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CPEB and two poly(A) polymerases control miR-122 stability and p53 mRNA translation

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Abstract

Cytoplasmic polyadenylation-induced translation controls germ cell development^{1,2}, neuronal synaptic plasticity³⁻⁵, and cellular senescence^{6,7}, a tumor-suppressor mechanism that limits the replicative lifespan of cells^{8,9}. The cytoplasmic polyadenylation element binding protein (CPEB) promotes polyadenylation by nucleating a group of factors including defective in germline development 2 (Gld2), a non-canonical poly(A) polymerase^{10,11}, on specific mRNA 3' untranslated regions (UTRs). Because CPEB regulation of *p53* mRNA polyadenylation/translation is necessary for cellular senescence in primary human diploid fibroblasts⁶, we surmised that Gld2 would be the enzyme responsible for poly(A) addition. Here, we show that depletion of Gld2 surprisingly promotes rather than inhibits *p53* mRNA polyadenylation/translation, induces premature senescence, and enhances the stability of *CPEB* mRNA. The *CPEB* 3'UTR contains two miR-122 binding sites, which when deleted, elevate mRNA translation, as does an antagomir of miR-122. Although miR-122 is thought to be liver-specific, it is present in primary fibroblasts and destabilized by Gld2 depletion. Gld4, a second non-canonical poly(A) polymerase, was found to regulate *p53* mRNA polyadenylation/translation in a CPEB-dependent manner. Thus, translational regulation of *p53* mRNA and cellular senescence is coordinated by Gld2/miR-122/CPEB/Gld4.

Keywords

CPEB; polyadenylation; translation; p53; Gld2; Gld4

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

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Author Contributions Experiments were performed by D.M.B. and A.D. S.N. designed and constructed luciferase reporter constructs. D.M.B. and J.D.R. designed the experiments and wrote the manuscript.

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in WT MEFs⁷. Human foreskin fibroblasts depleted of CPEB also bypass senescence and divide ~270 days compared to WT cells, which 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 glycolysis⁶.

To investigate the possibility that CPEB control of *p53* polyadenylation requires *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) induced an increase in both *p53* protein levels (Fig. 1c) and *p53* mRNA polyadenylation (Fig. 1d; Supplemental Fig. 1). 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 ~50% reduction in *p53* protein levels⁶.

These paradoxical results prompted us to examine CPEB levels in *Gld2* deficient cells since CPEB is required for normal *p53* mRNA translation⁶. After comparing the amounts of CPEB nuclear pre-mRNA by intron-specific quantitative RT-PCR and mostly cytoplasmic mRNA by exon-specific quantitative RT-PCR, we found that the pre-mRNA levels, which generally reflect transcription, were nearly unchanged while cytoplasmic mRNA levels increased by about five-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, the entire CPEB 3' UTR was translated about 40% less efficiently compared to a reporter lacking the 3' most 455 nucleotides (Mock). However, in *Gld2*-deficient cells, the two reporters were translated equally. Additional deletions () of the CPEB 3' UTR suggested that there might be multiple regions that elicited increases in reporter translation following *Gld2* knockdown (i.e., E translation was about two fold greater than B, C, or D translation) (Fig. 2d).

Analysis of the regions of the CPEB 3' UTR that mediated translational repression by *Gld2* revealed the presence of two potential miR-122 binding sites (Supplementary Fig. 2). Although miR-122 is thought to be liver-specific and account for ~70% of the total population of microRNAs in that tissue¹³, deletion of these specific sites, either individually or combined, alleviated translational repression 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 is more widely distributed than originally thought. Indeed recent evidence shows that miR-122 is present in human skin¹⁴ and even HEK293 cells¹⁵.

To assess directly whether miR-122 might repress *CPEB* mRNA expression, we first cloned and sequenced it from human foreskin fibroblasts and found that it contained a nontemplated 3' monoadenylate residue (Fig. 3b; see discussion). Next, 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 about 3.25 fold relative to control (Fig. 3c), but had no stimulatory effect on a reporter whose 3' UTR contained no miR-122 sites (Supplementary Fig. 3). Based on evidence from Katoh et al¹⁶, who demonstrated that in murine liver, *Gld2* is essential for miR-122 stability, we suspected that *Gld2* might influence *CPEB* expression and possibly *p53* mRNA translation by controlling miR-122 steady-state levels. Indeed, Fig. 3d demonstrates that depletion of *Gld2* from skin fibroblasts reduced the level of miR-122 by nearly 40-fold. Importantly, when miR-122 LNA antagomir-transduced cells were incubated with the proteasome inhibitor MG132 and pulsed-labeled with ³⁵S-methionine for 15 min followed by p53 immunoprecipitation, there was a two-fold increase in the synthesis rate of p53 (Fig. 3e, 3f). Taken together, these data demonstrate that human primary skin fibroblasts contain miR-122 and that *Gld2* controls its steady state levels or activity.

