CPEBs STUDIES IN THE CELL CYCLE: MAPPING CPEBs NETWORK AND UNVEILING A NEW FUNCTION FOR CPEB-MEDIATED CAP-RIBOSE METHYLATION

Valeria Giangarra'

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Thesis director

Dr. Raúl Méndez

Institute for Research in Biomedicine (IRB)

a mia madre Franca e mio padre Luigi

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ABBREVIATIONS

A: adenosine

Adohcy: S-adenosylhomocysteine

Adomet: S-adenosylmethionine

APA: alternative polyadenylation

APC/C: Anaphase-Promoting Complex/Cyclosome

ARE: AU-rich element

AS: alternative splicing

ATP: adenosine triphosphate

bp: base pairs

C: cytidine

cDNA: mRNA complementary DNA

C-terminal: carboxy-terminal

CF I: cleavage factor I

CF II: cleavage factor II

CPE: cytoplasmic polyadenylation element

CPEB: cytoplasmic polyadenylation element-binding protein

CPSF: cleavage and polyadenylation specificity factor

CstF: cleavage-stimulating factor

CTD: carboxy-terminal domain

DNA: deoxyribonucleic acid

DTT: dithiothreitol

EDTA: ethylenediamine tetraacetic acid

eIF: eukaryotic initiation factor

eEF: eukaryotic elongation factor

eRF: eukaryotic release factor

ESE: exonic splicing enhancer

ESS: exonic splicing silencer

G: guanosine

G1: gap 1

G2: gap 2

GT: guanylyltransferase

GTP: guanosine triphosphate

GV: germinal vesicle

GVBD: germinal vesicle breakdown

Hex: hexanucleotide

IP: immunoprecipitation

IRES: internal ribosome-entry sequence

Kb: kilo base pairs

KDa: kilodalton

M: mitosis

MI: meiosis-I

MII: meiosis-II

m7Gppp: 7-methyl-guanosine

mRNA: messenger RNA

MT: N7G-methyltransferase

N: any nucleotide

N-terminal: amino-terminal

nt: nucleotide

ORF: open reading frame

PABPN1: poly(A)-binding protein

PAGE: polyacrylamide gel electrophoresis

PAP: polyA-polymerase

PARN: poly(A)-specific ribonuclease

PAS: polyadenylation signal

PB: processing body

PBE: Pumilio Binding Element

PBS: phophate buffered saline

PCR: polymerase chain reaction

PEST: Pro-, Glu-, Ser- and Thr-rich region

Pi: inorganic phosphate

PIC: preinitiation complex

Pol II: RNA polymerase II

poly(A): polyadenosine

pre-mRNA: premature mRNA

RE: regulatory element

RFM: Rossmann-fold methyltransferase

RNA: ribonucleic acid

RT: RNA 5'-triphosphatase

RT-PCR: reverse transcription-polymerase chain reaction

siRNA: small interfering RNA

SDS: sodium dodecyl sulfate

SF: splicing factor

SG: stress granule

ASF: alternative splicing factor

snRNA: small nuclear RNA

snRNP: small nuclear ribonucleoprotein particle

T: thymidine

TC: ternary complex

TMG: 2,2,7-trimethyl guanosine

T7: T7 bacteriophage

tRNA: transfer RNA

U: uridine

UTP: uridine triphosphate

UTR: untranslated region

VP39: vaccinia virus protein 39 KDa

WB: western blot

WT: wild type

3'end: three prime end

5'end: five prime end

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ABSTRACT

CHAPTER I

Early animal development is programmed in part by messanger RNAs (mRNAs) inherited by the egg at the time of fertilization. These silent maternal mRNAs are not translated at the same time or in any cellular location; instead, their expression is often regulated both temporally and spatially. CPEB1 (for Cytoplasmic Polyadenylation Element Binding protein 1) is a known regulator of meiotic progression in Xenopus laevis oocytes. Its function is to mediate cytoplasmic polyadenylation and translational regulation of a specific subset of mRNAs, leading to the activation of a self-sustainable circuit, which drives the progression of the oocytes from prophase I to metaphase II.

However for some mRNAs the increase in the poly(A) length does not appear to be sufficient to stimulate translation and additional CPEB-mediated events are required. These observations suggest that events other than polyadenylation may drive the translation of such mRNAs. Another structural element involved in the regulation of mRNA translation is the cap. The 7-methylguanosine structure, which is referred as a cap 0, can be further methylated at the 2'-O-position of the second ribose (cap I) and, in addition, at the third ribose (cap II). It was shown that in the case of mos mRNA, the polyadenylation-dependent cap I and cap II stimulates the translation and oocyte maturation in Xenopus laevis.

Here we characterized the functional cap I ribose methyltransferase in Xenopus laevis. This enzyme is a nucleo-cytoplasmic shuttling protein, which interacts with CPEB1 upon progesterone stimulation in a RNA-dependent manner. The modification of the cap is required for the translational activation of a reporter mRNA, more than the elongation of the poly(A)tail.

CHAPTER II

CPEB1 is only one of four members of the CPEBs family of proteins (CPEB1-4). CPEB4 was found to take over CPEB1 during *Xenopus laevis* oocytes meiotic progression, and both CPEB1 and CPEB4 were shown to mediate cytoplasmic polyadenylation in the somatic cell cycle. In the present study we narrowed down the functions of CPEB1, CPEB2 and CPEB4 during the cell cycle. We found that CPEB1 is needed for proper S phase, cell proliferation, cell-to-matrix attachment and for early steps of mitosis (prophase). In mitosis CPEB2 functions after CPEB1, being needed in metaphase, while CPEB4 is required for the last step of mitosis and cytokinesis. Moreover we found that CPEB1, CPEB2 and CPEB4 are interconnected during somatic cell cycle progression, showing that their relative levels and activities are tightly regulated to accomplish proper cell division.

Altogether these results add another step in the understanding of CPEBs role during the cell cycle, unveiling a new map in the CPEBs network during somatic cell cycle progression.

INTRODUCTION

THE MEIOTIC CELL CYCLE

Meiosis is a two-part division process by which diploid germ cells (oogonia or spermatogonia), gives rise to haploid gamets (eggs cells or sperm). Meiosis was mainly elucidated from the study of the *Xenopus laevis* oocyte maturation. Meiosis occurs over a prolonged period of time; oogonia enter meiosis, but become arrested at the diplotene stage of the first prophase (PI). During this first period, named oogenesis, primary oocytes grow in size and synthesize a reservoir of not translated mRNAs[1], permitting later on the re-entry in meiosis[2, 3].The transcription starts again in the mid blastula transition (MBT)[4]. The mRNAs of 45% of the mice genome[5] and 55% of Drosophila[6] is represented in the arrested oocytes. Meiotic maturation consists of two consecutive M-phases, metaphase I (MI) and metaphase II (MII), with no S phase occurring in between them.

In *Xenopus laevis*, immature oocytes are stimulated by progesterone. Once MII is reached, they arrest at metaphase II, where the egg awaits for fertilization. Importantly, the *Xenopus laevis* oocyte maturation is a transcriptionally silent period[7, 8] and all this process is mediated by the sequential translational activation of stored maternal mRNAs (reviewed in[3, 9]. This silencing period witnesses the important role of translational regulation and protein degradation. Through the meiotic maturation and the embryonic mitotic division the main body patterns are established, based on mRNA localization within the oocyte. Thus, repressed mRNAs are transported to the final destination, to be activated and translated at the right time and place.

Translationally repressed mRNAs harbor short poly(A) tails. Thus cytoplasmic polyadenylation is one of the key mechanisms that regulates translation during early development. Indeed meiotic progression occurs thanks to several waves of cytoplasmic polyadenylation, that needs to be very well regulated in time. The first (early) wave of cytoplasmic polyadenylation controls the translation of key molecules for meiotic progression, for example: mos, cyclin B5, cyclin B1, emi 1, c3h4, PP2C wee1, cdk2, cyclin A1, TPX2, Xkid and also histone B4, G-10, lamin B1 and FGF receptor[10-18].The first wave of polyadenylation occurs before GVBD and it is independent of protein synthesis and cdc2 activity, while the second stage wave of polyadenylation takes place in MI and it is dependent on protein synthesis and cdc2 activation[10]. The late-late takes place in Interkinesis and in MII and it is dependant on Aurora A and cdc2[9].

There are three key regulators of the meiotic progression: the M-phase promoting factor (MPF), an heterodimer of cyclin B (regulatory subunit) and cdc2 (serine/threonine kinase that constitutes the catalytic subunit), the anaphase promoting complex/cyclosome (APC/C), a big multisubunit complex[19], and finally the cytostatic factor (CSF), which arrests maturation at metaphase II (Fig. 1).Their activities are finely regulated by positive and

negative feedback loops. For example MPF activates APC/C and in turn APC/C inhibits MPF by triggering degradation of cyclins B. The inactive form of MPF (pre-MPF) is initially formed in PI arrested oocytes, with Cyclins B2 and B5[10, 20], and is activated by the dual specificity Cdc25 phosphatase as the result of new synthesis of Ringo and Mos induced by progesterone[3]. MPF activation mediates transition from PI to MI. It is the main actor responsible for changes during oocyte maturation such as GVBD[21, 22]. Then the level of MPF decreases, permitting the exit from MI and the entrance in interkinesis. This process is due by a negative feedback loop, where Cdc2 activates the APC/C, which in turn induces the ubiquitination and posterior degradation of Cyclins B[23]. Interestingly, in anaphase there is high APC/C activity, but it is required also partial MPF activity, to inhibit replication and by-pass the S-phase, which is achieved by the combination of degradation and high translation rate of cyclins B1 and B4[24]. At the end of the first meiotic division the polar body is extruded with half of the DNA content. For the second metaphase entry APC/C activity is decreased due to the activity of its inhibitors and then MPF rises again. The CSF activity, which begins before MII entry, will then establish and maintain the arrest at MII by stabilizing the MPF. The composition of CSF is not well understood, its establishment occurs before MII entry and required key molecules such as: mos/MAPK pathway, Emi2, cyclin E/cdk2. The CSF arrest maintenance is sustained by the activities of mos and Emi2.

The factors that drive meiotic progression are finely regulated, the differential rates of product accumulation, combined with the control of protein degradation, establish their phase–specific peaks of expression.

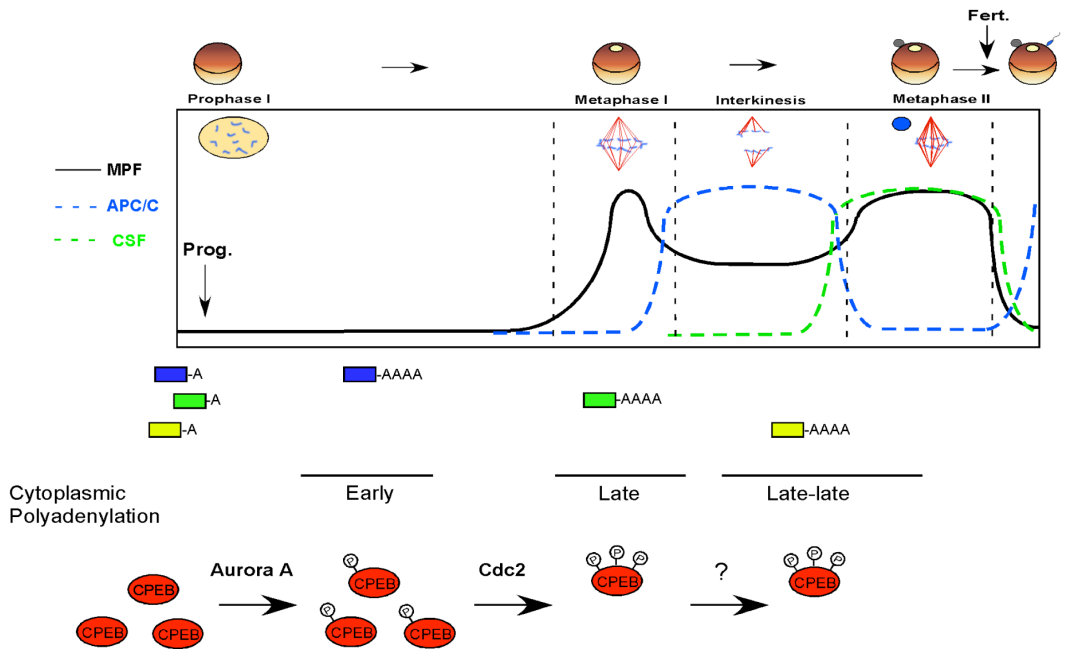


Fig 1. Schematic representation of meiotic progression from PI arrest to fertilization. MPF, APC/C and CSF activities are indicated. Oocyte morphology, chromosome dynamics, mitotic spindles

and polar body are shown. CPEB protein levels and phosphorylation regulation and the three waves of polyadenylation (early, late and late-late) are described. A indicates a short poly(A) and AAAA a long poly(A); P indicates phosphorylation; Fert. indicates fertilization (extracted from[9]).

THE MITOTIC CELL CYCLE

Mitosis is the process of nuclear division of eukaryotic cells to produce daughter nuclei that are genetically identical to the parent nucleus. In order to accomplish this task, the DNA in each chromosome must be replicated without errors and the replicated chromosome segregated precisely to the daughter cells, which receive a complete set of the genome.

As viewed in the microscope, the cell cycle is divided into two basic parts: mitosis and interphase. 95% of the cell cycle is spent in interphase: gap2 (G2), synthesis (S), and gap1 (G1) phases, while M-phase (mitosis and cytokinesis) lasts only about an hour. Interphase is the time during which both cell growth and DNA replication occur in an orderly manner in preparation for cell division. Mitosis is the nuclear division and cytokinesis is the process of cell cleavage that occurs at the end of mitosis (reviewed in[25]).

The cycle of eukaryotic cells is divided into four discrete phases (Fig. 2): the interphase begins with the G1-phase, which corresponds to the interval (gap) between mitosis and initiation of DNA replication. In this phase the cell is metabolically active and continuously grows but does not replicate its DNA. G1 is followed by S-phase (synthesis), during which DNA replication takes place. The completion of DNA synthesis is followed by the G2 phase (gap 2), during which cell growth continues and proteins are synthesized in preparation for mitosis[26]. It is not correct to consider the two gap phases just as time delays where the cell grows. In that time the cell monitors internal and external stimuli environment to decide its commitment in S and M-phases. For example, the length period for G1 is variable depending on external conditions and extracellular signals from other cells, the cell may even enter in a specialized quiescent state known as *G0* (G zero) and only in presence of specific mitogenic stimuli or signals they undergo through the progression of the cell cycle. At the end of G1 there is a commitment point, that is named in mammalian cells “restriction point”, and after this point, the cells are committed to enter in S-phase, even if the extracellular signals that stimulate cell growth and division are removed.

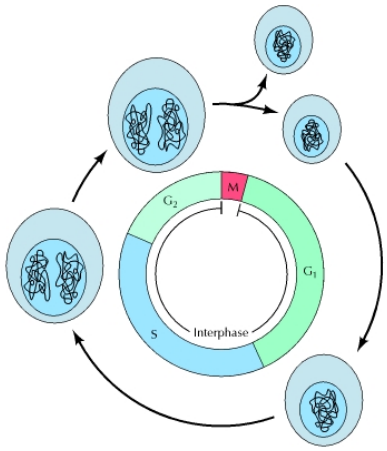


Fig 2. Phases of the cell cycle. The cells grows continuously in interphase, which includes three phases: S-phase is where the DNA is duplicated, G2 is the gap between S-phase and M-phase, M-phase is where the nucleus and then the cytoplasm divide and G_1 is the gap between M-phase and S-phase.

The duration of cell cycle phases depends considerably on the type of cells. It takes about 24 hours for a typical rapidly proliferating human cell, 90 minutes for budding yeasts, 30 minutes for early embryo cells after fertilization of the egg (Fig. 3). In this case, however, cell growth does not take place. Instead, these early embryonic cell cycles rapidly divide the egg cytoplasm into smaller cells. There is no detectable G1 or G2 phase, and DNA replication occurs very rapidly in these early embryonic cell cycles, which therefore consist of very short S phases alternating with M phases.

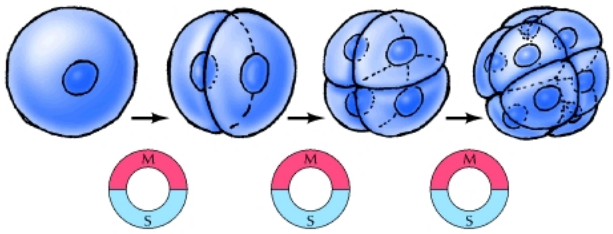


Fig 3. Embryonic cell cycles Early embryonic cell cycles rapidly divide the cytoplasm of the egg into smaller cells. The cells do not grow during these cycles, which lack G1 and G2 and consist simply of short S phases alternating with M phases.

However, some cells in adult animals cease division altogether (e.g., nerve cells) and many other cells divide only occasionally, as needed to replace cells that have been lost because of injury or cell death. Mitosis is the stage of the cell cycle in which occurs the biggest morphological changes in the cell. M-phase is usually divided into five distinct stages: prophase, prometaphase, metaphase, anaphase, telophase/cytokinesis (reviewed in[27]. It starts with prophase, as interphase chromatin condenses into well-defined chromosomes within the intact nuclear envelope and the previously duplicated centrosomes (the main

microtubule-organizing centre) migrate apart. The centrosome starts to nucleate two asters of dynamic microtubules, defining the poles of the future spindle apparatus.

Prometaphase begins with nuclear-envelope breakdown, the microtubules are captured by kinetochores (specialized proteinaceous structure associated with centromere DNA on mitotic chromosome). Chromosomes move to the spindle midzone, forming a highly dynamic metaphase plate. The sister cromatids (duplicated chromosomes) are connected to the opposite spindle poles.

In metaphase the chromosomes congress to the metaphase plate, where continue to oscillate throughout “metaphase”, suggesting that a balance of forces keeps them under tension. The degradation of key mitotic regulators begins, culminating in the activation of the anaphase-promoting complex/cyclosome (APC/C). Then a protease, known as separase, cleaves a key regulator of sister chromatids pairing, trigging the onset of anaphase.

In anaphase the sister chromatids are pulled to the opposite poles of the spindle. Then, in telophase the nuclear envelope reforms around the daughter chromosomes, and chromatin decondensation begins.

In cytokinesis, the process of cell cleavage, a contractile ring assembles at the cortex of the cell and the whole cytoplasm is divided in two daughter cells and the cell cycle is finished(reviewed in[25, 27].

The progression through the cell cycle is very well regulated and it is based on the complex network of regulatory proteins, called as the cell-cycle control system. Cell cycle progression relies mainly on two post-translational mechanism: protein phosphorylation and protein synthesis/degradation. The core of the cell-cycle control system is a family of protein kinases known as cyclin-dependent protein kinases (Cdks), whose activity depends on association with regulatory subunits called cyclins. The associations of different cyclin-Cdk complexes leads in the regulation of the different cell cycle transitions: Cyclin-D -CDK4/6 for G1 progression, Cyclin-E -CDK2 for the G1-S transition, Cyclin-A -CDK2 for S-phase progression, and Cyclin-A/B-CDC2 for entry into M-phase. Cyclins associate with CDKs to regulate their activity and also the progression of the cell cycle through specific checkpoints. For example the “DNA structure checkpoints” arrest cell at the G2/M transition in response to unreplicated DNA or DNA damage or the “spindle assembly checkpoint” prevents anaphase onset in the case that chromosome kinetochores do not show a correct bipolar attachment. All cyclins are degraded by ubiquitin-mediated processes and both synthesis and destruction of cyclins are important for cell cycle progression. For example the destruction of cyclin-B by APC is essential for metaphase-anaphase transition, and expression of indestructible Cyclin-B traps cells in mitosis[28].

FROM BIRTH TO DEATH: THE COMPLEX LIVES OF EUKARYOTIC mRNAs

Messenger RNAs (mRNAs) carry the information that needs to be transferred from DNA to protein. In eukaryotes, mRNAs are first synthesized in the nucleus as pre-mRNAs that are subjected to 5'-end capping, splicing, 3'-end cleavage, and nuclear polyadenylation. Once pre-mRNA processing is achieved, mature mRNAs are exported to the cytoplasm, where they serve as the blueprints for protein synthesis by ribosome and then are degraded.

Throughout their lifetime, mRNAs are escorted by host of associated factors, some of which remain stably bound while others are subjected to dynamic exchange (Tab. 1). Together with mRNA, this complement of proteins and small noncoding RNA (microRNA, miRNAs) constitute the messenger ribonucleoprotein particle (mRNP). It is the unique combination of factors accompanying any particular mRNA, as well as their relative position along the transcript, that dictates almost everything that happens to each mRNA in the cytoplasm [29].

The primary protein-coding transcripts that are produced by RNA polymerase II are termed pre-mRNAs (or, using the historical term that describes their size heterogeneity and cellular localization, heterogeneous nuclear RNAs; hnRNAs). The pre-mRNAs are associate with small nuclear RNP (snRNP) and a variety of different nucleocytoplamic shuttling hnRNP (heterogeneous RNP), SR (serine/arginine rich) and the exon junction complex (EJC) protein. The factors that determine the specific constellation of hnRNP proteins that assembles on each mRNA probably depend on the mRNA sequence and on the repertoire of hnRNP (including their relative abundance and the specific post-translational modifications that the receive) in the nucleus during transcription[30, 31].

Most, if not all, hnRNP proteins contain one or more of a small number of RNA-binding motifs. The most common of this are the RRM (RNA-recognition motifs), KH domains and RGG (Arg-Gly-Gly)[32].

HnRNP proteins participate in various nuclear events, such as transcriptional regulation [33, 34], telomere-length maintenance[35, 36], immunoglobulin gene recombination[37], splicing[30, 38], pre-ribosomal processing and 3'-end processing[39, 40]. HnRNP proteins are also important in nucleo-cytoplasmic transport of mRNA[41, 42], and in mRNA localization[43], translation [44] and stability[45].

The first mayor change in mRNP composition occurs as mRNAs are birthed from the nucleus through the nuclear pore complex (NPC). The NPC is a large, eight-fold symmetric supramolecular assembly (50 to 125 MD) that serves as the molecular gatekeeper for movement of proteins and protein-RNA complexes between the nucleus and the cytoplasm[46].

Many mRNAs enter the translationally active pool immediately upon export to the cytoplasm. At this stage, the 5'-cap is still largely bound by the nuclear CBC20/80 complex, whereas the poly(A) tail carries a mixture of nuclear and cytoplasmic poly(A) binding proteins PABPN1 and PABPCs (Tab. 1). In this newly exported mRNPs, CBC20/80 can functionally interact with translation initiation factor 4G (eIF4G), which serves to recruit the small ribosomal subunit and initiate 5'→3' scanning along the 5'UTR for an AUG start codon[47]. At some point CBC20/80 and PABPN1 are also replaced by eIF4E (the mayor cytoplasmic cap-binding protein) and PABPC, respectively. It is unknown whether the exchange occurs, a possibily could be that the low cytoplasmic concentrations of CBC20/80 and PABPN1 coupled with high concentration of eIF4E and PABPCs could naturally lead to the latter set replacing the former given reasonable dissociation rates[29]. In any event, once the translation is complete, a network of simultaneous interactions between the 5'-cap, eIF4E, eIF4G, PABPCs, and the poly(A) tail results in functional circularization of the message, an arrangement thought to facilitate translation control by regulatory elements in the 3'UTR, promote efficient ribosome reinitiation during active translation, and protect both ends of the transcript from the mRNA degradation machinery[48].

Upon export, not all mRNAs immediately enter the translationally active pool. Many are held instead in a translationally quiescent state awaiting either proper subcellular localization or some signal that the timing is now right to make the protein. Localization is associated with regulated translation, in order to produce the protein in a specific subcellular compartment. Mechanisms for mRNA localization include active transport along the cytoskeleton, diffusion and anchoring, local protection from degradation, and local synthesis by subsets of nuclei in syncytial cells. In many istances, a combination of mechanisms work on a single transcript.

The half-life of mRNA lives depends on how efficiently the mRNA degradation machinery is recruited to that mRNP. The general mRNA decay is required for the elimination of aberrant mRNAs containing a premature translational stop signal (nonsense mRNA) or lacking a translational signal altogether (nonstop mRNA)[49, 50]. In both yeast and mammalian cells, much of the mRNA decay machinery is concentrated in discrete cytoplasmic *foci.* These so-called cytoplasmic processing bodies, or "P-bodies" (PBs), appear to form around aggregates of mRNPs not actively involved in translation[51]. "Stress granules" (SGs) are related but distinct structures in mammalian cells, they are temporary *foci* for translationally inactive mRNPs upon exposition of environmentally stresses. When the stress is relieved, SGs disassemble and the sequestered mRNAs either return to the translationally active pool or are targeted for degradation in PBs[52, 53].

CBC20/80	The nuclear cap binding complex. A heterodimer of 20 and 80 kD subunits. Joins the mRNP coincident with cap formation during transcription and facilitates pre-mRNA splicing. In the cytoplasm, can serve as a translation initiation factor through interactions with eIF4G but is ultimately replaced by eIF4E.
eIF4E	Eukaryotic translation initiation factor 4E. The major cytoplasmic cap binding protein. Target of many translational regulators [eIF4E binding proteins (4E-BPs)] that disrupt its interaction with eIF4G.
eIF4G	Eukaryotic translation initiation factor 4G. A large scaffolding protein that can simultaneously interact with cap binding proteins, PABPCs, and eIF3 bound to the small ribosomal subunit.
PABPN1	The nuclear poly(A) binding protein. Binds poly(A) by a single RNA recognition motif (RRM) and an arginine-rich C-terminal domain. In budding yeast, the evolutionarily unrelated Nab2 protein serves this role.
PABPCs	Cytoplasmic poly(A) binding proteins. Single-celled eukaryotes contain a single PABPC, whereas human cells contain four. All PABPCs bind poly(A) RNA through four RRMs.
HnRNP proteins	A diverse set of factors loosely defined as all proteins associating with heterogeneous nuclear RNA (hnRNA, made up of pre-mRNA and nuclear mRNA) that are not stable components of other RNP complexes, such as small nuclear RNPs (snRNPs). Some hnRNP proteins accompany mRNAs to the cytoplasm; others are confined to the nucleus.
EJC	The exon junction complex. A set of proteins loaded onto mRNAs upstream of exon-exon junctions as a consequence of pre-mRNA splicing and which accompanies the spliced mRNA to the cytoplasm.
SR proteins	A family of structurally related, nuclear RNA binding proteins containing an RRM and a domain rich in serines and arginines (RS domain). The serines in the RS domain serve as sites of dynamic phosphorylation. Some SR proteins accompany mRNAs to the cytoplasm; others are confined to the nucleus. Many SR proteins play key roles in pre-mRNA splicing.
Y-box proteins	A family of multifunctional nucleic acid binding proteins containing a "cold-shock" domain. Along with PABPCs, Y-box proteins constitute the major mRNP structural components in somatic cells. They are thought to bind along the body of the message and have a packaging role that modulates translational activity. In *Xenopus* oocytes, Y-box proteins FRGY2 and mRNP3 are major components of stored mRNPs.
TIA-1/TIAR	Structurally related RNA binding proteins consisting of three RRMs and a C-terminal prionlike domain. The prionlike domain is thought to self-oligomerize in vivo and drive the formation of stress granules.
miRNAs	MicroRNAs. Small noncoding RNAs that imperfectly base-pair with recognition sites in 3′ UTRs. In combination with RISC (RNA-induced silencing complex), miRNAs negatively regulate protein synthesis by the cognate mRNA.

Table 1. List of mRNPs. mRNP is the messenger ribonucleoprotein particle (extracted from[29].

THE mRNA STRUCTURE

In eukaryotes, mRNAs are first synthesized in the nucleus as pre-mRNAs, primary transcript of the genomic DNA that contains protein-coding as well as protein-noncoding sequences. Pre-mRNAs undergo in the nucleus through co-trascriptional processing that consists in five prime end (5'-end) capping, splicing, three prime end (3'-end) cleavage and nuclear polyadenylation. While the pre-mRNAs is transcribed, it is immediately coated by several proteins needed for processing and mRNA export to the cytoplasm, where mRNAs are translated into proteins by ribosomes (please refer to "FROM BIRTH TO DEATH: THE COMPLEX LIVES OF EUKARYOTIC mRNAs"
The structural features of mature mRNA molecules include the canonical end modifications: cap structure in the 5'-end and polyadenosine tail (poly (A) tail) in the 3'end. The mRNA's body is composed of the open reading frame (ORF), which is in the middle of the upstream 5'untraslated region (5'UTR) and downstream of the 3'untraslated region UTR (3'UTR) (Fig 4).

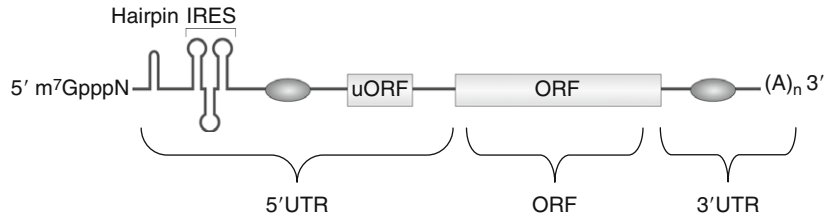


Fig 4. Cis-acting regulatory sequence in the 5'UTR, ORF and 3'UTR of mRNAs. m7GpppN is the cap structure, IRES are structured internal ribosomal entry site, uORF are upstream open reading frame and (A)n is the poly(A)tail (extracted from[54]).

