CPEB4 replaces CPEB1 to complete meiosis

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Dr. Raúl Méndez   **Ana Igea**

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A la meva família

"Anyone who has never made a mistake

has never tried anything new"

Albert Einstein

Contents

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Abbreviations

APC/C: Anaphase promoting complex/cyclosome

ARE: AU-rich element

AREBP: ARE binding protein

C3H-4: Cx8Cx5Cx3H fourth member

Cdc: cell division cycle

Cdk: cyclin dependent kinase/cell division kinase

CPE: cytoplasmic polyadenylation element

CPEB: cytoplasmic polyadenylation element binding protein

CPSF: cleavage and polyadenylation specificity factor

CSF: cytostatic factor

CstF: cleavage stimulation factor

EDEN: embryonic deadenylation element

eIF: eukaryotic initiation factor

eEF: eukaryotic elongatic factor

eRF: eukaryotic release factor

GCBD: germinal vesicle breakdown

Hex: hexanucleotide

IRES: internal ribosome entry site

M7Gppp: 7-methil guanosine

MI: metaphase I

MII: metaphase II

MBT: mid blastula transition

MPF: maturation (or M-phase) promoting factor

mRNA: messenger RNA

mRNP: messenger ribonucleoprotein; mRNA-protein complex

nt: nucleotides

ORF: open reading frame

PI: prophase-I

PABP: poly(A) binding protein

PARN: poly(A)-specific ribonuclease

PBE: pumilio binding element

poly(A): polyadenosine

RBP: RNA binding protein

RRM: RNA recognition motif

UTR: untranslated region

WT: wild type

Key words

3'UTR

poly(A)

polyadenylation

CPEB1

CPEB4

C3H-4

Cyclin B1

Cyclin E

Development

maternal mRNAs

meiosis

metaphase

translational control

feedback loop

Oocyte maturation

Xenopus laevis

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Abstract

In vertebrate oocytes, meiotic progression is driven by the sequential translational activation of maternal messenger RNAs stored in the cytoplasm. This activation is mainly induced by the cytoplasmic elongation of their poly(A) tails, which is mediated by the cytoplasmic polyadenylation element (CPE) present in their 3' untranslated regions (3´ UTRs). Sequential, phase-specific translation of these maternal mRNAs is required to complete the two meiotic divisions. Although the earlier polyadenylation events in prophase I and metaphase I are driven by the CPE-binding protein 1 (CPEB1), 90% of this protein is degraded by the anaphase promoting complex in the first meiotic division. The low levels of CPEB1 during interkinesis and in metaphase II raise the question of how the cytoplasmic polyadenylation required for the second meiotic division is achieved. In this work, we demonstrate that CPEB1 activates the translation of the maternal mRNA encoding CPEB4, which, in turn, recruits the cytoplasmic poly(A) polymerase GLD2 to "late" CPE-regulated mRNAs driving the transition from metaphase I to metaphase II, and, therefore, replacing CPEB1 for "late" meiosis polyadenylation.

Introduction

1. Gene Expression Regulation

Gene expression regulation gives the cell control over structure and function, and is the basis for cellular differentiation, morphogenesis and the versatile adaptability of many organisms. Gene regulation may also serve as a substrate for evolutionary change, since control of the timing, location, and amount of gene expression can have a profound effect on the functions of the gene in the organism.

1.1. Operons and Regulons

Operons are clusters of genes physically ordered in the genome in a manner enabling them to be regulated as groups (reviewed in (Beckwith, 1996)). The clustering of genes into operons allows prokaryotic organisms to co-ordinately express proteins involved in common processes, while greatly facilitating the ability to respond efficiently to environmental changes. Because transcription and translation are physically coupled in prokaryotes, operons provide a highly

efficient method of regulating the transfer of genetic information from DNA into protein. Although highly efficient, the clear disadvantage of the prokaryotic operon is the constraint placed upon gene expression by physically coupling the production of multiple proteins in a fixed group.

In eukaryotes, many proteins have become multifunctional (Hentze, 1994; Jeffery, 1999). Thus, producing a protein contained within an operon for a new function would be inefficient because the other proteins in the operon would have to be expressed concurrently, and the coordinated regulation of multiple genes is needed in higher eukaryotes to accomplish complex phenotypic functions such as cell growth and differentiation (Niehrs and Pollet, 1999; Qian et al., 2001).

In eukaryotes transcription is not directly coupled to translation and the two processes are physically separated by the nuclear membrane. While transcription is a significant contributor to eukaryotic gene expression, post-transcriptional regulation must also function to maintain coordinated protein production (Gygi et al., 1999; Keene, 2001; Klausner et al., 1993). Translational control is achieved by the combination of regulatory *trans*-acting factors - primarily RNA-binding proteins (RBPs), but also non-coding RNAs - that recognise specific elements usually located in the 5' and/or 3' untranslated regions (UTRs) of the target mRNA (Colegrove-Otero et al., 2005; de Moor et al., 2005; Gebauer and Hentze, 2004; Keene, 2007; Kuersten and Goodwin, 2003; Mendez and Richter, 2001; Richter, 2007). These factors bind RNA transcripts belonging to functionally related groups to co-regulate them through the chain of post-transcriptional events such as splicing, nuclear export, stability, localization and translation (Dreyfuss et al., 2002; Keene, 2007; Maniatis and Reed, 2002; Mazumder et al., 2003). This co-regulation is achieved through multiple combinatorial binding of RBPs allowing greater regulatory flexibility than a simple operon. This structure of higher-order coordination can be defined as "RNA regulon" (Keene, 2007; Keene and Tenenbaum, 2002). These RNA regulons dynamically interchange specific mRNA components during different biological scenarios (e.g. proliferation,

differentiation or biological cycles), and the orchestration of multifaced networks is essential for efficient performance of both generic and specialized gene function in development (Davidson et al., 2002; Niehrs and Pollet, 1999).

Translational regulation plays a key role as modulator of numerous biological situations. Whereas in conditions of stress starvation, apoptosis or viral infection, a global response modifies the translational efficiency of most mRNAs in the cell, in other circumstances, such as embryonic pattern formation, sex determination and neural plasticity, the transcription of specific mRNAs is regulated, leaving most cellular transcripts unaffected (Abaza and Gebauer, 2008). The last decades witnessed an enormous progress deciphering the molecular mechanisms of translation, demonstrating that this control of gene expression takes place in early development, cell growth, proliferation, survival, metabolism, learning and memory, and it is even the cause of many human diseases (for reviews see (Abaza and Gebauer, 2008; Gebauer and Hentze, 2004; Krichevsky et al., 1999; Kuersten and Goodwin, 2003; Mendez and Richter, 2001; Richter, 2007; Sonenberg and Hinnebusch, 2007)). Translation regulation is particularly significant in animal germ cells and early stages of embryonic development, since transcription is largely quiescent and does not resume until some later time during development (de Moor et al., 2005; Mendez and Richter, 2001; Sonenberg and Hinnebusch, 2007).

In this introduction I will provide a description of the main regulatory elements present in the mRNAs and an overview of some molecular mechanisms of translation in eukaryotes. Special emphasise will be given to the mechanisms that govern gene expression by RBPs recruited to regulatory sequences located in the 3'UTR of maternal inherited mRNAs during meiotic cell cycle progression and early embryonic development, focusing on the cytoplasmic polyadenylation-induced translation. The role of the key regulator CPEB1 (i.e., cytoplasmic polyadenylation element binding protein) in repression, activation and localization is discussed, as well as the expression and function of the other CPEB family members (CPEB2, CPEB3 and CPEB4).

2. The mRNA

Protein-encoding genes are transcribed exclusively by RNA polymerase II (pol II) (Bentley, 1999), and pre-mRNA processing is specifically targeted to transcripts made by this polymerase. Accordingly, pol II is especially equipped to cooperate with processing factors and other nuclear proteins, largely via interactions with an unique domain that protrudes from the large subunit of the enzyme (Cramer et al., 2001) (Figure 1). This carboxy-terminal domain (CTD) of pol II is required for transcriptional activation and repression and for efficient capping, splicing and cleavege/polyadenylation of RNA transcripts (Du and Warren, 1997; Hirose and Manley, 1998; McCracken et al., 1997a; McCracken et al., 1997b; Yuryev et al., 1996).

Termination, the release of pol II from the DNA template, occurs at diffuse positions hundreds of bases downstream of the poly(A) site. Termination requires a functional poly(A) site, but not cleavage of the RNA (Osheim et al., 1999; Tran et al., 2001). This requirement ensures that termination occurs after the 3' end of the gene has been transcribed. Then, the pre-mRNA is cleaved at the poly(A) site and a poly(A) tail is added to the exposed 3'-end (Colgan and Manley, 1997; Edmonds, 2002; Wahle and Ruegsegger, 1999; Zhao et al., 1999a).

During each of the processing steps, protein complexes are deposited on the pre-mRNA. Therefore, mRNAs are never naked molecules, they are always coated by RNA binding proteins constituting the messenger ribonucleoprotein (mRNP) particles (Aguilera, 2005).
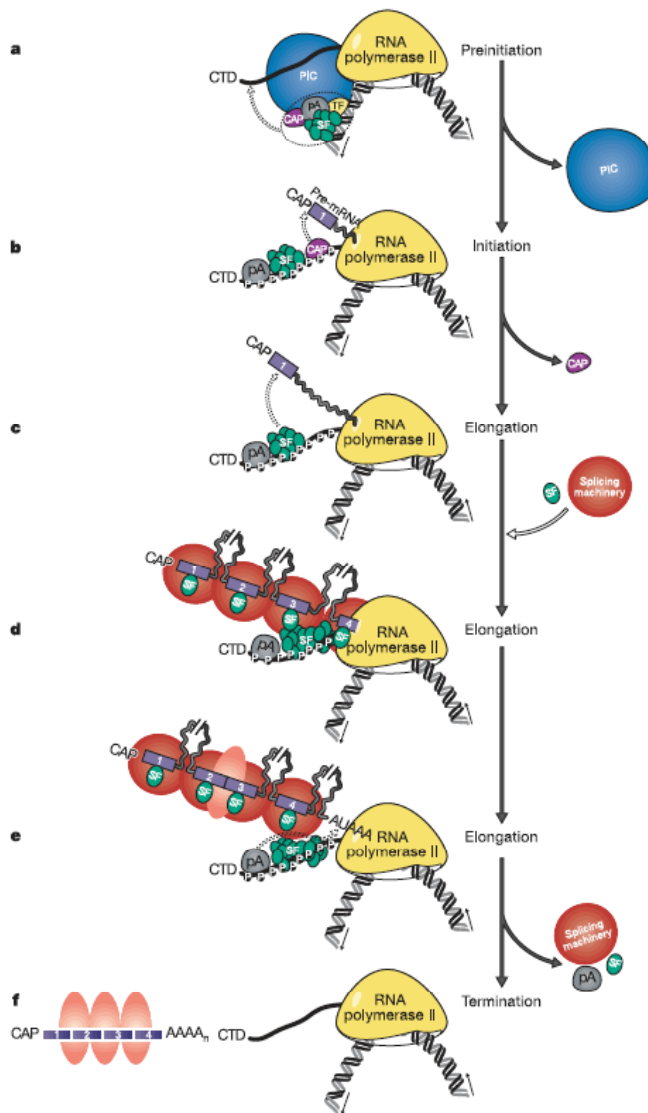
Figure1. Gene Expression factory model for coupling steps in gene expression. In this model the gene expression factory is anchored to the nuclear substructure and the DNA is reeled through the RNA polymerase as the nascent RNA is extruded through its exit channel. The machineries involved in transcription, capping, splicing and polyadenylation are shown. The shaded pink ovals over the spliced exons represent the mRNA complexes formed near the exon-exon junctions during the splicing reaction. PIC, preinitiation complex; TF, transcritption factors; CTD, carboxy-terminal domain; CAP, capping factor; SF, splicing factor; pA, polyadenylation factor; P, phosphorylated CTD. Taken from Maniatis and Reed, 2002

2.1. mRNA structure

Mature RNAs are exported to the cytoplasm only after all nuclear processing events are completed successfully (for review see (Maniatis and Reed, 2002; McKee and Silver, 2004; Stutz and Izaurralde, 2003)). The mRNAs consist of an RNA body flanked by two modifications, not coded in the DNA sequence, the 5'cap and the poly(A) tail. The RNA body contains a translatable region, the open reading frame (ORF), preceded and followed by two untranslated regions (UTRs), the 5'UTR and the 3'UTR.

A. The cap structure

The majority of eukaryotic mRNAs contain a 5'-terminal structure: the 7-methyl G5'ppp5'N cap. This cap consists in a 7-methyl guanosine linked by a 5'-5' triphosphate bridge to the first nucleoside of the transcript (Shatkin, 1976) (Figure 2). Capped mRNAs are present in yeast, moulds, plants, insects, and higher organisms. The only reported exceptions among cellular mRNAs are the poly(A)-containing mRNAs isolated from HeLa cell mitochondria (Dubin and Taylor, 1975; Grohmann et al., 1978), which may contain ppA at the 5' termini.
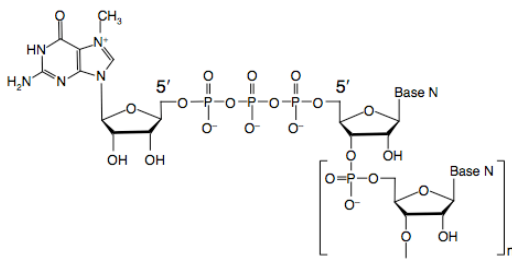
Figure 2. Chemical structure of the mRNA cap structure. The cap consists of N7-methyl guanosine linked by an inverted 5'-5' triphosphate bridge to the first nucleoside of the mRNA chain (base N can be adedine, guanine, cytosine or uracil). Taken from Gu and Lima, 2005

The cap is added when the RNA is about 25 bases long, soon after the 5' emerges from the RNA exit channel of the polymerase (Shuman, 1997) (Figure 1). The details of the capping/elongation connection remain to be clarified, but the cap probably needs to be in place by the time the first intron is spliced, because the cap binding complex (CBC), stimulates removal of this intron (Daneholt, 2001). The mRNA cap influences also the 3' end formation/polyadenylation (Cooke and Alwine, 1996; Flaherty et al., 1997), and nucleo-cytoplasmic transport (Izaurralde et al., 1995). In the cytoplasm, the cap structure serves as the binding site for the eIF4F complex of translation initiation factors (Jackson and Wickens, 1997) critically determining the quality and the quantity of mRNA translation (Filipowicz, 1978; Furuichi et al., 1977; Rhoads, 1988; Sonenberg, 1988). Although uncapped mRNAs are often very poorly translated, cap-independent mechanisms regulated by internal ribosome-entry sequences (IREs) present in the 5'UTR of mRNAs are able to recruit the small ribosomal subunit (reviewed in (Fraser and Doudna, 2007)).

B. The open reading frame

An open reading frame (ORF) is a portion of an organism's genome which contains a sequence of bases that could potentially encode a protein. The start-points and end-point of a given ORF are not equivalent to the ends of the mRNA, but they are usually contained within the mRNA sequence. The ORF is flanked by the 5' and 3'UTRs and is delimited by the AUG initiation codon (in an optimal context) and one of the three stop codons (UAA, UAG or UGA) (Kozak, 2002).

C. The untranslated regions

The untranslated regions are located upstream and downstream of the ORF, called 5' UTR and 3' UTR regions (Bashirullah et al., 1998; McCarthy and Kollmus, 1995; Pesole et al., 2000; Pesole et al., 1997; Sonenberg, 1994; van der Velden and Thomas, 1999)(Figure 3). UTRs play crucial roles in modulation of the transport of mRNAs out of the nucleus and translation efficiency (van der Velden and Thomas, 1999), subcellular localization (Jansen, 2001) and stability (Bashirullah et al., 2001). The ability to perform these functions depends on the *cis*-acting elements present within the UTRs (Figure 3).
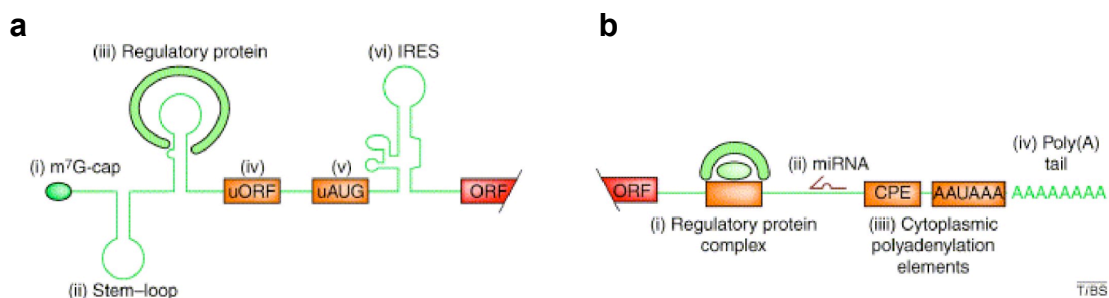
Figure 3. mRNA *cis*-acting elements in the mature mRNA. Schmatic representation of main regulatory elements in the UTRs. **(a)** 5'UTR: (i) modified cap structure; (ii) stem-loops or secondary structures; (iii) regulatory proteins interacting with specific elements in the 5'UTR; (iv) upstream ORF; (v) upstream AUG; (vi) internal ribosome entry sequences. **(b)** 3'UTR: (i) specific elements (structured or unstructured) can act as recognition sites for regulatory proteins (green); (ii) short antisense microRNA; (iii) cytoplasmic polyadenylation elements (CPE) and the hexanucleotide (AAUAAA). See text for more details. Taken from Gray and Wickens, 1998.

Regulatory sequences and structures within the 5'UTR can modulate translation. These include: internal ribosome entry sites (IRES), which direct cap-independent translation initiation; upstream open reading frames (uORFs), which act as negative regulators by diminishing translation from the main ORF; secondary or tertiary structures, like hairpins and pseudoknots, which often act by blocking translation initiation; and specific binding sequences for multiple regulatory factors (Gebauer and Hentze, 2004; Mignone et al., 2002) (Figure 3).

There are also regulatory motifs in the 3'UTR that include primary sequence for microRNA (miRNA) binding sites that affect translation and stability of the mRNA, and binding sites for regulatory proteins like the cytoplasmic polyadenylation element (CPE) and the hexanucleotide (Hex) (Figure 3). Examples of these UTR sequences and their binding proteins can be found in Table 1.

Table 1. Sequence elements identified in processed mRNAs and their interacting proteins. Taken from Keene and Tenenbaum, 2002

mRNA cis Element	Location	mRNA	RNA Binding Proteins
Iron response element (IRE)	5·UTR 3·UTR	H and L-ferritin, transferrin receptor	Iron regulatory proteins, Aconitase, Transferrin
Male specific lethal (MSL-2)	5·UTR 3·UTR	msl-2	Sex-lethal (SXL)
Internal ribosome entry site (IRES)	5·UTR	picornavirus, cellular mRNAs	PTB, UNA, PCBP-2, La/SS-B
5·-terminal oligopyrimidine tract (TOP)	5·UTR	ribosomal proteins, translation factors	La/SS-B, CNBP
AU-rich elements (AREs)	3·UTR	early response gene, cytokines, others	ELAV/Hu proteins, TTP
Selenocysteine insertion sequence (SECIS)	3·UTR	selenoprotein	SECIS binding protein
Histone stem loop	3·UTR	histone	Stem loop binding protein (SLBP)
Cytoplasmic polyadenylation elements (CPEs)	3·UTR	developmental, embryonic mRNAs, Myb	CPEBP
Nanos translational control element	3·UTR	nanos, hunchback	Smaug repressor, other factors
Amyloid precursor protein element (APP)	3·UTR	APP	Multiple cytosolic proteins
Translational regulation element (TGE)/ direct repeat element (DRE)	3·UTR	tra-2 ad GLI	Direct repeat factor
Bruno element (BRE)	3·UTR	oskar	Bruno
15-lipoxygenase differentiation control element (15-LOX-DICE)	3·UTR	lox	LOX-binding proteins, hnRNPK and E1
G-quartet element	5·UTR 3·UTR	FMRP, MAP-1B, Rab6,Sec-7 Munc, V1a/ GPC, others	FMRP

5'UTR regions are generally shorter than 3'UTR regions. However, the 5'UTR average length is roughly constant (between 100 and 200 nt) and more striking differences are observed for 3'UTRs, whose average length ranges between 200 nt (plants and fungi) to more than 1000 nt (human) (Pesole et al., 2000; Pesole et al., 2001). On the whole, the percentage of G+C content in 5'UTRs is greater than in 3'UTRs (Pesole et al., 2000). This difference is more marked in mRNAs from warm-blooded vertebrates probably due to the presence of CpG islands at the 5' of a large fraction of genes expressed in these organisms. UTRs can also contain introns and repeats, but while introns are much more frequent in the 5'UTRs, repeats are more abundant in the 3'UTRs (Pesole et al., 2000; Pesole et al., 2001).

D. The poly-A tail

Polyadenylation occurs after cleavage of the pre-mRNA at the 3' end, and consists on the addition of an average of 250 adenosine residues by the poly(A) polymerase (PAP) enzyme (Jensen et al., 2003; Zhao et al., 1999a). But in the cytoplasm, the length of the poly(A) tail can be modified by cytoplasmic proteins. Therefore, mRNAs are heterogeneous in poly(A) tail length, ranging from ~250 nucleotides (Brawerman, 1976) to ~30 nucleotides long (Ahlquist and Kaesberg, 1979; Sheiness and Darnell, 1973).

The poly(A) tail plays a key role in mRNA stability, export from the nucleus and regulation of translational efficiency mediated by multiple poly(A)-binding proteins (PABPs) (Bernstein and Ross, 1989; Fuke and Ohno, 2008; Gallie, 1991; Gallie et al., 1989; Jackson and Standart, 1990; Munroe and Jacobson, 1990a, b). However, some RNAs like histone mRNAs, do not contain poly(A) tail and they include other motifs that will recruit specific-factors, such as the Stem-loop Binding protein (SLBP), which perform the same function than PABP (Marzluff, 2005, 2007).

The poly(A) tail stimulates both cap-dependent and cap-independent translation initiation (Gallie, 1991; Preiss et al., 1998; Sachs et al., 1997). When both a cap and a poly(A) tail are present on an mRNA, they function together to induce an enhancement of translation through a mechanism involving protein-protein interactions between PABP, eIF4G, and the cap binding protein eIF4E (Gallie, 1991; Imataka et al., 1998; Tarun and Sachs, 1995, 1997). These interactions generate a closed loop by physical association between the 5'- and 3'- ends of an mRNA, resulting in circularisation of the mRNP (see section 2.2).

The removal of the poly(A) tail from an mRNA leads to its translation inhibition and it is used as a strategy to silence certain maternal mRNAs during oocyte maturation and early embryonic development, while regulated cytoplasmic polyadenylation of these mRNAs leads to their translation (Belloc and Mendez, 2008a; Belloc et al., 2008; Mendez and Richter, 2001; Pique et al., 2008; Richter, 1999; Sheets et al., 1995)

Most mammalian genes use alternative cleavage and polyadenylation to generate multiple mRNA isoforms differing in their 3'UTR (Beaudoing and Gautheret, 2001; Zhang et al., 2005). Alternatively polyadenylation occurs in both a splicing-independent manner (multiple polyadenylation sites in a terminal exon) and in a splicing-dependent manner (mutually exclusive terminal exons) (Edwalds-Gilbert et al., 1997). Very recently, has been observed that when two versions of a 3'UTR were possible, the extended one tends to show reduced protein synthesis by affecting mRNA translation, Also, the usage of short UTRs correlated with proliferating cells while extended UTRs correlate with arrested cells, suggesting that the mechanism or mechanisms dictating the alternative use of the 3'URs are regulated in a cell-cycle dependent manner (Sandberg et al., 2008).

