and senescence (Vasudevan et al. 2006). However, my work also demonstrates that the CPEB-complex composition and enzymatic partner proteins are perhaps different and less conserved in function than previously appreciated. Regardless, these experiments offer novel lines of questioning and further

experimentation. Also, several interesting predictions about CPEB function in other cellular contexts can be made from my work.

CPEB knockout mouse

Several lines of evidence had previously suggested the potential importance of investigating CPEB function in higher vertebrates. Namely, CPEB was found to regulate essential mRNAs in the early meiotic cell cycle, e.g. cyclin B and *mos* mRNAs. Furthermore, CPEB had also been shown to play a critical role the mitotic cell cycle (Stebbins-Boaz et al. 1996; Mendez et al. 2000a; Groisman et al. 2001; Groisman et al. 2002). While both female and male CPEB KO mice are sterile – CPEB KO females lack ovaries and CPEB KO males are afflicted with hypogonadism due to defects in germ cell development – CPEB KO mice appear otherwise normal (Tay and Richter 2001). Due to the predicted role of CPEB-complex function in the mitotic cell cycle, murine embryonic fibroblasts (MEFs) were cultured from these CPEB KO embryos (Groisman et al. 2006). Unlike WT MEFs which enter a cell cycle arrest termed cellular senescence, CPEB KO MEFs continue to proliferate, bypass senescence, and are immortal (Groisman et al. 2006). However, because CPEB is required for mitotic cell division one might have predicted that these experiments would have never been possible due to insufficient Cdk1 activity, principally due to defects in cyclin B mRNA translation. Interestingly, recent experiments from the Mendez Group show that CPEB4 may compensate for CPEB1 in function (Igea and Mendez 2010). Thus, other CPEB family members may compensate for the loss of CPEB1, as exemplified by the work concerning the D-type cyclins, where other family

member proteins can compensate for Cyclin D1 function when Cyclin D1 is knocked out in the mouse (Ciemerych et al. 2002; Kozar et al. 2004). However, the possibility of CPEB4 compensating in function for CPEB1 seems unlikely for two reasons: i) CPEB1 and CPEB4 bind different consensus sequences (Huang et al. 2006), and ii) CPEB4 seemingly requires CPEB1 for its translation (Igea and Mendez 2010) even though CPEB4 protein is detectable in mice (unpublished data). Regardless, further experimentation is required to determine whether CPEB4 compensates for the loss of CPEB1. Further breeding experiments between CPEB1 and other CPEB family members would either exclude the issue of compensation or, more interestingly, show some degree of compensation. Sterility of the CPEB1 KO mice present technical issues and the lack of a CPEB4 KO mouse make these experiments unfeasible. However, because CPEB4 also has been shown to bind the polyadenylation enzyme xGld-2 in *Xenopus* extracts (Igea and Mendez 2010), perhaps CPEB4 similarly interacts with Gld-2 or Gld-4. Perhaps a more feasible strategy would be to study the enzymes responsible for poly(A) addition, not merely the mRNA-binding specificity factors which merely nucleate these complexes.

Even though the CPEB KO mouse presents few abnormal phenotypes, ongoing studies in our laboratory suggest that some stress or state change may be required to reveal additional aberrant phenotypes. For example, cell culture stress was needed to reveal the bypass in senescence of CPEB KO MEFs. Thus, looking in the correct tissue(s) under the right stress-conditions may reveal additional defects or aberrant phenotypes. I will therefore speculate in the following sections, of other potential tissue(s) and biologically relevant contexts for further investigation.

Role of CPEB in senescence

Senescence, like apoptosis, limits the growth of aberrantly proliferating cells; cells that bypass senescence may go on to form malignant tumors (Collado et al. 2005; Collado and Serrano 2005; Collado et al. 2007). CPEB is required for the normal onset of senescence and normal polyadenylation of the tumor suppressor protein p53 (Burns and Richter 2008); it is possible CPEs are highly mutated in cancer cells, thus disrupting normal p53 function. While, mutation of the putative CPE motifs found in p53 mRNA has not been formally tested, in an alternative observation from tumor cells, the shortening of 3'-UTRs prior to transformation does occur (Mayr and Bartel, 2009). The mechanism for 3'-UTR shortening is alternative polyadenylation site selection (APA) by an as yet an unknown mechanism. Several mechanisms are proposed, such as increases in the concentration of poly(A) cleavage and specificity factors (Ji and Tian 2009), nucleosome positioning (Spies et al. 2009), and DNA methylation (Wood et al. 2008). The 3'-UTR is essential for contributing RNA sequences required for association of trans-acting factors, like cognate miRISCs and RNA-binding proteins. It is plausible that both CPEB and p53 mRNAs may experience 3'-UTR shortening. While CPEB and p53 do not have any obvious alternative poly(A) sites, expressed sequence tags (ESTs) from the UCSC genome browser predict that in different stages of development both may have a shortened 3'-UTRs. P53 mRNA 3'-UTR shortening would affect CPEB binding as well. No one has investigated 3'-UTR shortening of p53 and its associated consequences during tumorigenesis.