5'UTR

It is the noncoding or untranslated region at the 5'-end of an mRNA. It contains a variety of regulatory elements, which are able to influence its overall translation rate. These elements are: the length of the 5'UTR, its thermal stability and GC content, the locations of secondary structures and stem loops, multiple upstream ORFs (uORF), upstream AUGs (uAUG) and IRES, primary sequence binding sites for regulatory proteins[2, 54, 55]. The average length of the 5'UTR of mRNAs in humans is 210 nt and the minimum length has been shown to be 18 nt. Although some prokaryotic mRNAs may be leaderless, eukaryotic mRNAs always have UTRs. The 5'UTR of the Tre oncogene mRNA (2858 nt) is the longest known 5'UTR in humans (reviewed in[56]. The impairment of any of these features in mRNAs can alter translational regulation, leading to various diseases or disease susceptibility (reviewed in[57]).

CAP STRUCTURE AND SYNTHESIS

The 5'-end of most eukaryotic polymerase II (Pol II) transcripts is modified co-trascriptionally by addition of a 7-methyl guanosine (m7G) cap. This process occurs when the nascent transcript is 20-25 nucleotides in length[58] through the RNA polymerase II large subunit CTD (carboxy-terminal domain) [59] [60, 61]. The CTD is a docking site for factors and enzymes involved in mRNA processing and required for mRNA cap methylation, splicing and polyadenylation. Depending on the species, it contains about 50 repeats of the consensus sequence YSTPSPS and can be phosphorylated on multiple sites (Ser^2 and Ser^5 are the most characterized)[62, 63]. The phosphorylations and dephosphorylations on the CTD residues along the transcription, dictate which enzymes and factors are recruited, retained or removed from the transcribing polymerase[59-61].

The cap structure consists of a methylated guanosine at position 7, linked by a three phosphates group to the first transcribed nucleotide. This structure is defined as "cap 0" [64] (Fig. 5).

In both metazoans and unicellular eukaryotes capping proceeds by the sequential action of three enzymes [pppN(pN) is the 5'end of mRNA with three phospates (p), where N is any nucleotide and n is the number of nucleotides linked to N: Pi is inorganic phosphate; G is a guanosine; and m7GpppN(pN)n is mature capped mRNA][65].

First, RNA 5'-triphosphatase (RT) removes a phosphate from the 5'-end of the nascent transcript:

pppN(pN)n⇒ppN(pN)n + Pi (n= 20-25);

second, a guanylyltransferase adds a guanosine residue in an inverted position:

ppN(pN)n + GTP⇒GpppN(pN)n + PPi;

third, the inverted guanosine is methylated by N7G-methyltransferase:

GpppN(pN)n +S-adenosylmethionine (Adomet)⇒ m7GpppN(pN)n + S-adenosylhomocysteine (Adohcy).

Enzymes involved in capping have been identified in several species. The three enzymes are encoded separately in yeast, however in mammals the RT and the GT are replaced by a bifunctional capping enzyme (CE). Some viruses, like vaccinia virus, encode a single polypeptide containing all three activities. RNMT, the mammalian methyltransferase is a distinct protein that belongs to the Rossmann-fold MTase (RFM). In higher eukaryotes the cap 0 can be further methylated at the 2' hydroxyl group (2'-O) of the second ribose (counting 7-methylguanosine as the first), modification designated cap I, and also at the third ribose, cap II[66].Sometimes also the adenosine at the second base is methylated at position 6[67] (Fig. 5).

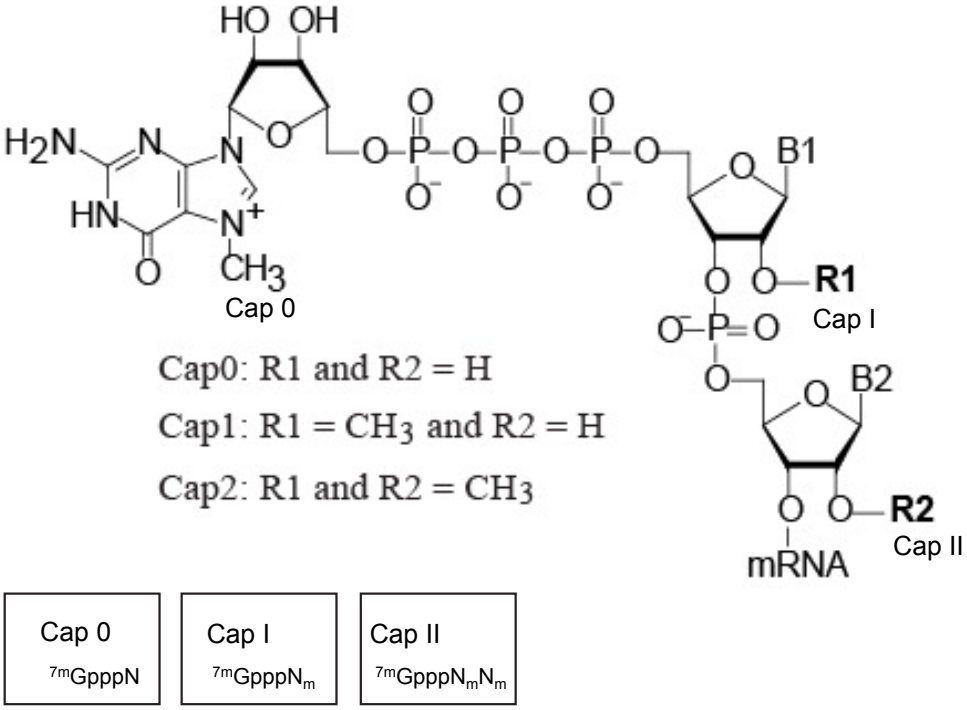


Fig 5. Chemical structure of the 5′-cap in eukaryotic mRNA. Cap 0 is the 7-methylguanosine cap; cap I and cap II occur when the methyl group (CH_3) is added to the ribose of the first and the second nucleotide, respectively (adapted from[68]).

CAP-RIBOSE METHYLTRANSFERASES

Cap-specific mRNA (nucleoside-2-O-)-methyltransferases I and II are a S-adenosyl-L-methionine-dependent (SAM) methyltransferases that mediates mRNA cap I or cap II 2'-O-ribose methylation to the 5'-cap structure of mRNAs. Position-specific iterative BLAST searches have identified several families of site-specific Rossmann-fold 2'-O-MTases[69], which have a common putative catalytic tetrad K-D-K-E [70].

Cap I-specific 2′-*O*-ribose MTases have been studied extensively in viral systems. One of the best characterized is the vaccinia virus protein VP39. It belongs to *poxviruses* family and it is a prototype of cap I-dependent 2'-O nucleoside methyltransferase; it transfers a methylgroup from AdoMet to the ribose 2'-OH of the first transcribed nucleoside of viral mRNA to form cap I[71, 72]. Moreover VP39 is a bifunctional enzyme, it functions as the smaller stimulatory subunit of poly(A)polymerase (PAP). Vaccinia PAP is a heterodimer in which the larger subunit VP55 catalyzes the poly (A) tail formation[73]. In the absence of the VP39 subunit, VP55 adds the first 30-35nt in a rapid, highly processive way, but then in a slow and a nonprocessive mode. VP39 possesses no independent PAP activity, but convert the nonprocessive polyadenylation to a rapid semi-processive reaction, anchoring the VP55 subunit to the growing poly (A) tail[73, 74]. The 1.85 A crystal structure of a VP39 variant complexed with its AdoMet cofactor is reported[75].

Based on crystal structures, mechanisms of action have been proposed for cap I MTase activities residing in domain I of Reovirus protein λ2[69] and the N-terminal portion of flavivirus NS5 proteins (reviewed in[76, 77]). The *orf 69* gene in the baculovirus *Autographa californica* nucleopolyhedrosis virus (AcNPV) also encodes a cap I 2′-*O*-ribose MTase[78]. The characterization of the NS5 protein from West Nile flavivirus (WNV) demonstrated its ability to perform both guanosine N^7 and ribose 2′-*O*-ribose methylations that complete the 5′ cap[79], as well as the SARS coronavirus[80]. Region VI of L protein in vesicular somatitis virus shows similar dual MTase activities with a single SAM binding pocket in the protein.

It has been found that among unrelated RNA and DNA viruses that replicate in the cytoplasm and contain 5'cap structures, the 2'-O-methylation of the viral mRNA enhances virulence through evasion of intrinsic cellular defence mechanisms, like type I interferon (IFN) signalling, throw the modulation of the proteins with tetratricopeptide repeats (IFIT), which are interferon-stimulated genes implicated in regulation of protein translation[81] [82, 83].

Cap I and cap II structures are present in the majority of mRNAs in higher eukaryotes, except for cases of trans-splicing in kinetoplastid protozoa, such as trypanosome. The trans-splicing consists in the addition of a mini-exon at the 5'-end of a mature mRNAs. Prior to trans-splicing, the mini-exon donor RNA is capped by the addition of a (5'-5') triphosphate-

linked 7-methylguanosine, followed by modification of the first four transcribed nucleotides (cap 4)[84]. In nematodes, in addition to cap 0 mRNAs, there is a certain amount of mRNAs containing an hypermethylated 2,2,7-trimethylguanosine (TMG) cap structures, in which 7-methyl guanosine is replaced by 2,2,7-trimethyl guanosine (m3 2,2,7GpppN). TMG is also present on the 5'-end of the majority of U small nuclear RNAs (snRNA), important molecule involved in splicing (U1, U2, U4 and U5)[85, 86].

The proteins responsible for ribose 2′-*O*-ribose methylations at positions 1 (*TbMTr1*), 2 (*TbMTr2*), 3 and 4 (*TbMTr3*) have been identified [87].

There was characterized also the human cap I 2'O-ribose methyltransferase (hMTr1) [68], as well as the human cap II 2'O-ribose methyltransferase (hMTr2)[88].

THE OPEN-READING FRAME (ORF)

The open reading frame is the mRNA sequence that is translated into protein . It starts with the initiation codon AUG and terminates with one of three stop codons, UAA, UAG or UGA. The mechanisms driving translation of the ORF are described below.

3'UTR

It is the noncoding or untranslated region at the 3'end of an mRNA. It immediately follows the stop codon and includes regulatory sequence elements, such as those that direct formation of the poly(A) tail, as well as sequence elements that regulate mRNA translation, mRNA stability, mRNA localization and binding sites for microRNAs (miRNAs). More than half of the mammalian mRNAs undergo to alternative processing events, causing the formation of multiple mRNA transcripts with a different 3'-end[89, 90]. The choice of the 3'UTR affects the inclusion or the exclusion of regulatory elements, which in turn influence tissue- and cell cycle-specific gene regulation[29, 91], or more dramatically, the translation of different proteins with different domains. Alternative 3'UTR formation is generated by alternative splicing events (AS) or alternative selection of cleavage and polyadenylation sites (alternative polyadenylation, APA) or the combination of both[92].

The shortening of 3'UTR by APA is correlated with increased protein expression, proliferation, transformation, and early developmental stages, in part as a result of exclusion of miRNA sites[93-97]. While longer 3'UTR are correlated with decreased protein expression, differentiation, localization and late developmental stages[98].

THE POLY(A) TAIL

The poly-A tail is a long chain of adenine nucleotides that is added to mRNA molecules. Nuclear polyadenylation is ubiquitous for al the mRNAs. However, more and more evidences from studies of the last 70 years show that mRNA-polyadenylation occurs also in the cytoplasm on a large set of mRNAs.

The nuclear polyadenylation is part of the 3'end processing. It is a two-step reaction, which involves an endonucleolytic cleavage of the pre-mRNA, followed by a synthesis of polyadenylated tail onto the upstream cleavage product (reviewed in[91]. The core molecular machine responsible for the 3'-end formation is composed by four multi-subunit protein complexes: CPSF (Cleavage and Polyadenylation Specificity Factor), CstF (Cleavage stimulation Factor), CFI and CFII (Cleavage Factor I and II). In addition the RNA polymerase II (POL II), PABP (poly(A) binding protein), Symplekin and the single subunit poly(A) polymerase (PAP), constitute the cleavage and polyadenylation complex. The assembly of the complex depends on the cooperative interactions of CPSF and CstF to specific sequences in the pre-mRNA. CPSF (specifically CPSF160 subunit) binds the canonical poly(A) signal (PAS) AAUAAA or AUUAAA located 25-30 nucleotides upstream of the cleavage site (CA), while CstF (through the CstF64 subunit) recognizes a less defined 30 nucleotides downstream U/GU-rich region. These events permit the recruitment of the cleavage factors to the correct cleavage site (Fig. 6a). After the cleavage, the poly (A) tail is firstly elongated in a distributive manner until PABP binds, and secondly in a processive manner, until it reaches a length of approximately 250-300 adenosine residues (reviewed in [99]) (Fig. 6b).

Once the mRNAs are exported to the cytoplasm, they could be immediatly available for translation, or could be rather de-adenylated and stored in a repressed status until their translation is needed. Cytoplasmic polyadenylation occurs on the last set of mRNAs and is involved in several biological processes such as meiotic progression[100], mitosis[101] and tumor progression[102].

The cytoplasmic polyadenylation targets mRNAs with a short poly(A) tail of 20-30 nucleotides. One of the most studied mechanism of cytoplasmic polyadenylation is dependent on CPEB1 (cytoplasmic polyadenylation element binding protein 1). This process requires the presence of two cis-element in the 3'UTR of the regulated mRNAs. The first is the PAS that, as for the nuclear polyadenylation, is recognized by CPSF, and the second is the cytoplasmic polyadenylation element (CPE), present in many mRNAs. Once CPEB1 is phosphorylated, it recruits CPSF to the PAS. Together CPEB1 and CPSF recruit the cytoplasmic poly(A) polymerase GLD-2, mediating the elongation of the poly(A) tail. Multiple copies of the embryonic poly(A)-binding protein (ePAB) seat on the elongating poly(A)tail

(reviewed in[99]) (Fig. 6c). The poly(A) tail is important in the regulation of the stability, transport and translation of mature transcripts (reviewed in[90]).

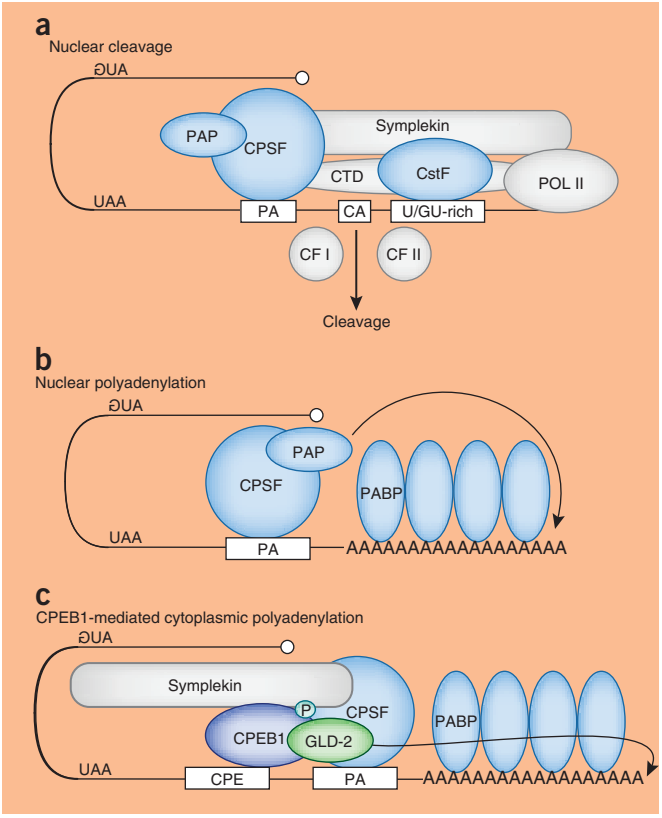


Fig 6. a, Nuclear cleavage. CA is the cleavage site, PA or PAS is the polyadenylation signal, A/GU-rich is the U- and GU-rich sequence. The core complex is formed by the *trans*-acting elements: CPSF (Cleavage and Polyadenylation Specificity Factor), CstF (Cleavage stimulation Factor), CFI and CFII (Cleavage Factor I and II), the carboxy-terminal domain (CTD) of RNA polymerase II (POL II), PABP, Symplekin and the single subunit poly(A) polymerase (PAP)
b, Nuclear polyadenylation. PABP is the poly(A) binding protein.
c, CPEB1-mediated cytoplasmic polyadenylation. CPE is the cytoplasmic polyadenylation element, CPEB1 is the cytoplasmic polyadenylation element binding protein 1, GLD-2 the cytoplasmic poly(A) polymerase GLD-2 (extracted from[99]

TRANSLATION

INITIATION

Translation initiation in eukaryotes is the most regulated and complex stage of gene expression (Fig. 7). It requires the action of at least 12 initiation factors (eIFs), many of which are known to be targets of regulatory pathways (Tab 2).

The goal is the identification of the initiation codon by the eukaryotic translational machinery.

The first step is the recruitment of the initiator methionyl-tRNA (Met-tRNA$_i$) to the (40s) ribosomal subunit to form the 43S preinitiation complex (PIC). The Met-tRNA$_i$ is delivered to the 40S subunit in the ternary complex (TC), which is formed by the Met-tRNA$_i$ and the GTP-bound form of eIF2. The binding between Met-tRNA$_i$ and eIF2•GTP is specific, infact the affinity of Met-tRNA$_i$ is greater for eIF2•GTP than for eIF2•GDP, and this affinity switch depends on the methionine moiety on the Met-tRNA$_i$[103]. Once the GTP in TC is hydrolyzed to GDP the eIF2•GDP must be recycled to eIF2•GTP for renewed TC assembly, a reaction catalyzed by the heteropentameric eIF2B complex. This interaction is enhanced by one of the eIF2α kinases, which are activated in stress condition to down-regulate general initiation[104]. Binding of TC to the 40s subunit requires the assistance of eIFs 1, 1A, 5, and the eIF3 complex[105] [106-108].

The second step is the mRNA recruitment to the 43S PIC. The 43S PIC binds the mRNA near the 5'-7-methylguanosine cap, a process that is promoted by eIF3, the poly(A)-binding protein (PABP), and eIFs 4B, 4H (in mammals), and 4F. The eIF4F complex is formed by the cap-binding protein eIF4E, the RNA helicase eIF4A and the scaffolding protein eIF4G. The binding of eIF4G to the 3' poly(A) tail-binding protein (PABP) stabilizes the recruitment of eIF4F and the resulting assembly of circular messenger ribonucleoprotein (mRNP), referred to as the "closed-loop" structure.

The third step is the scanning and AUG recognition. 43S PIC scans the mRNA leader for an AUG codon in a suitable sequence context. The initial event in start codon recognition is the base-pairing between the anticodon of Met-tRNA$_i$ and the AUG in the peptidyl-tRNA (P) site of the 40S subunit[109-111]. The scanning PIC is arrested after AUG recognition, eIF2•GDP and many other eIFs present in the PIC, are released.

The fourth step is the subunit joining. The joining of the large subunit (60S) is catalyzed by eIF5B to produce an 80S initiation complex (IC) containing Met-tRNA$_i$ base-paired to AUG in the P site and ready to begin the fifth step: the elongation phase of protein synthesis (reviewed in[111]).

Name	Number of subunits and their molecular mass (kDa)	Function
Core initiation factors		
eIF2	3 (36.1, 38.4 and 51.1)	Forms an eIF2–GTP–Met-tRNA$_i$ ternary complex that binds to the 40S subunit, thus mediating ribosomal recruitment of Met-tRNA$_i$
eIF3	13 (800 total)	Binds 40S subunits, eIF1, eIF4G and eIF5; stimulates binding of eIF2–GTP–Met-tRNA$_i$ to 40S subunits; promotes attachment of 43S complexes to mRNA and subsequent scanning; and possesses ribosome dissociation and anti-association activities, preventing joining of 40S and 60S subunits
eIF1	1 (12.7)	Ensures the fidelity of initiation codon selection; promotes ribosomal scanning; stimulates binding of eIF2–GTP–Met-tRNA$_i$ to 40S subunits; and prevents premature eIF5-induced hydrolysis of eIF2-bound GTP and P_i release
eIF1A	1 (16.5)	Stimulates binding of eIF2–GTP–Met-tRNA$_i$ to 40S subunits and cooperates with eIF1 in promoting ribosomal scanning and initiation codon selection
eIF4E	1 (24.5)	Binds to the m^{7}GpppG 5′ terminal 'cap' structure of mRNA
eIF4A*	1 (46.1)	DEAD-box ATPase and ATP-dependent RNA helicase
eIF4G‡	1 (175.5)	Binds eIF4E, eIF4A, eIF3, PABP, SLIP1 and mRNA (see FIG. 3a) and enhances the helicase activity of eIF4A
eIF4F	3 (246.1 total)	A cap-binding complex, comprising eIF4E, eIF4A and eIF4G; unwinds the 5′ proximal region of mRNA and mediates the attachment of 43S complexes to it; and assists ribosomal complexes during scanning
eIF4B	1 (69.3)	An RNA-binding protein that enhances the helicase activity of eIF4A
eIF4H	1 (27.4)	An RNA-binding protein that enhances the helicase activity of eIF4A and is homologous to a fragment of eIF4B
eIF5	1 (49.2)	A GTPase-activating protein, specific for GTP-bound eIF2, that induces hydrolysis of eIF2-bound GTP on recognition of the initiation codon
eIF5B	1 (138.9)	A ribosome-dependent GTPase that mediates ribosomal subunit joining
eIF2B	5 (33.7, 39.0, 50.2, 59.7 and 80.3)	A guanosine nucleotide exchange factor that promotes GDP–GTP exchange on eIF2
Auxiliary factors		
DHX29	1 (155.3)	A DExH box-containing protein that binds 40S subunit and promotes ribosomal scanning on mRNAs with long, highly structured 5′ UTRs
Ded1	1 (65.6)	A DEAD box-containing NTPase and RNA helicase that potentially promotes scanning in *Saccharomyces cerevisiae*
eIF6	1 (26.6)	An anti-association factor that binds 60S subunits and prevents them from joining to 40S subunits
p97	1 (102.4)	Closely related to the carboxy-terminal two-thirds of eIF4G; binds eIF4A and eIF3; and promotes initiation in a potentially mRNA-specific manner
PABP	1 (70.7)	Binds to the 3′ poly(A) tail of mRNA, eIF4G and eRF3; enhances binding of eIF4F to the cap; and might facilitate recruitment of recycled post-termination 40S subunits back to the 5′ end of mRNA

Table 2. Eukaryotic initiation factors. Ded1, DEAD box helicase 1; DHX29, DExH box protein 29; eIF, eukaryotic initiation factor; PABP, poly(A)-binding protein (extracted from [112].

ELONGATION

The ribosome contains three sites for the peptidyl tRNA: site A, is the point of entry for the aminoacyl tRNA (except for the first peptidyl tRNA, which enters at the P site), site P is where the peptidyl tRNA is formed in the ribosome and the E site, which is the exit site of the uncharged tRNA after it gives its amino acid to the growing peptide chain. Peptide chain elongation begins with a peptidyl tRNA in the ribosomal P site next to a vacant A site. Then, conformational changes occur and the A site is ready for the binding of a new aminoacyl-tRNA, process, that is activated by eEF1A/EF-Tu GTPase activity. The ribosomal peptidyl transferase center then catalyzes the formation of a peptide bond between the incoming amino acid and the peptidyl tRNA[29].

Then, the tRNA is deacylated and its acceptor end is in the exit (E) site of the large ribosomal subunit, while its anticodon end is in the P site of the small ribosomal subunit (reviewed[113]).

The peptidyl-tRNA istead, has its acceptor end in the P site of the large ribosomal subunit and its anticodon end in the A site in the small subunit. This complex is translocated by elongation factor 2 (eEF2)[114]. At the end of the translocation the deacylated tRNA is completely in the E site, the peptidyl-tRNA completely in the P site, and the mRNA moved by three nucleotides to place the next codon of the mRNA into the A site.

This cycle is repeated until a stop codon is encountered and the process of termination is initiated.

TERMINATION

The termination of translation is caused by the presence of a stop codon in the ribosomal A site. The peptidyl transferase center of the ribosome catalyze the hydrolysis of the ester bond linking the polypeptide chain to the P site tRNA, a reaction that permits the release of the completed polypeptide[115]. There are two mainly factors involved: the class 1 release factors, which decode stop codons presented in the A site and the class 2 release factors (GTPases), which stimulate the activity of class 1 release factors regardless of which stop codon the class 1 factor has engaged.

RECYCLING

The recycling of the ribosomal subunits is the fourth stage of translation, so they can be used in another round of initation. In eukaryotes and in archea the factors involved in the recycling are almost unknown. Significant information is available only for bacteria. One of

the factor proposed to be involved is eIF3, that binds to the side of the 40S subunit opposite the interface[116].

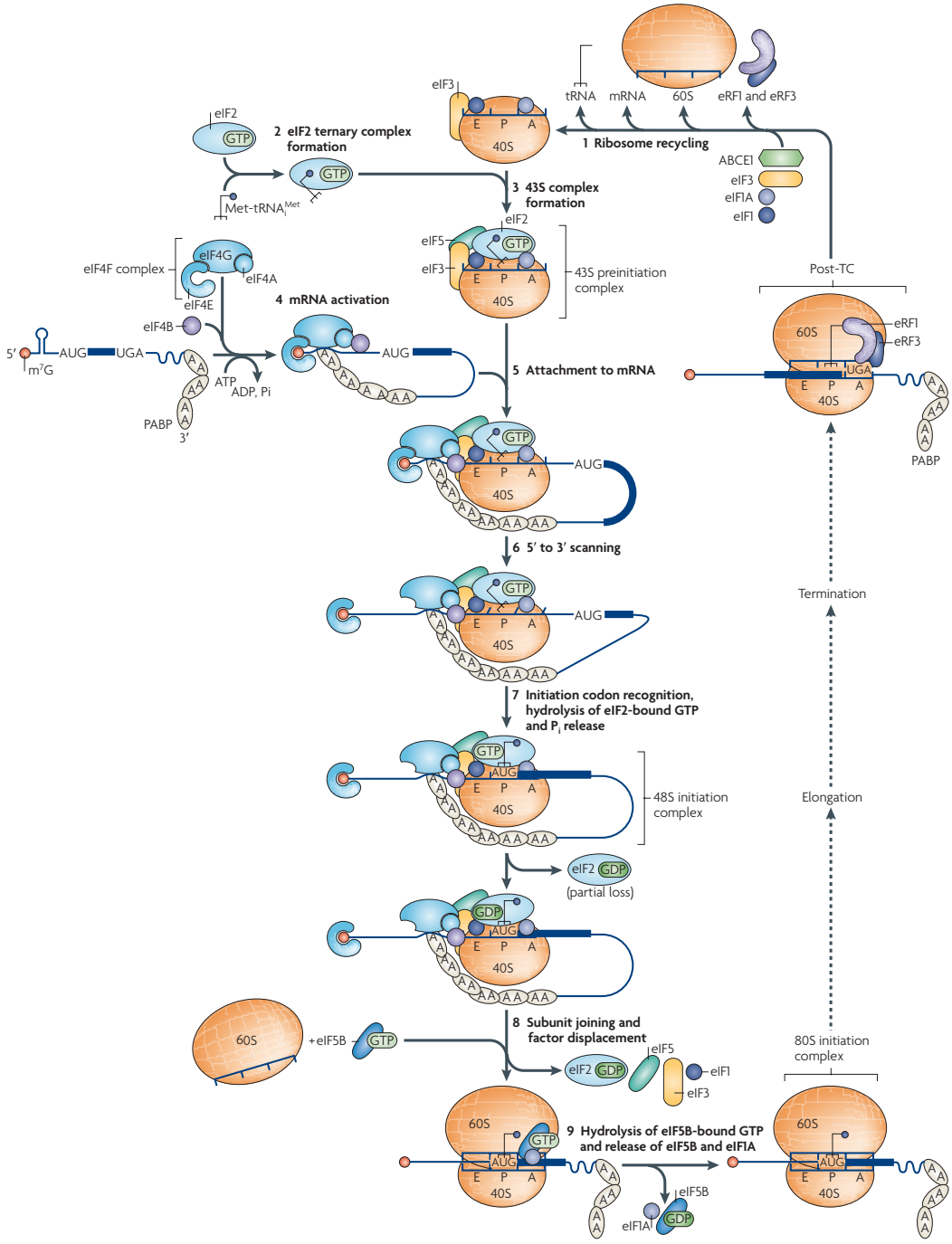


Fig 7. Current model of the canonical pathway of eukaryotic cap-dependent translation initiation. (Extracted from[112]).

SYNERGIC INTERACTION BETWEEN CAP STRUCTURE AND POLY(A) TAIL:THE CLOSED-LOOP MODEL

As previously mentioned, protein synthesis is usually regulated at the initiation stage, mediated by the 5′ ^{7m}GpppN mRNA cap structure bound by the translation initiation complex eIF4F, composed of eIF4E, eIF4A, and eIF4G, which has a consensus binding site Y*XXXX*Lϕ for eIF4E, and additional sites for eIF3 and the poly(A)-binding protein. eIF3 recruits the small ribosomal subunit, whereas the eIF4E-eIF4G-poly(A)-binding protein (PABP) relay results in the so-called "closed loop" model[117-119]. The mRNA circularization formed by the cap-eIF4E-eIF4G-PABP-poly(A) leads to the synergistic enhancement of translation by capped and polyadenylated mRNAs[120, 121]. Probably the vicinity of both ends enhances the translational efficiency by the new recruitment of the translation-terminating ribosome to the next translation initiation[120]. PABP is a 70kDa protein, which contains four RNA recognition motifs (RRMs) and a proline-rich C-terminal region [122, 123]. PABP is organized in a repeated structure, every 27 adenosine residues of the poly(A) tail[124]. The poly(A) ribonucleoprotein stimulates the 60S subunit joining step[125-127] in addition to the recruitment of the 40S ribosomal subunit to the mRNA[128](Fig. 8).