2.2. The closed-loop model of translation

The closed-loop model of translation refers to the bridging of the 5' end with the 3' end of the same mRNA, which circularizes the mRNA molecule (Figure 4). The 5' cap and 3' poly(A) tail will act synergistically to promote the stability and translatability of an mRNA (Gallie, 1991; Iizuka et al., 1994; Jacobson and Peltz, 1996; Preiss et al., 1998; Tarun and Sachs, 1995).
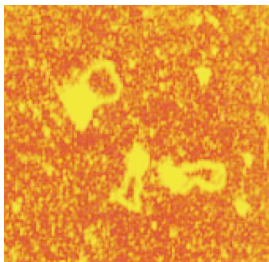
Figure 4. Visualization of circular RNA/Protein complexes by Atomic Force Microscopy. Representative AFM images of complexes formed on the capped, polyadenylated double-stranded RNA in the presence of eIF4G, PAPB and eiF4E. Picture adapted from Wells et al., 1998

There are at least two general closed-loop mRNPs: the initiation loop and the reinitiation mRNP. The initiation loop is formed during the first round of translation (Amrani et al., 2008), when the 5'-cap and the 3'-poly(A) tail are recognized by eIF4E and the poly(A)-binding protein (PABP), respectively, and eIF4G mediates their association (Imataka et al., 1998). These interactions result in the formation of a circularized mRNA (Figure 5a), which provides an efficient initiation of translation (Tarun and Sachs, 1997; Wells et al., 1998). After translation termination ribosomes are recruited to the next cycle of translation initiation (Sonenberg and Dever, 2003; Uchida et al., 2002). Although termination itself is not required and releasing factors eRF1 and eRF3 do not seem to affect the rate of translation initiation nor the formation of the closed-loop mRNP that includes only the 48S complex, they are required for the formation of the 80S ribosome to generate a second state of the closed-loop structure (Amrani et al., 2008) (Figure 5b).

At least, two PABPs molecules are required for a stable closed-loop structure because Pab1-poly(A) association requires a minimum of 12 adenosines, and multiple Pab1 molecules can bind the same poly(A) tracts in a 27-nt repeating

unit (Baer and Kornberg, 1983; Sachs et al., 1986; Sachs et al., 1987). In concord with this conclusion, previous studies showed that an A15 tail did not suffice to stimulate translation in *Drosophila* (Gebauer et al., 1999) and mammalian extracts (Munroe and Jacobson, 1990a), but that longer poly(A) tails promoted strong translational enhancement.

Interactions between the ends of an mRNA may play also an important role in quality control; if the mRNA has been partially degraded and has lost its 3'-end, it will not be translated efficiently. Thus, the system helps guard against the synthesis of truncated proteins that could be toxic to the cell.
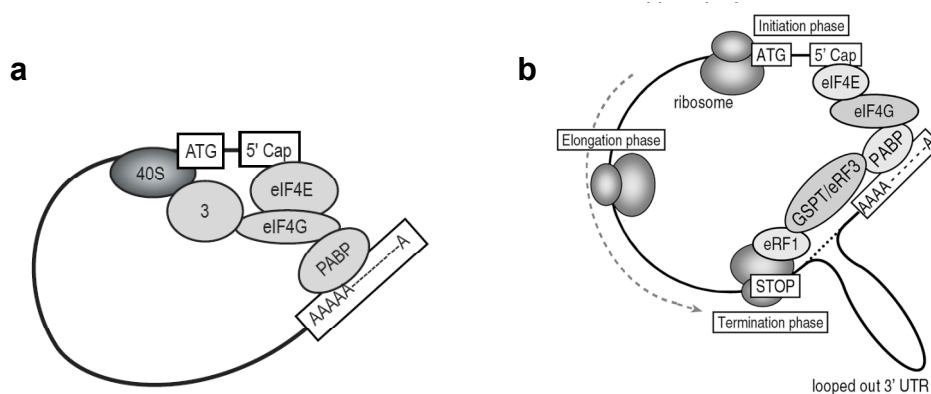
Figure 5. Closed-loop mRNPs. Schematic diagrams of **(a)** the initiation closed-loop formed by the interaction of PABP bound to the poly(A) tali with the eIF4G component of the eIF4F complex bound to the 5'-cap. Taken from (Jackson and Standart, 2007) **(b)** The reinitiation closed-loop formed by eRF1, eRf3, poly(A) tail, PABP, eIF4F and 5'-cap. Taken from Uchida et al., 2004

3. Molecular mechanisms of translation

Translation can be divided in four parts: initiation, elongation, termination and recycling. Although translation initiation has long been considered to be the major point of translation control, recent developments have revealed new insights as to how termination and elongation can also serve as points of translational regulation in protein synthesis.

Global translation regulation is normally driven by phosphorylation or proteolysis of key general translation initiation factors, and mRNA-specific regulation is exerted by proteins (or microRNAs) that recognize sequence elements usually located in the UTRs of the transcript (for reviews see Abaza and Gebauer, 2008; de Moor et al., 2005; Jackson and Standart, 2007; Piccioni et al., 2005).

Here I will briefly summarize how translation in eukaryotes is regulated, giving more emphasis to initiation because this is the step in which most of the regulatory events have been examined over the last years.

3.1. Initiation

Translation initiation in eukaryotes is a complex event requiring more than 30 polypeptides (not including the ribosomal proteins) that comprise 13 eukaryotic initiation factors (eIFs) (Sonenberg and Hinnebusch, 2007) (Table 2).

Table 2. Eukaryotic initiation factors.

Eukaryotic initiation factor	Function
eIF1	Processivity of scanning, AUG recognition, promotes dissociation of 80S ribosomes into 40S and 60S
eIF1A	Increases Met-tRNAi binding to 40S subunit, processivity of scanning, AUG recognition and 60S subunit joining, promotes dissociation of 80S ribosomes into 40S and 60S
eIF2 (α, β and γ)	Binds Met-tRNAi to 40S subunit; GTPase activity
eIF2B (α, β, γ, δ and ε)	Guanidine-nucleotide exchange factor for eIF2
eIF3	Promotes Met-tRNAi and mRNA binding to 40S subunit and promotes dissociation of 80S ribosome into 40S and 60S
eIF3j	Promotes dissociation of 80S ribosomes into 40S and 60S
eIF4A (I and II)	DEAD-box helicase; binding of pre-initiation complex to the mRNA and scanning
eIF4B	Promotes eIF4A activity
eIF4E	m7Gppp cap binding protein; binding of pre-initition complex to the mRNA
eIF4F	Cap-binding complex consisting of eIFs 4A, 4E and 4G
eIF4G (I and II)	Scaffold protein, interacts with eIFs 4E, 4A, 3 and PABP; binding of pre-initiation coomplex to the mRNA
eIF4H	Promotes eIF4A activity
eIF5	AUG recognition; promotes eIF2 GTPase activity; assembly of pre-initiation complex
eIF5B	60S subunit joining; GTPase activity stimulated by the 80S ribosome
eIF6	Promotes dissociation of 80S ribosomes into 40S and 60S

Two major pathways are involved in the recruitment of the small ribosomal subunit to the mRNA: the first one relies on the 5' terminal cap structure of mRNAs, while the second, known as cap-independent, relies on a series of elements of complex secondary structure, termed IRES, present in some mRNAs, (Gingras et al., 1999). The vast majority of eukaryotic transcripts are translated in a cap-dependent manner.

The rate-limiting step of translation initiation is the binding of the 43S pre-initiation complex (composed of the 40S ribosomal complex, initiation factors eIF3, eIF1, eIF1A, eIF5, and eIF2-GTP-met-tRNA) to mRNA via the eIF4Finitiation factor complex (eIF4E, eIF4A, eIF4G, and eIF4B) (for reviews see Gebauer and Hentze, 2004; Sonenberg and Dever, 2003) (Figure 6).
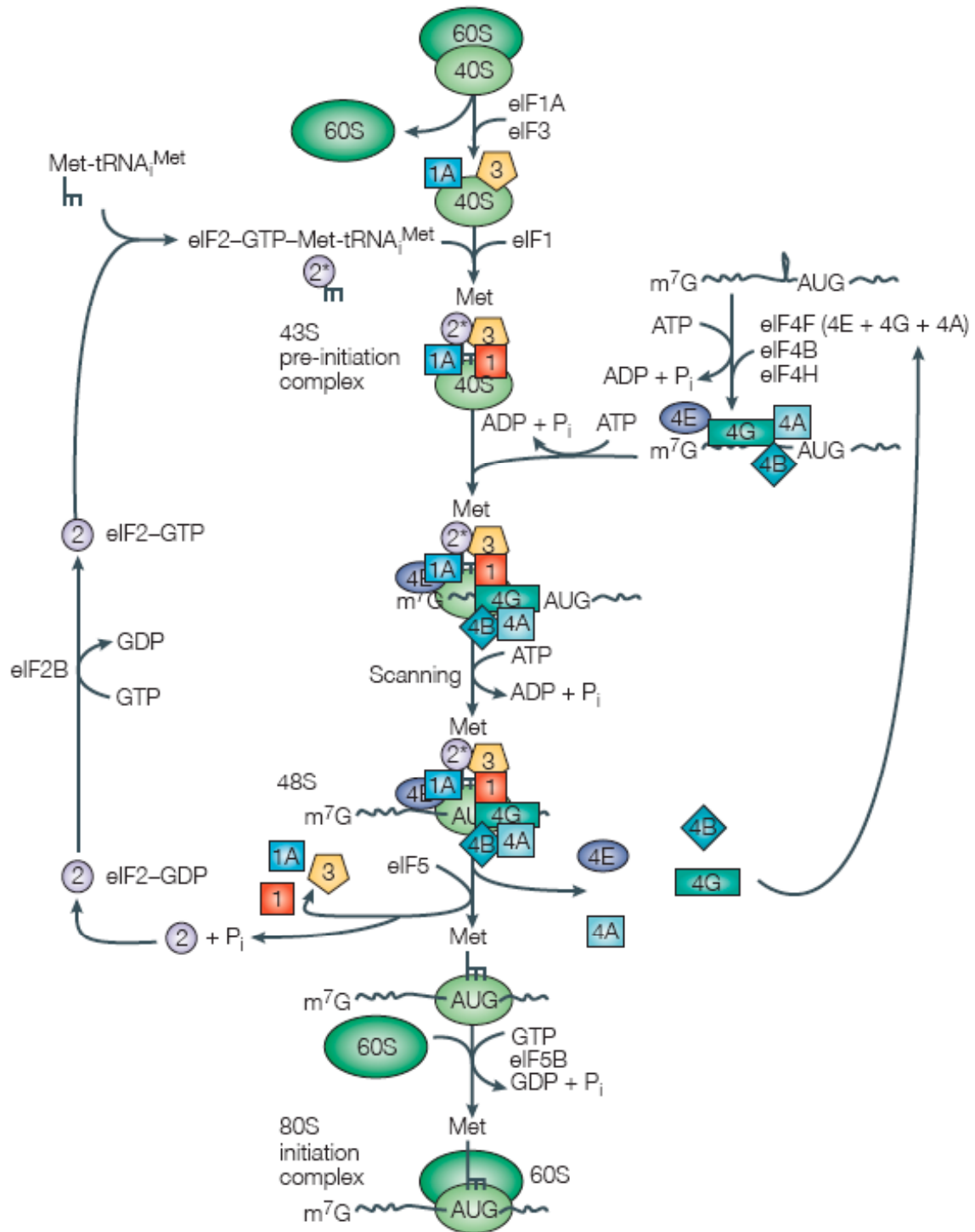
Figure 6. Translation initiation. The current model for translation initiation in eukaryotes requieres the assembly of the 80S ribosome on the mRNA. This process is mediated by proteins that are known as eukaryotic initiation factors (eIFs) (see text for details). Taken from Holcik and Sonenberg, 2005.

The eIF4F complex recognizes the 5'-cap of the mRNA through the eIF4E and unwinds structures found in the 5'UTR. This is accomplished through the ATP-dependent action of eIF4A assisted by the RNA-binding proteins eIF4B and eIF4H. eIF4F, in conjunction with eIF3 and the poly(A) binding protein (PABP) bound to the 3'-poly(A) tail, loads the mRNA onto the 43S complex ((Pestova et al., 2001; Takyar et al., 2005); for reviews see Kapp and Lorsch, 2004; Kozak, 1989; McCarthy, 1998). Then, the 43S complex begins scanning down the message in the 5' to 3' direction, searching for the initiation codon.

When the 43S complex encounters an AUG codon that is embedded in a favourable sequence context, usually the first AUG, codon-anticodon base pairing takes place between the initiation codon and the initiator tRNA in the ternary complex (Figure 6). The first AUG in the mRNA can be bypassed if it is in an unfavourable sequence context and a downstream AUG imbedded in a favourable context will be used instead. The optimal context surrounding the AUG which determines its use is GCC(A/G)CC**AUG**G in mammals (Kozak, 1994). Within this motif, the purine (A/G) in position –3 is the most highly conserved and functionally the most important position (Kozak, 1994, 2002). The binding of the 43S to the first codon results in the formation of a stable complex known as 48S initiation complex.

Once the initiator codon is identified, eIF-5 triggers hydrolysis of the GTP in the ternary complex, initiation factors are released, and the large (60S) ribosomal subunit enters, resulting in the formation of an elongation-competent ribosome (80S) able to catalyze the formation of the first peptide bond (Gebauer and Hentze, 2004; Kapp and Lorsch, 2004) and elongation begins.

Several proteins have been identified in different species which can compete for the assembly of a translationally active eIF4E-eIF4G complex and hence act as regulators of translation. For example the eIF4E-binding protein family (4EBP1, 4EBP2 and 4EBP3) contain a motif that resembles the site on eIF4G that binds eIF4E and they can compete with eIF4G for binding to eIF4E.

3.2. Elongation

The ribosome contains three sites: an acceptor (A site) where the aminoacyl-tRNA is placed, the peptidyl site (P site) which contains the tRNA with the growing polypeptide, and the exit site (E site) where the empty tRNA exits the ribosome (Ramakrishnan, 2002) (Figure 7).
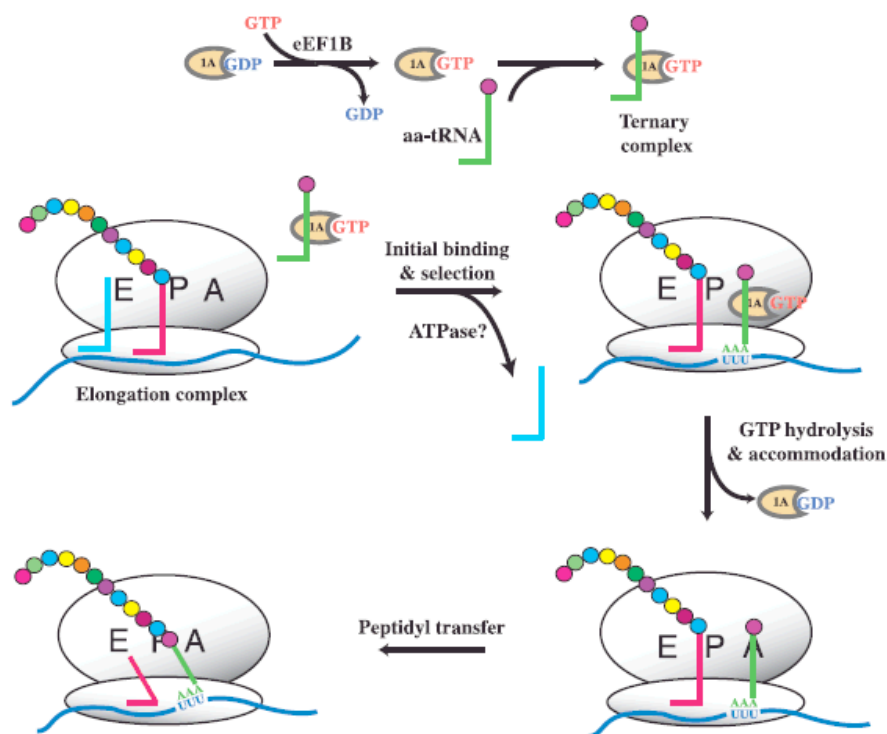
Figure 7. Translation elongation. Current model for translation elongation in eukaryotes. Elongation consists on the positioning of the peptidyl-tRNAs on the ribosomal A site, the formation of peptidyl bond by the large ribosomal subunit, and the translocation of the ribosome (see text for details). Taken from Kapp and Lorsch, 2004.

Peptide chain elongation begins with a peptidyl tRNA in the ribosomal P site next to a vacant A site. An aminoacyl tRNA is carried to the A site as part of a ternary complex with GTP and the elongation factor 1A. eEF1A-GTP-aa-tRNA ternary complexes with either the cognate or noncognate aminocyl tRNAs can bind to the ribosomal A site (Figure 7). However, several steps involving codon-anticodon base pairing between the mRNA and the tRNA, conformational

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changes in the decoding center of the small subunit, and GTP hydrolysis ensure that only the cognate tRNA is selected for entry into the next stage of elongation (for review see Rodnina and Wintermeyer, 2001).

Then, the ribosomal peptidyl transferase center catalyzes the formation of a peptide bond between the incoming aminoacid and the peptidyl tRNA (Moore and Steitz, 2003). The result is a deacylated tRNA in a hybrid state with its acceptor end in the exit (E) site of the large ribosomal subunit and its anticodon end in the P site of the small subunit (Green and Noller, 1997). The peptidyl-tRNA is in a similar hybrid situation with its acceptor end in the P site of the large subunit and its anticodon end in the A site of the small subunit. This complex must be translocated such that 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.

3.3. Termination

The termination is the least-studied step in the translation regulation (for review see Bertram et al., 2001; Inge-Vechtomov et al., 2003; Kisselev et al., 2003; Kisselev and Buckingham, 2000; Nakamura and Ito, 2003; Poole and Tate, 2000).

The termination of translation occurs in response to the presence of any of the three stop codons (UAA, UAG, or UGA) in the ribosomal A site. The end result of this process is the release of the completed polypeptide following the hydrolysis of the ester bond linking the polypeptide chain to the P site tRNA. The peptidyl transferase centre of the ribosome is believed to catalyze the hydrolysis reaction in response to the activity of the eukaryotic Release Factor 1 (eRF1) and eukaryotic Release Factor 3 (eRF3) (Arkov, 1998 #420;Arkov, 2002 #418;Arkov, 1999 #419;Caskey, 1971 #460;Seit-Nebi, 2001 #973; Zavialov,

2002 #974}. eRF1 recognizes all stop codons to release the completed polypetide chain from the ribosome (Frolova et al., 1994), and eRF3 is essential for the GTP-dependent releasing activity (Zhouravleva et al., 1995).

After the finished peptide is released from the ribosome, one molecule of GTP is hydrolyzed leading to the dissociation of the release factors from the ribosome (for reviewe see Kapp and Lorsch, 2004).

3.4. Recycling

The fourth stage of translation is the recycling of the ribosomal subunits so that they can be used in another round of initiation.

The closed-loop model of eukaryotic mRNAs has suggested the possibility that termination and recycling may not release the 40S subunit back into the cytoplasm. Instead, the 40S subunit may be shuttled across or over the poly(A) tail back to the 5'-end of the mRNA via the 5'- and 3'-end-associated factors. In this model, the closed-loop serves to facilitate reinitiation of translation rather than (or in addition to) the first initiation event. This proposal was first bolstered by the finding that eRF3 and PAB interact with each other (Hoshino et al., 1999), connecting the termination apparatus to the poly(A) tail.

4. mRNA specific translational regulation

Translational control over the steps described in the previous section will affect the translation of all cellular mRNAs. But a more specific mechanism targeting a subset of mRNAs can be achieved through *cis*-acting elements present in the 5' and 3'UTRs of particular subpopulations of mRNAs. A hallmark of mRNA-specific translational control is the participation of specific *trans*-acting factors that recognize and bind these *cis*-acting elements. They are key players that seem to mediate the majority of the best-characterized examples in early embryonic development, differentiation and cell cycle (Colegrove-Otero et al., 2005; de Moor et al., 2005; Mendez and Richter, 2001; Wilkie et al., 2003). Generally, these regulatory factors assemble onto an mRNA as a large multiprotein complex(es) concomitantly with transcription, splicing and 5'/3' end-processing in the nucleus, and can directly influence its future by affecting the subcellular localization, translational efficiency, stability or degradation (Dreyfuss et al., 2002; Keene, 2007; Maniatis and Reed, 2002).

During early embryonic development in *Xenopus* and *Drosophila*, prior to the re-establishment of embryonic transcription, translational regulation of mRNAs become extremely important, and a variety of different mechanisms of this regulation target dynamic changes of poly(A) tail mediated by polyadenylation and/or deadenylation, and translational repression by blocking the recognition of the cap by the eIF4F (Colegrove-Otero et al., 2005; de Moor et al., 2005) (Figure 8 a, b). Other mechanisms include regulation of ribosomal subunit binding (for reviews see Colegrove-Otero et al., 2005; de Moor et al., 2005; Gebauer and Hentze, 2004) (Figure 8 c,d); and an increasing number of studies

indicate that mRNA translation is also regulated by small miRNAs (e.g. post-initiation repression), although it function in meiosis and early development is still nuclear (Filipowicz et al., 2008; Jackson and Standart, 2007; Standart and Jackson, 2007) (Figure 8 e).
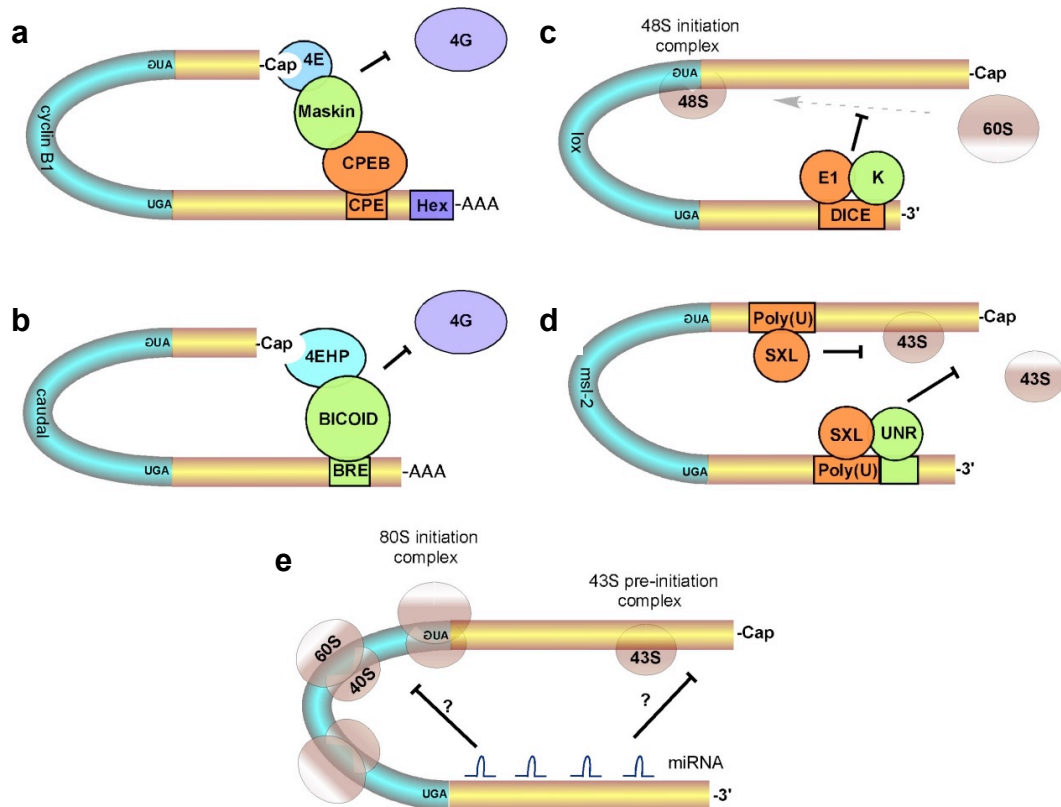
Figure 8. Mechanisms of mRNA-specific regulation. Three different mechanisms of translational regulation are schematized: interference with the eIF4F complex, though Maskin **(a)** or Bicoid **(b)**; inhibition of ribosomal subunit binding, either preventing the joining of the 60S ribosomal subunit **(c)** or of the 48S initiation complex **(d)**; miRNA mediated repression by imperfect base-pairing interaction with the 3'UTR **(e)**. Pictures adapted from Abaza et al., 2006 and Gebauer and Hentze, 2004.

The *cis*-acting elements also control a special and interesting case in the local regulation of translation that occurs in polarized cells allowing gene expression to be controlled in both spatial and temporal fashions (Bashirullah et al., 1998; Colegrove-Otero et al., 2005; Gebauer and Hentze, 2004; St Johnston, 2005).

Localization of mRNAs provides an universal mechanism to spatially restrict gene expression within individual cells. Over 500 cytoplasmically localized mRNAs have been identified so far and most of them are localized in oocytes, eggs, early embryos, or differentiating somatic cells (Bashirullah et al., 1998; Eberwine et al., 2001). The ability to localize a small subset of mRNAs to particular subcellular regions of the cytoplasm is achieved in part by different combinations of factors that distinguish primary sequence or secondary structure features of an individual mRNA and in part by temporal hierarchies in the assembly or localization of these factors. However, localizing the mRNA by itself is not enough to achieve local distribution of gene products. In most cases, a combination between mRNA localization and translational control is also required.