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 sites according to Targetscan.org or Microrna.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 and p53 immunoprecipitation. Fig. 3g shows that although miR-122 antagomir alone elicited an increase in p53 synthesis, the antagomir plus shRNA for *CPEB* induced no 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 control poly(A) tail length and translation.

If not *Gld2*, what poly(A) polymerase modifies *p53* mRNA polyadenylation and translation? We surmised that an alternative 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* (*PAPD5*)¹⁷ and *MitoPAP* (*PAPD1*)¹⁸. Both polymerases were depleted with shRNAs (Supplementary Fig. 4) but only the loss of *Gld4* reduced p53 protein levels (Fig. 4a). To investigate whether *Gld4* interacts with *p53* mRNA in a *CPEB*-dependent manner, FLAG-*Gld4* was expressed in cells (Supplementary Fig. 5) containing shRNA for tetracycline repressor (TETR, a control) or *CPEB*. *Gld4* was then immunoprecipitated and the extracted RNA was examined for p53 and GAPDH (a control) RNAs by RT-PCR (Fig. 4b). *p53* mRNA was detected only when *CPEB* was present, suggesting that *Gld4* is anchored to *p53* mRNA by *CPEB*, and indeed, *CPEB* co-immunoprecipitated *Gld4* but not *Mcl1*, a nonspecific control (Fig. 4c). Finally, depletion of *Gld4* reduced *p53* mRNA polyadenylation (Fig. 4d), which probably then induced p53 mRNA destabilization (Fig. 4e; depletion of *Gld4* reduced mostly cytoplasmic *p53* mRNA as examined by RT-PCR using exon-specific primers but had no effect on *p53* transcription as examined by RT-PCR using intron-specific primers).

The results presented here and in Katoh et al. (ref. 16) suggest a model for homeostatic control of p53 synthesis in human skin fibroblasts (Fig. 4f). Gld2 stabilizes miR-122 by catalyzing the addition of a single adenylate residue to its 3' end¹⁶. miR-122 then base-pairs to two regions of the CPEB 3' UTR, causing instability and/or translational inhibition of the mRNA. CPEB, whose levels are modulated by these events, binds to the *p53* 3'UTR and recruits Gld4, which in turn maintains *p53* mRNA polyadenylation and translation. We envision this hierarchical regulation of p53 to resemble a rheostat, where translation is turned up or down rather than a switch, where translation is turned on or off¹⁹, although *p53* mRNA translation can also be controlled by a switch mechanism in response to DNA damage^{20,21}. A 50% change in p53 synthesis can toggle a cell between growth and senescence⁶, demonstrating that drastic biological consequences result from a relatively modest change in protein level.

Although ectopically-expressed Gld2 immunoprecipitated from hepatocarcinoma cells adds a single adenosine to miR-122 in vitro¹⁶, Gld2 tethered to a small non-coding RNA by MS2 adds >300 adenylate residues in injected oocytes²², and about that same amount to mRNA when bound to CPEB¹⁰. How the enzyme can modulate its catalytic activity depending on the substrate is unknown, but we postulate that components of the RNA-induced silencing complex (RISC) might be responsible. In addition to our demonstration that miR-122 is 3' mono-adenylated in skin fibroblasts, ~20% of all RNA deep sequencing reads from cloned neuroblastoma miRNAs contain a non-templated adenylate residue²³, suggesting that miR-122 may be one of several miRNAs that are mono-adenylated by Gld2.

In conclusion, our results demonstrate that Gld2 control of miR-122 stability in human skin fibroblasts tunes CPEB expression, which in turn regulates *p53* mRNA polyadenylation and translation by Gld4. The coordinated activities of these factors then gate entry into senescence. These studies also bring up two additional aspects of CPEB-related activities: how does Gld4, but not Gld2, associate with CPEB on *p53* mRNA, and what molecular machinery is responsible for miR-122 destruction upon Gld2 depletion? Deciphering the mechanisms involved would likely require analysis of the combinatorial associations of factors on different RNA substrates.