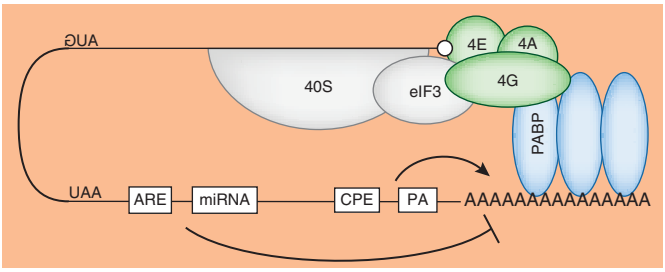


Fig 8. The "closed-loop" model. The mRNA circularization formed by the cap-eIF4E-eIF4G-PABP-poly (A) leads to the synergistic enhancement of translation by capped and polyadenylated mRNAs. miRNA binding site and AREs target this loop promoting deadenylation (extracted from[99]).

The 5'-cap and the 3' poly(A) tail stimulate translation on their own, but together they exert a synergistic effect. The mechanism by which the 5'-end functionally interacts with the 3'-end has been first elucidated in yeast[117]. For example, deadenylation-dependent decapping precedes exonucleolytic degradation of mRNAs in Saccharomyces cerevisiae[129], but in contrast deadenylation during oocyte maturation does not cause decapping in Xenopus oocytes[130]. In *Xenopus* oocytes, the cytoplasmic N7G-methyltransferase is independent of polyadenylation and increases at nuclear breakdown[130].

Moreover in frog oocytes the poly(A) tail and the cap stimulate translation synergistically upon progesterone stimulation[130]. This synergy was also demonstrated in plant, and mammalian cells in vivo[120, 131]. It was also recapitulated in vitro[132-135], and currently represents an attractive paradigm for control of translational initiation by 3'-end sequences. Many other studies contribute to understand the functional mRNA 5' and 3' interactions. Specific studies include reports of effects of poly(A) or the poly(A) tail upon cap-dependent translational initiation[126, 136], PAN1 (PAB-dependent poly(A) ribonuclease) in yeast is also required for translational initiation[137]; a deadenylation-dependent decapping step in the degradation of yeast mRNA[138]; translational repression through binding of a protein to the 5'UTR can cause deadenylation in somatic cells[139]; the sum of enhancement of translation of capped luciferase mRNAs, respect uncapped, in presence or in absence of the poly(A) tail and in a micrococcal nuclease-treated Krebs-2 cell extract[140]. High mRNA concentrations decreased both the cap- and poly(A) tail-dependence of translation and synergy. The reason for this is not immediately clear, but may reflect titration of general RNA-binding proteins[141] by mRNA excess.

GLOBAL CONTROL OF TRANSLATION

There are several reasons why the regulation of translation is a crucial event. In terms of time, the regulation at the translational level happens faster without the necessity of going through all the upstream processes of gene expression such as transcription, mRNA processing, and mRNA export. It is usually a reversible mechanism, because it is mediated through reversible protein modifications, such as the phosphorylation. It is crucial in those systems where the transcriptional control is not possible, like reticulocytes, which lack a nucleus, oocytes, or RNA viruses. It mediates the spacial control[142, 143] and the fine tuning of gene expression.

It is clear that translational regulation is involved in many physiological processes: in the response to cellular stress[144], in the mis-regulation of gene expression during cancer[145], in apoptosis[146], in development, and in the establishment of synaptic plasticity and, consequently, in learning and memory[147].

To simplify, the translational control can be divided into global regulation of translation and mRNA-specific regulation[54]. Global regulation affects the translational efficiency of the majority of mRNAs through a general tuning of translation, while mRNA-specific regulation only affects the translation of target mRNAs.

Global regulation of translational is often mediated by modifications of translation initiation factors.

The key target is the cap-binding protein eIF4E. This protein permits the assembly of eIF4F to the m7G cap, through the interaction via eIF4G and the helicase eIF4A. The disruption of the binding of eIF4E to eIF4G, through inhibitory protein, 4E binding proteins) (4E-BP) is a way to control translation initiation [148]. In mammals three 4E-BPs exist (4E-BP1,4E-BP2 and 4E-BP3), each containing the canonical eIF4G binding sequence for eIF4E: Tyr-X-X-X-X-Leu-φ, where φrepresents a hydrophobic amino acid. 4E-BPs is regulated by phosphorylation[149], when it is hypo-phosphorylated 4E-BPs bind to eIF4E and prevent translation initiation, but when it is hyper-phosphorylated, 4E-BPs binding to eIF4E is blocked. In addition to 4E-BPs, several other proteins can bind eIF4E in an mRNA-specific manner to inhibit translation initiation.

Another example for global downregulation of translation is the control of the availability of active ternary complexes, where the ternary complex permits the binding of Met-tRNAi to the 40S subunit.

After exposure of cells to stress conditions (e.g., oxidative stress, nutrient limitation, hypoxia, temperature stress), the a-subunit of eIF2 (eIF2a) is phosphorylated by specific kinases and inhibits the exchange of GDP for GTP by the guanine-nucleotide-exchange factor eIF2B and,

as a consequence, the formation of active ternary complexes is strongly reduced, and translation is downregulated globally[54, 144]. The molecular mechanism for this inhibition is based on the fact that eIF2B has a much higher affinity toward phosphorylated eIF2a–GDP than toward unphosphorylated eIF2a–GDP [150], causing the accumulation of blocked eIF2·GDP–eIF2B complexes and therefore the depletion of ternary complex. The mRNA itself can also be targeted to exert translational regulation, via cis-regulatory elements (mainly in the UTRs), that are bound by trans-acting factors. Also the ribosome could be a target, considering that several of its protein constituents can undergo posttranslational modifications, like ubiquitination[151], methylation[152], and NEDDylation[153].

mRNA-SPECIFIC CONTROL OF TRANSLATION

mRNA-specific control of translation is a layer of regulation of gene expression that affects the translation of selected sets of mRNAs. Different mechanisms have been described to drive mRNA-specific translation, mostly via RNA-binding proteins that recognize *cis*-regulatory elements of a given mRNA. So far, one of the most studied mechanism of mRNA-specific translational control is mediated by a family of RNA-binding proteins known as cytoplasmic polyadenylation binding proteins (CPEBs). These proteins are able to regulate translation of CPE-containing mRNAs in space and time. Approximately 20% of the mRNAs contain CPEs sequence. Indeed, upon nuclear export, not all the mRNAs are translated at once, but many of them are rather translationally repressed, eventually localized into the appropriate subcellular compartment, awaiting for the right time/stimulus to be activated and translated. An important event for mRNA translational activation to occur, is the cytoplasmic polyadenylation, which allows the formation of the "closed-loop" mRNA conformation, which is a requirement for translational competence.

MECHANISMS OF TRANSLATIONAL CONTROL BY CYTOPLASMIC POLYADENYLATION

In 2008 Pique' *et al.* defined a set of rules, named "CPE combinatorial code", that dictates whether and when a given mRNA will be polyadenylated in the cytoplasm, and thus activated for translation during Xenopus oocytes maturation[10] (Fig. 9).
She focused on the CPE, PBE (Pumilio Binding Element) and PAS. They generated a large set of 3'UTRs (cyclin B1, B2, B3, B4 and B5), focusing on the presence, the sequence and the relative distance of these three elements. They defined five rules: the first is that translational repression requires a cluster of at least two CPEs with a separation of less than 50 nucleotides (10-12 nt is the optimal distance) where most probably a CPEB dimer is bound, whereas a single CPE or two CPEs greater apart do not support translational repression even though cytoplasmic polyadenylation still occurs. The second is that translational activation requires a single consensus CPE or a nonconsensus CPE with a PBE. The distance between CPE and the PAS must be less than 100 nt, but no overlapping between these two sequences should occur. The third is that the distance CPE-PAS (25 nt is the optimal) mediates the extent of polyadenylation and translational activation, which could be "strong" or "weak". The fourth is that the "early" or CDC2-independent wave of polyadenylation requires CPEs not overlapping with the PAS, as for mos, cyclin B2 and B5, C3H4 and emi1 mRNAs, whereas the so called "late" or CDC2-dependent wave is mediated

by at least two CPEs, with one of them overlapping the PAS, as for cyclin B1, B4, emi2, and cyclin E mRNAs. The fifth is that the presence of an additional cis-element in the 3'UTR, ARE (AU-rich elements) defines, together with the different arrangements of CPE, the extent of polyadenylation[10].

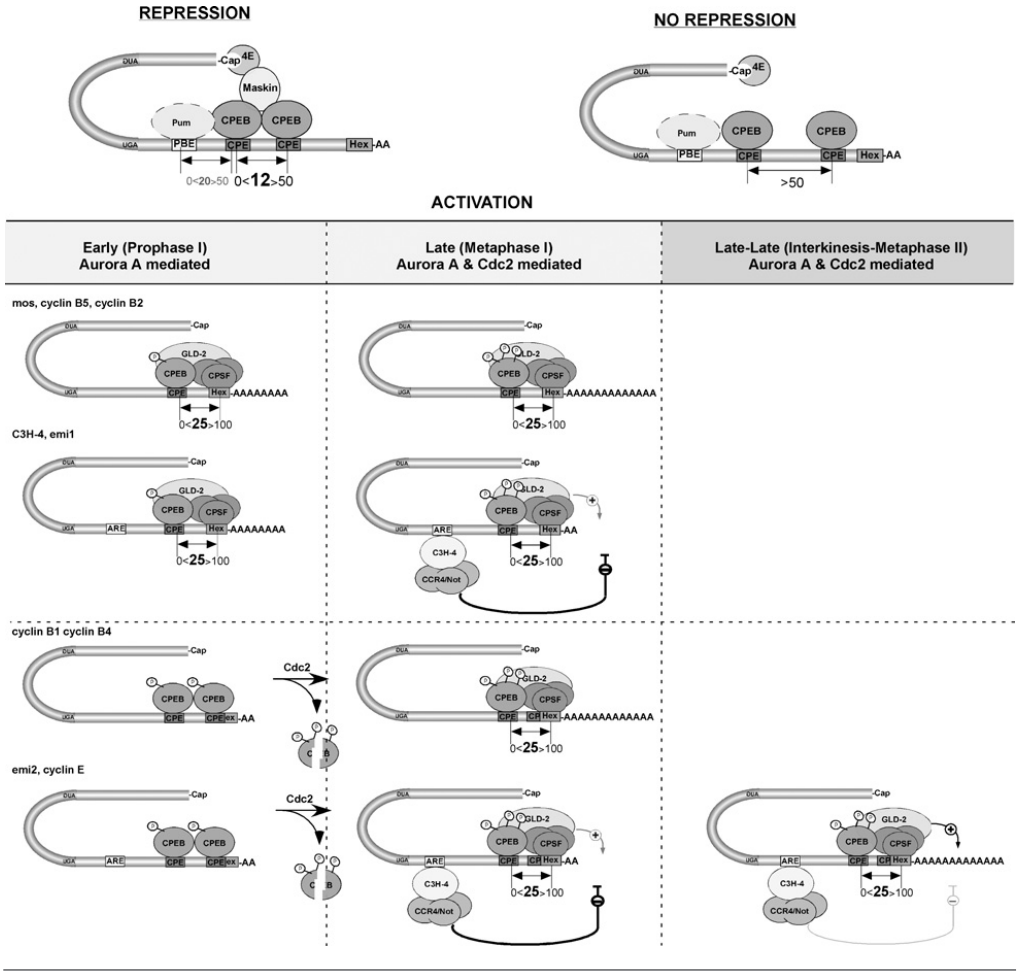


Fig 9. Model for CPE/ARE-mediated translational control. Schematic representation of the *cis*-elements and *trans*-acting factors involved. It is shown the distance (nt) necessary for the translational repression and activation, as well as the time of activation of different mRNA (early, late, late-late). Optional factors are shown with dotted line (extracted from[9]).

TRANSLATIONAL CONTROL BY CAP-RIBOSE METHYLATIONS

While the function of cap 0 is well defined, the function of capI and capII is not as clear.
Early experiments suggest that cap modification confers little if any translational advantage [154, 155].
In sea urchin embryo, the translational activation of maternal histone mRNAs coincide with cap ribose methylation and cytoplasmic polyadenylation after fertilization[156], however the link between cap modifications and translational regulation is not demonstrated[157]. However, in *X. laevis* oocytes Kuge and Richter showed that cytoplasmic polyadenylation stimulates mRNA cap-ribose methylation during oocytes maturation, and that cap-ribose methylation inhibition by S-isobutyladenosine (SIBA) diminishes translation. SIBA is an analog of the SAM metabolite that inhibits methyltransferases[158]. The globin-sB4 chimeric mRNA (5'UTR and coding region of globin mRNA fused to a part of the 3'UTR of B4 mRNA)[16, 159]undergoes to cytoplasmic polyadenylation, methylation to the first and the second ribose and translational activation upon progesterone stimulation. Ongoing polyadenylation rather than the mere presence of the poly(A) tail is necessary for cap-ribose methylations. Additionally the inhibition of these methylations by SIBA lowers the translational activation of a reporter mRNA, without affecting significantly the polyadenylation [160].
Some years after Kuge and Richter showed that cap ribose methylation has important implications for early development and it is sufficient to drive translational stimulation without the intervening of polyadenylation.
They studied mos mRNA, that has a key role in the control of meiosis in vertebrate's oocytes [21, 161]. Mos is a serine/threonine kinase, that initiates mos-MAPK pathway, which in turn activates and stabilizes MPF. In *X. laevis* oocytes, mos stimulates maturation[162-164], suppresses DNA replication after MI[165], and promotes meiotic arrest after MI [163]. Mos mRNA is one of the several mRNAs that is polyadenylated upon progesterone stimulation[54, 166]. They further demonstrated that it undergoes also cap-ribose methylation during oocyte maturation. The inhibition of the methylation by SIBA, on the one hand doesn't affect mos polyadenylation or general protein synthesis, on the other hand it prevents the translation of mos protein and oocyte maturation. Cap-ribose methylation enhances translation 4.4-fold in vivo in the absence of poly(A) and also stimulates the rate of oocyte maturation by mos mRNA in absence of progesterone [167].
However, in the same year another group showed that a reporter mRNA bearing a cyclinB1 3'UTR is very inefficiently ribose methylated. They demonstrated that cap ribose modifications are not required for poly(A)-mediated stimulation of translation, suggesting that these events are mechanistically independent.

CPEB-MEDIATED TRANSLATIONAL REPRESSION

At the moment there are three models about the composition of the complex that mediates CPE-dependent repression in immature oocytes, through the disruption of the cap-eIF4E-eIF4G-ePAB-poly(A) mRNA circularizing complex. (reviewed in[168, 169]) (Fig. 10).

In the first presented model, the closed loop mRNA is disrupted by preventing the association of ePAB with the poly(A) tail. The unphosphorylated CPEB1 directly recruits the poly(A)-ribonuclease PARN[170], which in turn shortens the poly(A) tail, having an opposing activity with respect to the polymerase GLD-2. Moreover ePAB binds to CPEB1 and not to the adenine homo-polimer[170] (Fig. 10a). In the other two models, the closed loop model is affected in the binding between eIF4E and eIF4G. According to the first, the recruitment of a CPEB1-binding protein, Maskin, binds eIF4E blocking eIF4G recruitment in late stage oocytes[171]. In the second model the same effect happens through 4E-transporter (4E-T), an eIF4E protein (Fig. 10b). In early-stage *Xenopus* oocytes 4E-T interacts with CPEB1 and surprisingly to eIF4E1b, a close homolog of the canonical eIF4E1a, that binds poorly to the cap and has low affinity for eIF4G. Always in early-stage oocytes, CPEB1 interacts with Xp54 and the P-body component P100 (Pat1) and RAP55B[172] (Fig.10b). In HeLa cells 4E-T is involved in the nuclear import of eIF4E in presence of leptomycin B[173]. 4E-T, at steady state is the only protein found in P-bodies, while eIF4G and eIF4A are distributed in the cytoplasm. 4E-T has a role in P-bodies formation and in the localization of eIF4E in P bodies[174, 175]. The overexpression of human 4E-T represses cap-dependent reporter mRNA translation, via the *consensus* binding site for eIF4E[175]. In Drosophila, Cup is the characterized paralog of 4E-T that binds eIF4E, mediating translational repression with the help of Smaug and Bruno[176, 177]. The described models appear mutually incompatible and it is not clear if they assemble in a sequence-specific manner on different mRNAs or at different developmental times or whether they are just intermediary complexes[178]. For instance, the deadenylase PARN directly and specifically interacts with the 5′ cap structure and is inhibited by eIF4E, suggesting that eIF4E is not present in that complex[179], 4E-T and Maskin recognize eIF4E, through the same motif.

It is even more obscure which are the components of CPEB1-mediated repression complexes in somatic cells. For example, TACC3, the mammalian homolog of Maskin, doesn't have the eIF4E binding domain, eIF4E1b expression is confined to oocytes, eggs, and early embryos in *Xenopus*, Zebrafish and mice[172]; PARN is mainly nuclear in somatic cells; in HeLa cells the RNA helicase p54, the homologues of Xp54, is sufficient for translational repression without CPEB1[180].

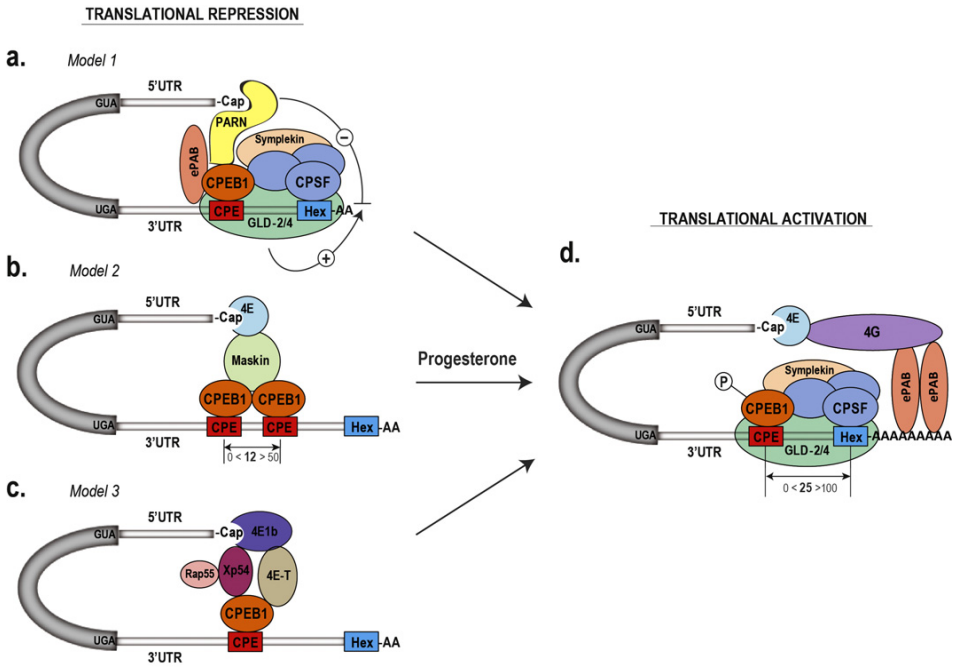


Fig 10. Proposed models for CPE-mediated translational repression and activation. (extracted from[168]).

CPEB-MEDIATED TRANSLATIONAL ACTIVATION

In *Xenopus laevis* oocytes, upon progesterone stimulation, a rearrangement of the CPEB1-mRNP occurs to achieve the cytoplasmic polyadenylation. Progesterone probably induces the action of both classical nuclear steroid receptors and a G protein coupled transmembrane receptor[181]; then a rapid drop in cyclic AMP leads to the inactivation of the Glycogen Synthase Kinase (GSK-3β) and also the Aurora A kinase [182]. Downstream of this event, CPEB1 is phosphorylated on Ser174 by Aurora-A (Eg2) kinase[184]. It has been shown that MAPK is implicated in priming CPEB for Ser174 phosphorylation or even in the activation of the possible Ser174 kinase[183]. Although CPEB, CPSF and Gld-2 are already in a complex in immature oocytes, this phosphorylation appears to induce a stronger association of CPEB with CPSF and Gld-2[184-186]. Importantly, Ser174 phosphorylation also induces the ejection of PARN from the polyadenylation complex, permitting the GLD2-mediated elongation of the poly(A) tail[170]. This complex is stabilized by Symplekin, the scaffolding protein, present in the nuclear and cytoplasmic polyadenylation complexes, which interacts with CPEB1[187]and the 100 kDa subunit of CPSF [188]. Then the kinase RINGO, a cyclin B1-like cofactor that activates cdk1, phosphorylates CPEB1, in multiple proline-directed sites, permitting the dissociation between the embryonic poly(A)-binding protein (ePAB) and CPEB1, allowing its binding to the poly(A) tail. This event is needed to protect the homopolymer from degradation by deadenylating enzymes. Poly(A)-bound ePAB also interacts with eIF4G, which instigates translation initiation of CPEB-bound mRNAs.
In the CPEB1-mRNP activation complex there are components with undefined role, such as CstF77, xGEF, APLP and Pumilio[10, 189-191]. It is not clear which and how many poly(A)polymerase are involved in the cytoplasmic polyadenylation. For example Orb, the *Drosophila* CPEB1 binds the canonical PAP during mid-oogenesis and subsequently GLD-2-type poly(A) polymerases (Wispy) during late-oogenesis[192]. Additionally, in human fibroblasts, CPEB1 recruits GLD4, a second non canonical poly(A) polymerase that mediates the elongation of p53 mRNA tail[193, 194]. About the other members of the family, it is clear that CPEB3 and 4 act as translational activators and CPEB4 recruits GLD2[101, 195, 196] [197].

THE CPEB-FAMILY OF PROTEINS

What was described until now was referred to CPEB1, the most studied protein among the CPEB-family of proteins.

In vertebrates, this family comprises four paralogs (CPEB1-4), where CPEB1 is the less related member of the family, in comparison with CPEB2-4[198]. CPEB orthologs, in different numbers, are found from nematodes to humans. For example, in Drosophila, Orb1-2 are the CPEB orthologs[199, 200], in Caenorhabditis elegans Fog-1 and cpb1-3[201, 202] [203, 204], at least one in the marine invertebrate, Spisula solidissima[205], in Aplysia Californica[206, 207], in human[208, 209] ().

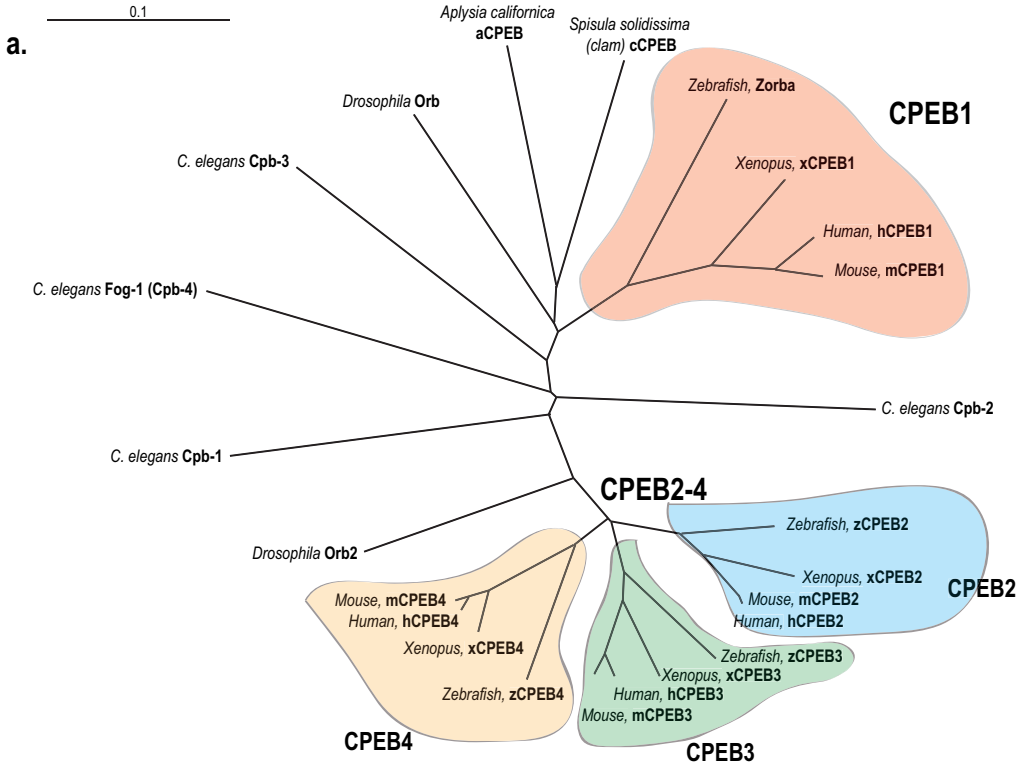


Fig 11. CPEB family of RNA-binding proteins. Phylogenetic tree of the most representative CPEB proteins; CPEB1 vertebrate orthologs (red ballon) are the less related members of the family, in comparison with CPEB2-4 orthologs, where CPEB2 is in blue, CPEB3 in green and CPEB4 in yellow (extracted from[168]).

The structure is similar among them, the C-terminal domain contains two RNA-recognition motifs (RRM1 and RRM2) and a binuclear zinc-binding domain[210, 211] and a regulatory N- terminal domain. Very recently it has been published that the zinc binding domain is a ZZ-type zinc finger, which participates in protein-protein interactions, rather than the recognition of sequence-specific in the mRNA[211]. The family members are more similar in the C-termini than the N-termini. For example, only CPEB1 in the N-termini, contains two phosphorylation site for Aurora A followed by a PEST (Pro-, Glu-, Ser- and Thr-rich region)

degradation motif[212]. The other CPEB isoproteins lack PEST sequence and Aurora A kinase phosphorylation sites. However, alternative splice isoforms of CPEB2-4 posses putative phosphorylation sites for cyclic AMP-dependent protein kinase (PKA), calcium-calmodulin-dependent protein kinase-II (CaMKII) and p70S6 kinase [213] (Fig. 12).

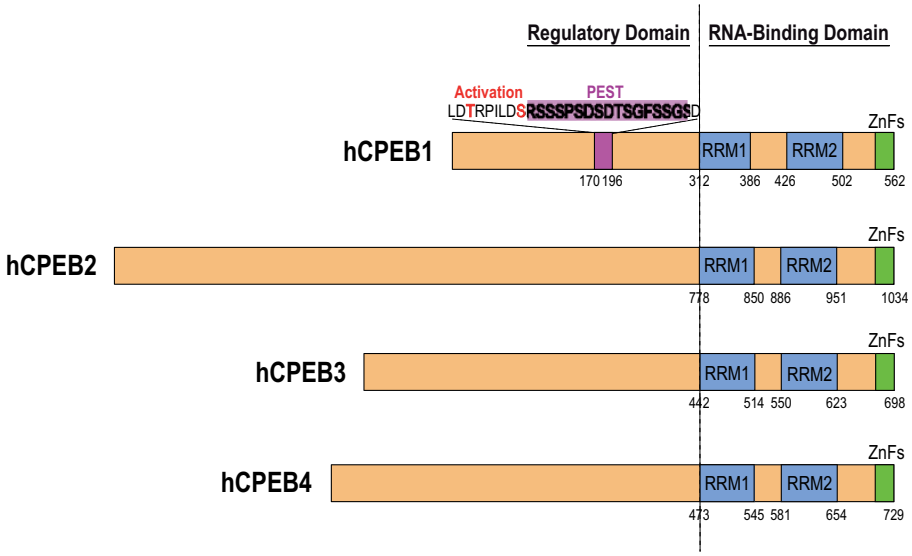


Fig 12. Protein structure of human CPEBs. The family members are more similar at the C-termini (RNA-binding domain) than at the N-termini (Regulatory Domain). CPEBs share a conserved RNA-binding domain at the C-terminal part and a highly variable regulatory domain at the N-terminal part. The RNA-binding domain comprises two RNA recognition motifs (RRMs, in blue) and two zinc-fingers (ZnFs, in green). Only the regulatory domain of CPEB1 contains two residues (in red) responsible for CPEB1 activation when phosphorylated by Aurora A, followed by a PEST-degradation motif (in purple) (extracted from[168]).

It is controversial if different CPEBs share the same CPE sequences. For example it has been published that CPEB3-4 and CPEB1 recognize distinctive elements by Selex [214], but there are opposing evidences, showing the contrary [101, 102, 196, 197].