4.1. Translational regulation through cytoplasmic polyadenylation

Maturing oocytes and early embryos are transcriptionally inactive (Gray and Wickens, 1998) and vertebrate development is directed by maternal inherited mRNAs that are synthesized and stored during the long period of oogeneis. In *Xenopus leavis*, maternal mRNAs are dormant in oocytes, and their mobilization into polysomes do not occur until later in development.

The regulation of translation by cytoplasmic polyadenylation was discovered some twenty years ago in the oocytes and the early embryos of clam, worms, flies, frogs and mice (Fox et al., 1989; McGrew et al., 1989; Paris et al., 1988; Rosenthal et al., 1983; Rosenthal and Wilt, 1986; Vassalli et al., 1989). The maternal mRNAs are stored in the growing oocyte with a short poly(A) tail of 20 to 40 nucleotides and are translationally repressed (masked). Upon oocyte maturation or after fertilization, the poly(A) tail of masked mRNAs is elongated to 80-250 residues and the mRNAs are translationally activated (Mendez and Richter, 2001; Richter, 2007). Cytoplasmic polyadenylation is generally

correlated with translational activation, and deadenylation with translational repression.

At least four different cytoplasmic polyadenylation elements have been identified in the 3'UTR of mRNAs in *Xenopus*. The C-rich cytoplasmic polyadenylation element and the U-rich embryonic cytoplasmic polyadenylation element normally mediate cytoplasmic polyadenylation in the zygote and early embryo (Paillard et al., 2000; Simon et al., 1992). The putative cytoplasmic polyadenylation factors binding to these elements are poly(rC) binding protein 2 (PCBP2) and Elav related protein A (the ortholog of HuR) respectively (Paillard et al., 2000; Simon and Richter, 1994; Slevin et al., 2007; Wu et al., 1997). A polyadenylation element that functions early in oocyte maturation, the polyadenylation response element (PRE) is recognized by the RNA binding-protein Musashi (Charlesworth et al., 2004; Charlesworth et al., 2002; Charlesworth et al., 2006). Mutating Musashi or its binding site blocks cytoplasmic polyadenylation, indicating that it is likely to be a cytoplasmic polyadenylation factor.

By far, the best characteristic cytoplasmic polyadenylation element is the CPE, which is required for the cytoplasmic polyadenylation of a number of mRNAs, including cyclin B1 mRNA, during oocyte maturation and embryonic cell cycle (Bardwell et al., 1991; Groisman et al., 2002; Paris and Philippe, 1990). The sequence of the CPE is variable. In *Xenopus* the CPE usually is UUUUUAU or UUUUAAU, although some variation may be tolerated in the context of specific mRNAs (Barkoff et al., 2000; Fox et al., 1989; McGrew et al., 1989; Pique et al., 2008; Richter, 2007; Stebbins-Boaz et al., 1996). CPE sequences have been shown to repress mRNA translation in immature *Xenopus* oocytes and to direct cytoplasmic polyadenylation and translational activation in maturing oocytes. Both aspects of CPE function require the CPEB (cytoplasmic polyadenylation binding protein). CPE activity can also be modulated by other sequences located in the 3'UTR (Belloc and Mendez, 2008; Belloc et al., 2008; Fox et al., 1989; McGrew et al., 1989; Stebbins-Boaz and Richter, 1994).

A. CPEB

CPEB is a critical regulator for gene expression in early development. It was first cloned and characterized in *Xenopus* oocytes as a 62 kDa protein that bounds specifically to CPEs mediating cytoplasmic polyadenylation (Hake and Richter, 1994; Stebbins-Boaz et al., 1996). Later on, *Xenopus* CPEB became the founding member of a large RNA-binding protein family, from *C.elegans* to humans (see section 6 for more details). As the founding member of the CPEB family proteins, CPEB is sometimes referred as CPEB1.

Xenopus CPEB1 protein is composed of three regions (Figure 9): the amino-terminal regulatory portion, two RNA recognition motifs (RRMs), and a cysteine-histidine repeat similar to metal-coordinating region or zinc-finger.

Figure 9. Structure of *Xenopus* CPEB1. The carboxy-terminal portion is devoted to RNA binding and contains two RRMs (blue boxes), and a zinc finger (Zif, green box), all of which are necessary for optimal interaction with the CPE. The amino-terminal portion of CPEB1 contains the regulatory information, such as a PEST (proline, glutamic acid, serine, threonine) box (red box) that mediates tubulin binding and, possibly, proteasome-induced destruction; the Eg2 phosphorylation site; and the Eg2-interacting element (yellow box). Taken from Mendez and Richter, 2001.

The N-terminal half contains consensus Eg2/Aurora A kinase phosphorylation sites (LDS/TR), which are phosphorylated early during meiotic maturation (Mendez et al., 2000a; Mendez et al., 2000b). This region also contains a PEST sequence, a sequence enriched in proline (P), glutamic acid (E), serine (S) and threonine (T) that target proteins for rapid destruction (Rechsteiner and Rogers, 1996) (Figure 9). CPEB1 PEST box mediates CPEB1 degradation by ubiquitination in response to Cyclin-dependent kinase (Cdc2) and *Xenopus* Polo-like Kinase 1 (Plx1) phosphorylation during later stages of meiotic

maturation (Mendez et al., 2002; Reverte et al., 2001; Setoyama et al., 2007; Thom et al., 2003).

The C-terminal half contains the two RRM domains (Hake and Richter, 1994) and two unusual zinc-finger regions ($C_4C_2H_2$) required to binding CPE-bearing mRNAs *in vitro* (Figure 9) (Hake et al., 1998).

B. CPEB1-interacting proteins

here, we summarize the CEPB1-interacting partners known to date; although there is some controversy surrounding the CPEB1-interacting proteins belonging to the functional complex(es) involved in the regulation of CPE-containing mRNAs.

Maskin

Maskin is a homologue of the transforming acid coiled-coil domain protein 3 (TACC3) and these proteins play a vital role in the formation of the mitotic spindle in multiple organisms (Barros et al., 2005; Bellanger et al., 2007; Gergely et al., 2003; O'Brien et al., 2005; Peset et al., 2005; Yao et al., 2007). Maskin was first identified in *Xenopus laevis* oocytes as a factor that interacts simultaneously with both CPEB1 (Cao and Richter, 2002; Stebbins-Boaz et al., 1999) and the eukaryotic initiation factor eIF4E (Richter, 2001). This interaction precludes the association of eIF4E with eIF4G, thereby preventing the 40S ribosomal subunit from being correctly positioned on the 5'end of the mRNA, repressing translation. The Maskin-eIF4E complex is disrupted by cytoplasmic polyadenylation triggered by CPEB1 phosphorylation (Cao and Richter, 2002). The key factor that modulates the Maskin-eIF4E association is poly(A)-binding protein (PABP), which binds eIF4G and increase its affinity for eIF4E, thus displacing Maskin (Cao and Richter, 2002). However, there is controversy about the presence of maskin

in the mature repression complex as a recent work failed to find Maskin bound to CPEB1 in PI-arrested oocytes (Minshall et al., 2007).

Maskin is phosphorylated during maturation, but its effects on translation at this time are modest (Barnard et al., 2005). CDK1 catalyzes several phosphorylations of Maskin, helping to its dissociation from eIF4E (Barnard et al., 2005; Cao et al., 2006; Nelson et al., 2004). And calcineurin (CaN) dephosphorylates Maskin causing it to reassociate with eIF4E and repress translation (Cao et al., 2006). A phosphorylation on Ser626 (Barnard et al., 2005; Kinoshita et al., 2005; Pascreau et al., 2005; Peset et al., 2005) by protein kinase A (Barnard et al., 2005) or Aurora A (Barnard et al., 2005; Kinoshita et al., 2005; Pascreau et al., 2005; Peset et al., 2005) is necessary for Maskin to become associated with spindles and centrosomes, confirming an earlier observation that this protein, as well as CPEB1, is found on the mitotic apparatus (Groisman et al., 2000).

Symplekin

Symplekin is a protein found in nuclear complexes containing CPSF and other processing factors and is thought of as a scaffold protein involved in 3' end RNA processing (Hofmann et al., 2002; Takagaki and Manley, 2000; Xing et al., 2004). In yeast and plants, homologues of symplekin are required for proper cleavage and polyadenylation, and this is possibly also the case in vertebrates (Herr et al., 2006; Preker et al., 1997; Zhao et al., 1999b). In vertebrate somatic cells symplekin is predominantly nuclear. However, in the *Xenopus* oocyte is also found in the cytoplasm in complexes with the 100kDa subunit of CPSF (Hofmann et al., 2002), as part of the citoplasmic polyadenylation complexes (Barnard et al., 2004; Kim and Richter, 2006). Symplekin interacts also avidly with CstF-64. This interaction, however, is mutually exclusive with an interaction with CstF-77, which is the subunit required for integrity of the CstF complex. Therefore it was proposed that symplekin functions to facilitate or maintain a properly assembled CstF, and

by extension, to help hold together the much larger, complete polyadenylation complex (Takagaki and Manley, 2000).

PARN

PARN was initially described as a poly(A)-specific 3' exonuclease in HeLa cell free extracts (Astrom et al., 1991, 1992). Active PARN is oligomeric, highly processive, and stimulated by the presence of the cap structure at the 5'-end of the RNA substrate (Dehlin et al., 2000; Gao et al., 2000; Martinez et al., 2001; Martinez et al., 2000). The interaction between PARN and the cap structure at the 5'-end of an mRNA affects mRNA decay and also initiation of translation by competing with eIF4E on 5'-cap binding (Gao et al., 2001). Moreover, translation-dependent protection of the cap by PABP inhibits the deadenylating activity of PARN under physiological conditions (Caponigro and Parker, 1995; Korner and Wahle, 1997; Korner et al., 1998; Wickens et al., 1997).

In *Xenopus* oocytes, two isoforms of PARN, 74 and 62kDa in molecular sizes, have been identified (Korner et al., 1998). The two isoforms differ in nuclear-cytoplasmic distribution, the 74-kDa form being exclusively nuclear while the 62kDa form is cytoplasmic (Korner et al., 1998). The 64kDa PARN isoform is thought to be responsible for the short poly(A) tails of CPE containing mRNAs (Kim and Richter, 2006). Thus, overexpression of catalytically inactive PARN can induce polyadenylation of CPE containing mRNAs in immature oocyes, while overexpression of wild type PARN represses the polyadenylation induced by Gld-2 overexpression (Kim and Richter, 2006). It has been proposed that PARN and Gld-2 are simultaneously bound to CPE-containing mRNAs balancing each other until CPEB1 phosphorylation induces the release of PARN. However, a recent work failed to find PARN bound to CPEB1 in PI-arrested oocytes (Minshall et al., 2007).

CPSF

The cleavage and polyadenylation specificity factor (CPSF) is a four-subunit complex that directly recognizes the hexanucleotide (AAUAAA) and mediates polyadenylation in the nucleus (for reviews see (Proudfoot and O'Sullivan, 2002; Wahle and Ruegsegger, 1999)). These functions are carried out, at least in part, by CPSF-160 (Murthy and Manley, 1995). The 160kDa subunit is the RNA binding protein that recognises the poly(A) signal (Keller et al., 1991), while the 73kDa subunit is the endonuclease that mediates the formation of the 3'ends of all mRNAs (Dominski et al., 2005; Kolev and Steitz, 2005; Mandel et al., 2006; Ryan et al., 2004). The 100kDa subunit resembles the 73kDa subunit but does not appear to have nuclease activity and its precise function in 3' end processing is unknown. The 30kDa subunit is a zinc finger protein that has been reported to have some affinity for RNA and it potentially has endonuclease activity (Barabino et al., 1997).

In *Xenopus*, CPSF directly interacts to CPEB1 through the 160-kDa subunit both in immature and mature oocytes (Barnard et al., 2004; Kim and Richter, 2006; Mendez et al., 2000b; Rouget et al., 2006; Rouhana et al., 2005). The 100 and 30 kDa CPSF subunits are also present in *Xenopus* oocyte cytoplasm, while the 73kDa endonuclease is absent from the cytoplasmic CPSF complex (Dickson et al., 1999). The complex between CPEB1 and CPSF/GLD-2 is stabilized in response to progesterone during meiotic maturation, which leads to translational activation of CPE-containing mRNAs (Kim and Richter, 2006; Mendez et al., 2000b).

GLD-2

Germ-line-development factor 2 (Gld-2) was first characterized as a PAP in *Caenorhabditis elegans* (Wang et al., 2002). This protein is widely conserved, having homologues from fission yeast to mammals. The GLD-2 protein belongs to the large family of DNA polymerase β nucleotidyl transferases, but has only limited additional homology to the classical poly(A) polymerases and it lacks the RNA binding domain.

The *Xenopus* GLD-2 protein (xGLD-2) lacks any recognizable RNA-binding domain like other members of the GLD2 family, suggesting that other factors associate with the polymerase to determine which RNAs will undergo polyadenylation. XGLD2 does not interact with the repressor factors Maskin and Pumilio, implying that PAP is not associated with this repressive complex (Rouhana et al., 2005). In contrast, XGLD-2 was shown to coimmunoprecipitate very efficiently with symplekin in both mature and immature oocytes. In addition, tagged xGld-2 was shown to bind the 160kDa CPSF subunit as well as CPEB1 synthesised in reticulocyte lysates (Barnard et al., 2004). The complex between CPEB1 and CPSF/GLD-2 is stabilized in response to progesterone-stimulated signalling during meiotic maturation, which leads to translational activation of CPE-containing mRNAs (Kim and Richter, 2006; Rouhana and Wickens, 2007).

XRbm9

The mammalian Rbm9 gene has multiple promoters and numerous alternative splicing events that give rise to a large family of proteins with variable N- and C-terminal and internal deletions. The *Xenopus* Rbm9 contains in its ORF a single central RNA recognition motif (RRM)-type RNA-binding domain with two RNP domains, two arginine/glycine-rich (RGG) motifs that are characteristic of RNA-binding proteins, and an alanine-rich carboxy-terminal sequence that could be involved in protein-protein interactions. XRbm9 shows a 59% similarity with hRBM9, which increases to 98% for the RNA-binding domain (Papin et al., 2008). XRbm9 is a 55kDa protein expressed throughout oogenesis, oocyte maturation and during embryogenesis up to stage 33. XRbm9 interacts directly with XGld2 N-terminal domain, and is in the polyadenylation complex with CPEB1 and CPSF (Papin et al., 2008).

Xp54

P54 helicases are found in P(rocessing)-bodies, distinct cytoplasmic foci that are sites of (reversible) RNA storage and RNA decay, composed of mRNA and factors mediating both RNA degradation and translational repression (Andrei et al., 2005; Cougot et al., 2004; Eulalio et al., 2007; Parker and Sheth, 2007; Sheth and Parker, 2003; Standart and Minshall, 2008; Wilczynska et al., 2005).

RCK/Xp54 is a DEAD-box RNA helicase (Minshall and Standart, 2004; Minshall et al., 2001), involved in splicing, RNA transport, degradation and translation (Weston and Sommerville, 2006). Xp54 is present at constant levels throughout oogenesis and is implicated in the nuclear assembly of stored mRNA particles in early oocytes, where it shuttles between nucleus and cytoplasm (Ladomery et al., 1997; Smillie and Sommerville, 2002; Thom et al., 2003). Xp54 associates with CPEB1 and with eIF4E suggesting a role in translation repression (Coller and Wickens, 2002; Minshall and Standart, 2004; Minshall et al., 2001). Also, Xp54 thethered to the 3'UTR of a reporter gene represses its translation (Minshall and Standart, 2004; Minshall et al., 2001). A role in translation repression has also been reported for other members of this highly conserved helicase family (Chu and Rana, 2006; Coller and Parker, 2005; Mair et al., 2006; Nakamura et al., 2001).

Pumilio

Xenopus Pumilio (Pum) is a RBP member of the Pumilio/Fem3-binding protein (PUF) family (Wickens et al., 2002) that specificaly-associates with maternal mRNAs (Nakahata et al., 2001), as well as CPEB1, via its PUF domain. Members of this family can mediate translational repression and mRNA destabilization in organisms from yeast to vertebrates (for reviews see Wharton and Aggarwal, 2006; Wickens et al., 2002). Pum binds directly to CPEB1, and contributes to repression (Nakahata et al., 2001; Nakahata et al., 2003) or activation (Pique et al., 2008) of CPE containing mRNAs. Many CPE-containing mRNAs also contain Pum binding sites, including cyclin B1

and Gld-2, indicating that the recruitment of Pum may be both by protein-protein and RNA-protein associations.

eIF4E1b and 4E-T

Xenopus 4E-T has two splice isoforms. The X4E-T long isoform is 67% identical to human 4E-T and contains all three sequences that have been reported to promote nuclear import/export (Dostie et al., 2000), while the short form lacks the N-terminal nuclear exporting signal. Both forms are cytoplasmic and contain potential eIF4E-binding site, but the short isoform is more abundant in oocytes than the longer form (Minshall et al., 2007). Tethered 4E-T represses translation (Minshall et al., 2007). *Xenopus* 4E-T is found in the CPEB1 RNP complex in early oocytes, and the only eIF4E1 protein in this complex is eIF4E1b, rather than the canonical cap-binding factor eIF4E1a (Minshall et al., 2007). In this complex is also found the RNA helicase RCK/Xp54, and the P-body components P100 (Pat1) and Rap55.

eIF4E1b is a close homolog of the canonical eIF4E1a cap-binding protein. Though eIF4E1b possesses all residues known to be required for cap- and eIF4G-binding, it binds m7GTP weakly, and rather than binding eIF4G, binds 4E-T. All eIF4E1 proteins are cytoplasmic (Minshall et al., 2007); but a distinguishing feature of eIF4E1b proteins is the presence of several tandem basic residues in their N-termini, reminiscent of proteins that undergo nuclear import (Evsikov et al., 2006; Joshi et al., 2005). The levels of eIF4E1b slowly decline during oogenesis, while the expression of eIF4E1a increase (Minshall et al., 2007). In the adult tissues eIF4E1b is only detectable in the ovary (Minshall et al., 2007).

CstF77

Mammalian CstF is involved in pre-mRNA cleavage before nuclear polyadenylation by recognizing the so-called downstream, or G/U rich. CstF contains three subunits, CstF-50, CstF-64 and CstF-77. The functions of

CstF-64 and CstF-77 include RNA recognition and protein-protein interactions, respectively (Takagaki and Manley, 1997, 2000). The third mammalian subunit CstF-50, consists of a short N-terminal extension and seven WD-40 repeats.

In addition to its nuclear function, CstF77 has been reported to be present in the cytoplasmic polyadenylation complex in Xenopus oocytes, associated with CPEB1, Gld-2 and CPSF (Rouget et al., 2006). It may have a role in translational repression, rather than in cytoplasmic polyadenyalation.

Aurora A

Aurora A/Eg2 is member if a family of Serine/Threonine protein kinases, which have important roles in cell cycle progression, bipolar spindle formation and chromosome segregation (for reviews see Crane et al., 2004; Ducat and Zheng, 2004; Marumoto et al., 2005). There are three types of Aurora kinases in vertebrates mammals (Aur-A, Aur-B and Aur-C); two in *Xenopus*, *Drosophila* and *C.elegans* (the A- and B-types); and a single one in budding yeast (Ipl1) and fission yeast (Ark1) which, so far, seem mostly B-like in their functions.

Aurora A is an important regulator of spindle formation, and therefore essential for accurate chromosome segregation. Moreover, in *Xenopus* oocytes seems to have a different role. Several studies strongly support the idea that Aurora A phosphorylates CPEB1 on S174 soon after progesterone stimulation (Mendez et al., 2000a), converting CPEB1 from a repressor to an activator and increasing the affinity of CPEB1 for the CPSF (Mendez et al., 2000b) and the release of PARN from the repression complex (Kim and Richter, 2006). This phosphorylation is crucial for the polyadenylation-dependent translation of specific mRNAs.

XGef

The Guanine nucleotide exchange factor for the Rho family of GTPases in *Xenopus* (XGef) interacts directly with the N-terminal half of CPEB1 in oocytes (Martinez et al., 2005; Reverte et al., 2003). XGef has two binding sites for CPEB1, and enhances the early phosphorylation of CPEB1. As XGef immunoprecipitates contain MAPK in both immature and mature oocytes it may be required to bring CPEB1 to the signalling complexes involved in its phosphorylation (Keady et al., 2007).

APLP1

The mouse CPEB1 is found to bind the small intracellular domain of the transmembrane Amyloid precursor-like protein 1 (APLP1) and its relatives (Cao et al., 2005). In *Xenopus* oocytes, Gld-2, the CPSF 100kDa subunit, symplekin and several other factors involved in polyadenylation, are all detected by immuno-electron microscopy on membranes in the same fractions as APLP1, CPEB1 and CPE-containing mRNAs (Cao et al., 2005). While the association with amyloid precursor proteins may have great significance for the role of CPEB1 in neurons, it is yet unclear whether APLP1 is required for polyadenylation in oocytes and even whether it mediates the membrane association of the polyadenylation machinery.

D. The dual role of CPEB1

CPEB1 in *Xenopus* performs a dual role: it represses cap-dependent translation in the oocytes (de Moor and Richter, 1999) and activates translation, via cytoplasmic polyadenylation, in meiotically maturing eggs and early embryos (Mendez and Richter, 2001). CPEB1 might reside in several ribonucleoprotein-complexes and accomplishes its dual role in translation regulation depending on the association with the above mentioned interacting factors (Figure 10). In arrested immature oocytes, these CPEB1-interacting proteins may function in

redundant repression mechanisms and, thus, at present it is difficult to choose among the multitude of models proposed for translational repression mediated by CPEB1. In addition, a given mRNA can exist in more than one complex depending on the combination of factors that are recruited in time and space.

CPEB1 translational repression

Translational silencing is the consequence of the cytoplasmic shortening of the long poly(A) tail, acquired during the nuclear cleavage and polyadenylation of the pre-mRNA, from 200-500 to 20-40 nucleotides. This deadenylation is the result of the direct recruitment of the deadenylase PARN by CPEB1 (Kim and Richter, 2006) (Figure 10a). Active repression (masking) is accomplished by the recruitment of Maskin through direct interaction with CPEB1. In turn, Maskin binds to the cap-bound eIF4E precluding the recruitment of the eIF4G and therefore of the 43S ribosomal complex (Stebbins-Boaz et al., 1999) (Figure 10b). One problem with the proposed model is that, although CPEB1 can recruit PARN to the mRNA, deadenylation by PARN requires the recognition of the 5'cap structure (Copeland and Wormington, 2001; Gao et al., 2000) leading to a conflict between having Maskin and PARN in the same complex.

But the closed-loop driven by CPEB1-Maskin-eIF4E is not the only repression complex assembled by CPEB1. In early oogenesis (where Maskin and PARN are not expressed), CPEB1 fractionates with very large mRNP complexes containing CPEB1 associated with eIF4E-T and an ovary-specific eIF4E1b that binds the cap weakly (Minshall et al., 2007). The identification of this complex, that does not contain Maskin, suggests an additional model for repression where the recruitment of eIF4E-T by CPEB1 and its association with eIF4E1b would compete for eIF4G association, thus blocking translation initiation. This large mRNAP also includes the RNA helicase RCX/Xp54, and the P-body components P100 (Pat1) and Rap 55 (Figure 1c). Interestingly, Xp54 has been described itself as a CPEB1 and

eIF4E interacting protein, providing an additional mechanism to repress translation (Minshall and Standart, 2004).