METHODS SUMMARY

Molecular biology and cell culture

Primary human foreskin fibroblasts obtained from the Cell Culture Core Facility of the Yale University Skin Disease Research Center were cultured as described²⁴ in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum. Amphotropic retroviruses and shRNA-containing 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 7µg/ml polybrene. Typically 70-90% infection efficiency was achieved as assessed by a GFP-encoding viral gene or by immunostaining with HA antibody (Covance). After infection, fresh medium was added to the infected fibroblasts. Some cells were analyzed by western blotting for p53 (DO-1,

Neomarkers) and β -actin (Abcam). Other cells were fixed with 0.2% glutaraldehyde and stained for β -galactosidase activity at acidic pH according to Dimri et al²⁵.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Methods

Analysis of p53, Gld2, and Gld4

Lentiviruses expressing shRNAs for Gld2, Gld4, and mitoPAP were generated as described⁶. shRNA against the TET repressor has also been described⁶. A retrovirus expressing Gld2-HA was generated as described⁷. 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 used for p53 immunoprecipitation and analysis by SDS-PAGE and phosphorimaging. Cells were also cultured in methionine/cysteine free media in the presence of MG132, a proteasome inhibitor, for 30 min. followed by a 15 min culture in 100 μ Ci ³⁵S-methionine and cysteine; p53 was then immunoprecipitated and analyzed as noted above.

Ligation-mediated polyadenylation test (LM-PAT) assays were used to detect the poly(A) tail of *p53* mRNA in WT cells, shGld2 knockdown cells (two shRNAs targeting different regions of Gld2 were used), cells expressing ectopic CPEB, and cells expressing ectopic that lacked a zinc finger and hence was unable to bind RNA (CPEB ZF)⁶. Quantitative RT-PCR analyses were normalized against *actin* RNA.

Oxygen Consumption and cellular senescence

To measure oxygen consumption, $\sim 4 \times 10^5$ cells were washed and suspended 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.

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.

miR-122 cloning and sequencing

Small RNAs from human foreskin fibroblasts were extracted with mirVANA PARIS kit (Ambion) and those corresponding in length to 18-24 nucleotides were resolved by urea-PAGE, extracted, and ethanol precipitated. miRNA cloning linker-1 (IDT) was ligated to the 3' ends and used to prime a reverse transcription reaction with Superscript III (Invitrogen) and the RT primer DP3 (5'-ATTGATGGTGCCTACAG-3'). cDNA was then PCR amplified with *miR-122* specific primer 5'-AGGGGCGCCTGGAGTGTGACAATG-3' and DP3. The PCR product was cloned into pGEM-T (Promega) and sequenced. The chromatogram shown is adapted from 4peaks (<http://mekentosj.com/science/4peaks/>).

Antagomir depletion of *miR-122*

Locked nucleic acid (LNA) antagomir against *miR-122*, or a scrambled sequence LNA (Exiqon) were electroporated (Amaxa, Lonza) at a final concentration of 4 nM into $\sim 10^6$ human foreskin fibroblast cells together with 0.8 μ g *firefly* (*pGL3*, Promega) and 1.0 μ g *Renilla* (*pRLTK*) luciferase-encoding plasmid, the latter harboring the full length CPEB 3' UTR or deletion mutations of the 3' UTR. Luciferase assays were performed 16-24 hours after electroporation according to methods described elsewhere²⁶. The amount of *Renilla* luciferase activity derived from RNA containing the entire CPEB 3' UTR (full) was arbitrarily set at 100. When employed, the CPEB 3' UTR deletions () were (in CPEB nucleotide number): A, 420-755; B, 480-755; C, 530-755; D, 565-755; E, 640-755.

CPEB-Gld4 Co-immunoprecipitation

Cells were transfected with plasmids encoding FLAG-CPEB and HA-Gld4 using Effectene (Qiagen). The cells were then harvested in PBS and lysed in lysis/wash buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 0.1% SDS, and Complete Protease Inhibitor (Roche)). Extracts (0.5 mg protein) were incubated with M2-FLAG antibody (Sigma)-coated Dynabeads (Invitrogen) for 2 hours at 4°C. The beads were then washed 3 times with lysis/wash buffer and the bound proteins eluted by boiling in SDS sample buffer. Coimmunoprecipitates were detected by western blotting with HA antibody (HA.11 16B12, Covance). Control IPs were performed with generic mouse IgG-coated Dynabeads.