CPEB1

CPEB1 is the only well-established mRNA specificity factor for cytoplasmic polyadenylation in vertebrates. It was first cloned and characterized in *Xenopus laevis* oocytes as a 62-kDa protein that binds specifically to the CPEs mediating cytoplasmic polyadenylation [215, 216]. Depletion of CPEB1 from an egg extract abolishes polyadenylation and injection of a CPEB antibody blocks it in oocytes and embryos [215-217] [184, 185]. N-terminal deletions and phosphorylation site mutants of CPEB act as dominant negative blockers of cytoplasmic polyadenylation[184, 185, 215-217]. In mice, knock out or knock down of Cpeb1 causes poly(A) tail changes in target mRNAs in early oogenesis and oocyte maturation[218, 219]. Among the family, only CPEB1 in the N-termini, contains two phosphorylation sites for Aurora A (LDS/TR) followed by a PEST (Pro-, Glu-, Ser- and Thr-rich region) degradation motif[212]. The PEST domain mediates CPEB1 degradation by ubiquitination in response to cell division cycle 2 (Cdc2) and *Xenopus* Polo-like Kinase 1 (Plx1) phosphorylation during later stages of meiotic maturation[220, 221] [222, 223]. Very recently it has been published that its zinc binding domain in reality is a ZZ-type zinc finger, which participates in protein-protein interactions, rather than the recognition of sequence-specific in the mRNA[211].
CPEB1 mRNA is highly expressed in the reproductive system [219], and in the brain[224, 225], but not in the corresponding tumour tissues.
CPEB1 controls the polyadenylation of CPE-containing mRNAs and their translational eccifiency in oocytes, fibroblast, HeLa cells and during early embryonic divisions (reviewed in [226].
Interestingly, CPEB1 has been shown to be able to shuttle into the nucleus[97, 227, 228] where it recruits CPSF to the PAS, as it does in the cytoplasm. Indeed, CPEB recruits CPSF to proximal PAS to mediate 3'UTRs shortening genome-wide[97]. Such shortening of 3'UTRs is associated with proliferation, de-differentiation, and transformation[94]. In this way nuclear CPEB1 selects from the nucleus the regulatory elements to be included in the mature 3'UTRs, which are needed for cytoplasmic regulation as for mRNA-specific translational regulation. In this way CPEB1 coordinates alternative 3'UTR formation with translational regulation[97].

CPEB2

CPEB2 binds the same CPE as CPEB1, although with less affinity[101, 196] and is expressed mostly in testis[208].

An identified target of CEPB2 is HIF-1 α mRNA, which is target also for CPEB1. It has been shown that both of them are involved in positive regulation of hypoxia-inducible factor 1α following insulin stimulation[195].

In particular CPEB2 interacts with the elongation factor, eEF2, to reduce eEF2/ribosome-triggered GTP hydrolysis in vitro and slow down peptide elongation of CPEB2-bound RNA *in vivo*. The interaction of CPEB2 with eEF2 down-regulates HIF-1α RNA translation under normoxic conditions; however, when cells encounter oxidative stress, CPEB2 dissociates from HIF-1α RNA, leading to rapid synthesis of HIF-1α for hypoxic adaptation. This study delineates the molecular mechanism of CPEB2-repressed translation and presents a model for controlling transcript-selective translation at elongation[80].

Moreover, it has been found that CPEB2 and CPEB1 act as negative regulators of TWIST1 expression in a sequence-specific and additive/cooperative manner, together with miR-580 [229].

CPEB3

In neurons, CPEB3 is a regulator of local protein synthesis. It is one the mouse homolog of ApCPEB, a functional prion protein in Aplysia[213, 214]. In NMDA treated hippocampal cultures, CPEB3 is activated by monoubiquitination through Neuralized1, an E3 ubiquitin ligase, and leads to the growth of new dendritic spines as well as to the increase of the subunits of AMPA receptors: GluA1 and GluA2 (two essential target for synaptic plasticity). This result suggests that CPEB3 has a role in hippocampal plasticity and hippocampal-dependent memory storage[197].

The AMPA receptor GluR2 mRNA is a target of CPEB3 regulation; CPEB3 binds this RNA in vivo, but the CPEB3 knock-down in neurons results in elevated translation of GluR2 mRNA . Moreover CPEB3 does not mediate the cytoplasmic polyadenylation and the recruitment of CPSF100 [214].

CPEB3 also binds Tob, a member of the anti-proliferative protein family, together they recruit Caf1 deadenylase, accelerating deadenylation and decay of its mRNA[230].

CPEB4

In meiosis CPEB1 and CPEB4 are functionally exchangeable, through the binding of the same CPE-containing mRNAs[196]. CPEB4 is encoded by a maternal mRNA, which is activated in the “early” wave of cytoplasmic polyadenylation by CPEB1, upon progesterone stimulation. This, in turn, leads to the gradual accumulation of the protein from MI to MII. While CPEB1 is degraded by the kinases Cdc2 and Plx1 at MI, CPEB4 is required for MI-MII transition and for the recruitment of the poly(A)polymerase GLD2, in order to drive the “late” or “late-late” waves of polyadenylation. Some of the targets are XKid, TPX2, cyclin E, emi2, cyclins B1/B4, that encode for proteins required for the second meiotic division and needed to prevent DNA replication after MI[20] [100] [10, 14]. Moreover, CPEB4 activates the translation of its own mRNA, generating a positive feedback loop[196]. The kinases responsible for CPEB4 activation are still not known. In mitosis CPEB4, together with CPEB1, regulates the poly(A)tail of 467 mRNAs (mainly cell-cycle regulator factors) in a phase-specific manner, a process that is required for cell proliferation and mitotic entry [101]. CPEB4 expression is heightened in glioblastomas and pancreatic ductal carcinomas (PDA), where is involved in tumor growth, vascularization and invasion.

CPEB4 is up-regulated in pancreatic tumours, as compared to healthy pancreas or undifferentiated tumors. CPEB4 seems to facilitate the expression of tissue plasminogen activator (TPA) mRNA, which in turn promotes tumor proliferation, migration, invasion and vascularization. In PDA cells when CPEB4 is knocked down, tPA levels are reduced, as a consequence of decreased cytoplasmic polyadenylation, but when tPA is overexpressed in this context, tumor growth is in part rescued. The mechanism by which CPEB4 regulates tPA is indeed an important event in pancreatic tumor formation. Additionally many others mRNAs enriched in this type of cancer, are related to tumorigenesis[102].

CPEB IN MITOSIS

CPEB, through the regulation of cyclin B1 mRNA, is essential for the embryonic cell cycle. This kind of cell cycle consists of the alternating S and M phases without intervening of G1 and G2, in absence of transcription. In cycling extracts from *Xenopus laevis* embryos, the progression into M phase requires CPEB-mediated polyadenylation of cyclin B1 mRNA. The activation of CPEB is mediated by phosphorilation on Ser174 by the oscillating Aurora A, leading to the cytoplasmic polyadenylation of cyclin B1 mRNA and the dissociation between Maskin and eIF4E. Exit from M phase seems to require deadenylation and subsequent translational silencing of cyclin B1 mRNA by the association of Maskin and eIF4E, whose expression is cell cycle regulated[217].

The regulation of the poly(A) tail is not only required to overcome the lack of transcription in specialized cell division, but it is also a general mechanism of gene expression control in mitosis. Increasing number of data is expanding the knowledge of CPEB family also in mitosis. In mitotic cell cycle the change in the poly(A) tail, is similar to what occurs in the meiotic temporal translational control. In 2010 Novoa *et al.* found that hundreds of mRNAs have different poly(A) tail length, depending on the phases of the cell cycle in which they were collected (G2/M or S), in tumor-derived cell lines (HeLa)[101]. In order to do this, they performed a genome-wide screening based on two differential purification of mRNA, depending on their poly(A) tail. Moreover, that mRNAs that contain differential poly(A) tail in the cell cycle encode protein related to cell death, cell cycle, cellular growth and proliferation. For example, Cdc20 mRNA, which encode a key regulator of the cell cycle[231], contains a short poly(A)tail in G2/M transition and long in S-phase, while CDKN3, another important cell-cycle regulator[232], shows a short poly(A) tail in S, but longer in G1. Additionally, the cell-cycle changes in polyadenylation follow protein translation. In HeLa cells the levels of CPEB1 and CPEB4 mRNAs are the most abundant, while CPEB2 mRNA is much less expressed and CPEB3 mRNA is nearly undetectable. They demonstrated that CPEB1 and CPEB4, but not CPEB2, were able to mediate cytoplasmic polyadenylation and translational activation, by recruiting CPSF, through the recognition of the same target mRNAs, even if with different affinity. Then, microarray data showed that the depletion of CPEB1 affects the cytoplasmic polyadenylation of 324 mRNA and the absence of CPEB4 regulate the poly(A) tail of 199 mRNAs, with an overlapping of 56 mRNAs in mitosis. A bioinformatic analysis reveals that the 3'UTR of these mRNAs were statistically enriched for *cis*-acting elements, such as CPEs, AREs, PBEs and microRNA target sequences. Moreover the depletion of CPEB1 alone, and to a greater extent the absence of CPEB1 and CPEB4 together have an

important effect in the inhibition of proliferation, especially in the mitotic entry. The fact that the double knock-down had a stronger phenotype indicates that the function of CPEB1 and CPEB4 are redundant and it is a consequence of translational misregulation of multiple targets [101].

CPEB1 could also have a possible role in the localized translation in non-germ cells, due to its presence in the mitotic spindle[170]. Even if conducted in egg extracts from *Xenopus tropicalis*, Sharp *et al* identified ~450 mRNAs that showed significant enrichment on microtubules (MT-RNAs). MT-RNAs are enriched with transcripts associated with cell division, spindle formation, and chromosome function, demonstrating an overrepresentation of genes involved in mitotic regulation[233]. In this group they found mRNAs like cyclin B and xkid, which are demonstrated targets for CPEB[14].

Interestingly the role of Orb2 (CPEB2-4 ortholog in *Drosophila*) has been related to asymmetric cell division in neuroblasts. For example in the embryonic CNS, Orb2 is mainly concentrated in cell bodies, while in adult brain is accumulated more in axonal and dendritic terminals [234]. In another study using a candidate gene approach and a genome-wide analysis, 28 mRNA targets of *Drosophila Orb2* were identified, aPKC mRNA is one of them and it is an essential regulator of asymmetric division[235] [236].

CPEB in senescence

Senescence is the phenomenon by which primary cells exit the cell cycle and prevent malignancy[237, 238].

CPEB1 is required to induce senescence in primary cells. Proliferation is a balance between pro-proliferative pathways and cell death/senescence pathways. Surprisingly CPEB1-knockout mice are fully viable, though sterile[219]. Soon after they are plated for cell culture, mouse embryo fibroblasts (MEFs) from wild-type animals display a similar proliferation rates to the constitutive CPEB1 KO. After 8-10 passages the wt MEFs stop dividing and enter into senescence, as expected, while the MEFs belonging to the KO, escape senescence, become immortal, showing longer telomeres[239, 240]. Also human lung and skin fibroblasts primary culture depleted for CPEB1 by short hairpin RNA (shRNA) bypass senescence[241]. This phenotype is rescued after reintroduction of CPEB1 at early passages, indicating that CPEB1 is one of the factors important in cellular senescence. Depletion of CPEB1 in primary cells affects the translational regulation of hundreds of transcripts. CPEB1 binds a conserved CPE sequence in the p53 mRNA 3'UTR, and recruits a non–canonical poly(A) polymerase GLD4, driving its polyadenylation and translation[194]. Knock-down of CPEB1 reduces the synthesis of p53 protein by about 50%, that it is enough for the cells to bypass senescence and become immortal, preventing may be the telomere shortening[241].

In MEFs CPEB1 regulates senescence by mediating the translational repression of myc mRNA, which encodes for a potent proto-oncogene[239]. The fact that the absence/presence of a single protein (CPEB1) could regulate the translational activation of a target mRNA (p53) in a type of cell where it is completely deleted (KO), or repress the translation (myc) when is partially deleted (KD), may reflect the importance of a CPEB dose-dependency in translational regulation. Taking into account that translational repression is a stoichiometric event and requires high CPEB1 levels, while the translational activation requires instead lower levels, CPEB dosage in the cell could affect the formation of both the repression and activation complex.

Another example of dose dependent effect come from Orb2, where Orb2 RNAi flies have an impair viability, whereas mutant flies survive but show locomotion and behavioral problems [234].

CPEB in cancer

The direct connection between the altered expression levels of CPEB-family members and cell proliferation/senescence, raised new questions about their involvement in cancer. At least two members: CPEB1 and CPEB4 mediate malignant transformation. CPEB1 mRNA is highly expressed in the brain [224, 225] and in the reproductive system[219], its level is reduced in several tumors, such as ovarian, breast and gastric cancer, as well as in colorectal cancer and myeloma cell lines [242, 243]. Gastric cancer is associated with an unusual methylation of the CPEB1 promotor[242]. Interestingly the lower levels of CPEB1 expression are associated with an increase of cellular invasiveness and angiogenesis. Additionally CPEB1 controls the translation of HIF-1a, a transcription factor implicated in VEGFA transcription. When CPEB1 is over-expressed, HIF-1a mRNA is repressed and the level of VEGFA decreased, as well as the metalloproteinase MMP14 mRNA[195, 244].

Even if Cpeb1-knockout mice are normal and seem not to develop spontaneous cancer, after exposition of the tumour initiator DMBA or the phorbol ester TPA to the skin there is a faster and larger papilloma formation in comparison with wt mice[241]. All these evidences suggest that CPEB1 is a tumour suppressor. However recent studies showed that the nuclear role of CPEB1 is rather associated with transformation. Indeed CPEB1-mediated 3'UTR shortening leads to the formation of short 3'UTRs in oncogenes and particularly evident in tumoral tissues, as compared to differentiated tissues[97].

Regarding CPEB4, there is a direct evidence between its altered expression and the tumoral development. CPEB4 expression is heightened in glioblastomas and PDA, where is involved in tumor growth, vascularization and invasion.

CPEB4 protein is up-regulated in tumors of the pancreas, in comparison to healthy pancreas or undifferentiated tumors. CPEB4 seems to facilitate the expression of tissue plasminogen activator (TPA) mRNA, which in turn promotes tumor proliferation, migration, invasion and vascularization. In PDA cells when CPEB4 is knocked down, tPA levels are reduced, as a consequence of decreased cytoplasmic polyadenylation, but when tPA is overexpressed in this context, tumor growth is in part rescued. The mechanism by which CPEB4 regulated tPA is indeed an important event in pancreatic tumor formation. More than 800 mRNAs enriched in this type of cancer, are related to tumorigenesis and encode for RAS-related molecules, cell signaling, chromatin-remodeling, cyclins, apoptosis, stress/inflammation, cell migration/metastasis proteins[102]

CHAPTER I

OBJECTIVES

I. Identifying and characterizing *Xenopus laevis* cap-ribose methyltransferase (xMTr1).

II. Studying xMTr1 activity, influence on translational control of gene expresisom and regulation by CPEB1.

RESULTS

Identification of a *Xenopus laevis* cap I 2'O-ribose methyltransferase (xMTr1).

Domain architecture of xMTr1 among species

VP39 is a 333-amino acid protein, whose domains were characterized by mutagenesis studies[245, 246]. T.Brucei cap I 2'O-ribose methyltransferase (TbMTr1) is a 370-amino acid protein first identified following a database search for sequence similarities to the *E.Coli* FtsJ/Rrmj 2'-O-ribose methyltransferase [247]. The human cap I 2'-O-ribose methyltransferase (hMTr1) is 835-amino protein; it contains a nuclear localization signal (NLS), a G-patch RNA binding domain, a Rossmann-fold MTase (RFM) with a conserved K-D-K triad characteristic for 2'O-ribose MTases, a GTase-like domain lacking catalytic residues[247, 248] and a WW protein interaction domain[68]. The human cap II 2'O-ribose methyltransferase (hMTr2), it is composed by two RFM domains, where one lacked conserved residues required to bind S-Adenosyl methionine (SAM) and the K-D-K triad [88]. The domain boundaries of hMTr1 and hMTr2 were predicted according to protein fold recognition analyses carried out through GeneSilico metaserver[88].

The sequence of the putative *Xenopus laevis* cap I methyltransferase was identified by comparative genomic homology search with TbMTr1. Structure prediction in combination with multiple sequence alignment analysis suggested that the putative xMTr1 harbors a probable nuclear localization signal (NLS), a G-patch RNA binding domain, a Rossmann-fold MTase (RFM) with a conserved K-D-K-E catalytic triad, characteristics for 2'-O-ribose MTase, a RFM-like domain lacking catalytic residues and a WW domain potentially involved in protein-protein interactions (Fig 13). We investigated the possibility that this protein was the *Xenopus* cap I 2'O-ribose methyltransferase, (henceforth called xMTr1).

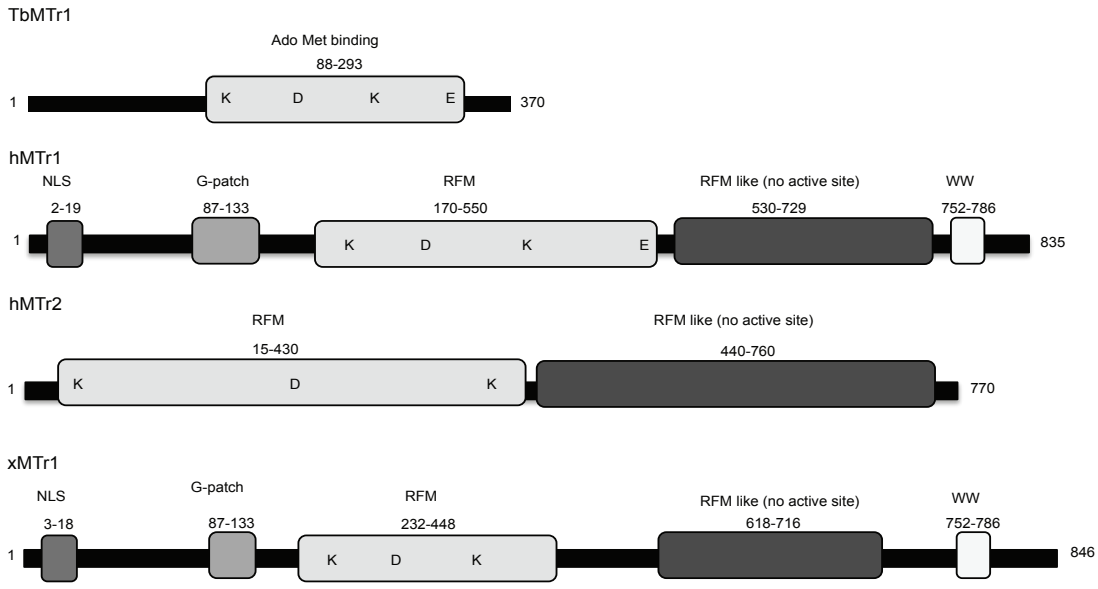


Fig 13. Putative xMTr1 shows common features to other methyltranferases. Domain architecture comparison of the cap-ribose methyltransferases: VP39, TbMTr1, hMTr1, hMTr2 and xMTr1. Predicted domains of xMTr1 includes a nuclear localization signal (NLS) a G-patch RNA binding domain, a Rossmann-fold MTase (RFM) with a conserved K-D-K-E catalytic triad, characteristics for 2'-O-ribose MTase, a RFM-like domain lacking catalytic residues and a WW domain potentially involved in protein-protein interaction.

Cloning strategy, protein expression and purification

The open reading frame of the putative xMTr1 was retro transcribed from Stage VI oocytes, using an oligo-dT primer. The putative ORF was then amplified using specific primers (see materials and methods for sequence) and cloned into the pET30 vector by restriction with HindIII and XhoI, as His_{10}-tagged fusion protein. E. Coli (strain BLB21) were transformed and grown at 30C for 4 hrs. The protein was then purified from a soluble extract by adsorption to Nickel-column, and dyalized for imidazole removal (see materials and methods for detailed description of the procedure). A major band was observed at 97kDa, as expected from the theoretical molecular weight (Fig 14a). The peptide sequence was confirmed by mass-spectrometry analysis (Fig 14b).

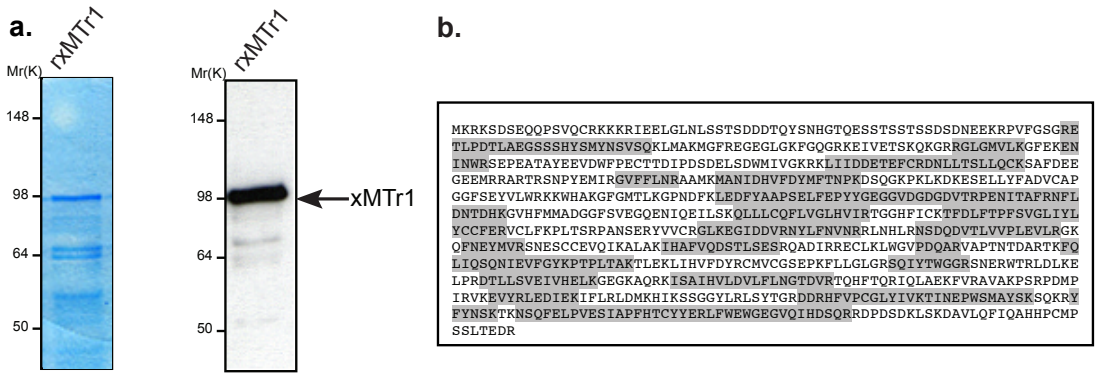


Fig 14. xMTr1 purification. a, xMTr1 was purified and run in a 8% SDS-PAGE. Gels were stained with Comassie blue [left panel] or transferred to a membrane for xMTr1 western-blot [right panel].
b, peptide coverage sequences (in grey) of the recombinant xMTr1 identified by mass spectrometry.

Generation and validation of xMTr1 antibodies

In order to study the function of the putative xMTr1, a rabbit polyclonal antibody was raised against the full-length protein. To this purpose, the recombinant protein was overexpressed in large-scale in *E.coli* BLB21 (DE3/pLysS), where the majority of the antigen was found in the insoluble fraction and sent for the antibody production (Fig. 15).

Two of the raised antibodies recognized clearly a band at 97kDa, corresponding to the recombinant protein. Moreover they were able to recognize the xMTr1, when over expressed in *Xenopus laevis* oocytes, as well as at the endogenous one. This data assayed that the xMTr1 was equally expressed in oocytes with or without progesterone stimulation.

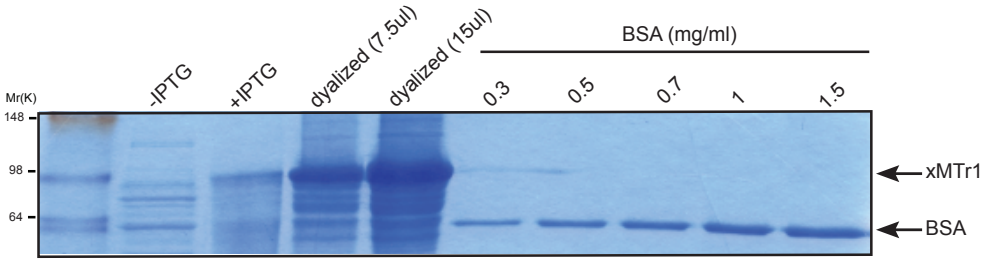


Fig 15. Overexpression recombinant xMTr1 to arise antibodies against the full lenght protein. Comassie-stained SDS-PAGE 8% gel of His-tag recombinant xMTR1 expression in E.coli (BLB21) after induction with IPTG. Two different quantity of the dyalized protein are loaded. Standard curve with bovine serum albumine (BSA).

Recombinant xMTr1 exhibits cap I methyltransferase activity

Set up of the *in vitro* methyltransferase assay

Once the putative xMTr1 was cloned and purified, we wanted to evaluate whether it had a cap I ribose-methyltransferase activity. To this purpose we set up an *in vitro* methyltransferase assay. This experiment is based on the resistance conferred by 2'-O-methylation to the RNase T2 digestion[160, 249] (Fig 16). We used as a substrate part of the 3'UTR of Cyclin B4 mRNA[160], an mRNA that undergoes through polyadenylation and translational activation upon progesterone stimulation during oocyte maturation[16].

The mRNA is 44-nucleotide-long; it starts with a guanosine (the G of the cap) followed by an adenosine and it contains one CPE sequence followed by one PAS.

As positive control, the same RNA was methylated *in vitro* by addition of VP39, the cap I methyltransferase from vaccinia virus[74, 250]. Indeed VP39 was able to methylate the first ribose of the first transcribed nucleotide (cap I, ^{7m}GpppGmpG), as observed in lane 1 of Fig.17. Interestingly, also the putative xMTr1 retained the same activity (lane 3), demonstrating that we actually purified the active form of the *Xenopus laevis* xMTr1. Other lower minor bands were observed, corresponding to cap 0 (^{7m}GpppG) and 3'- nucleotide monophospates (Fig 17).

Overall these results, showed that the *Xenopus laevis* xMTr1 is a 97 KDa protein, which is present in both stage VI and MI of *Xenopus laevis* oocytes. They also showed that its function is indeed to methylate the first ribose of the first transcribed nucleotide of a given RNA. We then asked if it could show any substrate specificity.

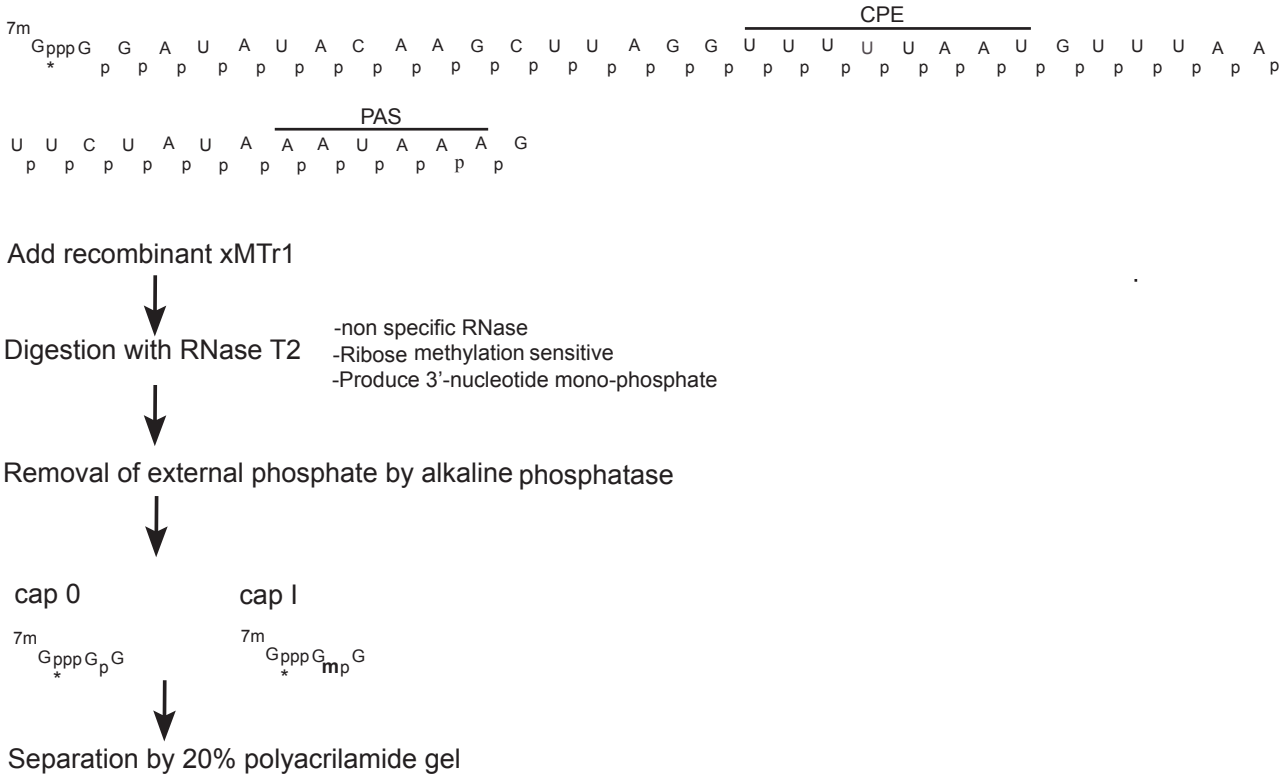


Fig 16. Schematic diagram of the cap ribose methylation assay. The p with the asterisk refers to the ^{32}P-labeled phosphate and the m to the ribose-methylation.

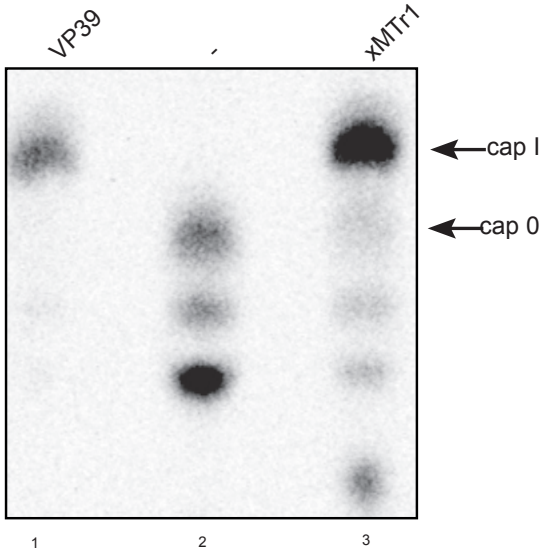


Fig 17. Recombinant xMTr1 exhibits cap I methyltransferase activity. RNA containing one CPE alone, followed by PAS, was transcribed *in vitro* and then a labeled cap was added. It was then incubated with enzymes as indicated in the presence of SAM. Purified product RNA was digested with RNase T2 and the treatment with alkaline phosphatase was used to remove the external phosphate. Digestion products were resolved in a 20% polyacrylamide-8M UREA gel and visualized by autoradiography. The absence of the enzymes was our negative control. The cap I created by VP39 was used as a reference.

xMTr1 is a nucleo-cytoplasmic shuttling protein in *Xenopus laevis* oocytes

The function of the known cap-ribose methyltransferases, seem to be predominantly nuclear. The isolated xMTr1 harbors in its sequence a putative NLS. In order to study its subcellular localization in Xenopus laevis, we performed a nucleo/cytoplasmic fractionation of the oocytes.. The nuclear and the cytoplasmic fractions were manually isolated from immature *Xenopus laevis* oocytes and their respective lysates were analyzed by SDS-PAGE gel (Fig 7). The correct isolation between the two fractions was proven by the different pattern of the total protein expression in both fractions and by the identification of a nuclear-specific protein (Fig. 7, asterisk). Interestingly we found this enzyme in both the cytoplasmic and the nuclear fraction, suggesting that the xMTr1 is a nucleo-cytoplasmic shuttling protein. Moreover the cytoplasm and the nucleus displayed similar levels of xMTr1 in stage VI oocytes, suggesting that it could play a role in both these compartments. CPSF is localized in the nucleus and in the cytoplasm of X. laevis oocytes[251].