Another trans-acting factor recruited by repressed CPE-containing 3'UTRs is *Xenopus* Pumilio (Pum) that interacts with CPEB1. Although Pum has a very weak effect on translational repression on CPE-containing reporters (Nakahata et al., 2003; Pique et al., 2008), it may play a critical role in the silencing by deadenylation. Accordingly, Pum is present in CPEB1-complexes containing Maskin but not in the ones containing the cytoplasmic poly(A) polymerase GLD2 (Rouhana et al., 2005) (Figure 10b).

a. Translational silencing
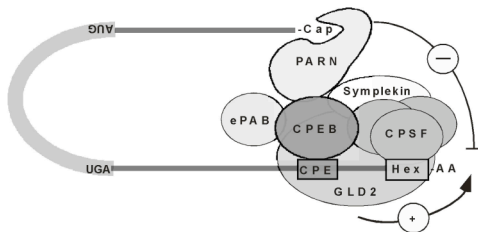
b. Translational repression
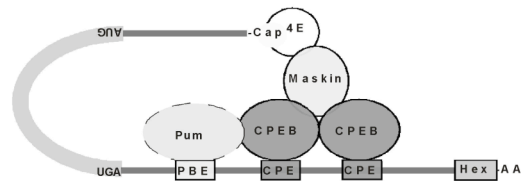
c. Early oogenesis translational repression
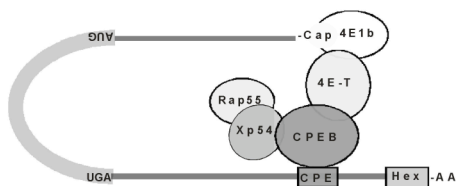
d. Translational activation by cytoplasmic polyadenylation
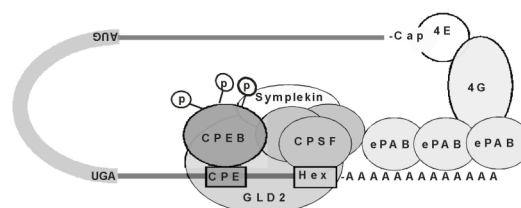
Fig10. CPEB1 mediated translational control. (a,b,c) In immature oocytes, messenger RNAs containing a cytoplasmic polyadenylation element (CPE) are translationally dormant (masked) and reside in a complex containing CPEB together with other repression proteins. **(d)** Once maturation begins, newly phosphorylated CPEB recruits CPSF and PAP, which elongates the poly(A) tail. At the time coincident with this elongation, proteins mediating repression dissociate from the complex allowing translation initiation. Taken from Novoa et al.

CPEB1 translational activation

The conversion of CPEB1 from translational repressor to a translational activator involves both Aurora A/Eg2- and cdc2-dependent phosphorylation (Mendez et al., 2002; Mendez et al., 2000a).

Exposure of oocytes to progesterone translationally activates Ringo mRNA (Ferby et al., 1999; Lenormand et al., 1999; Padmanabhan and Richter, 2006) and Glycogen Synthase Kinase (GSK-3β) inactivation, leading to an activation of Aurora A kinase (Andresson and Ruderman, 1998; Sarkissian et al., 2004). Aurora A phosphorylates CPEB1 at serine residue 174, an event that increases the affinity of CPEB1 for the cleavage and polyadenylation specificity factor (CPSF) (Mendez et al., 2000b). CPSF binds to the AAUAAA sequence (Bilger et al., 1994; Dickson et al., 1999; Fox et al., 1992), an interaction that is probably stabilized by CPEB1, recruits poly(A) polymerase XGld2 to the end of the mRNA (Barnard et al., 2004; Mendez et al., 2000b; Rouhana et al., 2005) and induces the ejection of PARN form the complex (Kim and Richter, 2006). This complex is stabilized by Simplekin, that contacts directly with CPEB1 and CPSF. Concomitantly, Maskin is phosphorylated by Cdc2 (Barnard et al., 2005) or Aurora A (Pascreau et al., 2005), promoting the dissociation from the eIF4E but not from CPEB1 (Cao and Richter, 2002). In addition, embryonic poly(A)-binding protein (ePABP) is recruited to the 3' end of the mRNA and helps to eIF4G to displace Maskin form eIF4E, enabling the initiation of translation (Cao and Richter, 2002; Kim and Richter, 2006; Wakiyama et al., 2000) (Figure 10).

However, recently there has been some controversy surrounding the kinase mediating the early phosphorylation of CPEB1. Different studies failed to detect active Aurora A in early meiosis (Frank-Vaillant et al., 2000; Ma et al., 2003), and MAPK has been shown to be also implicated in priming CPEB1 for Ser174 phosphorylation, or even in activating the possible Ser174 kinase (Keady et al., 2007).

4.2. Translational regulation through deadenylation

The cellular concentrations of specific mRNAs are controlled by the rates of both synthesis and degradation, and deadenylation is often the initial and rate-limiting step of mRNA decay of many, but not all mRNAs (Shyu et al., 1991; Wilson and Treisman, 1988). In the nucleus, deadenylation restricts newly added mRNA poly(A) tails to their proper lengths. In the cytoplasm, extensive deadenylation of an mRNA initiates its degradation or repression (for reviews see Garneau et al., 2007; Goldstrohm and Wickens, 2008). (Figure 11) Deadenylation rates vary widely among mRNAs, but also the length of the poly(A) tail of a single mRNA can vary under different conditions or depending on the cell cycle phase.
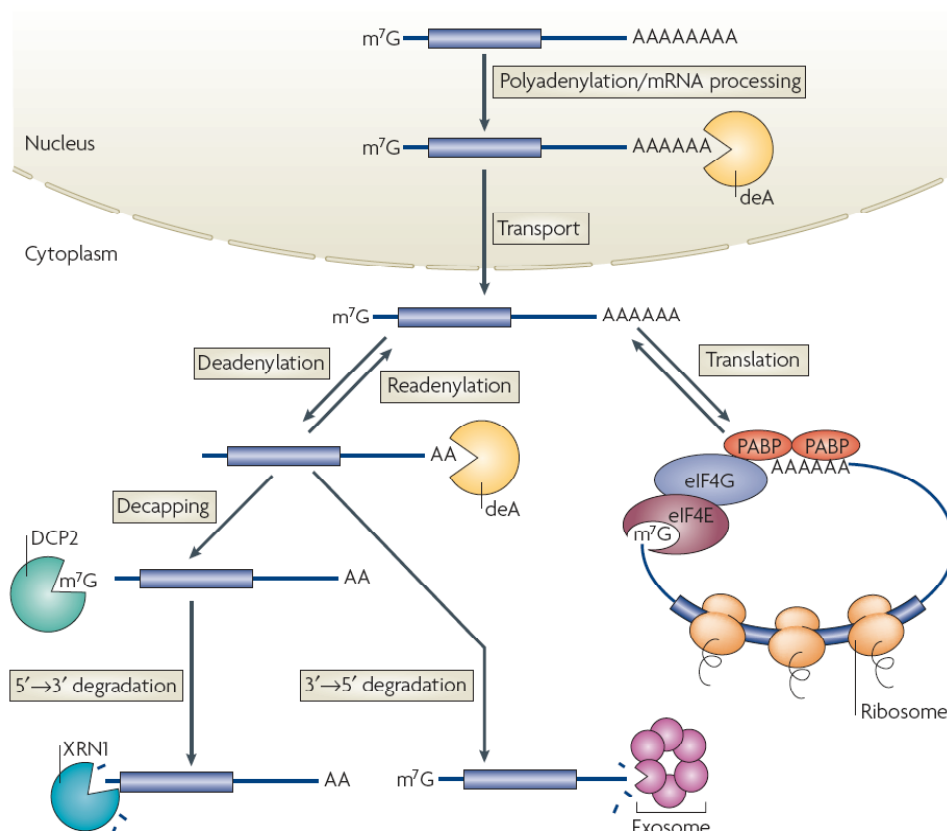
Figure 11. Pathways of translation and degradation. The deadenylase (deA) trims nuclear tails to their proper length. In the cytoplasm, the mRNA poly(A) tail enhances translation and stabilizes the mRNA. By removing the poly(A) tail, deadenylases elicit mRNA decay or translational repression, whereas readenylation can activate some mRNAs. Taken from Goldstrohm and Wickens, 2008.

A. Deadenylases

There are several characterized eukaryoic deadenylases, each with unique properties, but the range varies among species. Four major deadenylase complexes have been described in vertebrates:

a) **CCR4-NOT** complex is the main catalytic activity responsible for cytoplasimc deadenylation in yeast and in mammals (Dupressoir et al., 2001; Tucker et al., 2001; Yamashita et al., 2005). The CCR4 complex is constituted by different proteins in different cell types or conditions (Chen et al., 2001).

b) **PAN2-PAN3** is a PABP-dependent poly(A) nuclease involved in trimming the poly(A) tails of nascent mRNAs (Brown and Sachs, 1998; Brown et al., 1996; Yamashita et al., 2005).

Recently, CCR4-NOT and PAN2-PAN have been shown to interact between them and with PABP (Zheng et al., 2008). Also, both complexes have been shown to interact with eRF3, suggesting a coupling of termination with deadenylation (Funakoshi et al., 2007).

c) **PARN** is unique in that it has cap-dependent deadenylase activity (Dehlin et al., 2000; Gao et al., 2000; Martinez et al., 2001).

d) **Nocturnin** is implicated in circadian regulation of mRNAs by deadenylation (Baggs and Green, 2003; Liu and Green, 2002).

B. *Cis*-acting elements promoting deadenylation

All transcripts are affected at some time by default deadenylation since it is the first step of the mRNA turnover pathway but the underlying mechanism is not well known. Still, specific transcripts can be targeted for deadenylation by several sequences in their 3'UTRs, such as EDEN (embryo deadenylation element), or AREs (AU-rich elements).

EDEN

The consensus sequence is five repetitions of the UGU rinucleotide in a window of 35 nuceotides (Graindorge et al., 2006). These sequences are bound by the *Xenopus* protein EDEN-BP, a 53/55 kD doublet protein, which can also bind AREs (Paillard et al., 2002). CUG-BP is the EDEN-BP human homolog (Paillard and Osborne, 2003), which interacts with PARN to mediate deadenylation (Moraes et al., 2006).

AREs

By far the most well studied class of mRNA-stability element is the AU-rich element (ARE) found in the 3'UTR of many transcripts that encode cytokines, proto-oncogenes and transcription factors, among others (Khabar, 2005). Based on their structural and functional properties AREs can be divided into three classes (Table 3).

Table 3. Classification of ARE motifs. Taken from Espel, 2005

Class	Motif	Examples
I	Dispersed AUUUA and a U-rich region	c-fos, c-myc
II	WWWWAUUUAWWWW	GM-CSF, TNFα COX-2
III	U-rich, GUUUG repeats	c-jun

Class I and II contain copies of the pentameric AUUUA motif. Class I AREs contain one to three copies of scattered AUUUA motifs with nearby U-rich regions and control synchronous cytoplasmic deadenylation of mRNAs by generating intermediates having poly-A tails of 30-60 nucleotides, which are then completely degraded. Class II AREs contain multiple pentamers, with some overlapping and control asynchronous cytoplasmic deadenylation generating poly-A(-) mRNAs. Class III includes AREs that lack the pentamer sequences but contain U-rich regions and show degradation kinetics similar to mRNAs containing class I. However, it seems that no two ARE are

identical; even when the ARE sequences are similar, the flanking sequence can influence the overall effect on mRNA stability (Moraes et al., 2006; Stoecklin et al., 2002; Ueno and Sagata, 2002).

AREs are bound by ARE-binding proteins (ARE-BPs). To date, many proteins have been described to belong to this family; some of them promote destabilization by binding to an mRNA, but others prevent destabilization (Barreau et al., 2005).

C. Deadenylation in development

Deadenylation is an evolutionarily conserved mechanism to translationally silence maternal mRNAs during oocyte maturation and early embryogenesis in diverse species (Sachs et al., 1997). During development, the total amount of RNA remains unchanged (Golden et al., 1980), but the total amount of polyadenylated RNA is reduced ~40% upon maturation (Darnbrough and Ford, 1976; Sagata et al., 1980), due to selective deadenylation of a subset of mRNAs.

mRNAs suffer a default deadenylation and specific deadenylation in both *Xenopus* and mouse oocytes. The default pathway does not require specific *cis*-sequences and it is produced in mRNAs that are not protected from deadenylation by active extension of their poly(A) tails (Fox and Wickens, 1990; Varnum and Wormington, 1990). In contrast, certain mRNAs which are polyadenylated during meitotic maturation contain 3'-UTR elements that promote their deadenylation during meiotic progression and/or after fertilization (Belloc and Mendez, 2008; Bouvet et al., 1994; Legagneux et al., 1995).

Recently, our laboratory has characterized the role of a zinc-finger protein named C3H-4 during meiotic progression in *Xenopus* oocytes. C3H-4 is an ARE-binding protein that mediates deadenylation of ARE-containing mRNAs in response to progesterone-induced meiosis recruiting the CCR4/Not deadenylase complex (Belloc and Mendez, 2008). C3H-4 activity opposes

CPEB1 activity in MI on mRNAs containing both CPEs and AREs, and constitutes a new translation negative feedback loop inactivating early polyadenylated mRNAs and displacing their polyadenylation. Thus, for an mRNA polyadenylated by the "early" activation of a weak CPE, C3H-4 inactivates the mRNA after MI. However, for mRNAs containing a late-strong CPE arrangement, C3H-4 causes a delay in the poly(A) elongation, generating a third wave of polyadenylation in interkinesis (Figure 13).

After fertilization and until mid-blastula transition (MBT), EDEN-BP is activated by phosphorylation and mediates the deadenylation of a specific subset of mRNAs (Detivaud et al., 2003; Legagneux et al., 1992). Some of the identified EDEN-mediated deadenylation targets are the mRNAs of Aurora A (Eg2), Aurora B, c-Mos, Cdk1, Cdk2 (Eg1), casein kinase 2 beta, Bub3, Wee1, MELK (Eg3), Eg5/KLP and NEK2B and CPEB1 (Graindorge et al., 2006).

In all of these developmental situations, deadenylation does not destabilize mRNAs immediately, but appears to be a prerequisite for their subsequent degradation at later stages (Audic et al., 1997; Gillian-Daniel et al., 1998; Voeltz and Steitz, 1998). The uncoupling of deadenylation from mRNA decay in gametes and embryos contrasts with both yeast and metazoan cells in which poly(A) removal rapidly promotes mRNA degradation.

5. Meiotic progression in *Xenopus laevis*

Most of the knowledge accumulated to understand meiosis has been achieved from the study of the *Xenopus laevis* oocyte maturation. An important characteristic of the meiotic cell cycle is the occurrence of two consecutive M phases without an intervening S phase, which is essential for generating haploid germ cells.

Vertebrate oocytes undergo a round of DNA replication before the meiotic cell cycle, and then they enter meiosis only to be arrested at the prophase of the first meiotic division (Sagata, 1996). This first meiotic arrest may last up to a few years in *Xenopus* or several decades in humans, and is characterized by synthesis and storage of large quantities of dormant mRNAs (LaMarca et al., 1973; Rodman and Bachvarova, 1976). The resumption of meiosis marks the onset of oocyte maturation and is stimulated by progesterone in *Xenopus* (Bayaa et al., 2000; Tian et al., 2000) and by gonadotropin in mouse and human (Faiman and Ryan, 1967; Rao et al., 1974). As soon as maturation starts, transcription is actively repressed, and a complex network of translational activation of stored maternal mRNAs accompanies oocyte maturation (Gebauer et al., 1994; Mendez et al., 2000a; Mendez and Richter, 2001; Oh et al., 2000; Stebbins-Boaz et al., 1996). The transcriptional silencing that begins with oocyte maturation persists during the initial mitotic divisions of the embryo, which, unlike any other, lack an appreciable G1 or G2 phase. In *Xenopus*, after 12 rapid synchronous cleavages, when the developing embryo is composed of ~4000 cells, the mid-blastula transition occurs and is characterized by

lengthening of the cell cycle, inclusion of G1 and G2, and activation of zygotic transcription (Newport and Kirschner, 1982a, b).

Three key activities are needed for meiotic progression in *Xenopus* oocytes: the maturation promoting factor (MPF), the anaphase promoting complex/cyclosome (APC/C), and the cytostatic factor (CSF) (Figure 12).
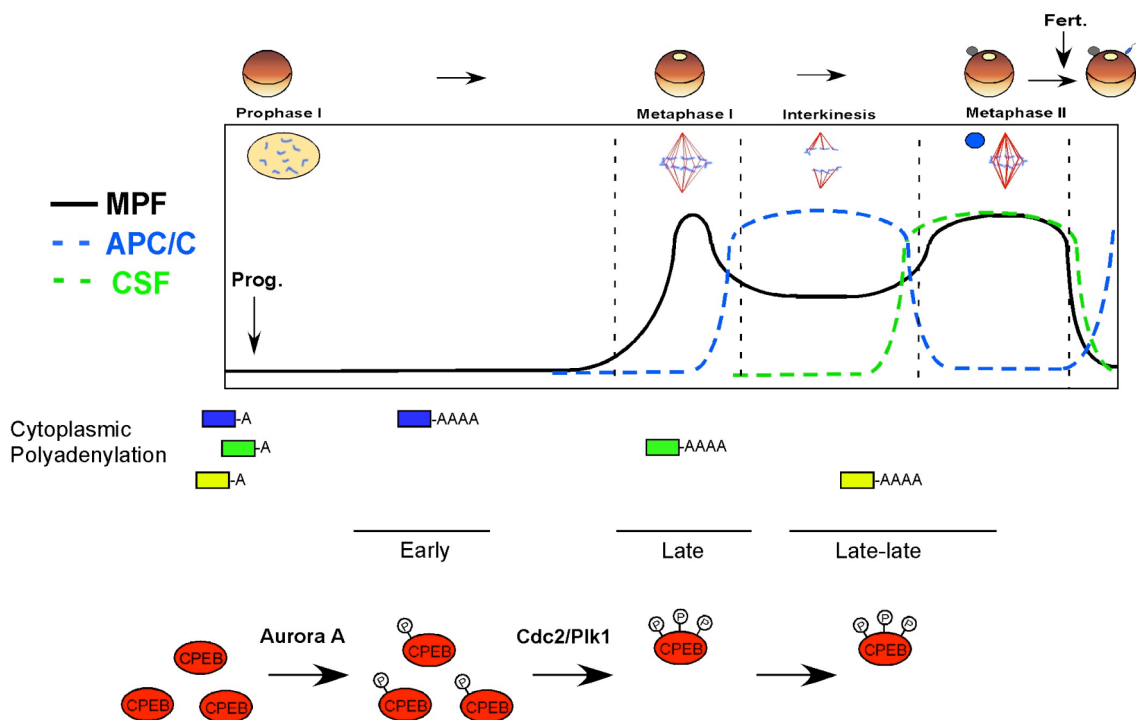
Figure 12. Meioic cell cycle progression in *Xenopus* oocytes. Schematic representation of meiotic progression of *Xenopus laevis* oocytes from PI-arrest to fertilization. Maturation-promoting factor (MPF), anaphase-promoting complex (APC) and Cytostatic Factor (CSF) activities are indicated. CPEB1 levels and regulation, and the three waves of cytoplasmic polyadenylation are also depicted.

The Maturing Promoting Factor (MPF), a heterodimer of Cyclin B and Cdc2 kinase, catalyzes the entry into M-phase of meiosis-I and meiosis-II, and is the responsible for many of the manifestations during oocyte maturation such as the germinal vesicle breakdown (GVBD) (Sagata, 1997; Vasudevan et al., 2006). This heterodimer is initially formed in PI arrested oocytes as an inactive pre-MPF, with Cyclins B2 and B5 (Hochegger et al., 2001; Pique et al., 2008),

and is activated by the dual specificity Cdc25 phosphatase as the result of new synthesis of Ringo and Mos induced by progesterone (Schmitt and Nebreda, 2002). MPF activation mediates transition from PI to MI. The subsequent decrease in MPF levels, required to exit from MI to enter in interkinesis (the transition phase between MI and MII), is induced by a negative feedback loop, where Cdc2 brings about the activation of the Anaphase-Promoting Complex (APC), which induces the ubiquitination and posterior destruction of Cyclins B (Peters, 2006). However, during the interkinesis, activation of APC is combined with the increased synthesis of Cyclins B1 and B4 by cytoplasmic polyadenylation (Hochegger et al., 2001; Pique et al., 2008) resulting in only a partial inactivation of MPF at anaphase-I, thus preventing entry into S-phase (Iwabuchi et al., 2000). Full reactivation of MPF in MII requires re-accumulation of high levels of Cyclin B as well as the inactivation of the APC by newly synthesized Emi2 and other components of the CSF, such as Cyclin E and high levels of Mos (Liu et al., 2007).

Meiotic progression not only requires the translational activation of specific mRNAs at specific phases of the cell cycle, the extent of translational activation is also finely regulated resulting in differential rates of product accumulation that, combined with the control of protein degradation, establish phase-specific peaks of expression of the factors that drive meiotic progression.

5.1. The combinatorial code of *cis*-acting elements

Individual CPE-containing mRNAs display specific translational behaviors during meiosis, suggesting that individual features within their 3'UTRs determine their response to CPEB1-mediated translational control. Thus, not every CPE-containing mRNA is masked (Barkoff et al., 2000; de Moor and Richter, 1999) and the activation of CPE-containing mRNAs does not occur en masse at any one time. Instead, the polyadenylation of specific mRNAs is temporally regulated (Ballantyne et al., 1997; de Moor and Richter, 1997;

Mendez et al., 2002). Despite the knowledge accumulated on the composition and regulation of the protein complexes that mediate translational repression and activation of CPE-containing mRNAs, the 3'UTR features that define whether an mRNA is a target for CPEB1-mediated translational repression and how the time and extent of cytoplasmic polyadenylation-dependent translational activation is controlled were still unclear.

In two recent works from our laboratory (Belloc and Mendez, 2008; Pique et al., 2008), a systematic analysis of the combinations of *cis*-acting elements that define, qualitatively and quantitatively, the differential translational control of CPE-regulated mRNAs has been performed (Figure 13). The authors postulate a set of rules that can be used to predict the translational behavior of CPE-containing mRNAs during meiosis:

1. **Translational repression** requires a cluster of at least two CPEs, irrespective of its position along the 3'UTR, where the distance between adjacent CPEs defines the extent of repression with an optimal distance of 10-12 nucleotides.

2. **Translational activation** requires, at least, a single consensus CPE or a non-consensus CPE together with a Pumilio Binding Element (PBE). The CPE must be closer than 100 nucleotides from the Hex, but not overlapping.

3. The distance CPE-Hex determines the **extent of polyadenylation and translational activation** (either "weak" or "strong"), with an optimal distance of 25 nucleotides, which would represent the more relaxed positioning of the CPEB1-CPSF complex interacting respectively with the CPE and the Hex. Other less optimal distances would likely involve bending of the RNA, introducing tension that would destabilize the binding of the CPSF-CPEB1 complex. Additional PBEs or CPEs have a positive effect except for an overlapping CPE, which has a negative effect.
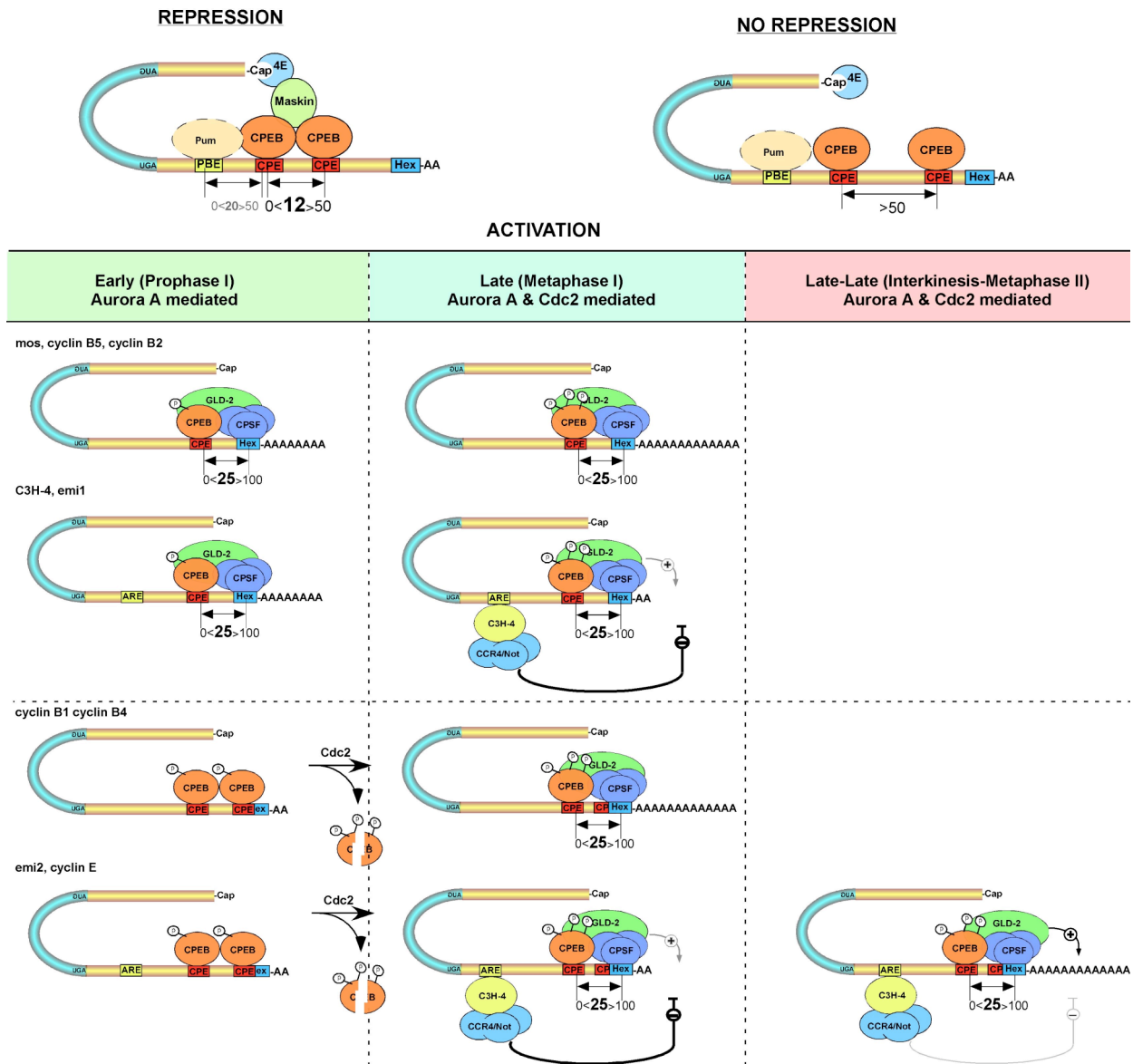
Figure 13. Model for CPE/ARE-mediated translational control. Schematic representation of the *cis*-elements and *trans*-acting factors recruited to the 3'UTR of specific mRNAs, with their covalent modifications. The distances required for translational repression and activation, as well as the time of activation are indicated. Optional factors/elements are displayed with dotted lines. Taken from Belloc et al., 2008.