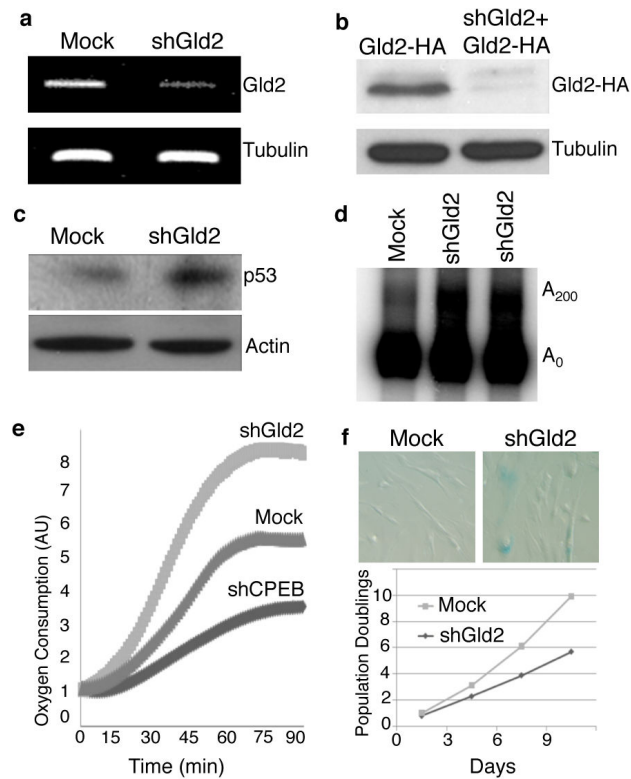
Gld4-RNP Co-immunoprecipitation

Mock or CPEB-depleted human foreskin fibroblasts⁶ (Burns and Richter, 2008) were electroporated (Amaxa, Lonza) with a plasmid encoding FLAG-Gld4 according to the manufacturer's instructions. Immunoprecipitation with the FLAG antibody followed the procedure of Peritz et al²⁷ with the following modifications: a.) M2 anti-FLAG (Sigma)-coated Dynabeads were used instead of agarose beads and b.) washes with buffer containing 1 M urea were omitted. *p53* RNA was detected by RT-PCR as described⁶.