A similar cellular distribution was observed in HeLa cells for the human cap II methyltransferase (hMTr2), tested by immunostaining of epitope-tagged protein, where the enzyme was present in both the cytoplasm and the nucleus[88]. Differently, the human cap I methyltransferase (hMTr1) was confined to the nucleus with almost no signal detectable in

the cytoplasm, in Hela cells[68]. These differences between a human cell line and X. Laevis oocytes may be indicative of a divergence in the evolution or rather a functional discriminant (as an example: mitosis in Hela vs. meiosis in the oocytes).

Interestingly the nuclear xMTr1 was found to be slightly shifted in its elecrophoretic mobility (Fig. 18), suggesting that this protein could be regulated by post-translational modifications.

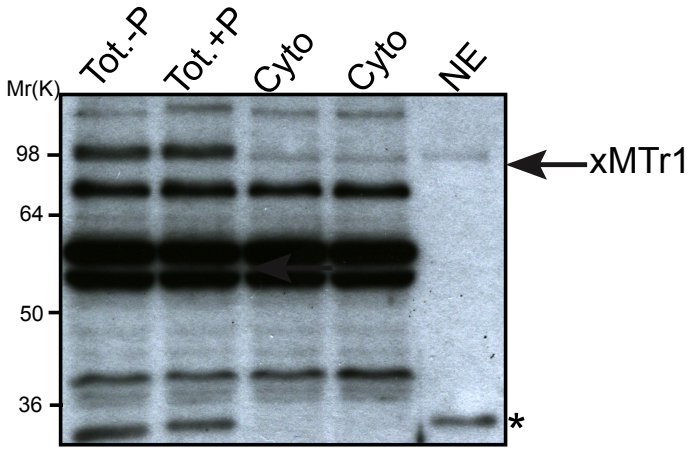


Fig 18. xMTR1 is a nucleo-cytoplasmic shuttling protein in Xenopus laevis oocytes. Xenopus oocytes, untreated or stimulated with progesterone (P) and the cytoplasmic (Cyto) and nuclear (NE) fractions were analysed by western blotting using anti-xMTr1. The asterisk indicates a band specific of the nuclear fraction. Oocytes treated with P, were collected at GVBD (germinal vesicle breakdown), which was determined by the appearance of the white spot at the animal pole of the oocyte.

Progesterone-dependent meiotic progression to meiosis I induces xCPEB1-xMTr1 interaction, in a RNA-dependent manner

The proposed role of cap ribose methylation is the regulation of the translation of specific mRNAs[167]. An hallmark for translational competence is the acquisition, by the mRNAs, of the "closed-loop" conformation (see introduction paragraph "Synergic interaction between cap structure and poly(A) tail)**.** To this purpose, the mRNAs need to have a "free-cap" and a polyA tail. In some cases however this is necessary but not sufficient (please refer to the "discussion" section, below). Additional signals are required to orchestrate the translation of specific mRNAs. In *Xenopus laevis* oocytes, CPEB1 drives the meiotic progression through the translational regulation of hundreds of mRNAs. However the binding of CPEB1 to the target mRNAs, and thus its function (for example in the translational activation vs repression) changes depending on several factors. To test the hypothesis that the cap ribose methylation could act as a signal for translation in the CPEB-regulated transcripts and in a CPEB dependent manner, we tested if the endogenous CPEB1 was able to take contacts with the endogenous xMTr1. We wanted also to address if this association was dependent on the presence of the RNA and at which stage of the oocytes maturation it could eventually take place (Fig 19). Our results showed that progesterone-dependent meiotic progression to meiosis I, induced xCPEB1-xMTr1 interaction in a RNA-dependent manner. This suggested that xMTR1 was one of the component of the CPEB-mediated activation complex *in vivo,* where the association with CPEB1 appeared stable in presence of the RNA molecule. However, it seemed that the xMTr1 was not part of the CPEB1-repression complex, even if the endogenous protein was found also in immature oocytes, leaving open the question of the role of xMTr1 in stage VI oocytes.

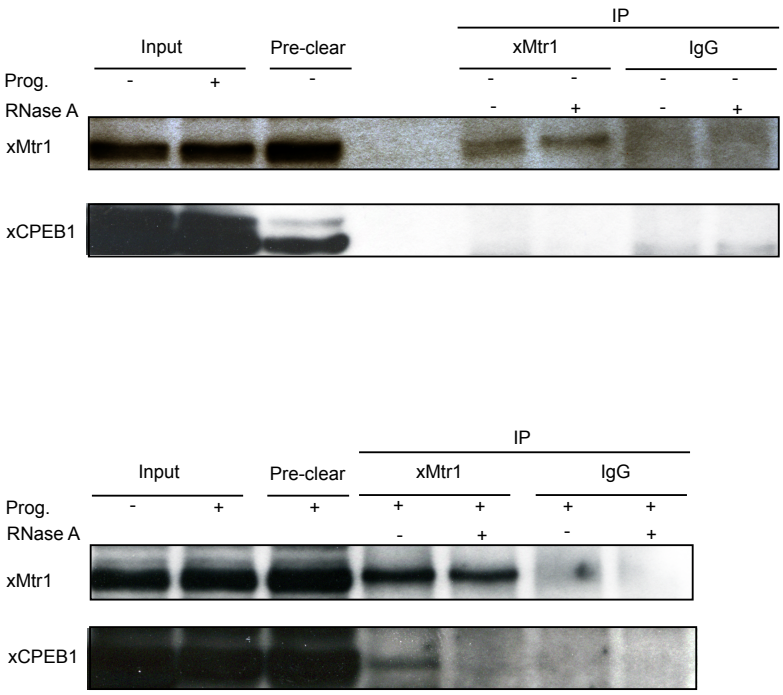


Fig 19. Progesterone-induced meiotic progression to meiosis I induces xCPEB1-xMTr1 interaction, in a RNA-dependent manner. Cytoplasmic extracts from oocytes untreated or treated with progesterone were subjected to immunoprecipitation with anti-xMTr1 and control IgG in presence or absence of RNase A. The co-immunoprecipitates were analysed by western blotting for the presence of CPEB1 protein.

xCPEB1 stimulates cap1 2'O-ribose methylation by xMTRr1, *in vitro*

CPEs and xCPEB1 stimulate cap I ribose methylation *in vitro*

To get further insights into the functional interaction between xCPEB1 and xMTr1, we performed the *in vitro* methyltransferase assay in presence or not of recombinant xCPEB1. We used as a substrate two capped RNAs, one containing a wild-type CPE sequence (CPE-wt) and the other harboring CPE-inactivating mutations (CPE-mut, Fig 20). We expressed and purified the GST-tagged xCPEB1 (Fig 21). The RNAs were treated as described above, using a costant amount of recombinant xMTr1 and incubated with three different amounts of xCPEB1. We found that a precise amount of xCPEB1 (17nmol) is able to drive cap I 2'O-ribose methylation on the CPE-wt substrate RNA (Fig 20 lane 5). However when the binding of CPEB1 to the RNA was prevented by inactivating mutations, CPEB1 lost the synergic activity and no cap I 2'O-ribose methylation was observed (Fig 20 lane 8). Interestingly, in the mentioned lane, an unexpected upper band appears, suggestive of a cap II structure, simirarly to what observed by Kuge *et al.* during oocyte maturation in *Xenopus laevis*[160]
These results suggested also that the stoichiometry of the complex xMTR1/xCPEB1 plays an important role for the functional interaction *in vitro*, because the addition of different amounts of xCPEB1 molecules prevented cap I ribose methylation.

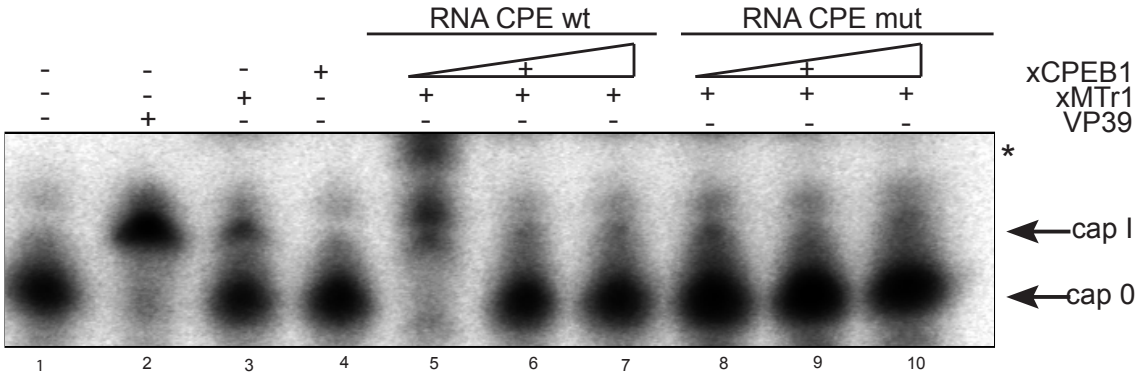


Fig 20. xCPEB1 stimulates cap1 2'-O-ribose methylation by xMTRr1, *in vitro.* *In vitro* transcribed RNAs CPE wild type and mutated with the labeled cap 0 structure were incubated with the xMTr1 and 17,102 and 170nmol of xCPEB1, respectively. Cap structure modified with VP39 is used as a positive control. The asterisk indicates the possible cap II structure formation.

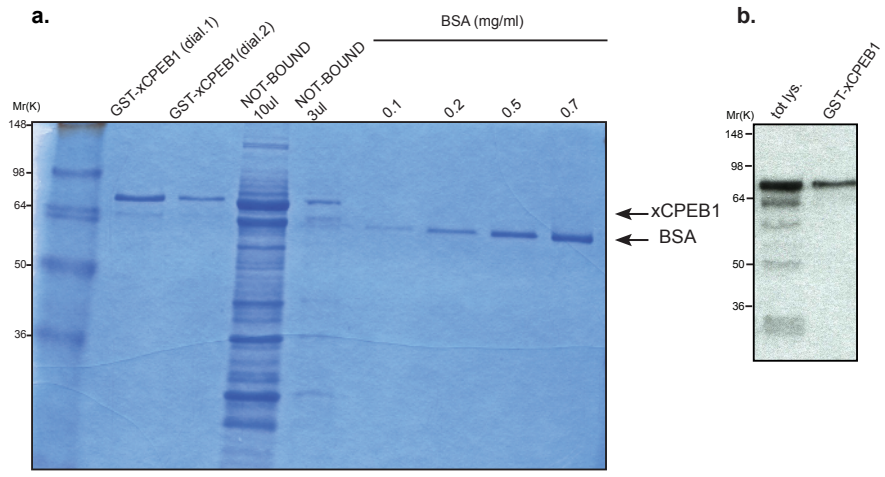


Fig 21. xCPEB1 purification. GST-CPEB1 was purified and run in 10% SDS-PAGE together with total lysates. Gels were stained with Comassie blue and the standard curve with bovine serum albumine (BSA) was performed [left panel] or transferred to a membrane for anti-GST western-blot [right panel].

cap I 2'O-ribose methylation induces translation of a "weak" mRNA

A nonconsensus CPE of cyclin B1 drives its polyadenylation, but CPEB1-induced cap I methylation is needed for translation.

CPEB1 binding to the CPE elements largely depends on the *in-cis* element sequence[215, 252]. While a consensus CPE (UUUUAAU) efficiently recruits CPEB, a non consensus CPE (UUUUACU) shows lower binding efficiency. Thus the primary sequence, the amount and the relative distance (also with respect to the PAS) of the CPEs, define the translational behavior of the CPEB-regulated mRNAs[10]. Interestingly, in the case of cyclin B1 mRNA a single non-consensus CPE is sufficient for driving cytoplasmic polyadenylation, but not translational activation[10].

To address the possible involvement of 2'-O-ribose methylation in translational regulation, we tested the efficiency of different 3'UTRs in mediating the translation of a Luciferase reporter, when injected in *Xenopus laevis* oocytes.

The objective of this experiment was to compare the translational stimulation of a 3'UTR harboring just a non-consensus CPE and a PAS, in presence or not of cap I methylation (Fig 22). In this way we could address the role of cap I in translation.

Wild-type cyclin B1 3'UTR, referred as "B1", contains a PBE (that is known to stabilize CPEB binding to the CPE), two consensus CPEs (CPE1 and CPE3), a non-consensus CPE (CPE2) and a PAS. A modified version of this 3'UTR harbors only the non consensus CPE (CPE2) and the PAS: it is referred as "B1[-1:3:P]". Both of them were fused downstream to the Firefly luciferase coding sequence, injected into *Xenopus laevis* oocytes together with Renilla Luciferase as control, and their translation and polyadenylation in presence or absence of progesterone were examined[10].

These are the conditions in which, in the cited study, Pique' et al. found that while B1 mRNA was translationally stimulated and polyadenylated upon progesterone stimulation, B1[-1:3:P] was polyadenylated, but not translated[10].

Both chimeric mRNAs were incubated in presence or absence of the recombinant xMTr1. Identical amounts were injected into immature oocytes that were then stimulated (translational stimulation) or not (translational repression) with progesterone.

The luciferase assay showed that in absence of progesterone the methylation of both 3'UTRs B1 and B1[-1:3:P] had not significant effect in the repression (Fig 22, upper panel, lane 1-2 and 3-4); as expected, the translational efficiency of B1 increased upon progesterone stimulation (lower panel, lane 5), and is completely abolished in the B1[-1:3:P] (lane 7). The cap methylation of B1 had not an effect in translation stimulation (lane 6), however it had a great effect in the B1[-1:3:P]. This result suggests that in presence of "weak" mRNA the 2'-O cap I modification is now sufficient to drive translational stimulation. When

CPEB1 is poorly recruited on the mRNA, it needed the methylation on the cap, than the binding with CPSF, to enhance translation of the mRNA.

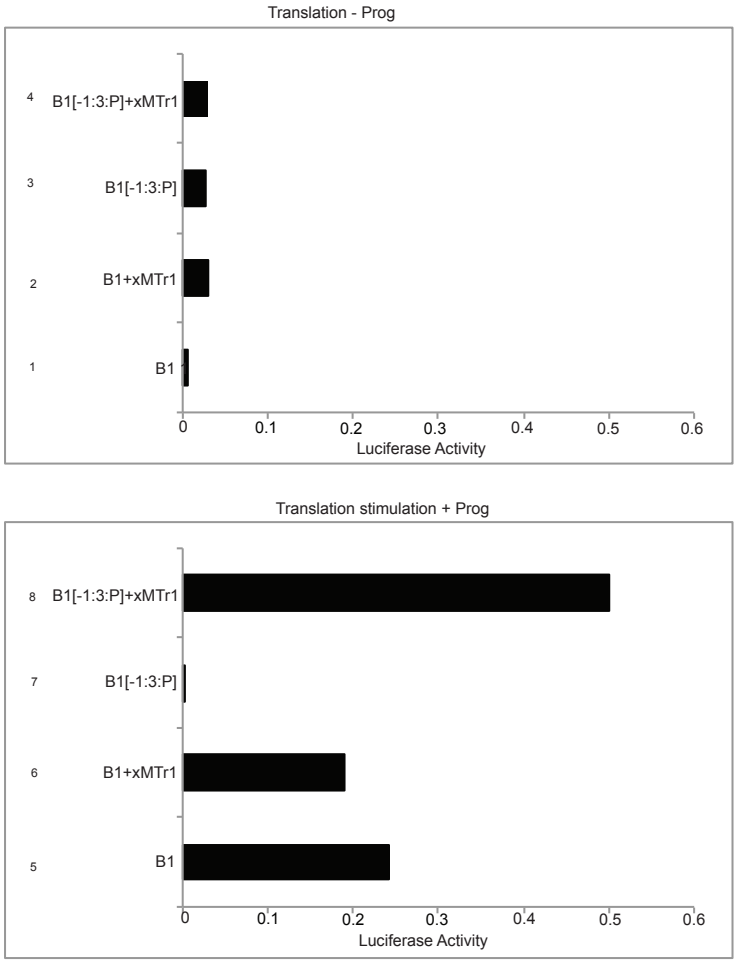


Fig 22. Cap I 2'O-ribose methylation induces translation of a "weak" mRNA. In vitro transcribed mRNAs of synthetic 3'UTR cyclin B1 wild type (B1) or with only a nonconsensus CPE fused to the Firefly luciferase ORF, were incubated or not with xMTr1, then they were co-injected into oocytes together with Renilla luciferase as a normalization control. Oocytes were then incubated in absence (repression) or presence (activation) of progesterone and the luciferase activities were measured.

DISCUSSION

In the present study we identified and characterized the cap I 2'-O methyltransferase, xMTr1. It can be classified as a component of the Rossmann-fold MTase (RFM) superfamily, as it contains the ubiquitous structural motif for the binding of adenosine-containing cofactors. We demonstrated, for the first time, that the xMTr1 catalyses the methylation of the 2'-O-ribose of the first transcribed nucleotide of a capped RNA (cap I) *in vitro,* in presence of the methyl donor SAM (Fig 17). We demonstrated that xMTR1 interacts with xCPEB1 both *in vitro* and *in vivo*. This interaction is suggestive of a role for the xMTr1 in the CPEB mRNP, during the translational regulation. Interestingly their interaction seems to occur upon progesterone stimulation, suggesting that the xMTr1 could play a role in the translational activation of specific mRNAs. According to our results, such mRNAs will be the ones harboring a weak binding site for CPEB1 (i.e. non-consensus CPE) and a PAS. As first, this *in-cis* arrangement is sufficient for triggering CPEB-mediated cytoplasmic polyadenylation according to the classical model. In these conditions, CPEB1 phosphorylation at serine 174 by Aurora-A (Eg2) kinase is the first signal that mediates the recruitment of CPSF to the PAS and consequent poly(A) tail elongation by GLD2. Symplekin serves as scaffolding protein to stabilize the transient interactions of this complex (Fig 23 upper panel). According to our model this specific class of "weak" mRNAs need a second signal in addition to the first one by Aurora-A. This second signal would be provided by xMTr1, as the 43S ribosomal subunit is not able to start the translation initiation at the 5'-end. In order to be translated, these mRNAs need to be modified on the cap structure. In *Xenopus laevis* oocytes we found that CPEB1 is able to recruit the enzyme responsible for the site-specific 2'-hydroxyl methylation of ribose moiety of the first transcribed nucleotide (Fig 20). Interestingly a precise amount of the recombinant xCPEB1 together with the xMTr1 promotes the formation of the cap I, meaning that their association has a synergic functional role, dependent on the presence of CPE sequence in the target mRNA. The stoichiometry of CPEB1 interactions is known to play a crucial role in the translational regulation of gene expression. Thus, high levels of CPEB1 will favor the formation of a stable repression complex, while lower levels are needed for the transient interactions that occur during translational activation. In such a frame the relative amounts of CPEB1 with respect to the xMTr1 are also important.

The activity of xMTr1 is restricted to the first transcribed nucleotide (cap I). However we don't discard the possibility of the action of a second putative xMTase (xMTr2) in the regulation of translation. Kuge et al. demonstrates that capII, together with polyadenylation, stimulates the translational activation of mos mRNA, which is necessary for the induction of oocyte maturation. Inhibition of this methylation by S-isobutylthioadenosine (SIBA), has a little effect on progesterone-induced mos mRNA polyadenylation or general protein synthesis, but prevents the synthesis of Mos protein as well as oocyte maturation[167].

According to our model, cap I methylation increases the affinity of the cap binding protein eIF4E to the cap. This event in turn leads to the recruitment of eIF4G and eIF4A, bringing the whole translation machinery to the mRNA. Only at this stage the mRNA would be competent for translation (Fig. 23, lower panel).

Moreover, In *Xenopus laevis* oocytes, the endogenous xMTr1 is present in oocytes stimulated or not by progesterone; it is a nucleo-cytoplasmic shuttling protein (coherently with the presence of a NLS in its sequence), is able to interact with the endogenous xCPEB1, probably through its WW domain. The tridimensional structure of cocrystals of eIF4E and 7mGDP reveals that the protein contains a cleft that could accomodate 7mGpppN (where N is any nucleotide), and thus could potentially recognize methylated ribose moiety of cap I and cap II, as well as the cap I methyltransferase VP39[253]. It has been also demonstrated that the phosphorylated form of eIF4E increases its affinity for the cap 0[254], and maybe it is triggered by the presence of the cap I. Interestingly among the higher eukaryotes, the xMTR1 is the only cap I MTase that was present both in the nucleus and in the cytoplasm. In other organisms the methylation of cellular mRNA cap I occurs in the nucleus (and the corresponding methyltransferase is only nuclear), whether cap II formation occurs in the cytoplasm (being the cap II methyltransferases only cytoplasmic)[255, 256]. The distribution of xMTr1 as a nucleo-cytoplasmic protein, opens new questions about the role of cap-ribose methylation during the coordination of nuclear and cytoplasmic events. With this respect, CPEB1 was recently found to be a shuttling protein[228]able, already in the nucleus, to recruit CPSF to the PAS on the 3'UTRs of the pre-mRNAs. Such recruitment mediates the cleavage of the regulated mRNAs to proximal and weak PAS.

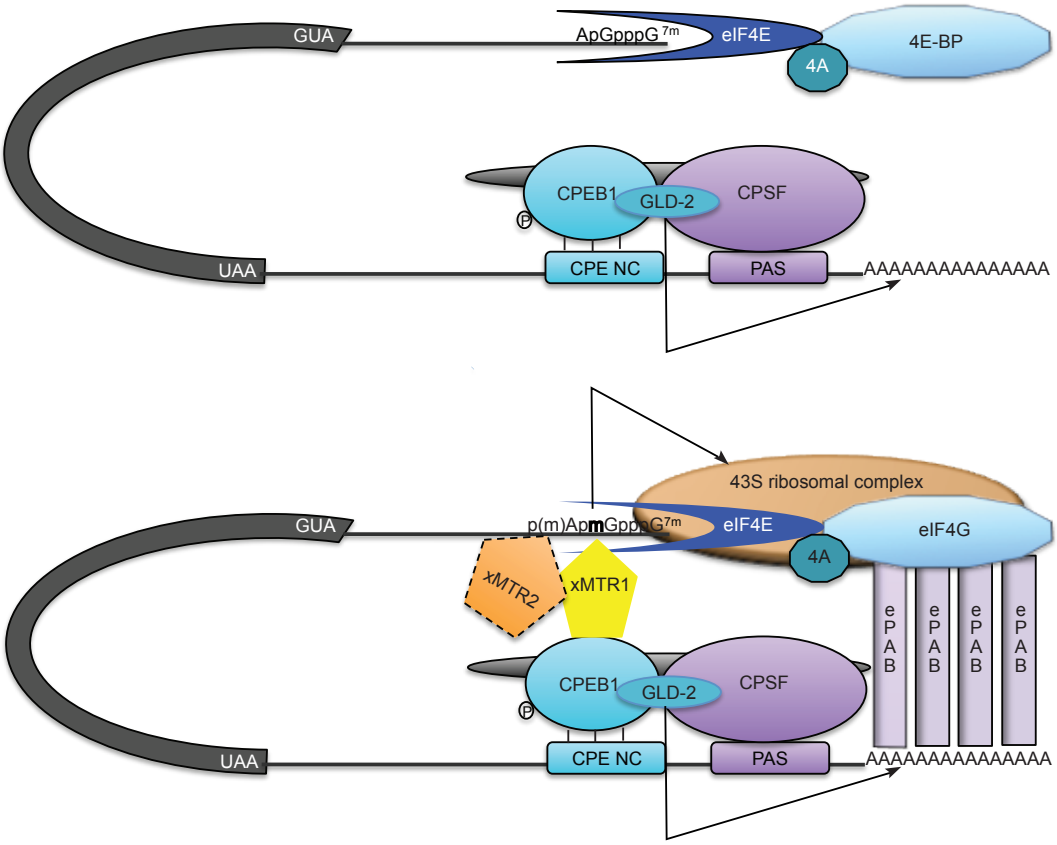


Fig 23. Model for xMTr1 and CPEB1-mediated translational stimulation of a "weak" mRNA. Schematic representation of the activation complex in the absence (top) and presence (bottom) of xMTr1. Abbreviations: CPE NC, nonconsensus cytoplasmic polyadenylation element; PAS, polyadenylation signal; xMTr1, *Xenopus* methyltransferase 1; xMTr2 in dotted line, possible involvement of *Xenopus* methyltransferase 2; CPSF, cleavage and polyadenylation specificity factor; CPEB1, cytoplasmic polyadenylation element binding protein 1; GLD-2, germline development 2; eIF4E, eIF4G, 4A, eukaryotic initiation factor 4E, 4G, 4A; 4E-BP,eIF4E-binding protein; ePAB, *embryonic poly(A)-binding* protein; symplekin in grey; $ApGpppG^{7m}$, CAP 0; $p(m)ApmGpppG^{7m}$, cap I or (capII) structure; AAAAA..., long poly(A) tail.

MATERIALS AND METHODS

Cloning, expression and purification of xMTr1. xMTr1 cDNA was cloned by RT-PCR from total RNA of stage VI oocytes using primers 5'-GGGAAGCTTTAATGAAGAGAAAATCCGACTCCGAAC-3' and 5'-GGGCTCGAGCTACCTATCCTCTGTCAGGCTGGAGG-3', digested with HindIII and XhoI and cloned in pET30. His_6-xMTr1 expression was induced in E.coli BLB21 (DE3/pLysS) with 1mM IPTG for 3 h at 30°*C. Bacteria pellets were sonicated in lysis buffer (PBS containing* 1% Triton X-100, 0.3 mM PMSF, 20 mM imidazole, and complete Protease Inhibitor Cocktail Tablets (Roche). Bacterial lysate was centrifuged 30 min at 20,000 × *g at 4°C*, the supernatant was collected, and the NaCl concentration was adjusted to 1.5 M. The supernatant was incubated for 1 h at 4 °C with 1 ml of nickel-nitrilotriacetic acid resin (Qiagen) equilibrated with lysis buffer. After incubation, the flow-through was collected, and the resin was washed with 10 ml of wash buffer (10 mM Tris-HCl, pH8, 500 mM NaCl, 1% Triton X-100, 0.3mM PMSF, 20 mM imidazole, and complete Protease Inhibitor Cocktail Tablets (Roche), followed by a wash with 10 ml of wash buffer containing 100 mM NaCl. Elutions of 0.5 ml were analyzed by SDS-PAGE, and fractions containing hMTr1 were combined and dialyzed against 20 mM Tris-HCl, pH 8, 100 mm NaCl, 15% glycerol, 0.3 mM PMSF and stored at −80 °C.

Expression and purification of CPEB1. GST-xCPEB1 expression was induced in Rosetta™(DE3) with 0.5 mM IPTG for 1 h at 30°*C and O/N at* 16°*C. Bacteria pellets were sonicated in CelLytic™ B Cell Lysis* Reagent (Sigma), supplemented with 0.2 mg/ml Lysozime, 0.2 mg/ml PMSF and protease inhibitors. Bacterial lysate was centrifuged 10 min at 16,000 × *g at 4°C*.The protein was purified accordingly to the standard protocol of glutathione magnetic beads (Pierce). Elutions of 0.5 ml were analyzed by 10% SDS-PAGE and stored at −80 °C.

Substrates RNA preparation. The "RNA GA" was created by annealing of 5'-AGCATTTAGGTGACACTATAGAATACAAGC3-3' and 5'-GACTTTATTTATAGAATTAAACATTAAAAACCTAAGCTTGTATTCTATAGTGTCACCTAAATGCT-3'. The "RNA GG" was created by annealing of 5'-ATTTAGGTGACACTATAGGATAC-3' and 5'-AGTTTATTTATAGAATTAAAGATTAAAAAGGTAAGGTTGTATCCTATAGTGTCACCTAAAT-3'. The "RNA CPE mut" was created by annealing of 5'-ATTTAGGTGACACTATAGGATAC-3' and 5'-AGTTTATTTATAGAATTAAAGATCCCAAAGGTAAGGTTGTATCCTATAGTGTCACCTAAAT-3'. They were boiled at 95°*C,* chilled on ice for 1min, add 25mM $MgCl_2$, and incubated 30 min at 37°C.

The RNA substrate was transcribed *in vitro* with Sp6 RNA polymerase (Promega). The RNA was purified by phenol/chloroform extraction and ethanol precipitation. The cap was added by ScriptCap m7G Capping System (Epicentre), adding ^{32}P-GTP for 60 min at 37°C. Subsequently, the RNA was purified by phenol/chloroform extraction and ethanol precipitation.