4. **"Early" or Cdc2-independent cytoplasmic polyadenylation** requires CPE(s) non-overlapping with the Hex, whereas **"Late" or Cdc2-dependent polyadenylation** is driven by at least two CPEs, with one of them overlapping the Hex. This effect is directly mediated by the fact that a CPE overlapping with the Hex has a dominant negative effect in polyadenylation and subsequent translational activation detected only in the presence of high CPEB1 levels. Thus, during the PI to MI transition, where the levels of CPEB1 are very high, multiple CPEs are occupied, including the one overlapping the Hex, preventing the recruitment of CPSF to the Hex. However, after Cdc2 is activated at MI most of the CPEB1 is degraded (Mendez et al., 2002) and stochastically only one CPE would be occupied. Because the non-overlapping CPE has a higher affinity for CPEB1 than the overlapping CPE-Hex that would imply that now the single CPEB1 would be preferentially recruited to CPE and free to recruit CPSF to the Hex and promote polyadenylation.

5. The **presence of AU-Rich Elements** further defines the effect on polyadenylation dictated by the different arrangements of CPEs. During meiosis, these AREs recruit C3H-4, which in turn recruits the CCR4/Not deadenylase complex. The effect of the C3H-4-mediated deadenylation on the target mRNAs is defined by the arrangements of CPEs. Thus, for an mRNA that was polyadenylated by the "early" activation of a "weak" CPE, the deadenylation overrides the polyadenylation inactivating the mRNA after MI. For "early-strong" CPEs polyadenylation is displaced to MI, whereas for mRNAs containing a "late-strong" CPE arrangement, which would be polyadenylated in MI, C3H-4 is not able to completely neutralize the polyadenylation but causes a delay in the poly(A) tail elongation until later meiotic stages, generating a third wave of polyadenylation in interkinesis.

5.2. Sequential waves of polyadenylation and deadenylation drive meiosis

Meiotic progression is a switch-like irreversible process where the successive meiotic phases are discrete states sustained by multiple positive and negative feedback loops that require protein synthesis (Belloc and Mendez, 2008; Ferrell, 2002; Matten et al., 1996; Xiong and Ferrell, 2003) and keep the oocyte from slipping rapidly back and forth between cell cycle phases (Brandman et al., 2005; Ferrell, 2002). The hierarchical translation of specific subpopulations of mRNAs at each meiotic phase is regulated through sequential waves of polyadenylation and deadenylation (Figures 13 and 14).
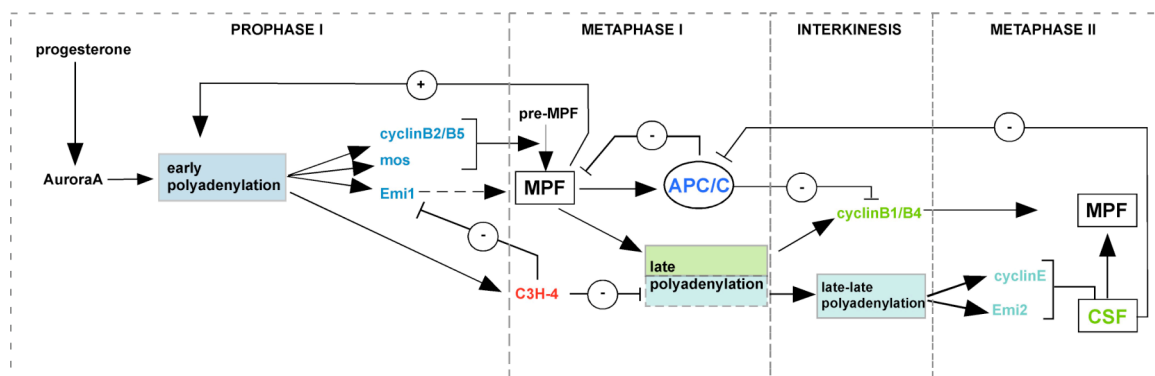
Figure 14. Sequential waves of polyadenylation and deadenylation drive meiosis. Schematic representation showing the sequential waves of polyadenylation and deadenylation driving meiotic progression. The three waves of cytoplasmic polyadenylation are depicted with boxes ("early", "late", and "late-late") and positive and negative feed-back loops are also indicated. Taken from Belloc and Mendez, 2008.

In PI arrested oocytes, the CPE-regulated mRNAs are either inactive with a short poly(A) tail or even actively repressed by a dimer of CPEB1. As the result of progesterone stimulation, CPEB1 is phosphorylated by Aurora A (Mendez et al., 2000a) inducing a first wave of "early" or Cdc2-independent cytoplasmic polyadenylation of mRNAs such as the ones encoding the MPF components Cyclins B2 and B5, the MPF activator Mos and the APC inhibitor Emi1 (Belloc

and Mendez, 2008a; Pique et al., 2008), which are required for the PI-MI transition. The switch-like activation of MPF is sustained by multiple positive feedback loops in the p42 MAPK/Cdc2 network (Ferrell, 2002; Matten et al., 1996), which require protein synthesis (Xiong and Ferrell, 2003) and that also target the re-activation of the "early" wave of polyadenylation through the synthesis and activation of Aurora A (Frank-Vaillant et al., 2000; Howard et al., 1999; Ma et al., 2003; Matten et al., 1996). At the same time, a negative feedback loop, which opposes CPEB1 activity on mRNAs containing both "early-weak" CPEs and AREs, is switch on through the "early" polyadenylation activated translation of C3H-4 mRNA. C3H-4 generates a deadenylation wave that inactivates Emi1 translation in MI allowing for the activation of the APC and the transition to interkinesis. As the result of MPF activation in MI, CPEB1 is sequentially phosphorylated by Cdc2 and Plk1 triggering its partial destruction by the proteasome (Mendez et al., 2002; Reverte et al., 2001; Setoyama et al., 2007) and generating the second wave of "late" or Cdc2-dependent polyadenylation of mRNAs such as the ones encoding Cyclin B1 and B4. These cyclins are required to sustain an intermediate MPF activity during interkinesis, and for the reactivation of MPF in MII (Mendez et al., 2002; Mendez et al., 2000a; Mendez et al., 2000b; Pique et al., 2008; Setoyama et al., 2007). In addition, the partial destruction of CPEB1 together with the synthesis of C3H-4 generates the third wave of "late-late" cytoplasmic polyadenylation. This wave targets mRNAs containing "late-strong" CPEs and AREs, such as the ones encoding the CSF components Emi2 and Cyclin E, which are synthesized during interkinesis. CSF, in turn, inhibits the APC allowing the full reactivation of the MPF, now with Cyclins B1 and B4, and maintaining the oocyte arrested in MII until fertilization takes place (Belloc and Mendez, 2008).

6. CPEB Family members

CPEB was first identified in *Xenopus* oocytes, where it regulates mRNA polyadenylation and translation (Hake and Richter, 1994) and nowadays is a family of proteins of four members: CPEB1, CPEB2, CPEB3 and CPEB4 (Figure 15). The founding member of the CPEB proteins is referred as CPEB1.

Whereas the carboxy-terminal portion is highly conserved among different groups, the amino-terminal portion varies considerable (Figure 15). Unlike mCPEB-1, the other CPEB-like proteins lack PEST sequence and Aurora kinase phosphorylation sites (Theis et al., 2003). In the so-called B region (whose presence depends on alternative splicing) mCPEB2, -3, and -4 possess putative phoshorylation sites for cyclic MP-dependent protein kinase (PKA), CaMKII (Kemp and Pearson, 1990; Kennelly and Krebs, 1991), and p^{70}S6 kinase a growth-factor-stimulated serine threonine kinase that acts on components of the transcriptional apparatus (Gingras et al., 2001a; Pinna and Ruzzene, 1996). Thus, mCPEB2, -3 and -4 could potentially be regulated by these other kinases. The binding motifs seem to be also different. mCPEB3-4 bind an U-rich motif within a secondary RNA structure distinct from CPE (Huang et al., 2006).

The CPEB-related proteins are expressed in many metazoans as well as vertebrates. CPEB homologues have been identified in humans (hCPEB1, hCPEB2, hCPEB3, hCPEB4) (Kurihara et al., 2003; Welk et al., 2001); mice (mCPEB-1, mCPEB-2, mCPEB-3, mCPEB-4) (Gebauer and Richter, 1996; Theis et al., 2003); *Caenorhabditis elegans* (CPB-1, CPB-2, CPB-3, and FOG-

1) (Jin et al., 2001; Luitjens et al., 2000); clams (p82) (Walker et al., 1999); flies (Orb and Orb2) (Lantz et al., 1994); and zebrafish (*Zorba*) (Bally-Cuif et al., 1998). By sequence comparison within and across phyla, it has been shown that CPEB1 and CPEB2-4 constitute different branches of the CPEB family proteins (Mendez and Richter, 2001) (Figure 16). For example, mouse CPEB2 (mCPEB2), mCPEB3 and mCPEB4 isoforms are more similar between them and show less homology to mCPEB1.
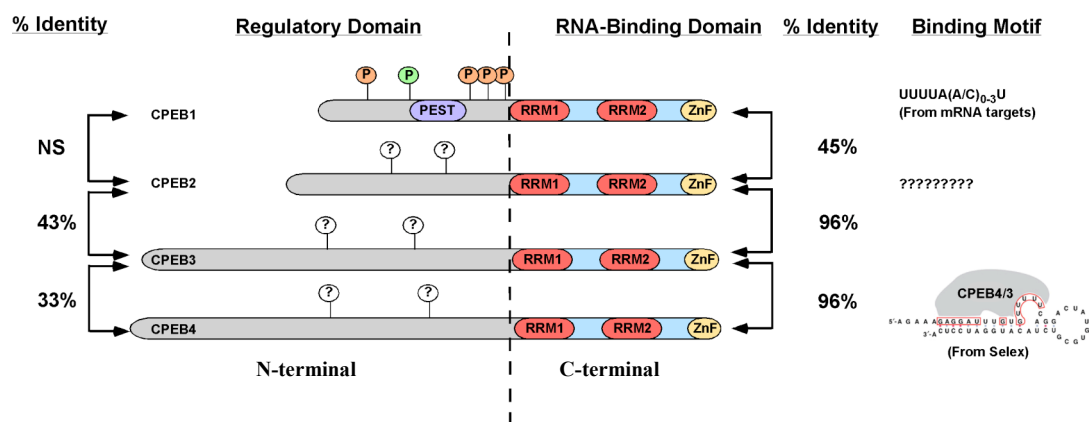
Figure 15. Comparison of CPEB family members. Schematic representation of the CPEB family of proteins. RNA recognition motifs (RRM1, RRM2, red), Zinc-finger domain (ZnF, yellow), PEST box (blue), Aurora A phosphorylation site (P, green) and Cdc2 phosphorylation sites (P, organge) and putative phoshporylation sites are shown (circled question marks). The percentages (%) of identity among the CPEB proteins as well the binding motifs in the target mRNAs are also indicated.

Northern blot analysis of mouse tissues showed that the four CPEBs are expressed in different tissues (Theis et al., 2003). The transcript of mCPEB1 is abundantly expressed in brain and weakly expressed in kidney, lung, heart and oocytes (Gebauer and Richter, 1996; Tay et al., 2000; Theis et al., 2003; Wu et al., 1998); mCPEB2 in testis and brain (Kurihara et al., 2003; Theis et al., 2003); mCPEB3 in heart and brain (Theis et al., 2003); and mCPEB4 in embryos, adult brain, kidney, lung and heart (Theis et al., 2003).
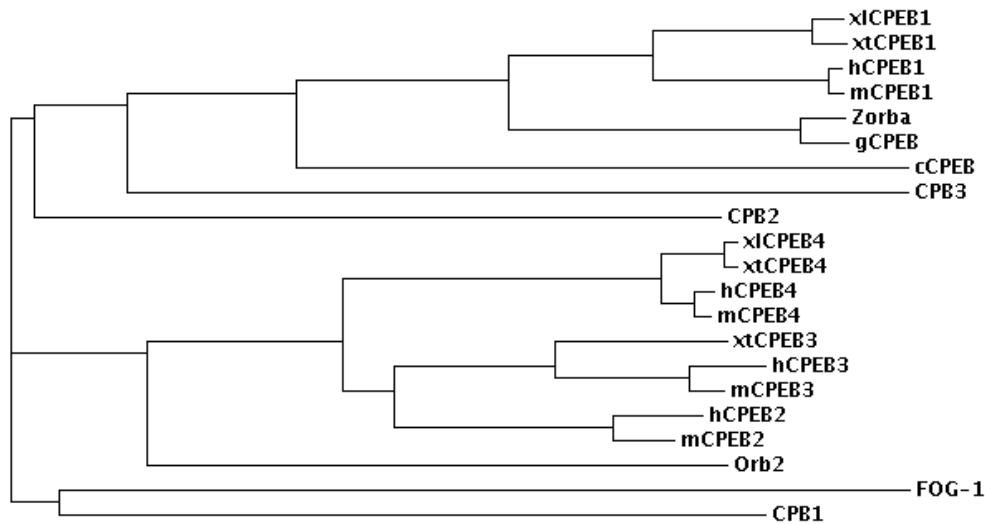
Figure 16. Unrooted tree of CPEB homologues. *Xenopus laevis*: xlCPEB1, xlCPEB4; *Xenopus tropicalis*: xt CPEB1, xtCPEB3, xtCPEB4; human: hCPEB1, hCPEB2, hCPEB3, hCPEB4; mouse: mCPEB1, mCPEB2, mCPEB3, mCPEB4; Zebrafish: Zorba; goldfish, *Carassius auratus*: gCPEB; *Caenorhabditis elegans*: CPB1, CPB2, CPB3, FOG-1; *Drosophila melanogaster*: Orb2; clam, *Spisula solidissima*: cCPEB.

6.1 CPEB1

In mammals, CPE/CPEB1 translational control not only regulates meiosis and development, but also cellular sencescence, axon guidance, synaptic plasticity and long-term memory consolidation (for review see Richter, 2007). In non-stimulated neurons, CPEB1 promotes repression of specific CPE-containing mRNAs before they reach the dendrites (Figure 17). Synaptic stimulation leads to CPEB1 phosphorylation by Aurora A and the subsequently enhanced cytoplasmic polyadenylation and translation of the target transcripts (Atkins et al., 2004; Derkach et al., 2007; Du and Richter, 2005; Huang et al., 2002; McEvoy et al., 2007; Wells et al., 2001) (Figure 17). The newly translated proteins then act as effectors to control experience-dependent modifications of denditric arbor structure, dendritic spine morphology, synaptic connectivity, cerebellar long-term depression (LTD) and hippocampal long term potentiation (LTP) (Bestman and Cline, 2008; Frey et al., 1988; McEvoy et al., 2007; Nguyen et al., 1994). CPEB1 knockout mice have defects in LTP, although LTD is still intact (Alarcon et al., 2004; Berger-Sweeney et al., 2006). CPEB1 may

mediate these brain functions through activity dependent translational activation of CaMKIIα (Welk et al., 2001; Wu et al., 1998) and other mRNAs (Du and Richter, 2005).

About 7% of brain mRNAs are estimated to be targets of CPEB1, although only relatively small number has been confirmed experimentally (Du and Richter, 2005). Apart fom αCaMKII, mRNA targets include key plasticity genes, such as BDNF (Du and Richter, 2005), tPA (Shin et al., 2004), engrailed (Di Nardo et al., 2007), Homer (Wells et al., 2001), and insulin-receptor substrate p53 (McEvoy et al., 2007). Proteins from theses mRNAs, plus many identified in a recent screen of CPE-containing mRNAs (Du and Richter, 2005), are capable of altering synaptic strength and neuronal structure. However, partial phenotypes have been observed in mCPEB1 deficient mice suggesting that lack of mCPEB-1 may be compensated by other CPEB family members, or by other proteins that mediate local protein synthesis.

CPEB1 has also a role in senescence, since mouse embryonic fibroblast cells (MEFs), that normally senescence after several passages in culture, become immortalized when CPEB1 is removed by gene targeting (Groisman et al., 2006). Exogenous CPEB1 restores senescence in the KO MEFS and also induces precocious senescence in wild-type MEFs. CPEB1 can not stimulate senescence in MEFs lacking the tumor suppressors p53, p19ARF or p16 (INK4A); and CPEB1 acts as a translational repressor protein to control myc translation and resulting cellular senescence.

In addition to regulating mRNA translation, CPEB1 also mediates mRNA transport in dendrites. CPEB1 colocalizes with Maskin in CPE-containing RNA particles that are transported along microtubules to dendrites by binding, directly or indirectly, to the motor proteins: kinesin and dynein (Huang et al., 2003). During the early development of *Xenopus laevis*, CPEB1 localizes at the animal pole of oocytes and later on at embryonic spindles and centrosomes. Spindle-localized translational activation of CPE-regulated mRNAS, encoding for proteins with a known function in spindle assembly and chromosome segregation, is essential for completion of the first meiotic division and for

chromosome segregation in *Xenopus* oocytes (Eliscovich et al., 2008). Overexpression of CPEB1 enhances RNA transport, whereas overexpression of a CPEB1 mutant protein, which is unable to associate with kinesin and dynein, inhibits transport (Huang et al., 2003). Disruption of embryonic CPEB1-mediated translational regulation results in abnormalities in the mitotic apparatus and inhibits embryonic mitosis (Groisman et al., 2001; Groisman et al., 2000).
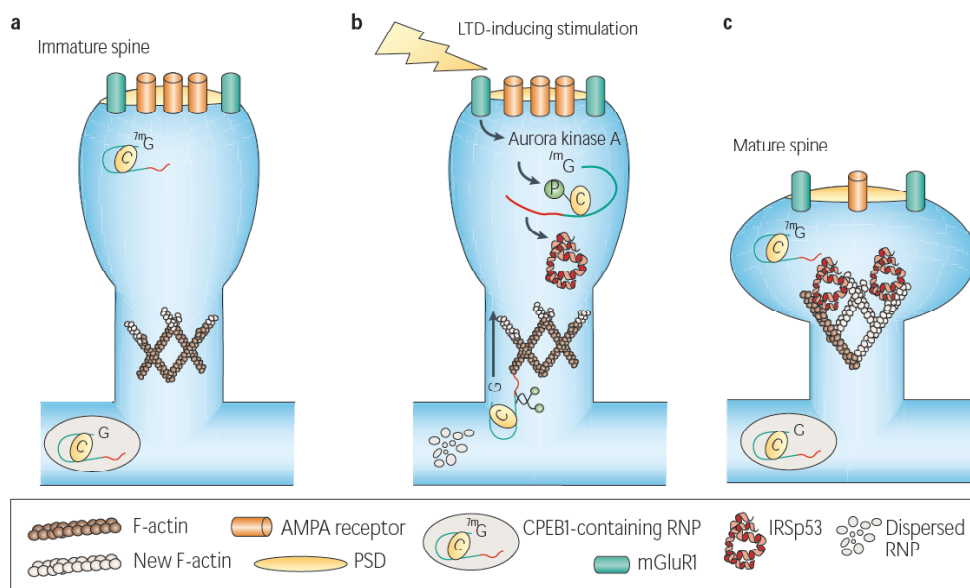
Figure 17. A proposed model for the role of CPEB1-mediated protein synthesis in neurons. (a) CPEB1 is bound to mRNAs in an unphoshporylated state, and represses translation. This complex is localized to dendrites in a ribonucleoprotein particle (RNP). **(b)** CPEB1 is phoshporylated by Aurora kinase A and translation of CPE-containing dendritic mRNAs. CPEB1-bound mRNA would be translocated into the spine head **(c)** A change in spine morphology occurs and CPEB1-mediated long-term differentiation contribute to this process. Taken form (Bramham and Wells, 2007).

6.2. CPEB2

CPEB2 is the least characterized CPEB family member and was first described in germ cells (Kurihara et al., 2003). In spermatogenesis, the length of poly(A) in mRNAs also correlates with translational activity. Since CPEB2 binds to poly(U) RNA oligomers, is abundantly expressed in testis and its expression is

restricted to cytoplasm of haploid spermatids, it has been proposed that CPEB2 could regulate the translation of stored mRNAs during spermiogenesis (Kurihara et al., 2003).

6.3. CPEB3 and CPEB4

CPEB3-4 are expressed in partially overlapping regions of the brain and are found in dendrites. CPEB3 colocalizes with a synaptic marker whereas CPEB4 does not. SELEX (systematic evolution of ligands by exponential enrichment) analysis shows that CPEB3-4 recognize a secondary structure and interact with uridines that are single-stranded as well as double-stranded stems (Huang et al., 2006). CPEB3 represses translation of a reporter RNA in transfected neurons and stimulates translation in response to NMDA (Huang et al., 2006). Although the mechanism of translational control by CPEB3 is not yet known, it does not bind CPSF nor require an AAUAAA *cis*-element, implying that, in contrast to CPEB1, it does no promote cytoplasmic polyadenylation (Huang et al., 2006). CPEB3 has also been implicated in LTP in mouse hippocampus (Theis et al., 2003) and in short-term memory performance and neural processes and circuits related to emotional memory, such as the amygdale (Vogler et al., 2009).

A recent work in our laboratory has characterized a role in cytoplasmic polyadenylation of CPEB4. Tethering experiments to reporter mRNAs in *Xenopus* oocytes showed that CPEB4 mediated translation stimulation by cytoplasmic polyadenylation, and gel shift assays showed binding of CPEB4 to CPEs (Novoa et al., submited). CPEB4 together with CPEB1 also regulates cytoplasmic polyadenylation in a mitotic cell cycle specific manner, affecting mitotic entry and cell proliferation (Novoa et al., submited). The mitotic defect of the double knock down of CPEB1 and CPEB4 suggested compensatory functions between the two proteins. Moreover CPEB4 shows higher levels in colonic tumours than in normal tissues (Saaf et al., 2007) and it has also been

described as a putative glucocorticoid-response gene (Wang et al., 2004). Cells microinjected with CPEB4 show the ability to inhibit the S phase entry through AP-1 dependent activity (Yamamoto et al., 2006). Although the above described, CPEB4 targets and function are currently unknown.

6.4. CPEB orthologues

A. *Drosophila* Orb

In *Drosophila* two CPEB homologues have been described Orb and Orb-2.

Drosophila Orb is 62% identical to CPEB1 and is required for proper localization and translation of gurken mRNA in dorsoventral axis formation and for translation of oskar mRNA in anteoposterior axis formation (Castagnetti and Ephrussi, 2003; Chang et al., 1999; Chang et al., 2001).

Orb2 is the orthologue of CPEB2-4 and also contains a poly-glutamine sequence in the N-terminal region. A null mutant for Orb2 is embryonic lethal, but it can be rescued by expressing full length Orb2. Flies expressing Orb2 without the poly-glutamine region are normal in learning and short-term memory but defective for maintaining long-term memory courtship behaviour. The long-term memory can be only rescued by expression full length Orb 2 during or shortly after the training session. This suggests that the poly-glutamine sequence is specifically required for long term memory but not other general functions (Keleman et al., 2007).