References

1. Mendez R, et al. Phosphorylation of CPE binding factor by Eg2 regulates translation of c-mos mRNA. *Nature*. 2000; 404:302–307. [PubMed: 10749216]
2. Tay J, Richter JD. Germ cell differentiation and synaptonemal complex formation are disrupted in CPEB knockout mice. *Dev Cell*. 2001; 1:201–213. [PubMed: 11702780]
3. Alarcon JM, et al. Selective modulation of some forms of schaffer collateral-CA1 synaptic plasticity in mice with a disruption of the CPEB-1 gene. *Learn Mem*. 2004; 11:318–327. [PubMed: 15169862]
4. Wu L, et al. CPEB-mediated cytoplasmic polyadenylation and the regulation of experience-dependent translation of alpha-CaMKII mRNA at synapses. *Neuron*. 1998; 21:1129–1139. [PubMed: 9856468]
5. Zearfoss NR, Alarcon JM, Trifilieff P, Kandel E, Richter JD. A molecular circuit composed of CPEB-1 and c-Jun controls growth hormone-mediated synaptic plasticity in the mouse hippocampus. *J Neurosci*. 2008; 28:8502–8509. [PubMed: 18716208]
6. Burns DM, Richter JD. CPEB regulation of human cellular senescence, energy metabolism, and p53 mRNA translation. *Genes Dev*. 2008; 22:3449–3460. [PubMed: 19141477]
7. Groisman I, et al. Control of cellular senescence by CPEB. *Genes Dev*. 2006; 20:2701–2712. [PubMed: 17015432]
8. Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol*. 2007; 8:729–740. [PubMed: 17667954]
9. Stewart SA, Weinberg RA. Telomeres: cancer to human aging. *Annu Rev Cell Dev Biol*. 2006; 22:531–557. [PubMed: 16824017]
10. Barnard DC, Ryan K, Manley JL, Richter JD. Symplekin and xGLD-2 are required for CPEB-mediated cytoplasmic polyadenylation. *Cell*. 2004; 119:641–651. [PubMed: 15550246]
11. Kim JH, Richter JD. Opposing polymerase-deadenylase activities regulate cytoplasmic polyadenylation. *Mol Cell*. 2006; 24:173–183. [PubMed: 17052452]
12. Kim JH, Richter JD. RINGO/cdk1 and CPEB mediate poly(A) tail stabilization and translational regulation by ePAB. *Genes Dev*. 2007; 21:2571–2579. [PubMed: 17938241]
13. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of tissue-specific microRNAs from mouse. *Curr Biol*. 2002; 12:735–739. [PubMed: 12007417]
14. Holst LM, Kaczkowski B, Gniadecki R. Reproducible pattern of microRNA in normal human skin. *Exp Dermatol*. 2010; 19:e201–205. [PubMed: 20201961]
15. Liao JY, et al. Deep sequencing of human nuclear and cytoplasmic small RNAs reveals an unexpectedly complex subcellular distribution of miRNAs and tRNA 3' trailers. *PLoS One*. 2010; 5:e10563. [PubMed: 20498841]
16. Katoh T, et al. Selective stabilization of mammalian microRNAs by 3' adenylation mediated by the cytoplasmic poly(A) polymerase GLD-2. *Genes Dev*. 2009; 23:433–438. [PubMed: 19240131]
17. Schmid M, Kuchler B, Eckmann CR. Two conserved regulatory cytoplasmic poly(A) polymerases, GLD-4 and GLD-2, regulate meiotic progression in *C. elegans*. *Genes Dev*. 2009; 23:824–836. [PubMed: 19339688]
18. Mullen TE, Marzluff WF. Degradation of histone mRNA requires oligouridylation followed by decapping and simultaneous degradation of the mRNA both 5' to 3' and 3' to 5'. *Genes Dev*. 2008; 22:50–65. [PubMed: 18172165]
19. Santos SD, Ferrell JE. Systems biology: On the cell cycle and its switches. *Nature*. 2008; 454:288–289. [PubMed: 18633407]
20. Ofir-Rosenfeld Y, Boggs K, Michael D, Kastan MB, Oren M. Mdm2 regulates p53 mRNA translation through inhibitory interactions with ribosomal protein L26. *Mol Cell*. 2008; 32:180–189. [PubMed: 18951086]
21. Takagi M, Absalon MJ, McLure KG, Kastan MB. Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. *Cell*. 2005; 123:49–63. [PubMed: 16213212]

22. Kwak JE, Wang L, Ballantyne S, Kimble J, Wickens M. Mammalian GLD-2 homologs are poly(A) polymerases. *Proc Natl Acad Sci U S A*. 2004; 101:4407–4012. [PubMed: 15070731]
23. Schulte JH, et al. MicroRNAs in the pathogenesis of neuroblastoma. *Cancer Lett*. 2009; 274:10–15. [PubMed: 18639376]
24. Rangarajan A, Hong SJ, Gifford A, Weinberg RA. Species- and cell type-specific requirements for cellular transformation. *Cancer Cell*. 2004; 6:171–183. [PubMed: 15324700]
25. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O, et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A*. 1995; 92:9363–9367. [PubMed: 7568133]
26. Nottrott S, Simard MJ, Richter JD. Human let-7a miRNA blocks protein production on actively translating polyribosomes. *Nat Struct Mol Biol*. 2006; 13:1108–14. [PubMed: 17128272]
27. Peritz T, Zeng F, Kannanayakal TJ, Kilk K, Eiriksdottir E, Langel U, Eberwine J. Immunoprecipitation of mRNA-protein complexes. *Nature Protocols*. 2006; 1:577–580. [PubMed: 17406284]

**Figure 1.**

Depletion of Gld2 enhances *p53* expression. (a) RT-PCR of Gld2 and tubulin RNAs following infection of human foreskin fibroblasts with lentiviruses expressing shRNA against Gld2 or GFP (mock, same in all panels). (b) Knockdown of Gld2-HA in cells expressing ectopic Gld2-HA. Tubulin served as a loading control. (c) Western blot showing 2.5 fold enhanced expression of *p53* relative to tubulin following Gld2 depletion. (d) Poly(A) tail analysis of *p53* mRNA in WT and Gld2 depleted cells (two shRNAs targeting different regions of Gld2 were used). (e) Oxygen consumption in cells infected with shCPEB, shGld2, or empty vector (Mock). (f) Mock or shGld2 infected cells stained for β -galactosidase, which denotes cellular senescence. Population doublings were determined in wild type or Gld2 depleted cells.