***In vitro* methyltranderase assay.** Methylation reactions with xMTr1 were carried out in 10X ScriptCap™ Capping Buffer, 0.1 mM SAM, purified enzyme and 2pmol substrate RNA in a total volume of 30ul. Reactions were carried out for 1 h at 37°*C. VP39 enzyme from the* ScriptCap™ 2'-O-Methyltransferase kit (Epicentre) was used as a positive control, and the methylation with VP39 was performed following manufacterer recommendations. The modified RNA was purified by phenol/chloroform extraction and ethanol precipitation. The RNA was suspended in 60 ul of 50 mM Na acetate pH 5.2, 0.01% Triton, 0.1 mM EDTA, 2 mM $MgCl_2$ and denatured by heating to 9*5*°C for 2 min and then rapidly chilled. The RNA was digested with 20U of RNase T2 (MoBiTec) for 16hr at 37 °C, then with 12 U of alkaline phosphatase AP (Roche) for 2 h at 37 °C. The digest was concentrated to 20 ul by speed vac, and 10 ul of the sample was applied to a 20% Sequagel (National Diagnostics). After elecrophoresis, the radioactive bands were detected by PhosphorImager.

Xenopus oocytes preparation. Stage VI oocytes were obtained from Xenopus females and induced to mature with progesterone (10 mM, Sigma), as described earlier[257].

Western blot analisis. For experiment described in Fig.18 enucleation[258] were performed according to published procedures. Oocyte lysates, prepared by homogenizing 6–10 oocytes in histone H1 kinase buffer containing 0.5% NP-40 and centrifuged at 12 000 *g* for 10 min, were resolved by 10% SDS–PAGE. Equivalents of 1–2 oocytes were loaded onto each lane. The antibody used was rabbit anti-serum affinity purified against the full protein xMTr1.

Immunoprecipitation. The anti-serum affinity purified against the full protein xMTr1, together with the pre-immune serum of the same rabbit were incubated with dynabeads protein A (Invitrogen) during 3 h at room temperature on wheel, washed with PBS and resuspended in 0.2 M triethanolamine pH 8.2 and 20 mM dimethyl pimelidate · 2HCl (DMP) was added and incubated for 30 min at room temperature on wheel. Reaction was stopped with two 5 min washes at room temperature with 50 mM Tris-HCl pH 8, and two extra washes with histone H1 kinase buffer. Fresh oocytes lysates from stage VI and GVBD (20 oocytes per conditions) were divided in two, treated or not with 50 ug RNase A for 30 min at

4 °*C* on the wheel. The lysates were added to the cross-linked antibody beads and incubated for 2 h at 4 ℃ on wheel. Immunoprecipitates were washed four times in lysis buffer with 100 mM NaCl, four times with 300 mM mM NaCl, two times with 100 mM NaCl and eluted *with sample buffer* (200 mM Tris–HCl pH 6,8, 40% glycerol, 8% SDS, 20 mM DTT), separated by SDS–PAGE and analysed by western blotting.

Luciferase assay. The plasmids B1 and B1[-1:3:P] were a kind gift from M. Pique'[10]. The reporter RNAs were transcribed in vitro with T3 mMessage machine kit (Ambion), precipitated with LiCl. Methylation reactions with xMTr1 were carried out in 25 mM Hepes, 2 mM DTT, 50 uM SAM, 40 U RNase inhibitor, 10 pmol of purified enzyme and 2pmol substrate RNA in a total volume of 20ul. Reactions were carried out for 1 h at 28°*C.* The modified RNA was purified by phenol/chloroform extraction and ethanol precipitation. Translation and polyadenylation of reporter mRNAs were assayed as described earlier [10]. Briefly, oocytes were injected with 0.0125 fmols of reporter mRNA (Firefly luciferase containing the indicated 3′ UTR or control 3′ UTR) together with 0.0125 fmols Renilla luciferase RNA as a normalizing RNA. Luciferase activity was measured using the Dual-Luciferase Reporter Assays System (Promega), according to the manufacturer's instructions.

CHAPTER II

OBJECTIVES

I. Developing a reporter system to study the translational regulation of the CPEB family of proteins during the somatic cell cycle.

II. Mapping their function and time of action in the cell cycle progression.

III. Studying CPEBs cross-regulation along the cell cycle.

RESULTS

A new method for CPEBs translational studies during the somatic cell cycle

CPEB1 and CPEB4 were found to be able to control phase-specific mRNA poly(A) tail elongation and translation during mitosis[101]. However the relative contribution of the CPEBs in translational regulation along the cell cycle are largely unknown.

In the present study, we developed a method for translational studies during the somatic cell cycle, using HEK293 cells as a model.

We stably co-transfected two different plasmids in HEK293 cells: one of them encodes for a destabilized Green Fluorescent Protein (GFP) under the control of a CPE-containing 3'UTR, and the other encodes for a destabilized Red Fluorescent Protein (RFP) linked to a CPE-lacking (i.e. random) 3'UTR (Fig 24a). In such system CPEBs contribute to the regulation of GFP levels, whether RFP signal is constitutively expressed and was used as an internal control.

To start with, we wanted to analyze CPEBs RNA and protein levels in each phase of the cell cycle. Then, RFP-normalized GFP levels were measured by FACS and cells were visualized in live imaging experiments. FACS analysis allowed us to gain high statistic confidence when analyzing cell populations, while live imaging experiments gave us a detailed picture of the GFP, RFP and cellular dynamics, complementing FACS studies.

We could then narrow down the CPEBs-mediated cell cycle regulation, and link it to the cell morphology after system perturbation (i.e. CPEBs depletion) (Fig 24b).

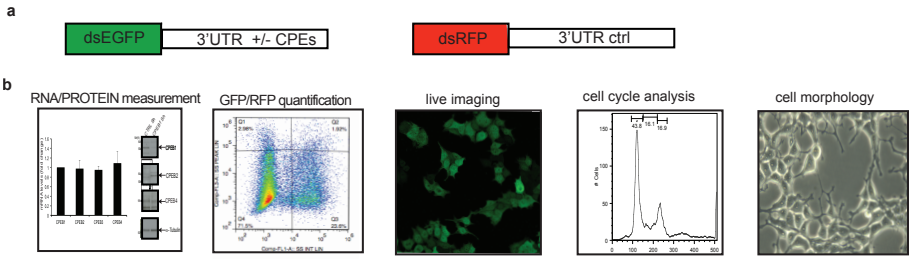


Fig 24. Rationale of this study.

a, Schematic representation of the transfected constructs. GFP open reading frame is showed in green, RFP open reading frame is showed in red. 3'UTRs are showed in white. **b**, From left to right: exemplificative representation of the output of FACS analysis (GFP vs. RFP plot), image showing GFP expression in GR-HEK293 cells, cell cycle profile of asynchronous GR-HEK293 cells, and phase contrast image of GR-HEK293 cells.

CPEBs interplay during the cell cycle

In order to assess mRNA CPEBs levels in GR-HEK293 cells, we performed RT-qPCR experiments in asynchronous cells and found that CPEBs showed similar mRNA levels (Fig 25 a). In order to study CPEBs protein levels, we performed Western Blot experiments. To test the specificity of CPEBs antibodies, we took advantage of an inducible knock down system that allowed us to induce CPEBs-specific short hairpin production upon addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) to the cell culture media. In this way we were able to efficiently knock down CPEB1, CPEB2 and CPEB4 in GR-HEK293 cells (Fig 25 b-d and Fig 26 a-c).

In order to study CPEBs protein levels during the cell cycle progression, GFP/RFP-HEK293 (GR-HEK293 cells) cells were synchronized by double thymidine blockade and then released. We then collected samples when cells were enriched in G1/S border (time 0), S phase (2,5 hours after release), G2-M phases (5 hours after release) and G1 phase (9 hours after release). Interestingly we found that CPEB1-depleted cells seemed to be insensitive to double thymidine treatment. Moreover, when we analyzed CPEBs relative protein levels, we found that all of them seemed to be interconnected during the cell cycle as depletion of any of the CPEBs had effects on the protein levels of the others.

In particular, depletion of CPEB1 led to an increase of CPEB2 levels (Fig 25 b), while depletion of CPEB2 increased CPEB1 protein levels (Fig 25 c). Finally, CPEB4-KD cells showed decreased levels of CPEB1 and CPEB2 (Fig 25 d). Moreover CPEBs knock down led to a slight increase in cell death, which was more evident in CPEB2-KD cells (Fig 25 e).

When analyzed by immunofluorescence experiments, all of the CPEBs showed a nucleo-cytoplasmic localization. Indeed CPEB1 was enriched into the nucleus but present also into the cytoplasm, CPEB2 was mostly nuclear, whether the relative cytoplasmic levels of CPEB4 were higher if compared to CPEB1 and CPEB2 (Fig 26 a-c).

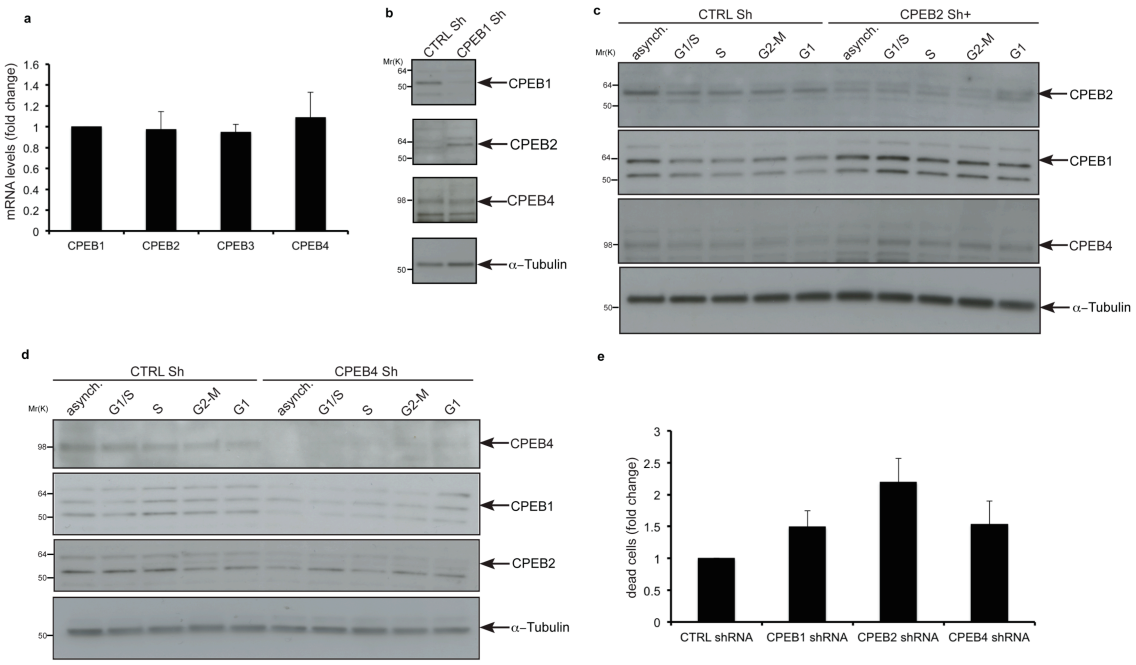
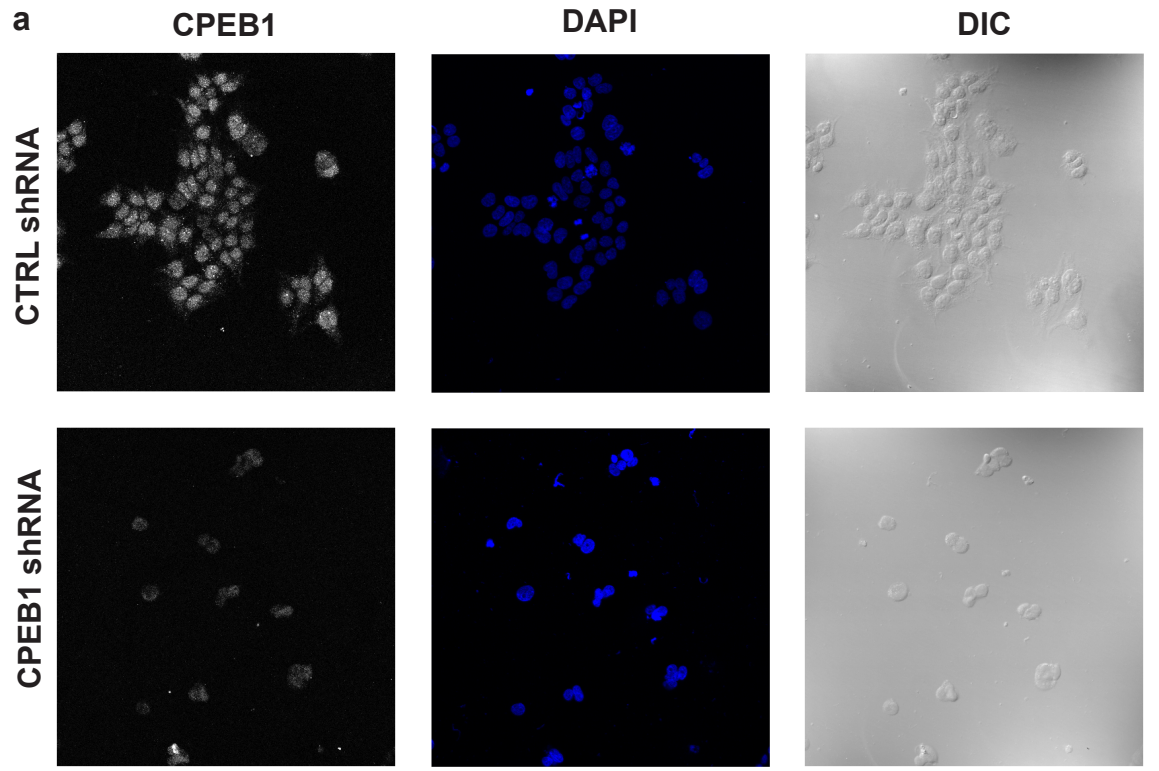
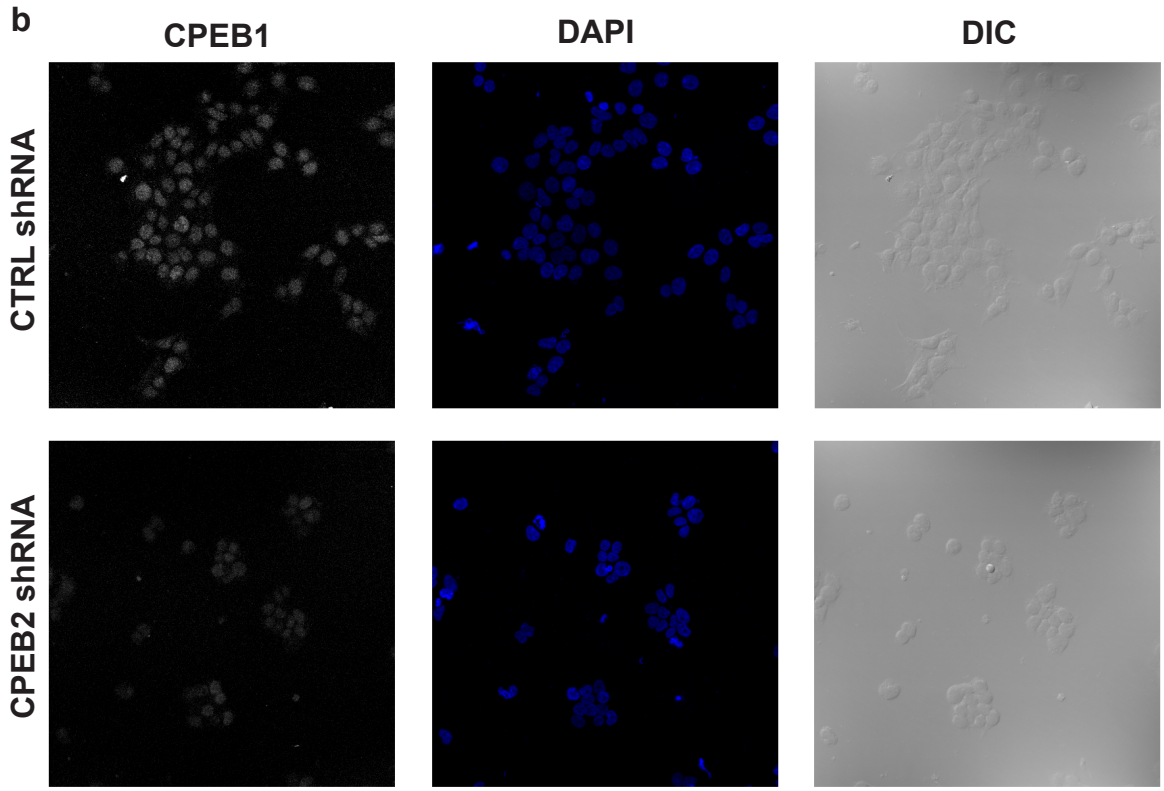


Fig 25. CPEB1, CPEB2 and CPEB4 interplay.
a, Total mRNA was purified from HEK293 cells and mRNAs retrotranscribed using oligo(dT). CPEB1-4 were amplified using specific oligos. Relative mRNA levels are represented. Error bars show standard deviations. **b-d**, GR-HEK293 cells were knocked-down for CPEB1, CPEB2 and CPEB4. Samples were collected at the indicated time-points and CPEB1, CPEB2 and CPEB4 protein levels were analyzed by SDS-PAGE. **e**, GR-HEK293 cells were knocked-down for CPEB1, CPEB2 and CPEB4. Living cells were stained with PI and its incorporation was analyzed by FACS. The fold change in dead cells number, as compared to control cells, is plotted. Error bars show standard deviations.


a
CPEB1
DAPI
DIC
CTRL shRNA
CPEB1 shRNA


b
CPEB1
DAPI
DIC
CTRL shRNA
CPEB2 shRNA

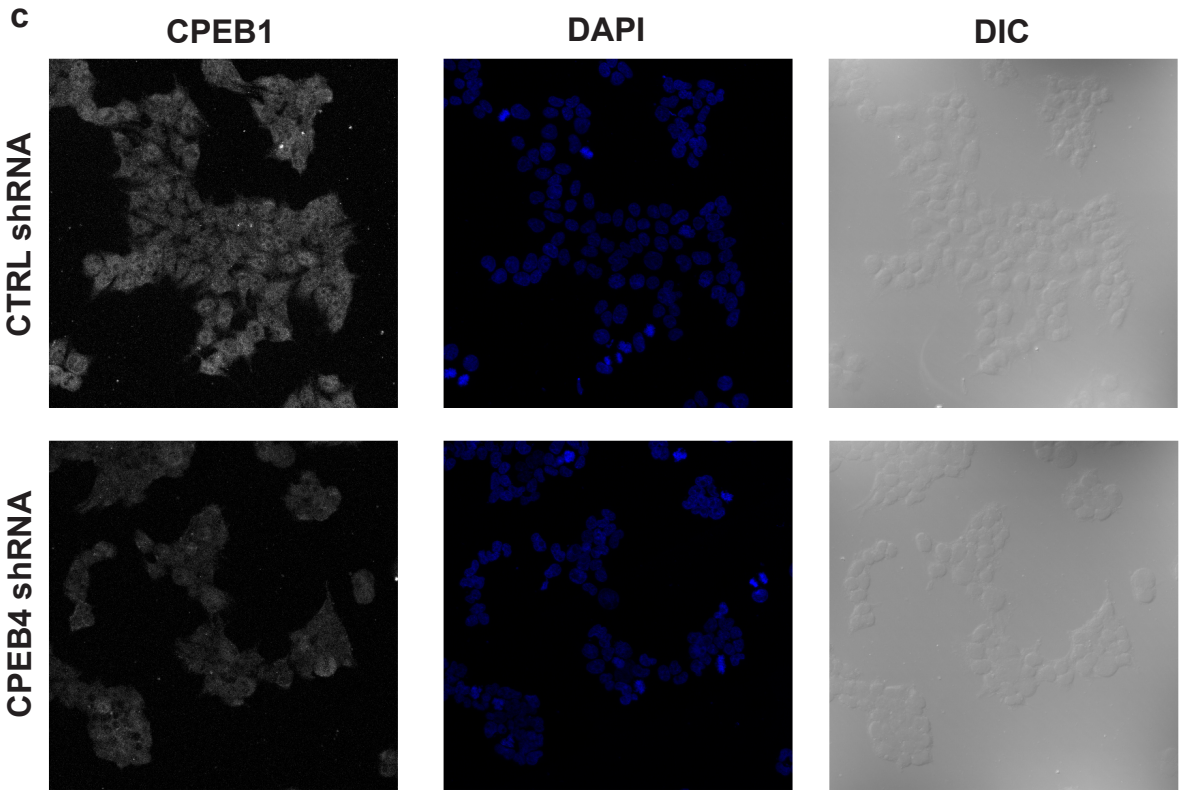


Fig 26. CPEB1,CPEB2 and CPEB4 localization in GR-HEK293 cells
CPEB1, CPEB2 and CPEB4 knock down in GR-HEK293 cells was induced by IPTG addition. Cells were stained after three days of induction. Immunofluorescence of CPEB1 (**a**), CPEB2 (**b**) and CPEB4 (**c**). From left to right: CPEBs staining in grey, DNA staining (DAPI) in blue, differential interference contrast (DIC).

All the CPEBs are required for GFP translation

We then wanted to assess whether the CPEBs have a role in the translational regulation of the CPE-containing reporter mRNA along the cell cycle.

We first asked whether GFP and RFP mRNA levels were fluctuating during the cell cycle, but we didn't observe any significant change (Fig 27 a,b), suggesting that translation would be driving any change in GFP/RFP protein during the cell cycle.

Thus, RFP-normalized GFP levels were measured by FACS, in control or CPEBs-KD cells at different phases of the cell cycle. We found that GFP, but not RFP, expression was specifically activated at G2 and M phases in control GR-HEK293 cells (Fig 28, white squares, Fig.29 a and interactive video 1).

In order to understand whether the observed GFP translational activation could be mediated by the CPEBs family of protein, we specifically mutated the CPE elements on the GFP 3'UTR. Accordingly, inactivating mutations of the CPE elements completely abolished GFP translation at G2/M phases (Fig 28, white diamonds, Fig. 18 b and interactive video 2).

We then wanted to study the individual contribution of each CPEB to GFP translational regulation. Indeed all the members of the CPEB family of proteins retain the ability to bind to the CPE elements[101], thus any of them could be in principle responsible for the observed phenotype.

As compared to control CPEB1-KD cells, even if not synchronous, showed decreased GFP levels (Fig 28, white triangles).

In the same vein, CPEB2 and CPEB4 KD cells showed lowered GFP levels, and GFP activation at G2-M phases was lost in these conditions (Fig 28, white circles and black crosses, Fig 29 d,e and interactive videos 3 and 4).

Altogether these results indicate that the CPEBs, through their binding to the CPEs, regulate GFP translation. In particular, CPEB2 and CPEB4 mediate GFP translation specifically at G2-M phases.

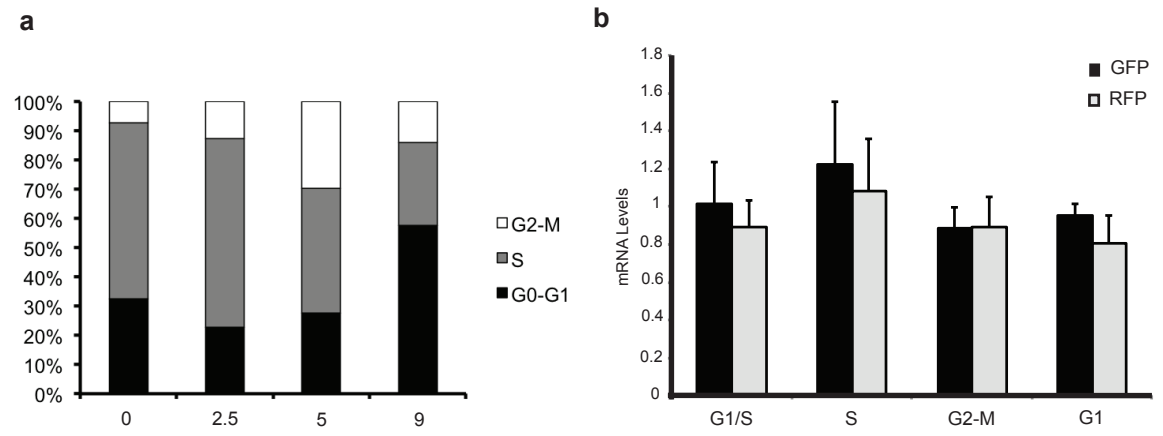


Fig 27. GFP and RFP mRNA levels don't change significantly in the cell cycle
a, GR-HEK293 cells were synchronized at G1/S by double thymidine blockade and then released. The samples were collected at the indicated times after release (X axis represents hours), and the DNA content was measured by propidium iodide staining and FACS analysis. Percentages of cells in each phase of the cell cycle are indicated. Results are shown as the average of five experiments. **b**, Relative mRNA levels for GFP and RFP were measured by qRT-PCR in control GR-HEK293 cells. The mRNA levels in the studied phases of the cell cycle are indicated. Results are shown as the mean value from three experiments, error bars indicate s.d.

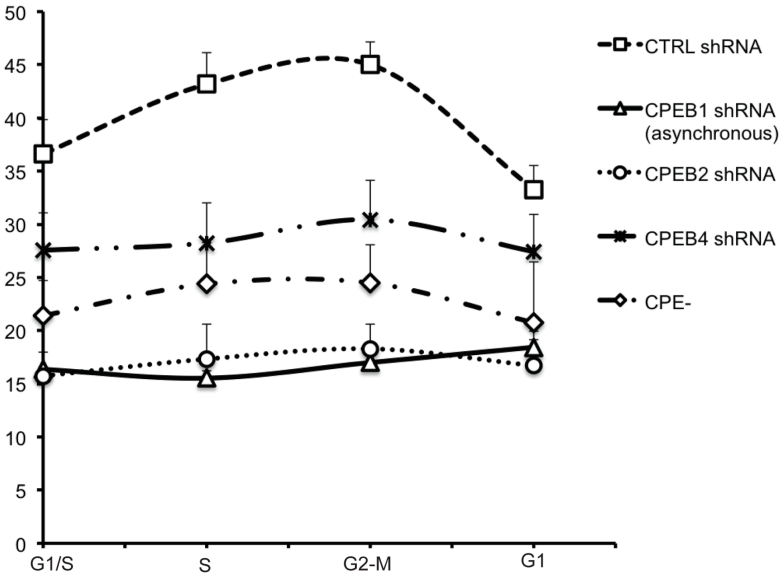


Fig 28. CPEs, CPEB2 and CPEB4 are responsible for GFP translational activation at G2-M phases.

GR-HEK293 cells were synchronized by double thymidine blockade and then released. RFP-normalized GFP expression was measured at the indicated time points. Squares indicate GFP levels in control cells. Crosses indicate GFP levels in CPEB4-KD cells. Circles indicate GFP levels in CPEB2 KD cells. Triangles indicate GFP levels in asynchronous CPEB1-KD cells. Diamonds indicate GFP levels in GR-HEK293 cells stably transfected with a GFP vector carrying CPE-inactivating mutations together with the control RFP vector. Results are shown as the mean value from six experiments, error bars indicate s.d.

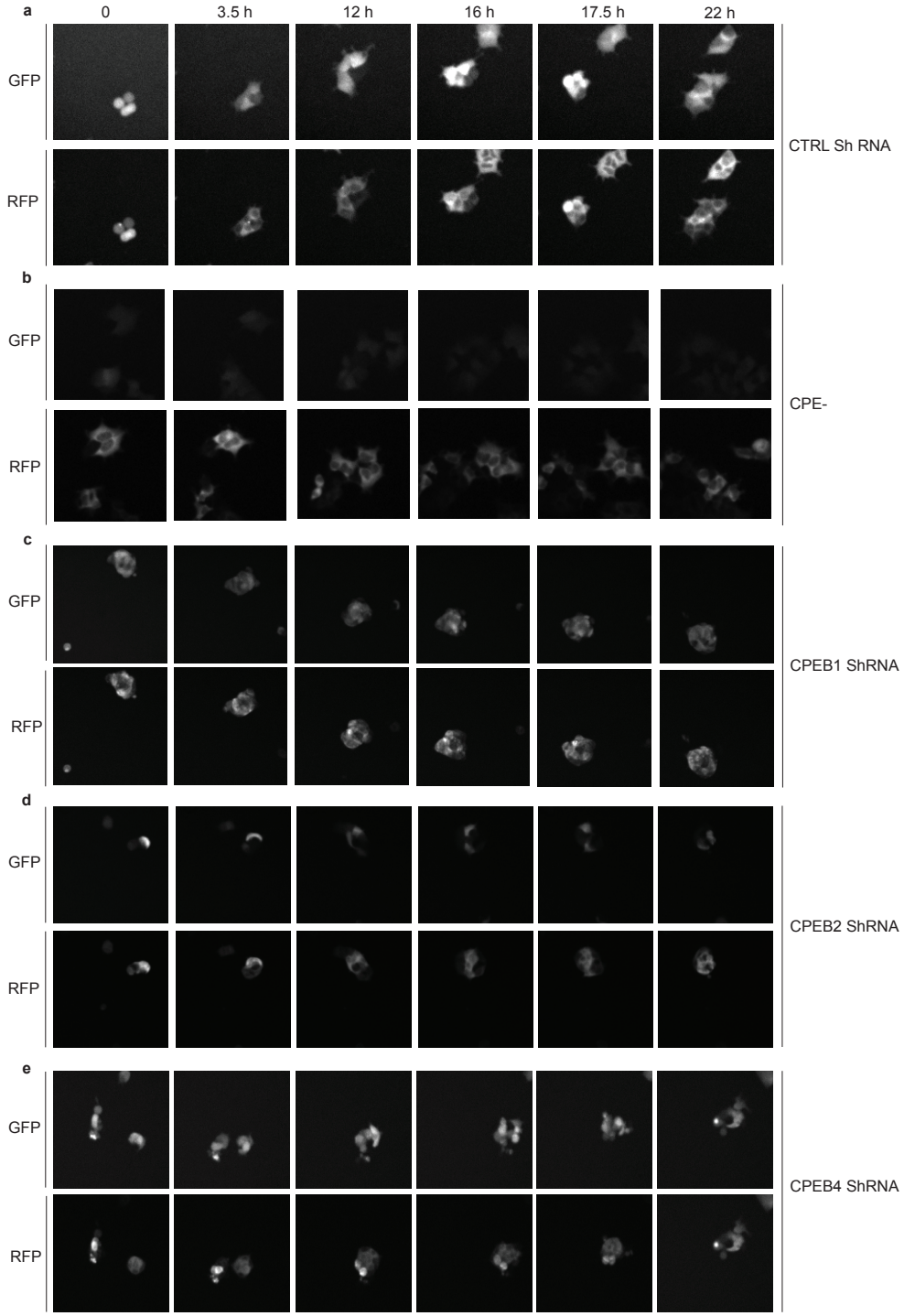


Fig 29. Frames from interactive videos 1-5 show GFP burst in control, but not in CPEB1-KD, CPEB2-KD, CPEB4-KD or CPE- GR-HEK293 cells.
GR-HEK293 cells were induced for 3 days with IPTG to induce individual CPEBs knock-down.
Control cells (**a**), CPE- cells (**b**), CPEB1-KD cells (**c**), CPEB2-KD cells (**d**) and CPEB4-KD cells (**e**) were plated and GFP and RFP signals recorded for 36 hours in live imaging experiments. Representative time points are displayed.