B. *C. elegans*

In *C. elegans* four CPEB homologues have been described: CPB-1, CPB-2, CPB-3 and FOG-1 (Hasegawa et al., 2006; Luitjens et al., 2000)

CPB-1 protein can be detected only in meiotic prophase cells undergoing spermatogenesis. RNAi directed against cpb-1 blocks spermatogenesis at the

primary spermatocyte stage (Barton and Kimble, 1990; Luitjens et al., 2000). *Cpb-2* mRNA is enriched in spermatogenic cells and is virtually absent in oogenic cells (Luitjens et al., 2000). CPB-3 is structurally most similar to the CPEB members implicated in oogenesis in *Drosophila*, Clam, Zebrafish, *Xenopus*, and mouse (Mendez and Richter, 2001). CPB-3 protein is highly expressed in the early meiotic region of a hermaphrodite germline and seems to be involved in at least two decisions of the germ cell fates: the sperm/oocytes switch and the mitosis/meiosis decision (Hasegawa et al., 2006; Stebbins-Boaz et al., 1996; Tay and Richter, 2001). FOG-1 protein has two isoforms. The long isoform of FOG-1 protein, which is the functional isoform, is expressed solely in spermatogenic cells and is involved in spermatogenesis as well as in proliferation of germ cells (Luitjens et al., 2000; Thompson et al., 2005). FOG-1 mutants makes functional oocytes but no sperm (Barton and Kimble, 1990; Luitjens et al., 2000), thus FOG-1 appears to mark germ cells destined for spermatogenesis (Lamont and Kimble, 2007). It has been proposed that FOG-1 may also bind CPEs (Jin et al., 2001) and promotes early larval germline proliferation in a dose-dependent manner: low FOG-1 promotes proliferation, whereas high FOG-1 promotes spermatogenesis (Thompson et al., 2005).

C. *Aplysia*

In *Aplysia*, a neuron-specific CPEB isoform is upregulated locally at activated synapses and is required for the maintenance of long-term facilitation, possibly through a prion-like mechanism (Si et al., 2003a; Si et al., 2003b). mCPEB-3 shares three common features with *Aplasya* CPEB (Liu and Schwartz, 2003; Theis et al., 2003): (1) a glutamine-rich region in the N-terminal domain; (2) they do not contain Aurora kinase phosphorylation sites; (3) their expression is up-regulated after neuronal stimulation.

Objectives

1. Characterization of other CPEB-family members in *Xenopus laevis*: CPEB4

2. Characterization of CPEB4 expression regulation during oocyte maturation and meiotic progression

3. Identification of CPEB4 targeted mRNAs and determination of CPEB4 function during meiotic progression.

Results

1. Identification of other CPEB family members in *Xenopus laevis*

At the beginning of this work, four CPEB homologues had been identified in humans (Kurihara et al., 2003; Welk et al., 2001) and mice (Gebauer and Richter, 1996; Theis et al., 2003), but in *Xenopus laevis* only one CPEB protein (CPEB1) was identified (Hake and Richter, 1994).

Based on the high homology of the CPEB family of proteins between species, CPEB2-4 mouse cDNA sequences were used as a reference in our blast search of databases from *Xenopus laevis* ESTs to identify possible candidate sequences codifying for other proteins of the CPEB family in *Xenopus*.

This search yielded two groups of multiple overlapping ESTs (Figure 18). The first group were ESTs corresponding to the Carboxyl-terminal part of a protein, which due to the high homology between CPEB2-4 (96%) was not possible to ascribe to any particular CPEB. The other group of ESTs produced a complete ORF of the putative *Xenopus laevis* CPEB4.
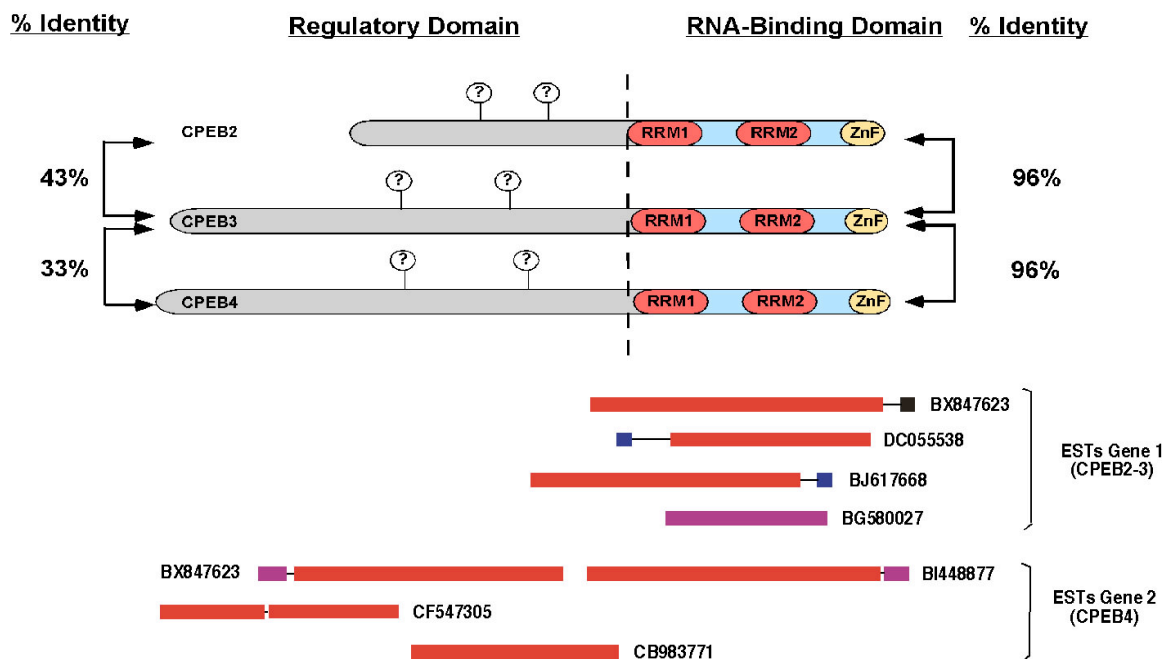
Figure18. Identification of _Xenopus laevis_ CPEB4. Schematic representation of the screening in an EST _Xenopus laevis_ database to identify CPEB-like proteins. The positive matching sequences are indicated.

1.1. Identification of _Xenopus laevis_ CPEB4

One of the ESTs from the group that codified for the putative CPEB4 was available and ordered through IMAGE GENE consortium (IMAGE: 4435269). This clone contained a sequence corresponding to a putative open reading frame (ORF) of 2Kb and a 3'UTR of 365 nucleotides (nt), which were subcloned in a pGEMT-Easy vector and sequenced.

A. The CPEB4 Open Reading Frame

The ORF codified in the EST corresponded to a truncated protein, with high homology in the N-terminal region to the mouse, human and _Xenopus tropicalis_ CPEB4. Due to the high homology between _Xenopus laevis_ and _tropicalis_, the
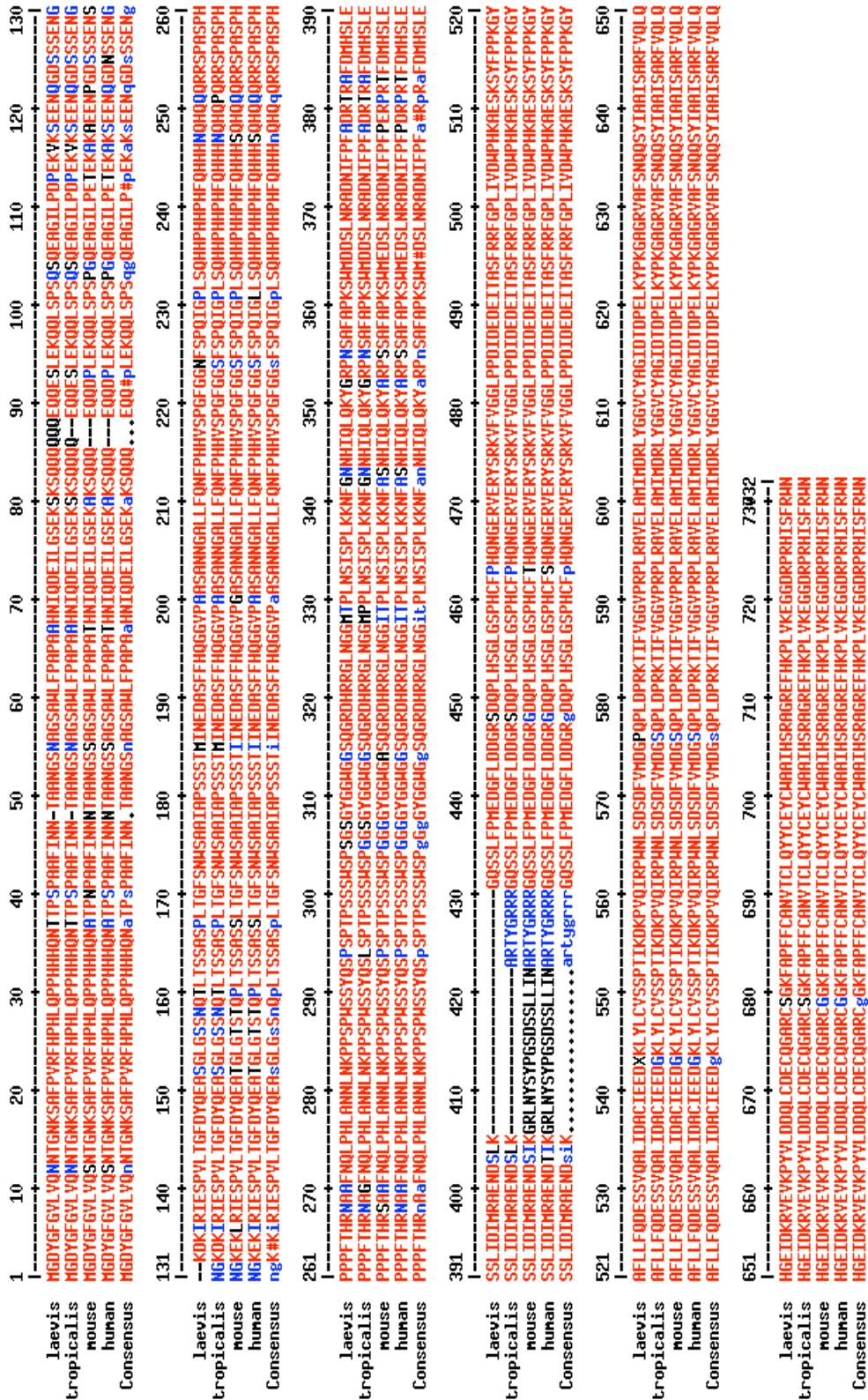
Figure 19. Alignment of CPEB4 homologues. CPEB4 is a highly conserved protein. The *Xenopus laevis* protein shows very high homology with its homologue in *Xenopus tropicalis* (98%), mouse (93%) and human (93%) (red, high consensus; blue, low consensus)

EST sequence was used as a reference in a second blast search in a *Xenopus tropicalis* database, and specific primers flanking the ORF were designed to amplify the endogenous *Xenopus laevis* CPEB4.

Total RNA from prophase-I (PI) arrested oocytes was used to perform oligo-specific reverse transcription, and a gene-specific PCR product was obtained was cloned and sequenced. Comparison of the endogenous protein with the ORF codified in the EST revealed a deletion of 82 nt in the EST, that produced a miss-reading and an altered reading frame, leading to a premature stop codon and as a result the production of a truncated protein. The endogenous CPEB4 mRNA encoded a 705-aminoacid polypeptide that showed 90% identity to the mouse, human and *Xenopus tropicalis* CPEB4 proteins (Figure 19). CPEB4 mRNA was detected by Northern blot analysis in stage VI, MI and MII oocytes .

B. The CPEB4 3' Untranslated Region

The 3'UTR contained in the EST corresponds to a 365 nt sequence rich in T/A stretches. Since the cDNA sequence contained in the EST was not complete, we decided to clone the endogenous full length 3'UTR sequence .

Total RNA from stage VI oocytes was obtained to amplify the endogenous *Xenopus laevis* 3'UTR CPEB4. Oligo(dT)-primed reverse transcription and PCR failed to amplify the endogenous 3'UTR, most likely due to high percentage of T/A stretches in the sequence. Thus, specific primers were designed based on the high homology with *Xenopus tropicalis* and a band of ~600 bp was obtained, cloned in the pGEM-T Easy Vector and sequenced. The CPEB4 3'UTR cloned contained 3 potential Hexanucleotides, 5 potential CPEs and 3 long AU-rich stretches with potential AREs (Figure 20).

a)

GCAACCCATTGCTTGGCTCACTTTGCAAGCATCCAAATAAGTGCACTCTTC
TGTTCTCTTAATCTCCCTCCCTCCTACCATCTTTAGGAACGCATGTCCTCTT
GTTGTAGTCTGTATTTTAACAATAGTATAATGAAAGAATGGCCGACACCATA
GGTATTTTGTAGAGTCTTGTGTCATTGAGAACTGTATTGGAACGCCTCTTGT
TCATAACAATATCACTCTGATGCATGCAAGTTTCATGCTGTCCTTTTCAAATA
GCAAGGGAGCAGGGGGACTGCAAGCCTTATTATTATTATTATTATAATAATA
TTTTTTTTTATATATAATTTTATCGGAAGAGCAAAGCTTAAAAGCAAGAAAAC
TATTGAGCGAGAAATAGAGTATTTTTTTTTTTATTAAATTATTGTTAAATATAAA
GTAACGTAAGAATACTGTTAATGTAACCATGCAACCACAATAACACTATGGG
TTTGTTGAACAGATTTTTTTTTTTAAATATATATATATATATTATTAGAGGGGAT
TTTTTAGCATCTATTTATTGTTTGTAATTTTTCCAGACTTTATTGAAAATAAAA
AGTCAGCAGTTGCCTATTAAAAGCATTAAGCA

b)

Figure 20. 3'UTR of *Xenopus laevis* 3'UTR. (a) Nucleotide sequence of the 3'UTR of Xenopus laevis obtained by RT-PCR. Putative *cis*-acting elements identified are shown: AREs (PolyU tracts; yellow bold letters), CPEs (consensus, red bold letters; non-consensus, pink bold letters); and hexanucleotides (blue boxes) **(b)** Schematic representation of *cis*-acting elements in the 3'UTR: Poly U tracts (orange ovals); Cytoplasmic polyadenylation elements (consensus CPEs, red hexagons; non-consensus CPEs, pink hexagons); Hexanucleotide (Hex, blue rectangle).

2. Expression of CPEB4 in meiosis and early embryogenesis

A polyclonal antibody raised against the human CPEB4 protein was available in the laboratory (Novoa et al., submited). When tested, this antibody only recognized a single band, with low affinity, in oocytes microinjected with a myc-tagged-*Xenopus* CPEB4 mRNA (Figure 21a).

Oocytes stimulated with progesterone and collected at different meiotic phases were also analysed with hCPEB4 antibody to detect endogenous CPEB4. Metaphase I occurred around 3 hours after progesterone treatment, and it was scored by the appearance of a white spot in the pigmented animal pole of the oocyte, as a result of the displacement of the cortical pigment granules upon nuclear and germinal vesicle breakdown (GVBD). The human antibody did not detect any band corresponding to the endogenous protein in any meiotic phase (Figure 21b).

Xenopus-specific antibodies were then produced against the N-terminal (from nucleotide 1 to 612; aminoacids 1-204) and C-terminal (from nucleotide 906 to 2115; aminoacids 403-705) regions of *Xenopus* CPEB4. Two antibodies against each region were produced, but only one of the antibodies against the N-terminal region of CPEB4 detected a band corresponding to the endogenous CPEB4 *Xenopus laevis* protein.
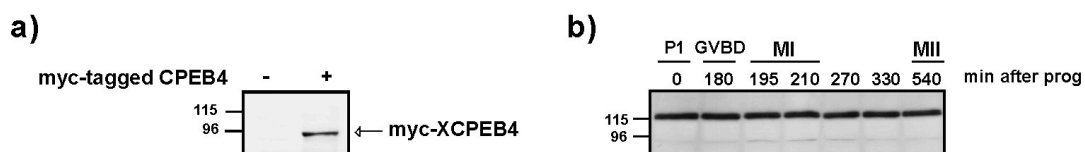
Figure 21. **hCPEB4 antibody does not recognize *Xenopus* CPEB4 protein. (a)** Extracts from oocytes non-injected and injected with a mRNA codifying for myc-tagged-*Xenopus* CPEB4 were analysed by Western Blot with anti-humanCPEB4. Arrow indicates the overexpressed *Xenopus* CPEB4 protein. **(b)** *Xenopus* oocytes stimulated with progesterone (prog) were collected at the indicated times and endogenous expression of *Xenopus* CPEB4 was analysed by western blotting with hCPEB4 antibody. The meiotic phases of the oocyte are indicated (PI, prophase-I; GVBD, germinal vesicle breakdown; MI, metaphase-I; I, interkinesis; MII, metaphase-II).

Expression of the endogenous CPEB4 protein was analyzed by Western Blot in a meiotic time course with this antibody. CPEB4 was present at very low levels (sometimes undetectable) in PI-arrested oocytes, and gradually accumulated in response to progesterone. CPEB4 reached maximal levels in the second meiotic division (Figure 22a), correlating with CPEB4 mRNA being translated from a maternal transcript during oocyte maturation.

Interestingly, CPEB4 followed an expression pattern complementary to CPEB1 during meiotic progression, which was highly expressed in PI-arrested oocytes and also in MI, but was degraded and virtually disappeared in MII-arrested oocytes (Figure 22b). Contrary to CPEB1 (Hake and Richter, 1994; Mendez et al., 2002), CPEB4 is not degraded during meiotic progression as seen upon microinjection of a myc-tagged-*Xenopus*CPEB4 mRNA into oocytes subsequently treated with progesterone and collected at different time points of the meiotic progression to analyse CPEB4 state (Figure 22c). CPEB4 levels also remained stable after fertilization and even after the mid-blastula transition (Figure 22d).

We concluded that CPEB4 is encoded by a maternal mRNA and that CPEB4 protein accumulates in the second meiotic division.

A SDS-PAGE mobility shift of CPEB4 protein was observed at later stages of the meioic progression, suggesting a possible posttranslational regulation by phosphorylation. Accordingly, overexpressed myc-tagged-CPEB4 shows a mobility change in response to progesterone without any effect on its stability (Figure 22c), and hCPEB4 is also phosphorylated when injected in oocytes stimulated with progesterone (Novoa et al., submited). Analysis of the CPEB4 sequence revealed putative phosphorylation sites for PKA, CaMKII and S6 kinases (Theis et al., 2003).
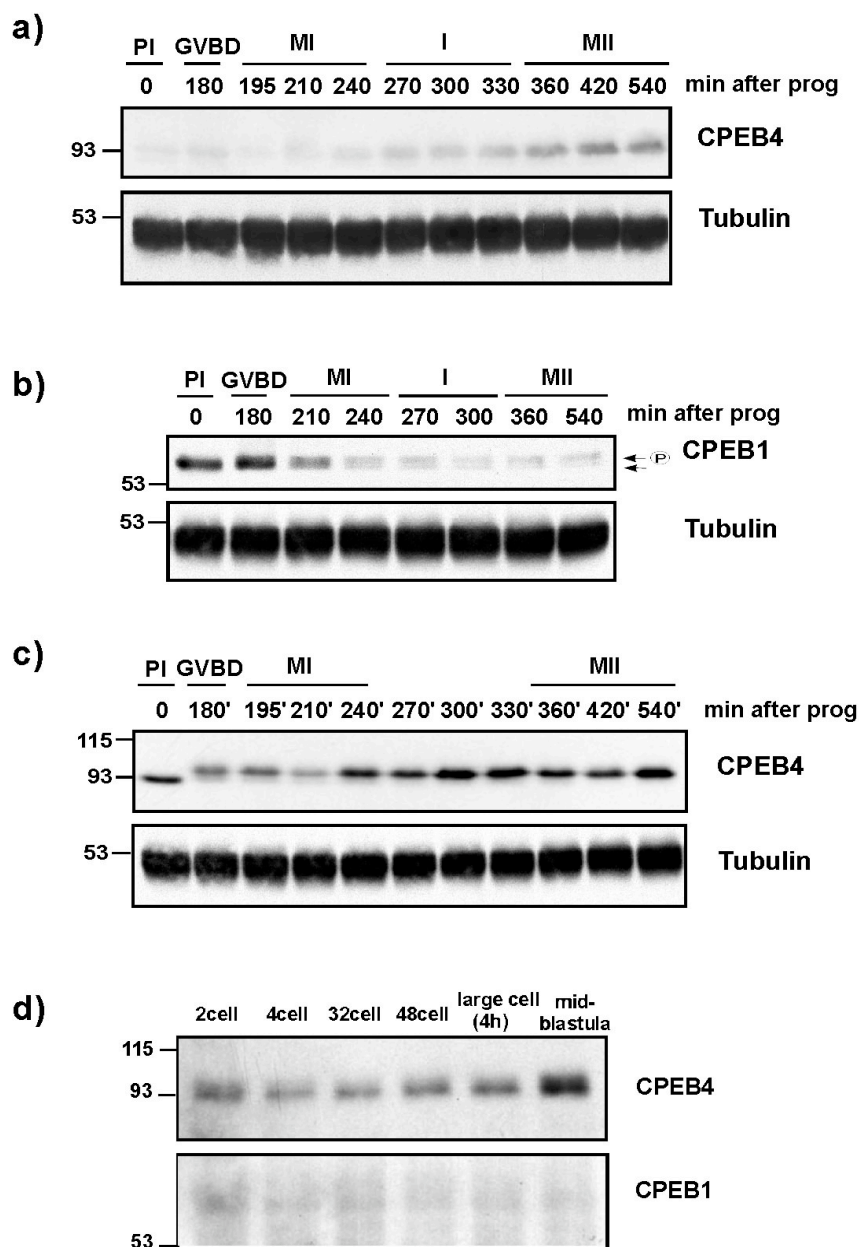
Figure 22. CPEB4 accumulates during the second meiotic division. *Xenopus* oocytes stimulated with progesterone (prog) were collected at the indicated times. Endogenous expression of **(a)** CPEB4 and **(b)** CPEB1 were analysed by western blotting with specific *Xenopus* antibodies. Tubulin is used as a loading control. The meiotic phases of the oocyte are indicated (PI, prophase-I; GVBD, germinal vesicle breakdown; MI, metaphase-I; I, interkinesis; MII, metaphase-II). **(c)** In vitro transcribed and polyadenylated mRNA encoding myc-tagged-*Xenopus*CPEB4 mRNA was injected into oocytes, treated with progesterone (prog) and collected at the indicated times. CPEB4 levels were analysed by western blotting using anti-CPEB4 and anti-Tubulin antibodies (2 oocyte equivalents were loaded per lane). **(d)** Fertilized eggs were collected at 2 cells, 4 cells, 32 cells, 48 cells, large cell (4 h) and mid-blastula stages and analyzed by western blotting using anti-CPEB4 and anti-CPEB1 antibodies.

3. Translational regulation of CPEB4 mRNA

3.1. CPEB4 mRNA polyadenyation

Because the expression pattern of the protein and the arrangement of *cis*-acting elements were consistent with this factor being encoded by a maternal mRNA, silenced in PI-arrested oocytes and translationally activated in response to progesterone (Belloc and Mendez, 2008; Pique et al., 2008), the poly(A) tail length of the endogenous CPEB4 mRNA was measured using a PCR-based assay (Charlesworth et al., 2004) (Figure 23).

A DNA oligonucleotide was ligated to the 3'-end of total RNA extracted from *Xenopus laevis* stage VI, MI and MII oocytes. A complementary primer was used to initiate cDNA synthesis by reverse transcription, and a gene-specific primer at the beginning of CPEB4 3'UTR was used to prime second-strand synthesis. The PCR products were resolved in a 2% agarose gel and detected by Southern blot. The CPEB4 transcript, which contained a short poly(A) tail in PI oocytes, was polyadenylated in metaphase I (MI) and partially deadenylated in the second meiotic arrest in metaphase II (MII) (Figure 23).