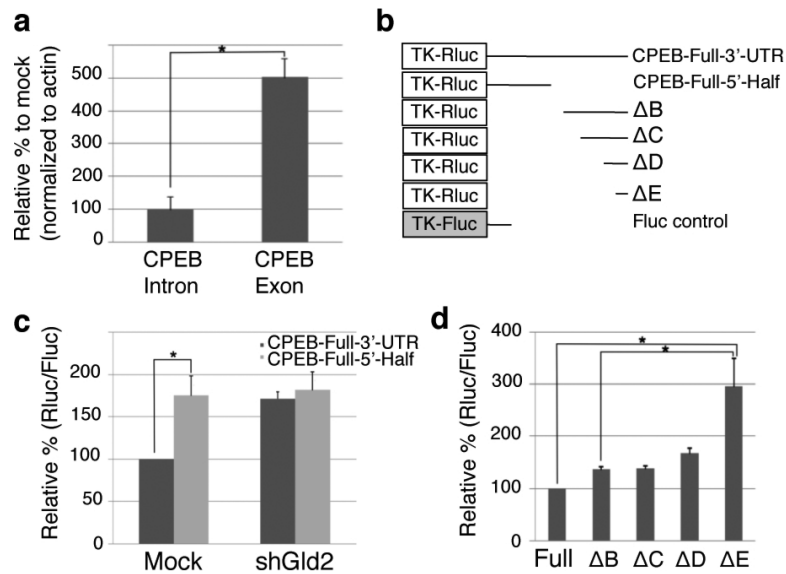
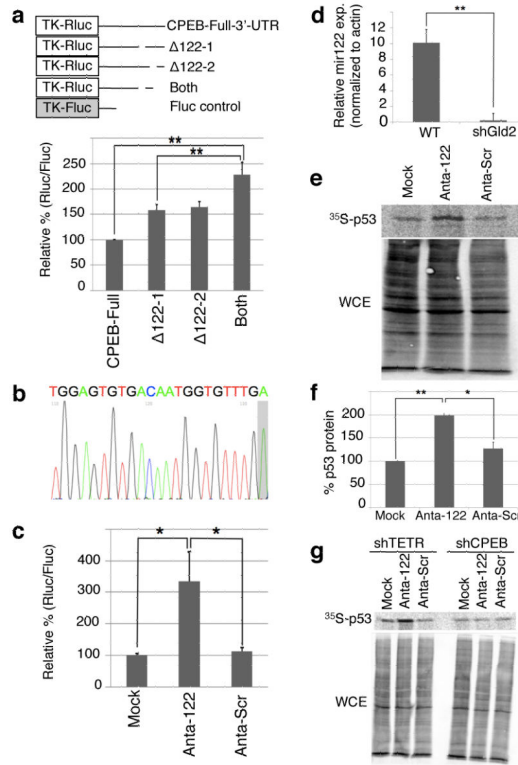


Figure 2.

Gld2 knockdown increases CPEB reporter mRNA and translation by a post-transcriptional mechanism. (a) Fold change of nuclear (intron-containing) or predominantly cytoplasmic (exon-containing) CPEB RNA following Gld2 depletion (n=3, bars are s.e.m.). (b) Schematic of reporter constructs used in the following experiments (numbers refer to nucleotides of CPEB 3'UTR). (c and d) Cells expressing *firefly luciferase* (*Fluc*) as a control and *Renilla luciferase* (*Rluc*) as noted in panel b were depleted of Gld2; the amount of *Renilla* luciferase activity (relative to firefly) was derived from RNA containing the entire CPEB 3'UTR (full) and set at 100. In all panels, n = 3 and the bars refer to s.e.m.; one or two asterisks refer to statistical significance (Students t test) at the p<0.05 or p<0.01 levels, respectively.