CPEBs knock down leads to cell cycle defects

Cell synchronization of control HEK293 cells allowed us to enrich for specific populations of cells at a given phase of the cell cycle (Fig. 30 a). We then asked if CPEBs knock-down could influence GR-HEK293 cell cycle progression.

Strikingly, CPEB1-KD cells were insensitive to double thymidine synchronization (Fig 30 b, compare to 30 a for control). CPEB2-KD cells, and to a lower extent CPEB4-KD cells, rather showed a delay in G1 entry when compared to control cells (Fig 30 c-d, 9h).

We then asked if the observed phenotypes were due to defects in cell proliferation. To answer this question, we performed 5-ethynyl-2'-deoxyuridine (EdU) incorporation and pulse-chase experiments. As shown in Fig 31 b (please compare to Fig 31 a for control), CPEB1-KD cells were almost not proliferating. Moreover, as compared to control cells, CPEB1-KD cells showed a much lower proportion of cells positive for EdU incorporation at time 0 (in the Y axis of figure 31 b, to be compared to 31 a. Mean =$1x10^4$ in CPEB1-KD cells vs. $5x10^4$ in control cells), suggesting that CPEB1-KD cells are defective in DNA replication. Moreover EdU^+-CPEB1-KD cells showed a severely impaired progression through the cell cycle (Fig 31 b, compare to 31 a for control). Both CPEB2-KD and CPEB4-KD cells showed equal rates of EdU incorporation if compared to control cells. However the progression through the cell cycle was slower than in control cells (Fig 31 c-d).

Coherently with what observed in fig 19c and d, CPEB2 knock down (and to a lower extent CPEB4 knockdown) led to significantly higher levels of cells in G2-M phases (Fig 31 c-d, right panels).

Altogether, these results showed that CPEB1 function in S phase is essential for HEK293-cells proliferation, whether CPEB2 and CPEB4 functions in G2/M phases are required for proper cell cycle progression.

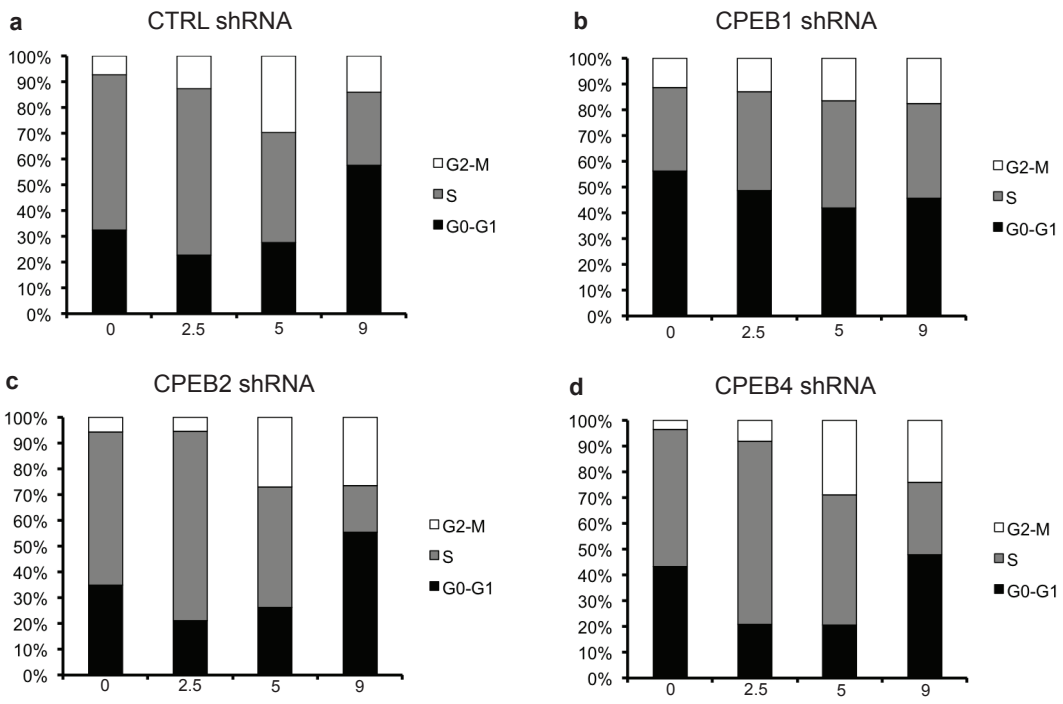
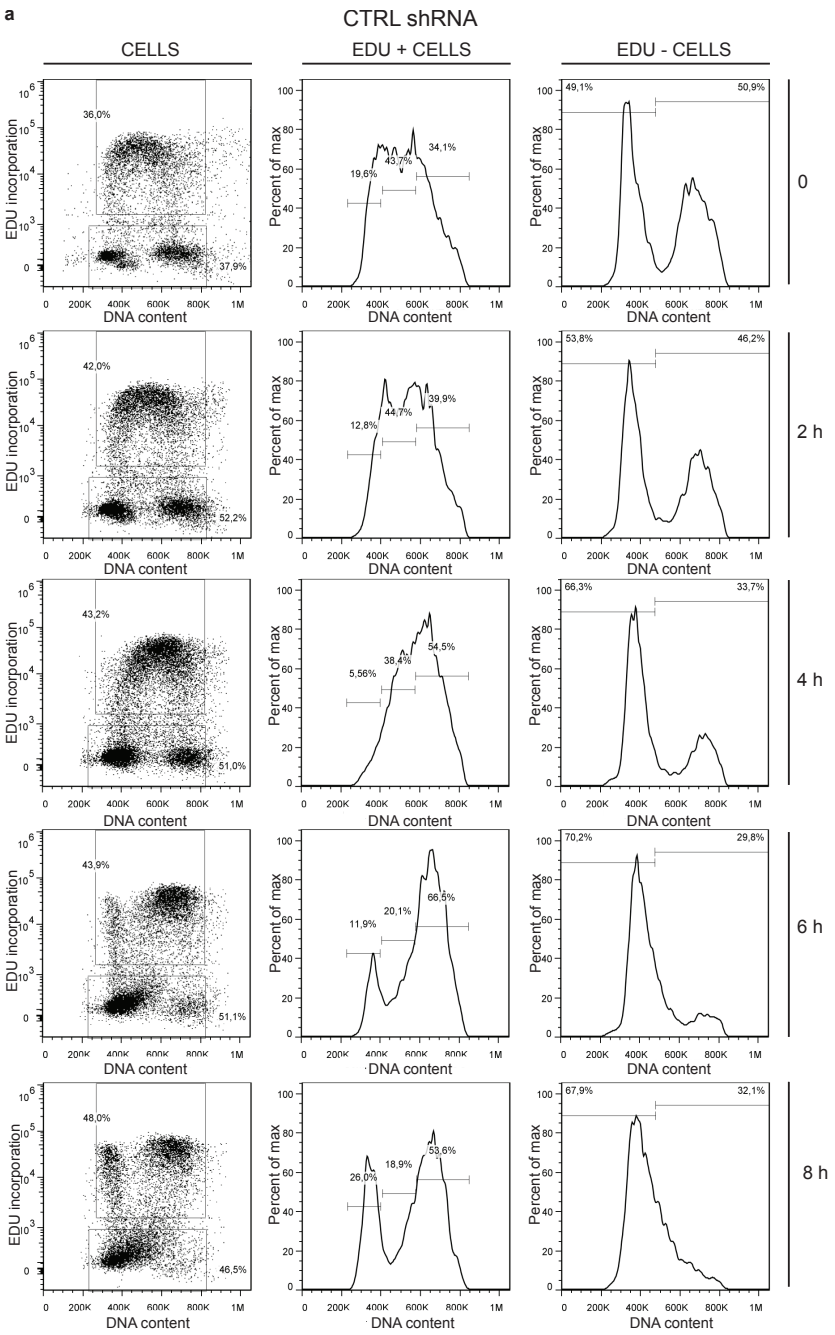


Fig 30. CPEB1-KD cells are not sensible to double-thymidine blockade. CPEB2- and CPEB4-KD cells show a delay in G1 entry.
GR-HEK293 cells were exposed to double thymidine treatment and then released. The samples were collected at the indicated times after release (X axis represents hours), and the DNA content was measured by propidium iodide staining and FACS analysis. Percentages of cells in each phase of the cell cycle are indicated. Results are shown as the average of five experiments.


a
CTRL shRNA
CELLS
EDU + CELLS
EDU - CELLS
EDU incorporation
DNA content
Percent of max
36,0%
37,9%
19,6%
43,7%
34,1%
49,1%
50,9%
0
42,0%
52,2%
12,8%
44,7%
39,9%
53,8%
46,2%
2 h
43,2%
51,0%
5,56%
38,4%
54,5%
66,3%
33,7%
4 h
43,9%
51,1%
11,9%
20,1%
66,5%
70,2%
29,8%
6 h
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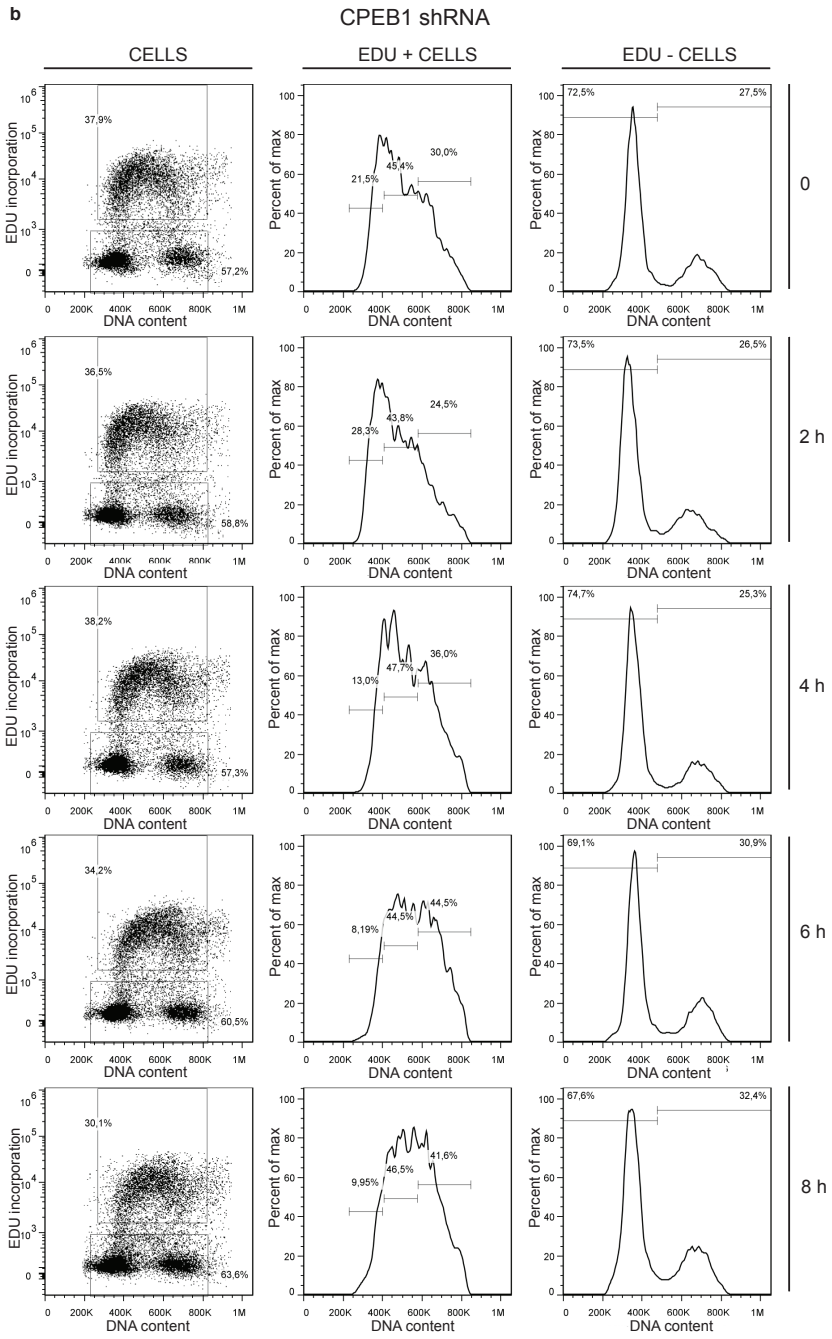
26,0%
18,9%
53,6%
67,9%
32,1%
8 h


b
CPEB1 shRNA
CELLS
EDU + CELLS
EDU - CELLS
EDU incorporation
DNA content
Percent of max
37,9%
57,2%
21,5%
45,4%
30,0%
72,5%
27,5%
0
36,5%
58,8%
28,3%
43,8%
24,5%
73,5%
26,5%
2 h
38,2%
57,3%
13,0%
47,7%
36,0%
74,7%
25,3%
4 h
34,2%
60,5%
8,19%
44,5%
44,5%
69,1%
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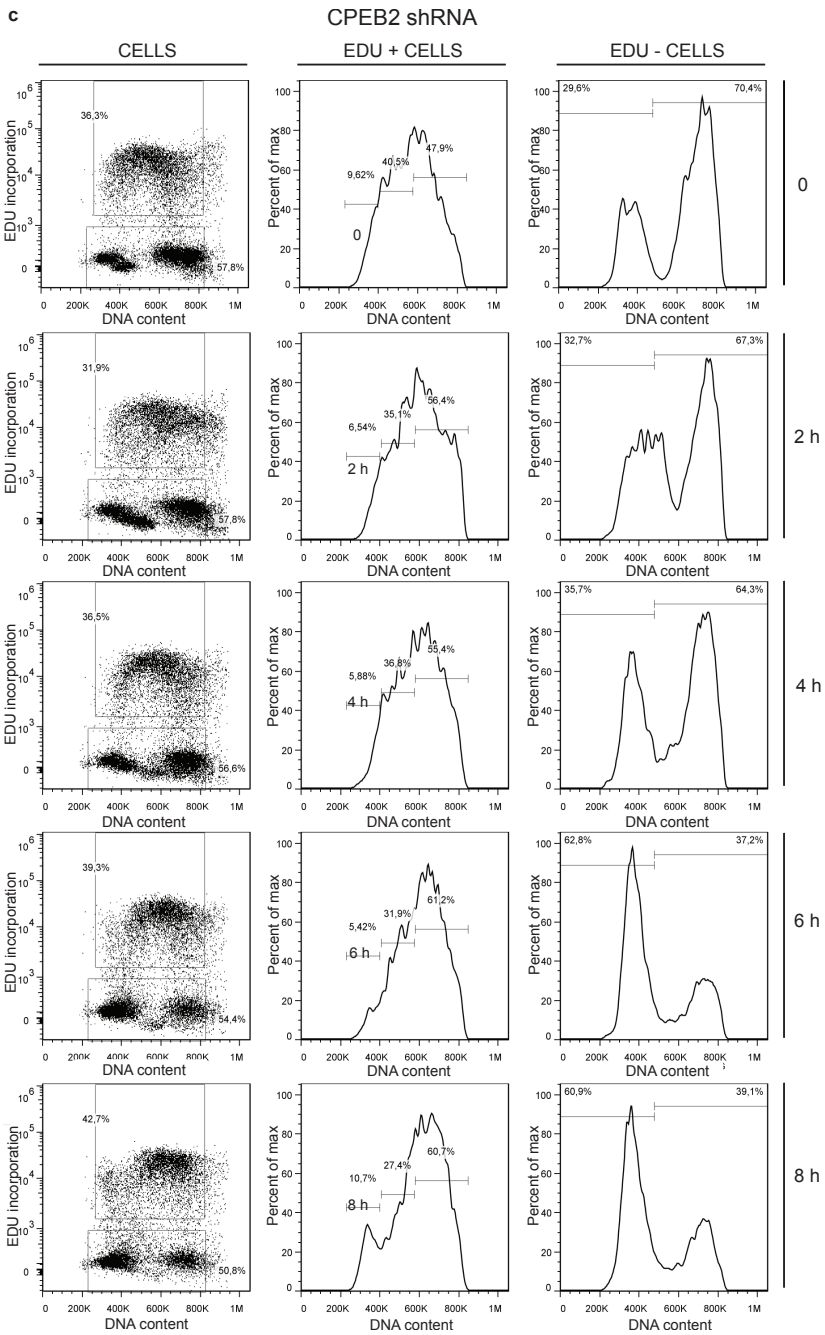
67,6%
32,4%
8 h


c
CPEB2 shRNA
CELLS
EDU + CELLS
EDU - CELLS
EDU incorporation
DNA content
Percent of max
36,3%
57,8%
9,62%
40,5%
47,9%
0
29,6%
70,4%
0
31,9%
57,8%
6,54%
35,1%
56,4%
2 h
32,7%
67,3%
2 h
36,5%
56,6%
5,88%
36,8%
55,4%
4 h
35,7%
64,3%
4 h
39,3%
54,4%
5,42%
31,9%
61,2%
6 h
62,8%
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60,7%
8 h
60,9%
39,1%
8 h

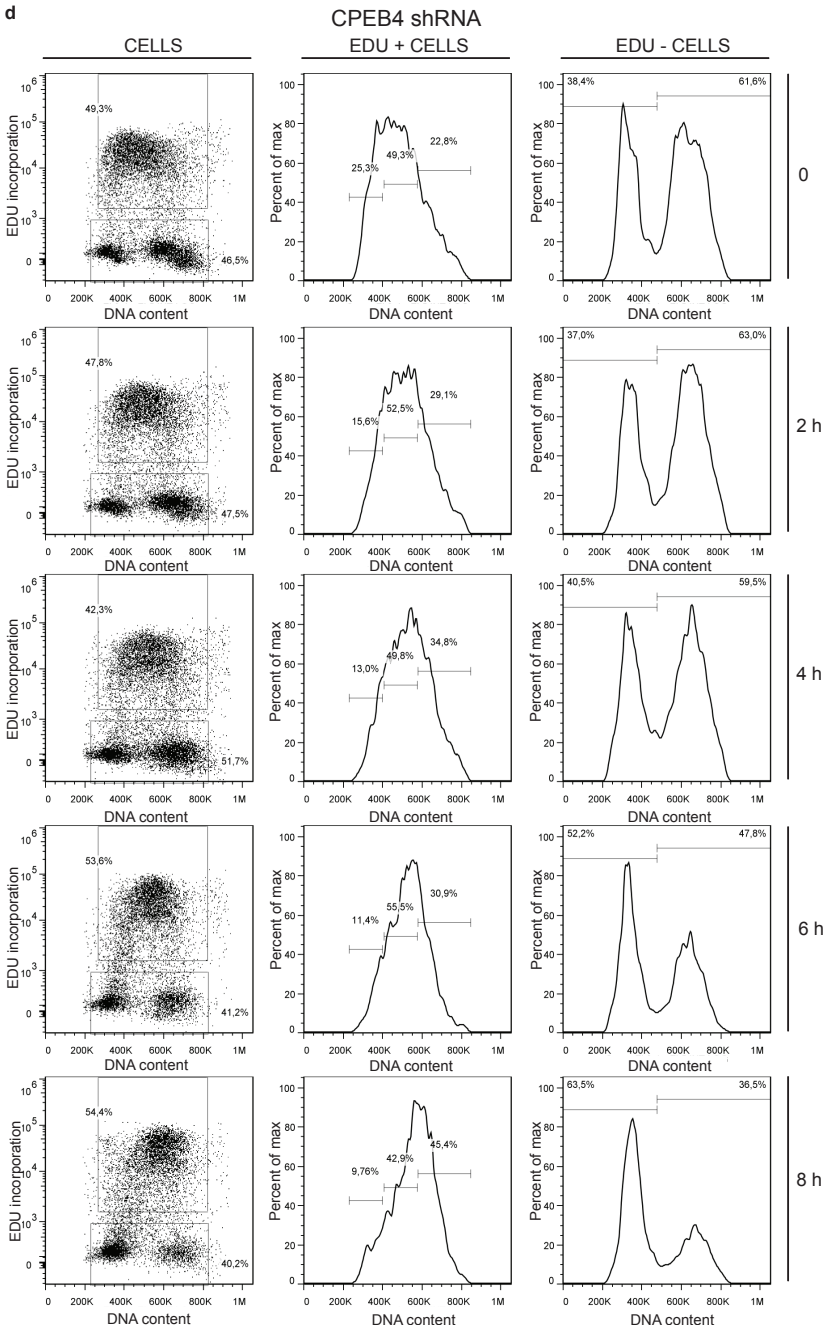


Fig 31. CPEB1-KD cells show DNA replication and cell cycle progression defects. CPEB2-KD and CPEB4-KD are partially arrested in G2-M phases and show defects in cell cycle progression.

GR-HEK293 cells were labeled with EdU and then released. Samples were collected at the indicated time points, stained for DNA content with Propidium Iodide and analyzed by FACS. Plots indicate EdU incorporation vs DNA content. **a**, CTRL GR-HEK293 **b**, CPEB1-KD GR-HEK293 **c**, CPEB2-KD GR-HEK293 **d**, CPEB4-KD GR-HEK293.

CPEB1 is required in early prophase, CPEB2 in metaphase, CPEB4 in late telophase/cytokinesis

Given the importance of CPEB1, CPEB2 and CPEB4 for cell cycle progression, we aimed at understanding and further characterizing their role during mitosis. To this purpose we overexpressed a fluorescent histon-H2B protein in control or CPEBs-depleted GR-HEK293 cells. In this way we could follow GR-HEK293 nuclear dynamics along the cell cycle by live imaging.

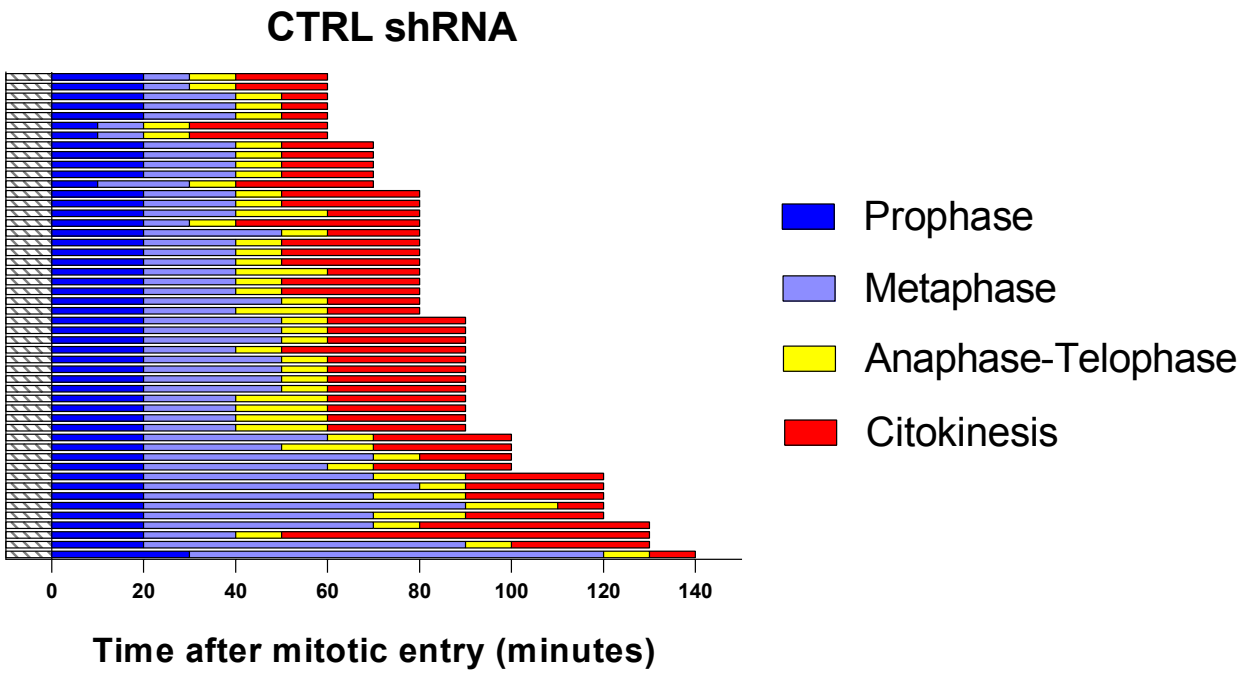
Coherently with what observed by the previously described cell cycle studies, only a small proportion of CPEB1-KD cells reached mitosis. Live imaging experiments allowed us to observe that those CPEB1-KD cells that did enter mitosis, showed defects in the initial steps of chromosome condensation (Fig 32 b, 33 b and interactive video 7). Thus, more than 70% of them were blocked in early prophase during the whole time of the movies (about 30 hours). We also observed few cases of cells going back from prophase to interkinesis (interactive video 6). These data suggest that CPEB1 is not only needed in S phase, but also in early prophase.

CPEB2-KD cells were able to condense chromosomes but showed defects in metaphase plate formation (Fig 32 c, 33 c and interactive video 8), suggesting that CPEB2 is needed in metaphase. About 70% of them spent more time in metaphase as compared to control cells, and about 50% were blocked in metaphase for the whole time of the experiments (about 30 hours). Interestingly, as for CPEB1-KD cells, a little percentage of them regressed to the previous phase (in this case prophase, interactive video 8).

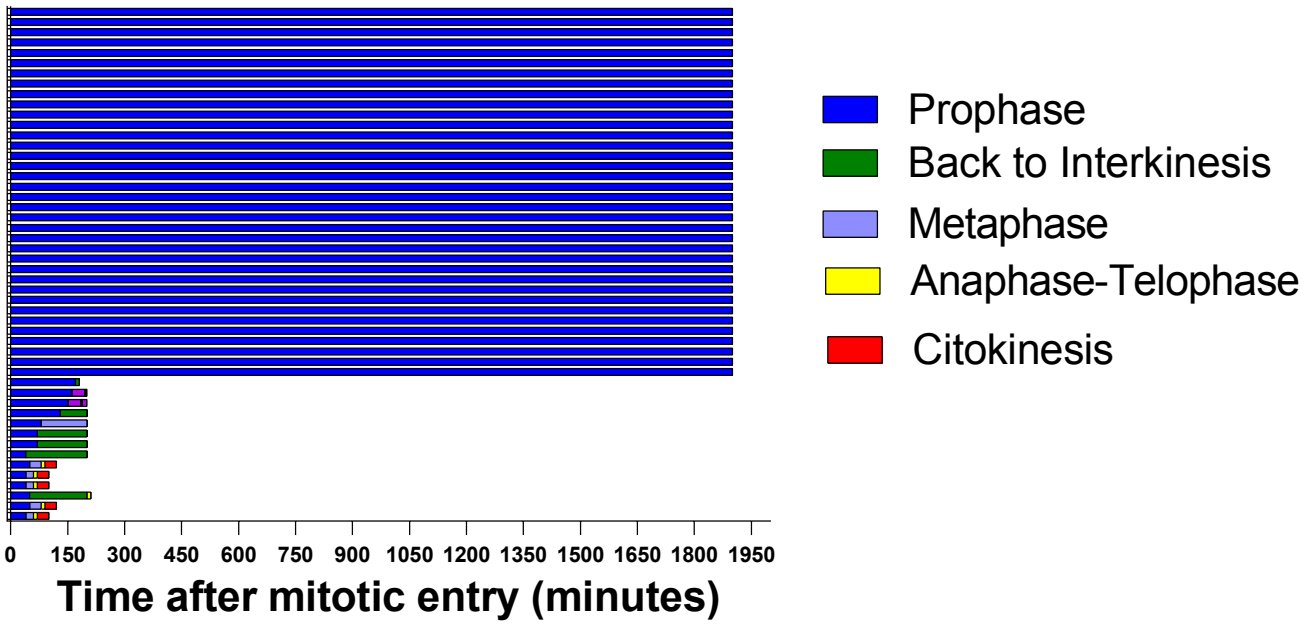
Even if CPEB4 KD cells were able to reach anaphase, more than the 50% of them showed defects in anaphase/telophase and cytokinesis (Fig 32 d, 33 d and interactive video 9). Such defects included chromosome bridges, formation of micronuclei, chromosome fragmentations, leading also to reverted cytokinesis (interactive video 9 and 10).