To assess whether the identified *cis*-acting elements of *Xenopus* CPEB4 3'UTR (CPEs, AREs and Hexs) mediated the polyadenylation behaviour observed for the endogenous CPEB4 mRNA, *in vitro* transcribed radiolabeled probes corresponding to the WT or mutant variants of CPEB4 3'UTR were microinjected into oocytes (see Appendix I for sequences of the 3'UTR

constructs). Oocytes were treated with progesterone and collected at different time points of the meiotic progression. Total RNA was extracted and resolved in a denaturing Acril-Urea gel. Autoradiography of the gel revealed the polyadenylation status of each probe. The WT probe (CPEB4 3'UTR) displayed the same polyadenylation pattern than the endogenous CPEB4 mRNA, being polyadenylated in MI and then partially deadenylated during interkinesis and in MII (Figure 24a). As a control, we microinjected the 3'UTR of cyclin B1 (cyclin B1 3'UTR), which contained CPEs but not AREs (Belloc and Mendez, 2008) and was polyadenylated in MI remaining polyadenylated thereafter.
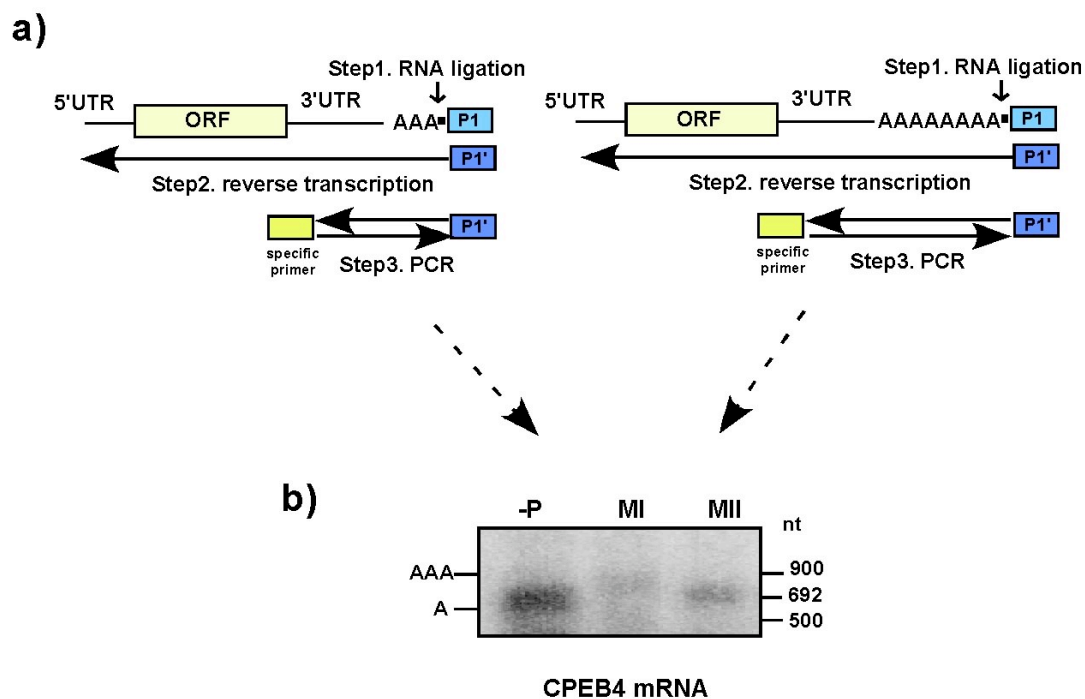
Figure 23. Endogenous CPEB4 is polyadenylated in MI and partially deadenylated in MII. **(a)** Schematic representation of PCR-based assay used to measure polyadenylation of endogenous mRNAs (Charlesworth et al., 2004) **(b)** Total RNA extracted from oocytes untreated (-P) or incubated with progesterone and collected at metaphase-I (MI) and metaphase-II (MII) were analysed by RNA-ligation-coupled RT-PCR followed by southern blot hybridization with a labelled probe for the 3'UTR of CPEB4 mRNA. The PCR products derived from the polyadenylated and non-polyadenylated CPEB4 mRNA are indicated.

Progesterone-induced polyadenylation of CPEB4 and cyclin B1 were abrogated when the putative CPEs were inactivated by point mutations (CPEB4 3'UTR-CPEs and Cyclin B1 3'UTR-CPEs). When the UTRs with the inactivated CPEs were microinjected with a long poly(A) tail [CPEB4 3'UTR-CPEs(polyA) and cyclin B1 3'UTR-CPEs(polyA)], the CPEB4 derived probe, but not the cyclin B1 derived probe, was specifically deadenylated after MI (Figure 24a).

These results indicate that CPEB4 is a maternal transcript stored inactive in PI arrested oocytes, polyadenylated in MI by CPEB1 and partially deadenylated between MI and MII.

Recently, our lab has described the role of C3H-4 during the meiotic progression in *Xenopus laevis* oocytes. C3H-4 is an ARE-binding protein that is synthesized from a maternal mRNA activated in the first wave of cytoplasmic polyadenylation and that modulates deadenylation of ARE-containing mRNAs after GVBD (Belloc and Mendez, 2008).

To test if C3H-4 could be mediating the deadenylation observed in the mRNA of CPEB4, oocytes were depleted of C3H-4 using an antisense oligo targeting the 3'UTR of C3H-4 mRNA. Then, oocytes were also microinjected with probes corresponding to CPEB4 3'UTR WT without a poly(A) tail or with CPEB4 3'UTR with the inactivated CPEs with a synthetic poly(A) tail. The deadenylation of the WT probe was partially prevented in C3H-4 depleted oocytes, both when the WT UTR-probe was injected deadenylated or when the CPE-mutated UTR-probe with a synthetic poly(A) tail was injected (Figure 24b). Therefore, we concluded that C3H-4 is implicated in the partial deadenylation of CPEB4 seen in the second meiotic division.

Figure 24. CPEB4 mRNA is polyadenylated in MI and partially deadenylated in MII. (a) Oocytes were injected with the indicated radiolabeled 3'UTRs. Total RNA was extracted from oocytes collected at the indicated times after progesterone stimulation and analyzed by gel electrophoresis followed by autoradiography. Schematic representation of the 3' UTRs is shown: CPEs as red hexagons, Hexanuclotide as blue boxes, PBEs as rhombus, putative AREs elements as yellow ovals. CPE point mutations are indicated with a cross. **(b)** Oocytes were injected with C3H-4 antisense oligonucleotide (asC3H-4) or C3H-4 sense oligonucleoitde (control), and the indicated radiolabeled 3'UTRs. Total RNA was extracted from oocytes collected at the indicated times after progesterone stimulation and analyzed as in **(a)**.

a)

CPEB4

cyclin B1

CPEB4 -CPEs

cyclin B1-CPEs

CPEB4 -CPEs (PolyA)

cyclin B1 -CPEs (PolyA)

b)

3'UTR CPEB4

CPEB4 (PolyA)

control   asC3H-4

control   asC3H-4
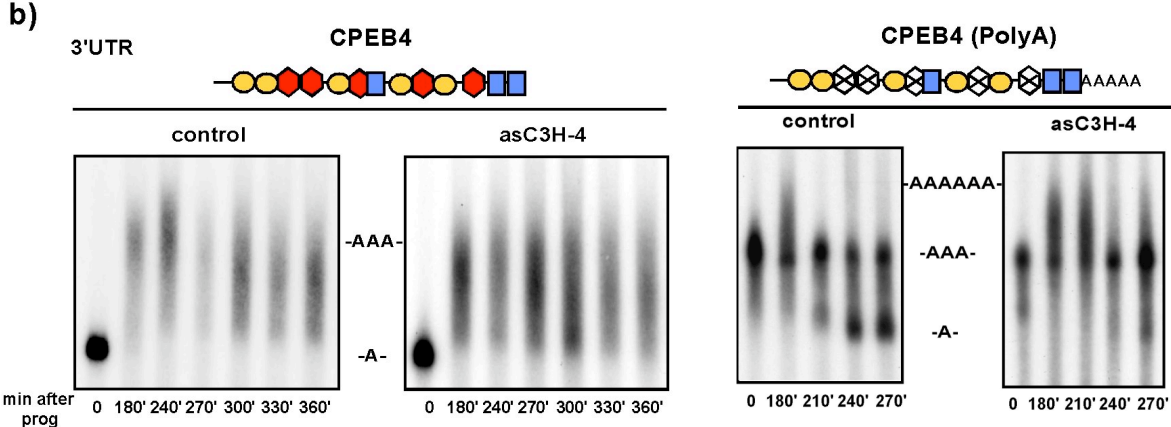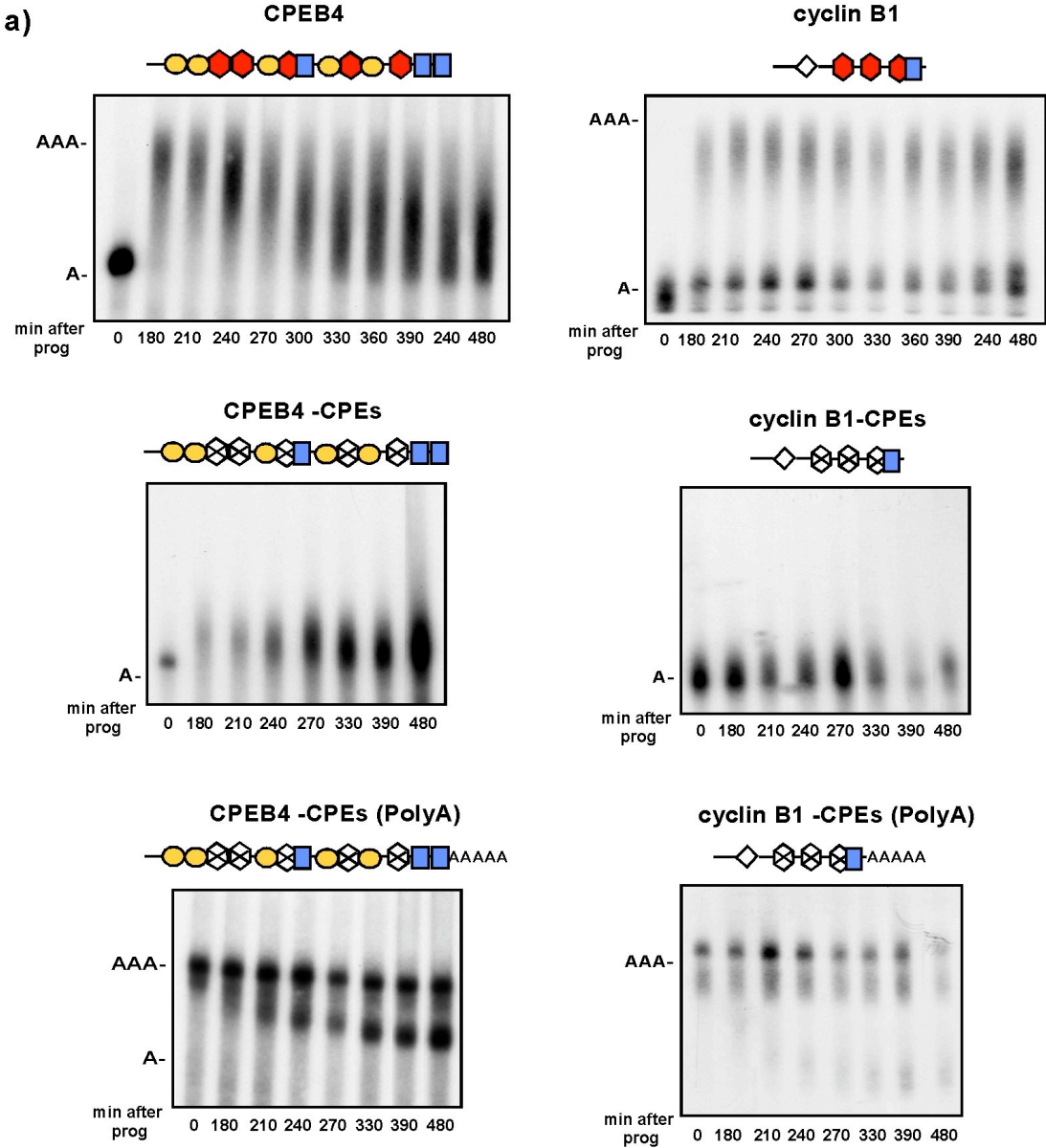
3.2. Translational control of CPEB4

To determine whether the observed changes in poly(A) tail length were reflected at the translational level, chimaeric mRNAs codifying for the Luciferase ORF followed by control random 3'UTR, CPEB4 3'UTR, mutant CPEB4 3'UTRs or cyclin B1 3'UTR, were microinjected into oocytes (see Appendix I for sequences of the 3'UTR constructs).

Oocytes were treated with progesterone and pools of 8 oocytes were collected at different time points during meiotic progression and analyzed for Luciferase activity in each condition. Both CPEB4 and cyclin B1 3'UTRs repressed translation in PI-arrested oocytes, compared with control 3'UTR (Figure 28a). After progesterone stimulation, both CPEB4 and cyclin B1 3'UTR mediated translational activation. But, even though the accumulation of Luciferase followed the same kinetics at early points (MI), the increase in Luciferase activity generated by CPEB4 3'UTR chimerical construct was slowed down during second meiotic division, whereas for cyclin B1 3'UTR chimerical mRNA continued increasing at a similar rate during the whole length of meiosis, until the MII arrest (Figure 25a). These translational kinetics were in agreement with the fact that cyclin B1 3'UTR remained polyadenylated during the two meiotic divisions and CPEB4 3'UTR was partially deadenylated in the second meiotic division (Figure 24).

To determine the contribution of the different *cis*-acting elements present in the CPEB4 3'UTR to the translation of the reporter, chimaeric mRNAs with point mutations in the indicated CPEs and Hexanucleotides were microinjected into oocytes and luciferase activity was analyzed after incubation in the presence or absence of progesterone.

The translational repression was dependent on the cluster of two consensus CPEs; if CPE 1 and 2 remain WT, the mutation of others CPEs slightly affect repression of the construct (constructs -C3, -C4, -C34, -C5) (Figure 25b). Thus, translational repression was most likely mediated by a CPE dimer, as shown

before for cyclins B 3'UTR (Pique et al., 2008). On the other hand, translational activation upon progesterone addition was sustained by either of the two more 3' CPEs (CPE 4 or CPE5) (constructs -C5, -C4, -C34). Mutation of the CPE 3 did not affect neither the repression nor the activation of the construct compared with the WT.

The second hexanucleotide was also essential for translational activation (construct –H2), although mutations of the third hexanucleotide (construct –H3) reduced significatively the translational activation (Figure 25b).

Thus, translational inactivation was only accomplished by the mutation of all the CPEs present in the 3'UTR or mutation of the second Hexanucleotide. The activation required the hexanucleotide and the nearby CPE, in agreement with being mediated by early cytoplasmic polyadenylation (Pique et al., 2008).
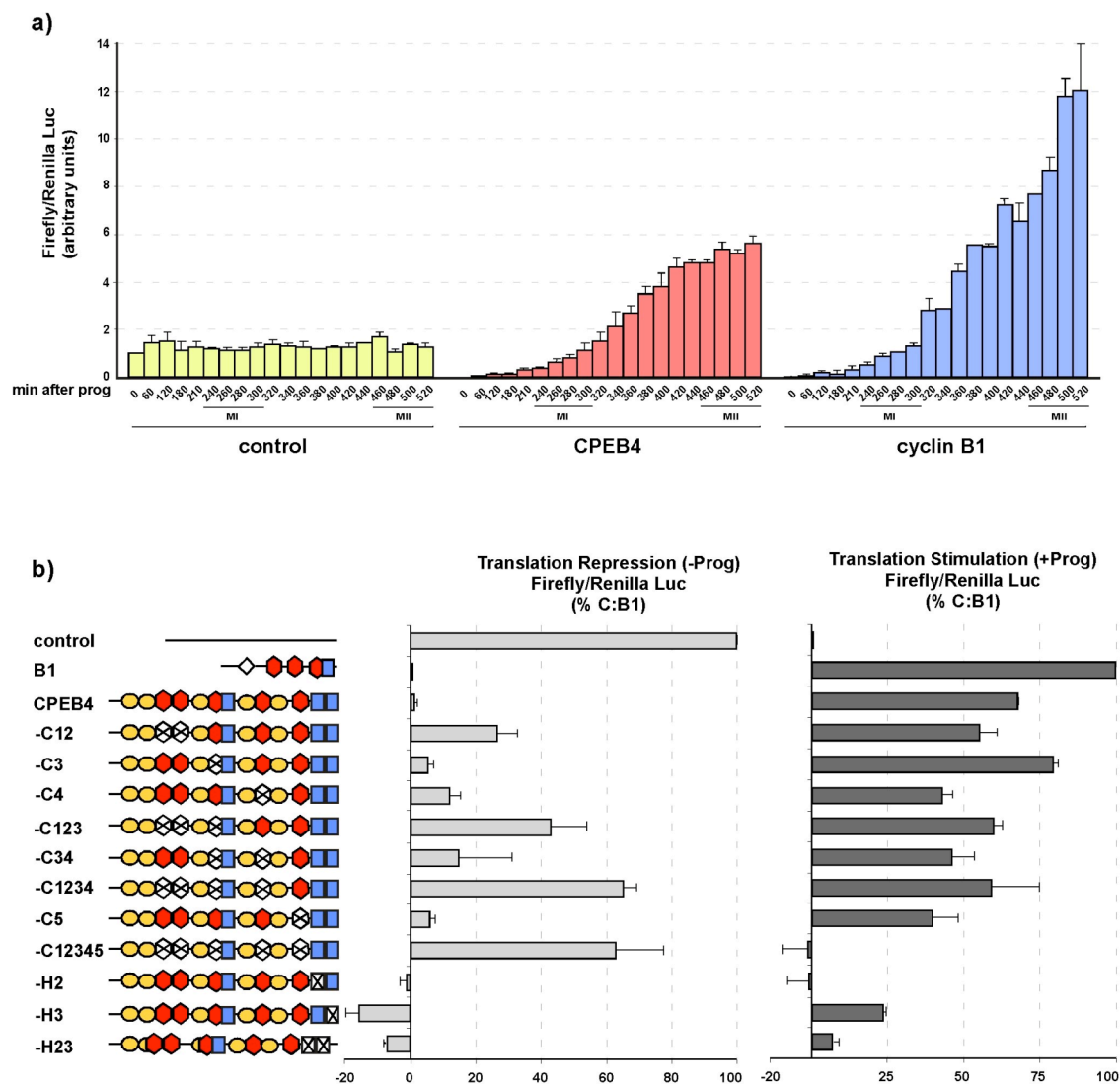
Figure 25. CPEB4 is translationally activated by CPEB1 during meiotic maturation. The indicated in vitro transcribed Firefly luciferase chimerical mRNAs were co-injected into oocytes together with Renilla luciferase as a normalization control. **(a)** Firefly luciferase ORF fused to a control 3'UTR of 470 nucleotides (control); cyclin B1 3'UTR wild-type (cyclin B1 3'UTR) and CPEB4 3'UTR wild-type (CPEB4). Oocytes were stimulated with progesterone, collected at the indicated times and the luciferase activities were measured. Data are mean±s.d. (n=4). **(b)** The indicated Firefly luciferase-3' UTR variants were injected in oocytes. Oocytes were then incubated in the absence (Repression) or presence (Activation) of progesterone and the luciferase activities determined after six hours. The percentage of translational repression in the absence of progesterone (left panel) was normalized to control (100% translation) and to the fully repressed B1 (0% translation). The percentage of translation stimulation was normalized to control (0% stimulation) and B1 (100% simulation). Data are mean±s.d. (n=5). A schematic representation of the 3' UTR, as in Figure 24, is shown.

4. Meiotic function of CPEB4

Meiotic resumption can be assessed at different levels, the first one and most evident is the oocyte external morphology. Metaphase I or GVBD can be scored by the appearance of a white spot in the animal pole of the oocyte, metaphase II can also be scored by a rearrangement in the white spot pigments; the white spot is reduced in size and a second dark ring appears. Chromosomal dynamics are the most direct method to follow meiotic progression and can be assessed by DNA staining and observation under the microscope. Finally, meitotic progression can be followed by a biochemical marker, MPF activity (Cdc2 kinase activity) that can be measured *in vitro* by the phosphorylation of histone H1 using oocyte extracts as the source of kinase. Cdc2 activity increases abruptly upon MI entry, then drops to low levels during the MI-AI transition, required for replication inhibition (Iwabuchi et al., 2000), and increases again reaching a maximum stable level at MII, held during the CSF-mediated arrest (Huchon et al., 1993).

4.1. CPEB4 is needed to complete meiosis

To determine if CPEB4 was required for meiotic progression, we inhibited its expression and analyzed the effect(s) of CPEB4 ablation in the meiotic phase transitions.

Oocytes were independently injected with four different CPEB4 antisense oligonucleotides targeting the ORF (as1 and as4), the 3' (as2) or the 5'UTR (as3), and a control sense oligonucleotide. After overnight (o/n) incubation at 18ºC, oocytes were treated with progesterone to trigger maturation. At the time that control oocytes reached MII, 4 hours after GVBD, the oocytes were collected and analyzed as indicated. Successful depletion of CPEB4 protein was confirmed by western blot (Figure 26a).

The effect of the antisense microinjections in oocytes was first assessed morphologically. While the MI white spot appeared to be the same in control oocytes than in antisense microinjected oocytes (data no shown), the MII white spot presented significant morphological differences for CPEB4 depleted oocytes (Figure 26b). Several dark rings appear around the white spot, and in some cases the oocytes enter apoptosis, indicative of abnormal meiotic progression.

The effect of CPEB4 depletion in meiosis was further assessed by analyzing chromosome dynamics monitored by direct visualization of stained DNA. Oocytes were fixed and stained with Hoecht and chromosomes were observed under the microscope. Control oocytes displayed the characteristic DNA staining with extruded polar body and chromosomes arranged in the metaphase II plate (Figure 26c). In CPEB4 depleted oocytes, the polar body was not detectable and the chromosomes were partially decondensed and not arranged in a metaphase plate, indicating that the oocytes did not complete the first meiotic division (Figure 26c).

Next, to further characterize the phenotype, we analyzed the effect of CPEB4 depletion at a biochemical level. For this we measured the cdc2 activity using oocyte extracts and Histone 1 (H1) as substrate for the kinase in an in vitro phosphorylation assay. As expected, control oligonucleotide injected oocytes showed that cdc2 activity increased in response to progesterone, partial inactivation after MI and reactivation at MII (Figure 26d). Cdc2 activity in CPEB4
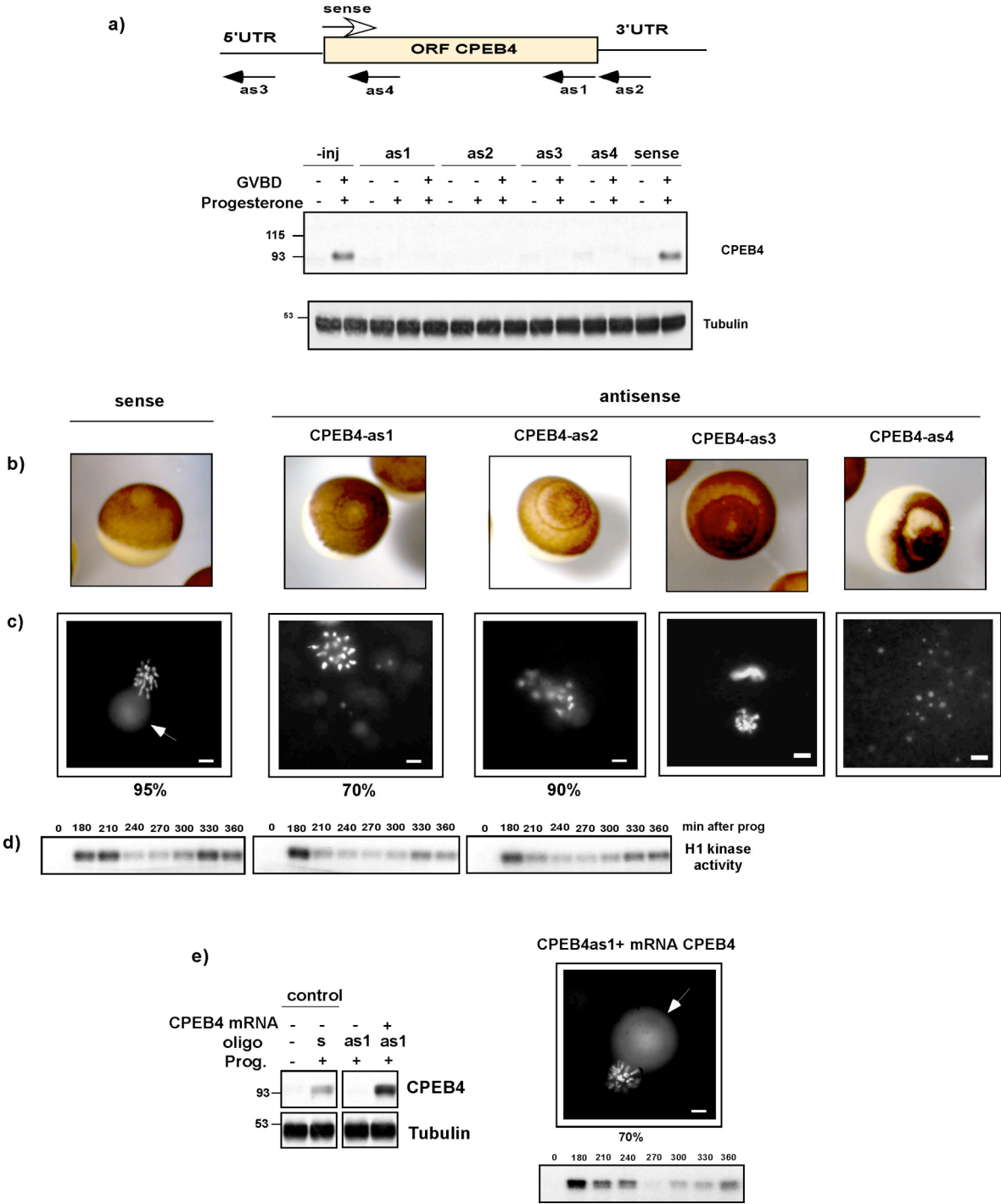
Figure 26. CPEB4 synthesis is required for the MI to MII transition. *Xenopus* oocytes were injected with CPEB4 sense (s) or antinsense (as1, as2, as3, as4) oligonucleotides as indicated and incubated for 16 hours. Then, they were stimulated with progesterone and collected 4 hours after control oocytes displayed 100% GVBD. Then analyzed for **(a)** CPEB4 levels by western blot using anti-CPEB4 and anti-Tubulin antibodies; **(b)** external morphology; **(c)** chromosomal arrangement; and **(d)** H1 Kinase activity. (e) CPEB4 depleted oocytes were microinjected with CPEB4-encoding mRNA non-targeted by antisense oligonucleotides, stimulated with progesterone and analyzed for CPEB4 by western blot, chromosomal arrengement and H1 kinase activity. Representative images and the percentage of appearance for each phenotype are shown. The arrow indicates the first polar body. Scale bar 10 μm.

depleted oocytes showed normal levels in GVBD, followed by a sharp decrease. At later times H1 kinase phosphorylation was partially reactivated, most likely as a consequence of oocyte apoptosis, which stimulates cdc2 and cdk2 (Figure 26d) (Zhou et al., 1998). Accordingly, the reactivation of H1 kinase was more evident with the CPEB4as2, which shows more apoptotic symptoms than the CPEB4as1. This indicates that MI was accomplished in the absence of CPEB4 but MI-MII transition was defective.