**Figure 3.**

miR-122 activates *p53* mRNA translation by repressing CPEB. (a) Gld2 depleted fibroblasts were transduced with *firefly* and *Renilla luciferase* with CPEB full length or deletion mutant 3'UTRs lacking putative miR-122 sites (Supplementary Fig. 2). The data are expressed as in Fig 2; in all panels, n = 3 and the bars refer to s.e.m. and one or two asterisks refer to statistical significance (Students t test) at the p<0.05 or p<0.01 levels, respectively. (b) Sequence of miR-122 from fibroblasts; a non-templated adenosine is shaded. (c) Fibroblasts expressing firefly and Renilla luciferase containing the CPEB 3' UTR were electroporated with miR-122 (Anta-122), scrambled, (Anta-Scr), or no LNA antagomir (Mock); data are expressed as in Figure 2. (d) Quantitative RTPCR for miR-122 in cells expressing GFP (WT) or shGld2. (e and f) Immunoprecipitation of ³⁵S-methionine-labeled p53 from MG132-treated cells transduced with no (Mock-GFP), miR-122 (Anta-122), or scrambled (Anta-Scram) LNA antagomirs. WCE refers to whole cell lysate. (g) Fibroblasts were treated as in panels e-g after first expressing either TET repressor (shTETR, a control) or *CPEB* shRNA.

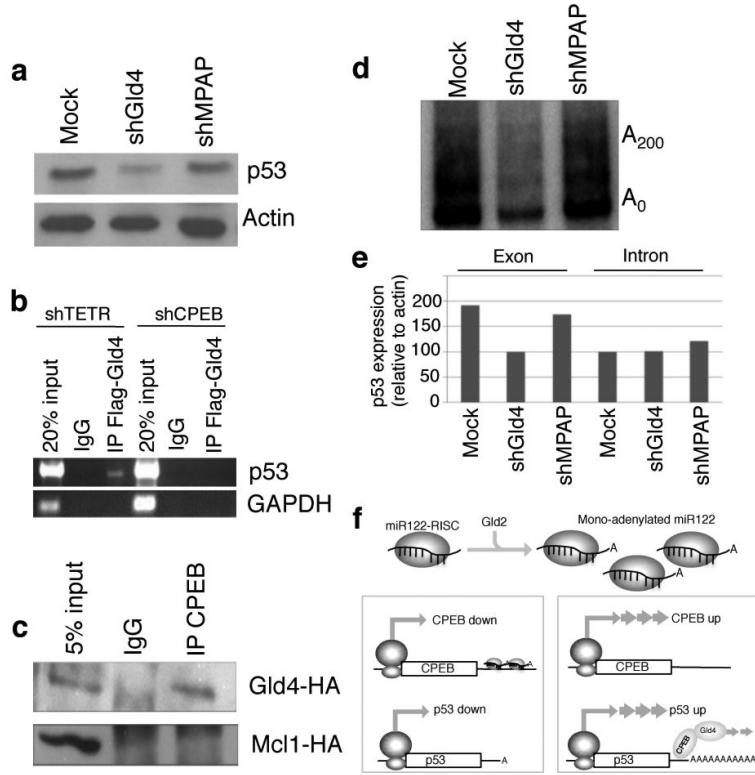


Figure 4. Gld4 controls *p53* mRNA expression. (a) p53 and actin western blots from fibroblasts expressing GFP (mock), shGld4, or shMitoPAP (shMPAP). (b) Fibroblasts containing *shTETR* or *CPEB* were transfected with Gld4-FLAG followed by FLAG antibody or IgG immunoprecipitation of RNA complexes and RT-PCR for *p53* or GAPDH (control) RNAs. (c) Protein from fibroblasts infected with Gld4-HA and CPEB-FLAG was FLAG or IgG immunoprecipitated and western blotted for HA. Other cells infected with CPEB-FLAG and Mcl1-HA (a nonspecific control) were processed similarly. (d) Examination of *p53* poly(A) tail6 from skin fibroblasts expressing GFP or (mock) Gld4 or mitoPAP shRNAs. (e) RT-PCR analysis of predominantly cytoplasmic *p53* RNA (exon-specific primers), or nuclear *p53* pre-mRNA (intron-specific primers) in cells expressing GFP (mock) or Gld4 or MitoPAP shRNAs. (f) Model for regulation of *p53* translation; see text for explanation.