Altogether these results show that CPEB1, CPEB2 and CPEB4 are sequentially required in mitosis. Depletion of any of these CPEB proteins leads to specific defects that, at least in part, are responsible for the observed general defects in cell proliferation and slightly increased cell death rates (Fig 29 e).

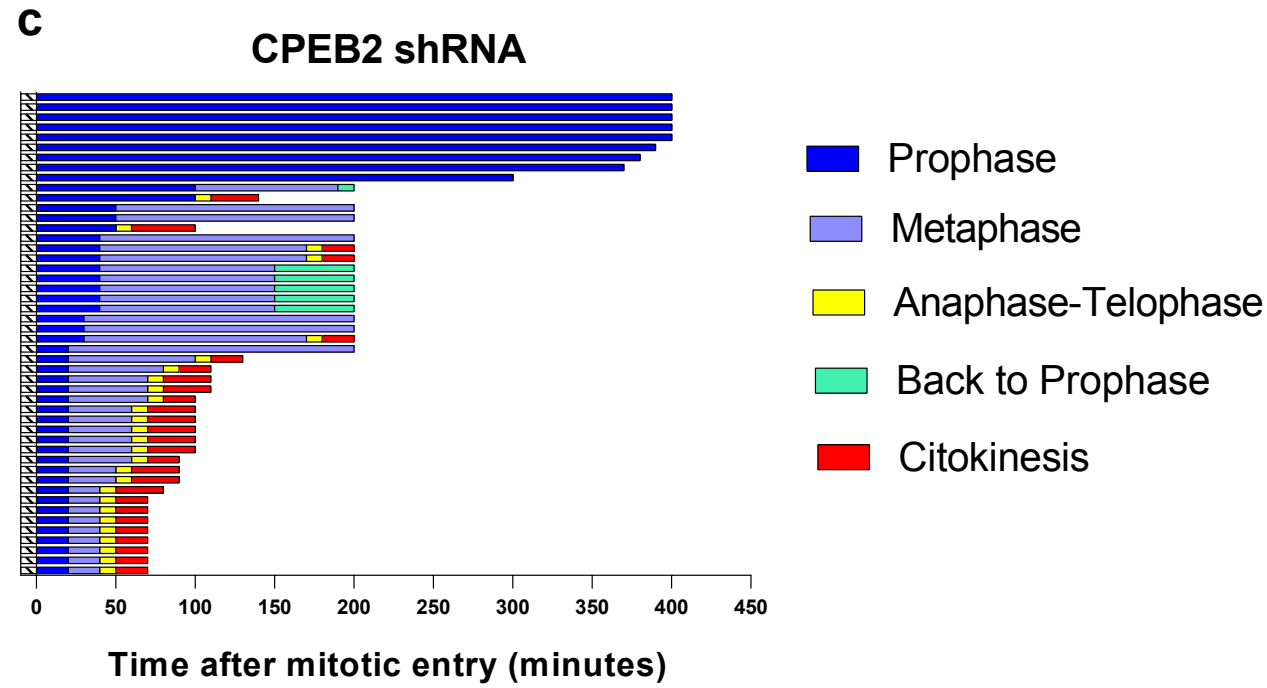
a


CTRL shRNA
0
20
40
60
80
100
120
140
Time after mitotic entry (minutes)
Prophase
Metaphase
Anaphase-Telophase
Citokinesis

b


CPEB1 ShRNA
0
150
300
450
600
750
900
1050
1200
1350
1500
1650
1800
1950
Time after mitotic entry (minutes)
Prophase
Back to Interkinesis
Metaphase
Anaphase-Telophase
Citokinesis

c


CPEB2 shRNA
0
50
100
150
200
250
300
350
400
450
Time after mitotic entry (minutes)
Prophase
Metaphase
Anaphase-Telophase
Back to Prophase
Citokinesis

d

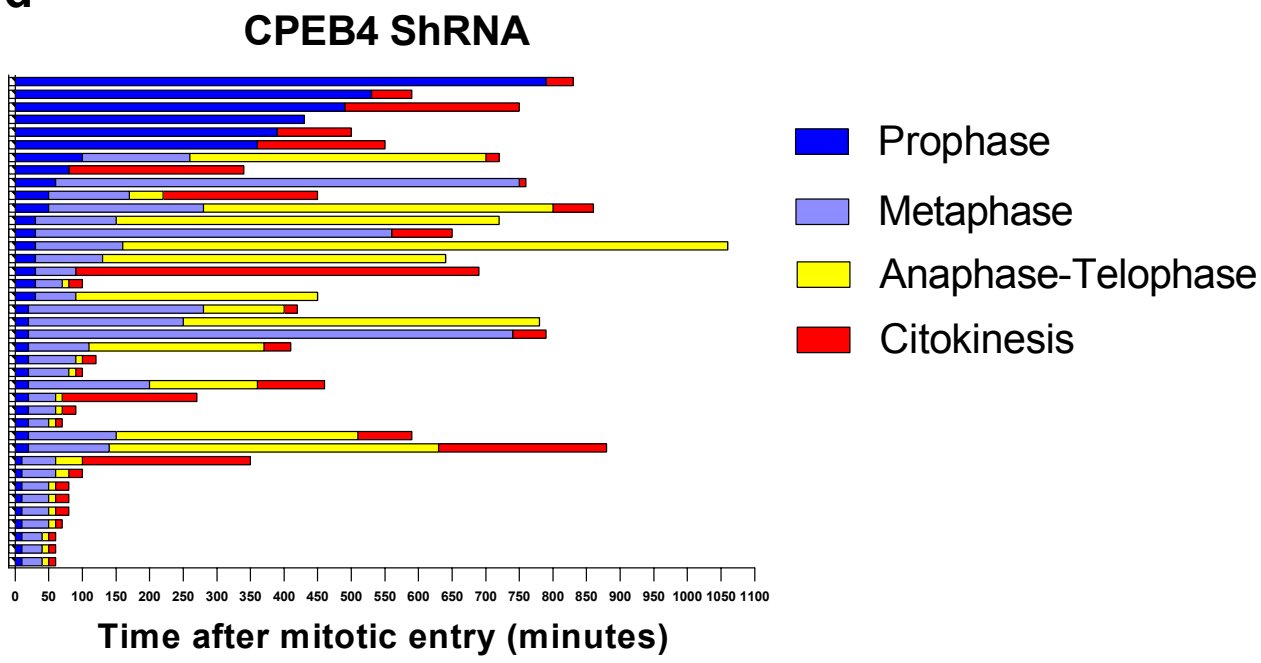

CPEB4 ShRNA
0
50
100
150
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000
1050
1100
Time after mitotic entry (minutes)
Prophase
Metaphase
Anaphase-Telophase
Citokinesis

Fig 32. CPEB1 is required in early prophase, CPEB2 in metaphase, CPEB4 in late telophase/cytokinesis
Control- (**a**) or CPEB1- (**b**), CPEB2- (**c**) or CPEB4- (**d**) knock down GR-HEK293 cells overexpressing H2B-fluorescent protein were visualized in live imaging experiments for 30 hours. DNA staining allowed mitotic division visualization. In Y axis each lane represents one cell. In X axis, time is represented as minutes. Colors represent the indicated mitotic phases.

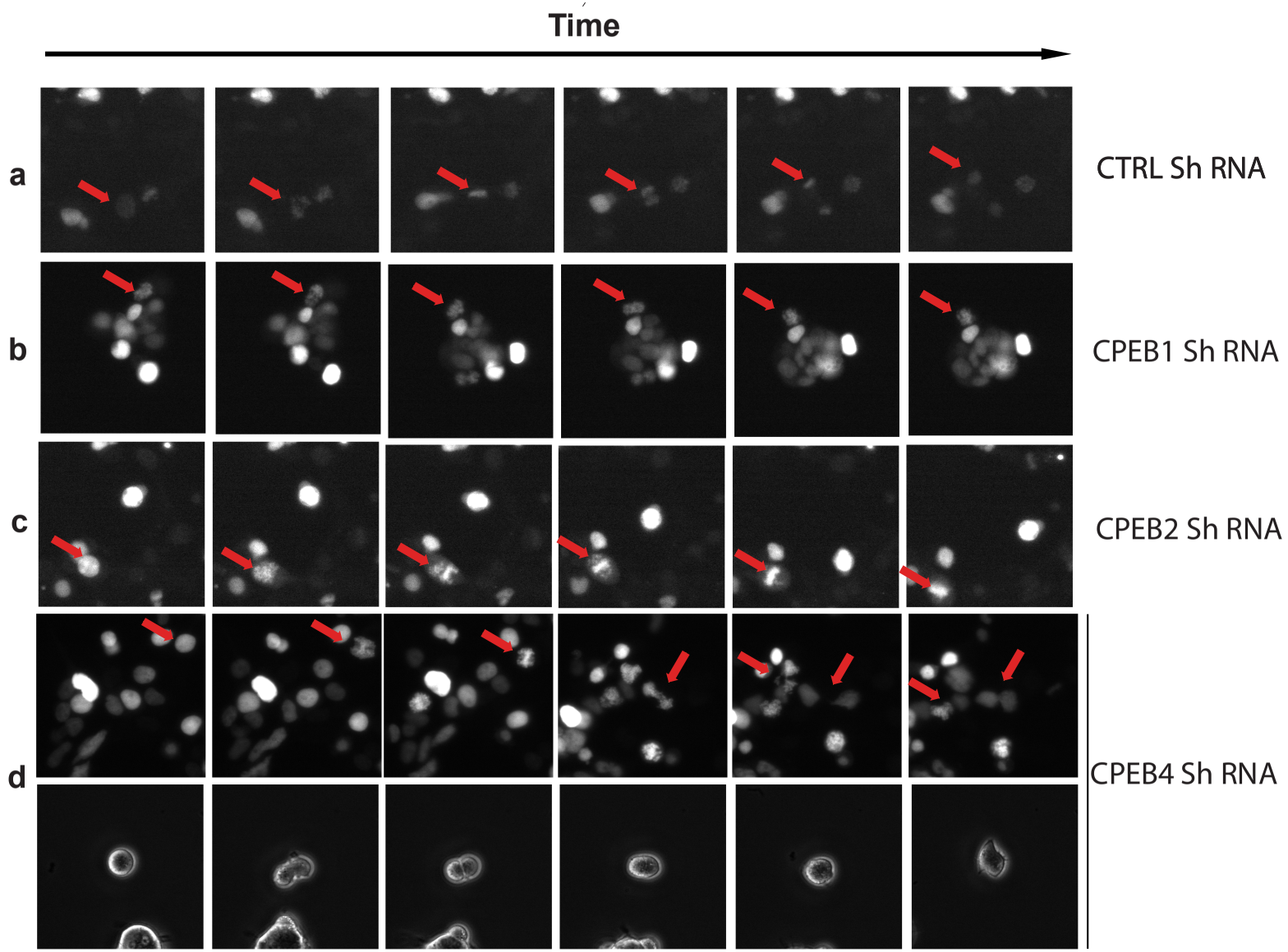

Time
a
b
c
d
CTRL Sh RNA
CPEB1 Sh RNA
CPEB2 Sh RNA
CPEB4 Sh RNA

Fig 33. CPEB1 is required in early prophase, CPEB2 in metaphase, CPEB4 in late telophase/cytokinesis: representative images
Control- (**a**) or CPEB1- (**b**), CPEB2- (**c**) or CPEB4- (**d**) knock down GR-HEK293 cells overexpressing H2B-fluorescent protein were visualized in live imaging experiments for 30 hours. Cell nuclei in gray; representative nuclei are pointed by red arrows. Each picture was taken at the indicated times.

DISCUSSION

CPEBs family of proteins has been shown to be involved in the regulation of important biological processes such as meiotic progression[10, 100, 196], mitosis[101], transformation and tumor development[97, 102, 168]. CPEB1 and CPEB4 act in relay to drive meiotic progression by orchestrating a self-sustainable circuit that accounts for positive and negative feedback loops. In meiosis, CPEB1 is needed for prophase I to metaphase I transition whether CPEB4 takes over CPEB1, sharing the same mRNA targets, to drive meiosis until metaphase II. As for times after fertilization, CPEB1 has been reported to be important during early embryogenesis, for early mitotic divisions, where cells lack G1 and G2 phases, to accomplish the need of fast divisions and embryo growth. Reminiscent of the importance of CPEB1 in early embryonic stages and fast cell division, is the role of CPEB1 in tumor biology. In this case it seems to be also linked to its nuclear role in 3'UTR shortening, which is associated with cellular proliferation and transformation[94, 97]. CPEB4 has a role in tumor development as well, contributing to pancreatic tumor progression through the translational control of the tissue plasminogen activator[102]. CPEB1 and CPEB4 have been also shown to mediate cytoplasmic polyadenylation of specific subsets of mRNAs during the mitotic cell cycle in a cell-specific manner[101]. From these studies it seems more and more clear that the CPEB family of proteins is implicated in the regulation of cell division, and perturbations of CPEBs action may lead to tumor development. Moreover they suggest that CPEBs play their role not as single isolated proteins, but rather their functions seem interconnected by compensatory and cooperative mechanisms.

We then aimed at shedding some light on the role the CPEBs interplay during the cell cycle. The reporter system here described revealed that the CPE elements are needed for translational activation of the GFP, specifically in G2-M phases (Fig. 34 b). RT-qPCR experiments showed that the GFP and RFP RNA levels didn't change significantly during the cell cycle (Fig 25 b), demonstrating that our system is appropriate for translational studies along the cell cycle.

Using HEK293 cells we showed that CPEB1, CPEB2 and CPEB4 have specific functions during the cell cycle (Fig. 34 b). CPEB1 is needed for DNA replication in S phase, as showed by EdU-incorporation experiments, and is implicated in cell proliferation, as cells lacking this protein show a very slow, if any, progression through the cell cycle. The fact that CPEB1 knock down in HeLa cells have milder effects on cell cycle progression as compared to HEK293 cells, may reflect the cell-line (and possibly tumor) specificity of CPEB1 and, in general, CPEBs functions. In fact different cell types show different relative levels of CPEB expression. In HeLa cells CPEB2 and CPEB3 mRNA levels are under-represented if compared to CPEB1 and CPEB4. They are almost not detectable by RT-PCR[101], suggesting that the CPEBs mediated cell cycle regulation is mainly achieved by CPEB1 and CPEB4. Instead, in HEK293 cells we found that all of the CPEBs have similar mRNA levels

(Fig 29 a), and knocking down CPEB individually gives distinct phenotypes, pointing to a cooperative effect. CPEB1-related strong effects in cell proliferation point also to the known role for CPEB1 in the coordination of 3'UTR processing (which occurs specifically on genes important for cell proliferation/transformation) with translational regulation of gene expression (probably of key regulators of cell cycle progression[97]). Interestingly we also noticed that CPEB1 knock down induces cell detachment from matrix and CPEB1-KD cells were aggregating in detached clumps, pointing to a role for CPEB1 in cell-to-matrix attachment (Fig 29 c and interactive video 5). We speculate that this phenotype could be mediated by local translation of specific mRNA targets in the focal adhesion. CPEB1 role in localized translation is a known mechanism of gene expression regulation[14] and, at least for ZO-1 mRNa, was shown to be important for epithelial tight-junction assembly and cell polarity[259]. For this reason we think that future experiments should be performed to explore the possibility of the involvement of CPEB1 in cell attachment.

During mitosis, CPEBs members were shown to be active in specific moments of the cell cycle, pointing to a temporally coordinated cooperation of the CPEBs to achieve proper cell cycle progression. Thus CPEB1 is required in early prophase, CPEB2 in metaphase and CPEB4 in late telophase/cytokinesis (Fig. 34 b). These defects resulted in slightly increased cell death rates of the studied CPEBs-KD cells, which were more pronounced in CPEB2-KD cells (Fig 29 e). This only mild phenotype in cell death could be due to the fact that these cells are already transformed. It would be interesting to perform similar studies in non-transformed cells to assess whether the observed mitotic defects would result in cell death or rather genomic instability and transformation.

Overall our results suggest that the relative levels and activities of each CPEB member in a given cell population establish the hierarchy of the nodes of CPEBs network. In turn these nodes participates to the regulation of somatic cell division. Studying such network in different tumors vs. healthy tissues could be useful to identify tumors that are susceptible to specific CPEBs perturbation with no consequences on healthy tissues. Once the tumor-specific CPEB-nodes will be identified, small compounds could be designed to specifically inhibit the activity of any member of the CPEBs to evaluate if they could be used to specifically inhibit tumor formation and progression.

Even if CPEB1, CPEB2 and CPEB4 functions are sequential in HEK293 mitosis, perturbing the system by single CPEB-KD experiments, leads to changes in the protein levels of other CPEBs, leading to phase specific phenotypes. In this vein, CPEB4 depletion leads to decreased CPEB2 levels (leading to mild defects in metaphase) and decreased CPEB1 levels (with defects in proliferation rates). Thus in HEK293 cells CPEB1 and CPEB2 work as repressors of other CPEBs (CPEB1 represses CPEB2, whether CPEB2 represses both CPEB1 and CPEB4). On the contrary CPEB4 role seems to positively regulate the

production (and function) of CPEB1 and CPEB2 (Fig 34 a). Still it has to be determined whether this functional relation are direct or through the action of other proteins/CPEBs partners.

Altogether our data show that the interplay between CPEB1, CPEB2 and CPEB4 proteins is required to mediate proper cell cycle progression, through sequential functions that are tightly connected and temporally coordinated. Ongoing studies will hopefully reveal the role of CPEB3 in this network.

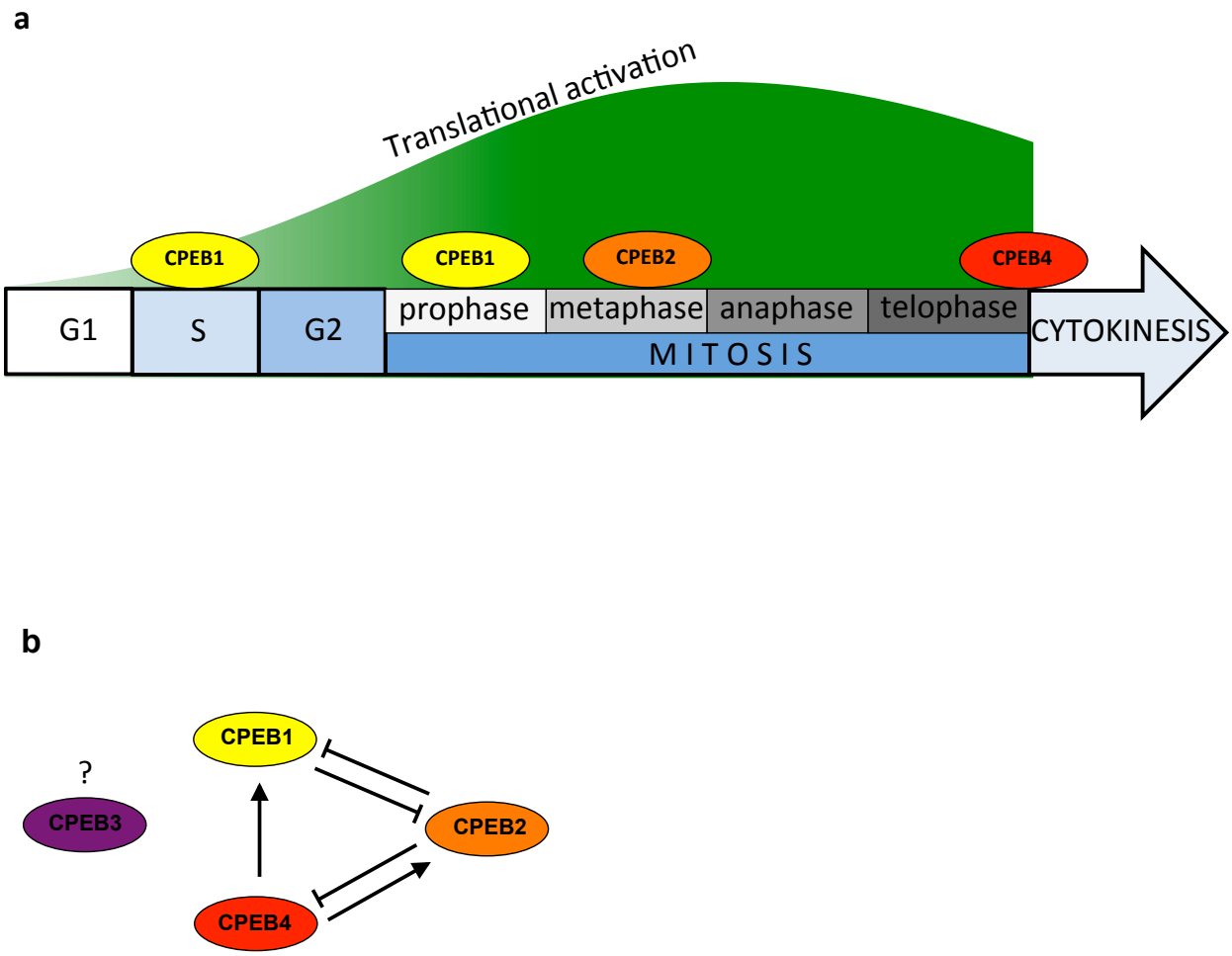


Fig 34. CPEB1-CPEB2-CPEB4 network in the cell cycle
The thick arrow represents the cell cycle phases. G1 stands for "GAP1", S stands for "synthesis", G2 stands for "GAP2". In green is represented GFP translational activation. In yellow is represented CPEB1, in orange CPEB2, in purple CPEB3 and in red CPEB4.

MATERIALS AND METHODS

Antibodies. Anti-CPEB1 antibody was from Proteintech (13274-1-AP). Anti-CPEB2 was from Abcam (ab126273). Anti-CPEB4 (NM_030627) rabbit polyclonal antibody was raised against amino acids 1-302[101]. Anti-α-tubulin was from Sigma (T902-6).

Oligonucleotides. For RT-qPCR: CPEB1, 5'- CACCTCTGCCCTTCCTGTC-3' (sense) and 5'- CCAGGTACAGGTGGCTTCAT-3' (antisense); CPEB2, 5'-GGCTGTATGGTGGAGTTTGT
-3' (sense) and 5'- GGCATTCATCACACATCTGG-3' (antisense); CPEB4, 5'-AGCTTGCGATGATAATGGAT-3' (sense) and 5'- CCCCTGACATTCATCACACA
-3' (antisense); α-galactosidase (housekeeping gene), 5'-CAGAAATCCGACAGTACTGCAA-3' (sense) and 5'-CATATCTGGGTCATTCCAACC-3' (antisense), GFP, 5'-ACGTAAACGGCCACAAGTTC-3' (sense) and 5'-AAGTCGTGCTGCTTCATGTG-3' (antisense); RFP, 5'- CGGCTCCTTCATCTACAAGG
-3' (sense) and 5'- GGTGATGTCCAGCTTGGAGT-3' (antisense).

Plasmid constructions. EGFP from EGFP-C1 plasmid was substituted with d2EGFP (AgI and BglII). Cyclin B1 –Pum 3'UTR[10] was cloned downstream to the d2EGFP.The SV40 polyadenylation signal was mutated from AATAAA to AAGGAA. CPEs were mutated from TTTTAAT to TTgggAT, from TTTTACT to TTggACT and from TTTTAAT to TTGGAAT. pDsRFP fas subcloned in pLHCX between HindII and ClaI. pmKate2-H2B DNA plasmid was from Evrogen.

Cell culture and DNA transfections. Exponentially growing human embryonic kidney (HEK) 293 cells were cultured at *37°C* in an atmosphere of 5% CO_2 in Dulbecco's Modified Eagle Medium (*DMEM*), 10% FBS (fetal bovine serum) and 1% penicillin/streptomycin. 293 cells were co-transfected at 50% confluence in 10 cm diameter dishes using a modified calcium phosphate method[260] and 10 µg of plasmid GFP DNA (wt or mut) and RFP DNA, or pmKate2-H2B DNA plasmid (Evrogen). The vectors were stably selected with 500 ug/ml Hygromicin B (for RFP) and 500 ug/ml G418 (for GFP) disulfate salt solutions. The cells were synchronized with RO-3306 and selected by cell sorting for RFP-positive and high GFP-positive, and with a double thymidine blockade and selected by cell sorting for RFP-positive and intermediate GFP-positive.

CPEBs knockdown cell lines. ShRNA for CPEB1 was directed against 5'-GGTACTGAGCATGCTCCATAA-3'; shRNA for CPEB2 was directed against 5'-

GTGTTCAGAACAGACAACAAT-3'; shRNA for CPEB4 was directed against 5'-GCTGCAGCATGGAGAGATAGA-3' and cloned into the lentivirus vector pLKO-puro-IPTG-3XLacO (Sigma), and virus production was performed as previously described[101]. Cells were infected with the different IPTG-inducible shRNA-producing viruses, selected by Hygromycin, and induced (shRNA +) or not (shRNA-) with 1 mM IPTG every day for 3 days for subsequent analysis. For protein, RNA, flow citometry analysis and DNA content, the different shRNA-expressing cells were DTB synchronized between day 1 and 3 of treatment with 1 mM IPTG, and samples were collected on day 3 of IPTG addition. For DNA content analysis cell were DTB synchronized (between day 1 and 3 of treatment with 1 mM IPTG), and samples were fixed on day 3 of IPTG addition.

Sinchronization of HEK293 cells. Synchronization at the G1/S border was done by double thymidine (Sigma) treatment (17 hours with 2 mM thymidine, released for 8 hours, and 16 hours with 2 mM thymidine). Cells were collected at 0, 2.5, 5 and 9 hours. Late G2 was achieved by treatment with 9uM RO-3306 (Calbiochem) for 12 h.

Cell extracts and Western-blot analysis. Cells were lysed in Triton buffer (20mM HEPES pH 7.0, 150mM NaCl, 1% Triton X-100, 10% glycerol, 1mM EDTA, 1mM phenylmethylsulphonyl fluoride, 1 X protease inhibitors (Sigma). The lysates were centrifuged at 16.000g and supernatants were resolved by 4%-20% Criterion precast polyacrylamide gel (Bio-Rad). 30 ug of lysate was loaded onto each lane.

qPCR. Total RNA was isolated using the RNAspin Mini Kit (GE Healthcare) and the mRNA levels for CPEB1, CPEB2, CPEB4, GFP and RFP were measured by qRT-PCR and normalized to the housekeping gene α-galactosidase. PCR was carried out in a LightCycler 480 (Roche) using SYBRGreen I Master (Roche) and the primers indicated in 'oligonucleotides' section above. Pair of primers monitored gene expression by amplifying a constitutive exon. Each experiment was performed in triplicate.

Flow citometry. The GFP and RFP levels were analysed by Gallios flow cytometer (Beckman Coulter). The cells were sorted by FACSAria III cell sorter (BD Biosciences). DNA content was measured by propidium iodide as indicated[261] and the samples were analyzed by FACS (Gallios (BD). Viabilty test was performed in live cells by propidium iodide incorporation and analyzed by FACS (Gallios, BD). Proliferation assay was performed using Click-iT EdU 647 flow citometry assay kit (Invitrogen), following the manufacturer's instructions. Flow cytometry data were analysed with FlowJo software (Tree Star, Inc., Ashland, OR, USA). For data analysis the mean of GFP and RFP signal of RFP^+/GFP^+ cells

was normalized to the mean of GFP and RFP signal of RFP^-/GFP^- cells at each time point.

Microscopy. For live imaging analysis, cells were plated in four compartment CellView™ cell culture dish (Greiner Bio One) and analyzed by confocal microscope spinning disk Andor for 36 h. Histone-H2B transfected cells were plated in six-well glass bottom dish (MatTek) and analyzed by automated inverted microscope TIRF, ScanR Olympus for 36 h. The images were processed using ImageJ.

Immunofluorescence. Cells were plated on poly-lysine pre-coated glass coverslips. 12 h later, cells were fixed in PBS/formaldehyde 4% (30 min) and permeabilized at room-temperature for 5 min in 0.1%Triton/PBS. They were then blocked with 1% BSA for 1 h and incubated with primary antibodies against CPEB1, CPEB2, CPEB4, washed, and then labeled with the matching secondary antibodies. Images were obtained on an inverted Leica TCS SP5 confocal microscope with a 633 1.4 NA PLAN APO objective.

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APPENDIX

The CD-ROM enclosed with this thesis contains 9 interactive videos and a Microsoft Office Power Point presentation (in case of any visualization problem, we suggest to download from the web the program “Fiji”, an open resource for imaging. To visualize them, please drag and drop each of the .avi files into the Fiji main bar).

GR-HEK293 cells were induced for 3 days with IPTG to induce individual CPEBs knock-down, then visualized in live imaging experiments for about 30 hours. RFP on the right, GFP on the left.
Interactive video 1= CTRL GR-HEK293
Interactive video 2= CPE- GR-HEK293
Interactive video 3= CPEB2-KD GR-HEK293
Interactive video 4= CPEB4-KD GR-HEK293
Interactive video 5= CPEB1-KD GR-HEK293

GR-HEK293 cells were transfected with a plasmid encoding for an H2B-fluorescent protein, induced for 3 days with IPTG and visualized in live imaging experiments for about 30 hours.
Interactive video 6= CTRL GR-HEK293-H2B
Interactive video 7= CPEB1-KD GR-HEK293-H2B
Interactive video 8= CPEB2-KD GR-HEK293-H2B
Interactive video 9 and 10= CPEB4-KD GR-HEK293-H2B