Beyond its primary role of tumor suppression, senescence also limits the efficiency of nuclear reprogramming in induced pluripotent stem (iPS) cells. Reprogramming Induced

Senescence (RIS) limits the efficiency of nuclear reprogramming by the four so-called Yamanaka factors – Oct4/Sox2/Klf4/c-Myc (Banito et al. 2009; Banito and Gil 2010). The discovery of iPS cellular reprogramming was a major scientific breakthrough for two major reasons: i) iPS cell induction will allow the study of cells from patients with various diseases in unprecedented ways (Nishikawa et al. 2008) and ii) it will allow the generation of stem cells that are isogenic to the patients, thus allowing them to be re-injected back into the same patient with less probability of host or xenographic rejection. Somatic cell reprogramming is a major scientific breakthrough for not only the study of developmentally related diseases, but potentially for regenerative and translational medicine (Yamanaka and Blau 2010).

One of the major limitations of nuclear reprogramming iPS cells is senescence; since CPEB KO MEFs and Kd foreskin cells clearly have defects in senescence onset, one interesting prediction is that both CPEB KO MEFs and Kd human foreskin cells should reprogram with much higher efficiency. Two lines of questions and experimentation could then follow: is RIS and cellular senescence regulation by CPEB similar, and is CPEB required for differentiation into a particular cell type, e.g. ovaries, testes, gametes or neurons? Additionally, iPS cellular reprogramming might allow for the study of CPEB function in human neurons.

Non-canonical 3' additions to microRNAs

Small non-coding RNAs called microRNAs regulate both the translation and stability of mRNA (Richter 2008). It is becoming more apparent that microRNAs are themselves regulated at both the level of processing by Dicer and Lin28 and stability by non-templated adenylate

addition (Kai and Pasquinelli 2010). microRNAs are primarily destabilized by the addition of multiple uridyates by Lin28 and Tut-4 (Heo et al. 2008; Hagan et al. 2009; Heo et al. 2009). Equally interesting is the stabilization of microRNAs by the addition of one non-DNA-templated adenylate. Surprisingly, Gld-2 poly(A) polymerase is shown by the Suzuki group via loss-of-function studies to stabilize miR-122 (Kato et al. 2009). Gld-2, first identified by Dr. Judith Kimble to be the poly(A) polymerase important for early germ line differentiation (Wang et al. 2002; Wang et al. 2004), was later demonstrated by the Richter group to polyadenylate mRNAs during oocyte maturation (Barnard et al. 2004). Furthermore, in an attempt to demonstrate the requirement for Gld-2 and p53 mRNA polyadenylation, I unexpectedly find that Gld-2 is not required for p53 mRNA polyadenylation. Instead, I confirm the findings from Suzuki group (Kato et al. 2009), that Gld-2 regulates the stability of miR-122, as well as establish that the stability of miR-122 is important for the translational repression of both CPEB directly and p53 indirectly by CPEB and Gld-4.

As my work demonstrates the biological importance of microRNA stabilization, it is therefore worth considering how widespread the stabilization of microRNAs by adenylate addition is, and what roles this may play in other biological contexts. Recent deep sequencing experiments suggest that non-templated adenylate addition is quite prevalent (Schulte et al. 2009). By bioinformatic analysis of RNA-seq data, Schulte et al. (2009) find that 20% of all microRNA reads have a non-templated adenylate (Schulte et al. 2009). Additionally, it is also likely Gld-2 is the principal non-canonical poly(A) polymerase responsible for the adenylate addition (Kato et al. 2009); this deserves further experimentation, i.e. small RNA cloning and next generation RNA-sequencing. The Gld-2 knockout mouse does exist and is viable indicating that Gld-2 mediated adenylate addition is not essential for embryonic development (Nakanishi et

al. 2007). Regardless, the question of how Gld-2 mechanistically adds only one adenylate and how this one adenylate stabilizes microRNAs is central to the study of miRNA-mediated regulation and should be investigated further. Because polyadenylate stretches both stabilize mRNAs and increase their translational efficiency by additional factors, namely PABPs, additional factors are most likely required for microRNA stabilization and await identification.

Predictions: CPEB in other cellular contexts

In the previous chapter, I demonstrated a mechanistic link between CPEB mRNA translation and microRNA-122 (miR-122). I will therefore consider possible roles for CPEB in relation to previously characterized miR-122 functions. One of the most provocative connections of miR-122 to CPEB is the requirement of miR-122 for Hepatitis C Virus (HCV) replication. Interestingly, HCV not only requires miR-122 for viral replication (Jopling et al. 2005; Sarnow et al. 2006) but also the DEAD-box helicase DDX6 for viral gene expression (Jangra et al.; Smillie and Sommerville 2002; Weston and Sommerville 2006; Jopling et al. 2008). Previous studies by the Standart group demonstrate CPEB co-immunoprecipitates with DDX6, also known as p54/RCK (Minshall et al. 2001; Minshall et al. 2009). Because DDX6 is required for HCV gene expression, one would predict that by ectopically overexpressing CPEB, DDX6 would potentially be titrated out and HCV gene expression inhibited. However, one must consider that upon ectopic expression of CPEB, most likely, p53 mRNA would increase in translation. Interestingly, miR-122 can regulate CPEB mRNA translation. MiR-122 is required for HCV stabilization and replication (Jopling et al. 2005). Thus, does CPEB increase in

expression after HCV infection due to the inhibition of normally expressed miR-122 by HCV? If this were the case, then one would predict a commensurate increase in CPEB/DDX6 interaction. Presumably, this increase interaction would benefit HCV by either increasing HCV gene expression or potentially decreasing p53 translation. The possibility of CPEB decreasing p53 translation is likely as DDX6 inhibits translation by interaction with the 7mG capped mRNA (Weston and Sommerville 2006).

MiR-122 has also been described as a gene under circadian control in liver cells, where the primary transcript for miR-122 oscillates after light-induced entrainment (Gatfield et al. 2009). Interestingly, no change in the overall levels of mature miR-122 was observed (Gatfield et al. 2009). Gatfield et al. (2009), as demonstrated by microarray experiments that newly transcribed mRNAs instead increase and therefore putatively regulated miR-122 mRNAs are under differential ratios of miR-122 to total mRNAs (Gatfield et al. 2009). Gatfield et al. (2009) surmise that due to dilution effects of cognate miRISC loaded with miR-122 relative to miR-122 targeted genes, the miR-122 targeted genes are de-repressed translationally. They further substantiate this hypothesis by demonstrating that miR-122 targeted reporter constructs are translationally derepressed (Gatfield et al. 2009). Perhaps CPEB is also differentially regulated in liver cells and plays a translational regulatory role in the circadian regulation of gene expression.

MiR-122 is also antagonized in function by the embryonic-like-abnormal-vision-like (ELAVL) protein Hu Antigen R (HuR) (Bhattacharyya et al. 2006). Studies from the Filipowicz group show the translational repression of CAT-1 mRNA by miR-122 is reversible. Inhibition of miR-122 via ectopic expression of HuR abrogates the translational repression of CAT-1 mRNA reporter (Bhattacharyya et al. 2006). Interestingly, HuR plays a significant role in mRNA stabilization during genotoxic stress, where it has been shown to translocate from the nucleus to

the cytosol to stabilize mRNAs and indirectly promote the translation of mRNAs (Gorospe 2003). Also, HuR has been shown to directly bind radiolabeled p53 RNA in UV-irradiation experiments which increases the stability and the translation of p53 mRNA (Mazan-Mamczarz et al. 2003). Because p53 does not contain any putative miR-122 binding sites, does HuR concomitantly antagonize miR-122 function on CPEB mRNA by de-repressing CPEB mRNA translation to promote p53 polyadenylation, stabilization, and translation? UV-irradiation experiments in HuR and CPEB knockdown versus WT cells could empirically test this question.

Conclusion of the Discussion

CPEB is an important trans-acting factor that can regulate transport and translation of mRNAs (Richter 2007). While CPEB has been rigorously studied in *Xenopus* oocytes in the context of oocyte maturation, CPEB function is conserved in other vertebrate metazoan somatic cells, such as neurons and skin cells. Because we know much of CPEB function and little of the specific mRNAs it might regulate, further non-biased approaches that utilize next-generation sequencing (RNA-seq) and new laboratory techniques like UV-cross-link-IP (CLIP) have the potential to reveal additional CPEB target mRNAs. However, regardless of how many new targets are identified by such methods, understanding the function of CPEB-associated complex members is critical for understanding the role of CPEB bound mRNAs within a given cellular context. The work presented here demonstrates a novel CPEB complex member and regulatory features of CPEB mRNA translation within the biologically relevant context of cellular senescence may provide additional biological contexts for further studies of CPEB function.

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