To confirm that the observed phenotype was due to the depletion of CPEB4 from the oocytes, we try to rescue it by overexpressing a synthetic CPEB4 mRNA not targeted by the antisense oligonucleotides. Oocytes were microinjected with CPEB4 antisense oligonucleotide 1, incubated overnight at 18ºC, and subsequently microinjected with the synthetic CPEB4 mRNA and treated with progesterne. Oocytes overexpressing CPEB4 successfully progressed through meiosis biochemically and at chromosomal level (Figure 26e).

All together we concluded that CPEB4 is required for MI-MII transition.

4.2. CPEB4 depletion induces DNA replication

An important characteristic of the meiotic cell cycle is the occurrence of two consecutive M phases without an intervening S phase, which is essential for generating haploid germ cells. Previous work has shown that when the meiotic cell cycle is blocked after GVBD, the oocytes proceed from meiosis I to an interphase-like state in which DNA replication is derepressed (Furuno et al., 1994; Nakajo et al., 2000). Thus, to further characterize the meiotic defect originated by preventing CPEB4 synthesis, DNA replication activation was analyzed.

It has been reported that depletion of Xkid causes meiotic exit and DNA synthesis after MI (Perez et al., 2002). Thus, Xkid depletion by antisense microinjection was used as positive control for DNA replication.

Oocytes were microinjected with sense or antisense oligonuceotide for Xkid, or several antisense oligonucleotides targeting CPEB4, and incubated at 18ºC overnight. Next morning, they were microinjected with α-32P-dCTP and subsequently treated with progesterone to induce maturation. Four hours after control oocytes reached GVBD, oocytes were collected and DNA was extracted to measure the incorporation of microinjected dCTP. Control oocytes did not synthesize DNA in the course of a normal meiosis, but new DNA was indeed generated in Xkid and CPEB4-depleted oocytes indicating that these oocytes entered an interphase-like state (Figure 27). The incorporation of labelled dCTP was sensitive to aphydicoline, indicating that DNA replication rather than DNA repair was occurring (Furuno et al., 1994).
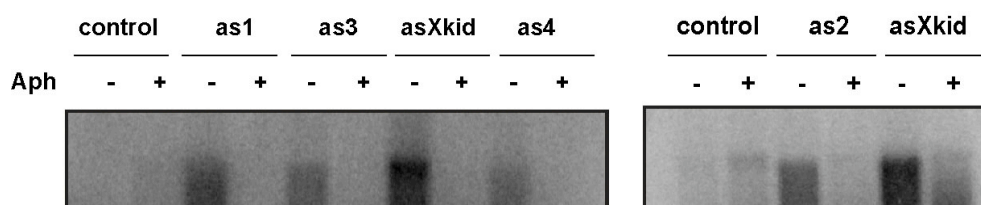
Figure 27. Depletion of CPEB4 induces exit of the meiotic progression and DNA replication. Oocytes injected with CPEB4 antisense oligonucleotide (as1, as2, as3, as4), CPEB4 sense oligonucleotide (control) and Xkid antisense oligonucleotide (asXkid) were injected with 0.4 µCi [α-32P]dCTP. Then, oocytes were stimulated with progesterone and incubated in the presence or absence of Aphydicolin (Aph) as indicated. Oocytes were collected 5 hours after control oocytes displayed 100% GVBD, DNA was extracted and analyzed by agarose gel electrophoresis followed by autoradiography.

5. Identification of CEPB4 mRNA targets

Translational activation of the CPE-regulated mRNAs during the "late" and "late-late" waves of cytoplasmic polyadenylation is required for MPF activation and CSF activity in the second meiotic division (Belloc and Mendez, 2008). In this meiotic phases CPEB1 levels are negligible. Thus, CPEB4 could play a role in polyadenylation of these "late" and "late-late" mRNAs, functionally substituting CPEB1.

5.1. CPEB4 binds to CPE elements

To determine whether CPEB4 could also bind CPE-containing mRNAs, the binding ability of CPEB4 to cyclin B1 3'UTR was tested. Cyclin B1 3'UTR contains 3 CPEs (2 consensus and 1 non-consensus sequences), one of them overlapping with the hexanucleotide, and a pumilio binding site (PBS). Accordingly with the CPE arrangement (Pique et al., 2008), cyclin B1 is a repressed mRNA in PI-arresed oocytes and polyadenylated in the "late" wave.

Oocytes were microinjected with cyclin WT 3'UTR or a mutant variant where the CPEs were inactivated by point mutations and stimulated or not with progesterone. The cytoplasmic extracts obtained from PI-arrested or MII oocytes were immunoprecipitated with either CPEB1 or CPEB4 antibodies, followed by specific RT-PCR for the microinjected probes. Both CPEB1 and CPEB4 co-immunoprecipitated the WT 3'UTR but not the mutant 3'UTR (Figure

28). Interestingly, for CPEB1 the amount of immunoprecipitated probe was larger in the first meiotic division than in the second, whereas for CPEB4 the proportion was reverted, with higher binding in MII.
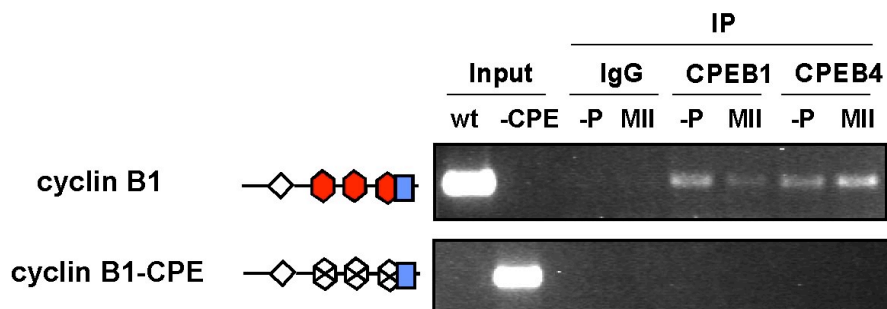
Figure 28. CPEB4 binds to the CPE elements. *Xenopus* oocytes were microinjected with in vitro transcribed RNAs derived from WT cyclin B1 3'UTR (cyclin B1) or the corresponding variant with the CPEs inactivated by point mutations (cyclin B1-CPE). Then, incubated for 8 hours in the presence (MII) or absence (-P) of progesterone and subjected to immunoprecipitation with anti-CPEB1, anti-CPEB4 and control IgG antibodies followed by RT-PCR for the microinjected RNAs.

5.2. CPEB4 binds to "late-late" polyadenylated mRNAs

Once determined that CPEB4 recognizes the same CPE sequence that CPEB1, representative "early" (CPEB4, Emi1 and mos), "late" (cyclin B1) and "late-late" (Emi2, cyclin E) polyadenylated mRNAs were tested for association with both CPEB1 and CPEB4 endogenous proteins by specific immunoprecipitation followed by RT-PCR.

The endogenous CPEB4 protein was bound to "late", "late-late" and CPEB4 mRNAs in MII, but not to the "early" mos and Emi1 mRNAs (Figure 29). CPEB1, however, was bound to all "early", "late" and "late-late" mRNAs in PI-arrested oocytes, but not in MII (Figure 29). As a negative control GAPDH was not associated with CPEB1 nor CPEB4. Thus, CPEB1 and CPEB4 regulate

overlapping, but not identical, subpopulations of mRNAs in the first and second meiotic divisions, respectively. Interestingly, CPEB4 was recruited to its own mRNA in MII, suggesting a positive feedback loop that may explain why CPEB4 mRNA is not completely deadenylated by C3H-4. Intriguingly, both mos and Emi1 are not only "early" polyadenylated mRNAs but also weak-polyadenylated (Belloc and Mendez, 2008; Pique et al., 2008).
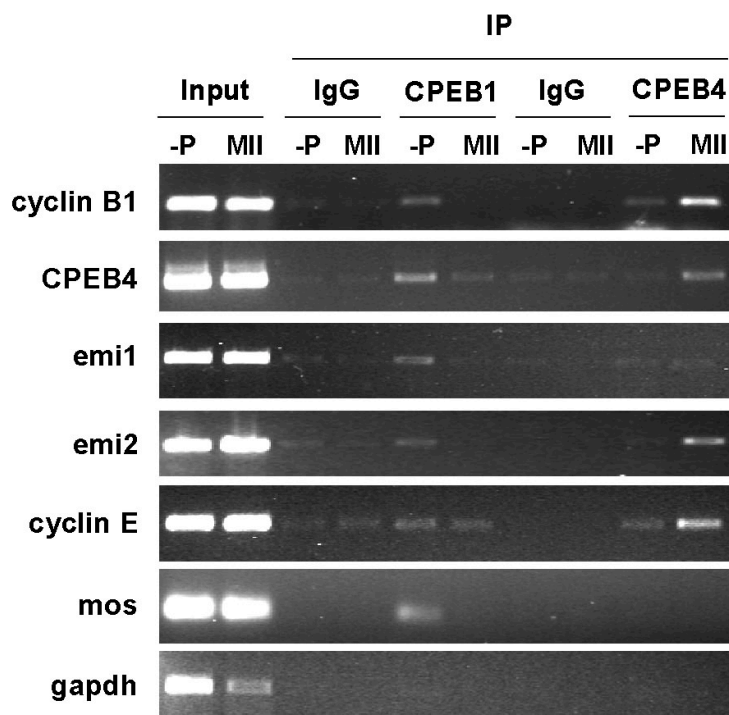
Figure 29. CPEB4 binds to late-late polyadenylated mRNAs. Cytoplasmic extracts from oocytes untreated (-P) or incubated with progesterone for 8h (MII) were subjected to immunoprecipitation with anti-CPEB1, anti-CPEB4 and control IgG antibodies. The coimmunoprecipitates were analyzed by RT-PCR for the presence of the indicated mRNAs.

A recent work from our laboratory had also characterized the binding of CPEB4 to CPEs by gel retardation assays (Novoa et al., submited), although the affinity for the CPEs is lower in the case of CPEB4 compared to CPEB1. This fact,

together with the weak polyadenylation pattern of the "early" Emi1 and mos, could explain the differential association of both proteins to CPE-regulated mRNAs.

5.3. CLIP: identifying target mRNAs

To detect protein-RNA interactions and isolate RNA binding sites in the context of an intact cell, in vivo cross-linking and immunoprecipitation (CLIP) provides a robust methodology (Ule et al., 2005) and has significant advantages over previous methods.

To obtain RNA CLIP tags of an appropriate size, pure cytoplasmic oocyte extract was UV cross-linked and partially digested with RNAse T1 prior to immunoprecipitation with CPEB1 and CPEB4 antbodies. We repeated the procedure several times but we were not able to see the formation of the shifted complexes neither for CPEB1 nor CPEB4, except for one experiment were we obtained a shifted band for CPEB4. The RNA from the band was isolated, purified and ligated to RNA-linkers. RNAs ranging in 100-200 nucleotides were purified and RNA-linker primers allowed PCR-based amplification of CLIP tags, cloning and sequencing. Unfortunately, the sequences obtained corresponded to the RNA-linker primers inserted several times in the vector used for sequencing and we did not further proceeded with this approach.

6. Translational control by CPEB4

6.1. CPEB4 functional complexes

Several CPEB1-interacting proteins that participate in the translational repression and activation of CPE-containing mRNAs have been described. To further characterize the functional significance of recruiting CPEB4 to CPE-containing mRNAs, we tested if any of the CPEB1 partners also interacted with CPEB4. Endogenous CPEB1 and CPEB4 were immunoprecipitated from PI-arrested and MII oocytes respectively, and analyzed for the presence of Maskin, PARN, CPEB1 and CCR4 (Figure 30).

Both proteins were equally able to recruit GLD2. To rule out if the co-immunoprecipitation of GLD2 by CPEB4 was through its association with CPEB1, we also analyzed the immunoprecipitates for the presence of CPEB1. CPEB4 did not co-immunoprecipitate CPEB1 in MII (Figure 30), indicating that both CPEBs are not bound to the same mRNA, and that the association of GLD2 and CPEB4 is not indirectly mediated through a potential dimerization with CPEB1. Thus, both CPEB1 and CPEB4 recruit the polyadeylation machinery to CPE-regulated mRNAs, but at different meiotic phases.

On the other hand, neither CPEB1 nor CPEB4 were able to immunoprecipitate the described CPEB1 partners maskin and PARN (Figure 30), confirming a recent observation by Minshall et al.
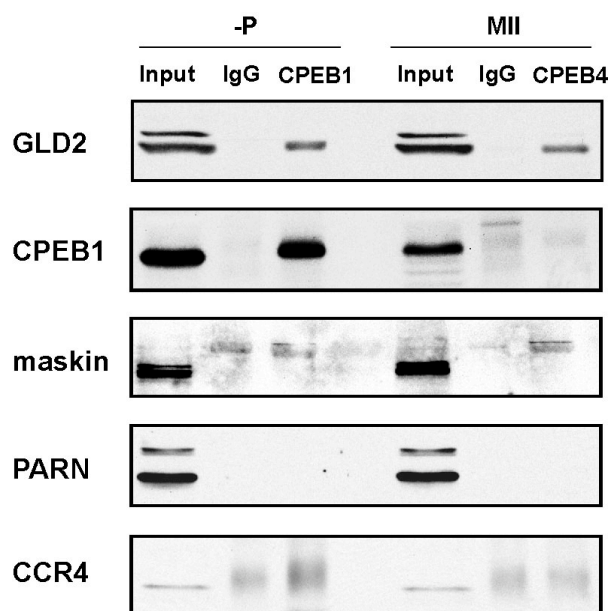
Figure 30. CPEB4 recruits GLD2 in MII. Cytoplasmic extracts from oocytes untreated (-P) or incubated with progesterone for 8h (MII) were subjected to immunoprecipitation with anti-CPEB1, anti-CPEB4 and control IgG antibodies. The coimmunoprecipitates were analyzed by western blotting for the presence of the indicated proteins.

Thus, we concluded that CPEB4 like CPEB1, can assemble a cytoplasmic polyadenylation complex by recruiting the cytoplasmic poly(A) polymerase Gld-2, but we found no evidence that CPEB4 can mediate deadenylation by recruiting PARN or CCR4 nor translational repression by recruiting maskin.

6.2. CPEB1 cannot rescue CPEB4 depletion in the second meiotic division

CPEB1 and CPEB4 bind to CPEs and recruit Gld-2 to CPE containing mRNAs activated during the "late" and "late-late" waves, but it was still an open question whether both proteins were functionally equivalent and had redundant functions.

To address this question, we substituted CPEB4 for a non-degradable mutant form of CPEB1. This CPEB1 mutant has the cdc2-phosphorylated residues substituted by Alanines (CPEB1-CA), but is still contains the regulatory Ser174, targeted by Aurora A kinase and required to activate CPEB1 and mediate cytoplasmic polyadenylation (Mendez et al., 2000a). Cdc2-phosphorylation is required for CPEB1 degradation at anaphase I (Mendez et al., 2002; Setoyama et al., 2007), thus this CPEB1 mutant is stable during meiotic progression. To avoid the meiotic arrest caused by overexpressing high levels of non-degradable CPEB1 in PI (Mendez et al., 2002), we microinjected a deadenylated mRNA encoding CPEB1-CA, which drove the accumulation of CPEB1-CA to similar levels than WT-CPEB in PI, but predominantly after GVBD (Figure 31a), and, therefore, without interfering with meiotic progression (Figure 31b). On the other hand, overexpression of CPEB1-CA in CPEB4 depleted oocytes did not rescue the MI to MII transition defect produced by the lack of CPEB4 (Figure 31b), if anything, CPEB1-CA microinjection aggravated the phenotype. As shown before, microinjection of a mRNA codifying for a CPEB4 not targeted by the antisense oligonucleotides was able to revert the phenotype and restore the normal meiotic progression (Figure 31b).

6.3. Both CPEB1 degradation and CPEB4 synthesis are needed to complete meiosis

To address the effect on the meiotic blockade caused by CPEB4 depletion, polyadenylation status of CPEB1 and CPEB4 target mRNAs was analyzed in control, CPEB4 depleted, CPEB1-CA overexpressed, and CPEB4 depleted and CPEB1-CA overexpressed oocytes.
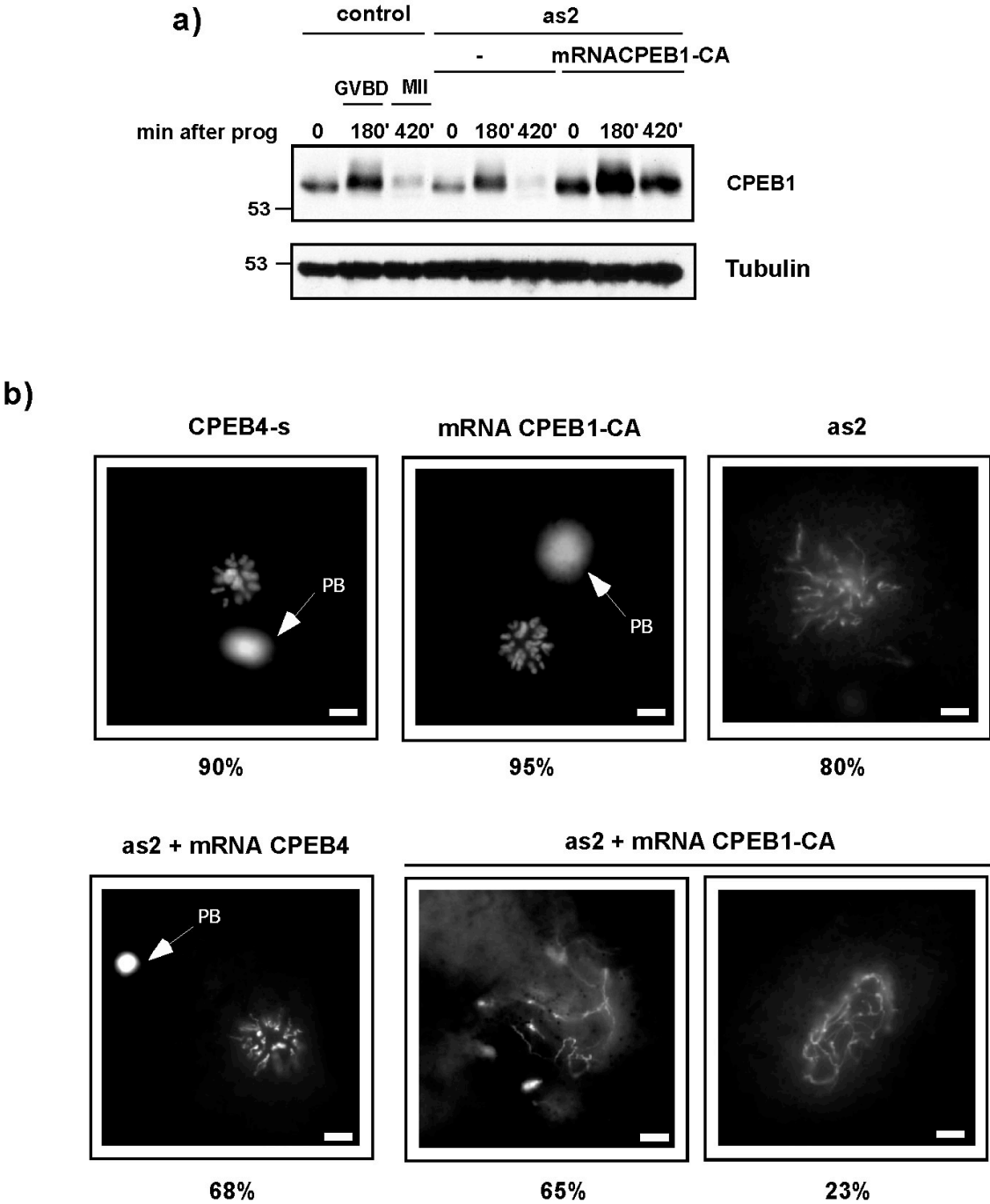
Figure 31. A stable CPEB1 mutant cannot replace for CPEB4 in the second meiotic division. *Xenopus* oocytes were injected with CPEB4 sense (control) or antisense (as2) oligonucleotides. After 16 hours, oocytes were microinjected with mRNAs encoding either CPEB4 or CPEB1-CA and incubated with progesterone. **(a)** Oocytes were collected at the indicated times and analyzed for CPEB1 levels by western blot using anti-CPEB1 and anti-Tubulin antibodies (1,5 oocyte equivalents were loaded per lane) **(b)** Oocytes were collected 4 hours after control oocytes display 100% GVBD and treated as figure 26c.

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As shown above, CPEB1-CA overexpression did not affect the meiotic progression, and CPEB1-CA levels remained constant during the meiotic progression (Figure 33a). As expected, analysis of the polyadenylation status of cyclin B1 revealed no major changes between control oocytes and oocytes overexpressing CPEB1-CA (Figure 32b). CPEB4 depletion partially prevented the polyadenylation of the "late-late" mRNA encoding Cyclin E (Figure 32c), but did not affect the polyadenylation of cyclin B1 mRNA (Figure 32b), consistent with this transcript being polyadenylated by CPEB1 in MI before CPEB4 accumulates. In concordance with the failure observed for CPEB1-CA to rescue the meiotic blockage due to CPEB4 depletion, polyadenylation of cyclin E mRNA was also not rescued (Figure 32c).

Surprisingly, substitution of CPEB4 by CPEB1-CA resulted in a shortened poly(A) tail for cyclin B1 (Figure 32b). Because cyclin B1 mRNA is normally polyadenylated by CPEB1 in MI, in the absence of CPEB4, we interpreted this result as deadenylation produced at later meiotic times caused by the presence of a non-degradable CPEB1 in the absence of CPEB4. Therefore, degradation of CPEB1 and new synthesis of CPEB4 in late meiosis seems to be required to prevent deadenylation during interkinesis of "early" and "late" mRNAs polyadenylated by CPEB1 during PI and early MI. Then, the third wave of "late-late" polyadenylation would be generated by the newly synthesized CPEB4.

a)

b)

cyclin B1

cyclin B1

c)

cyclin E1
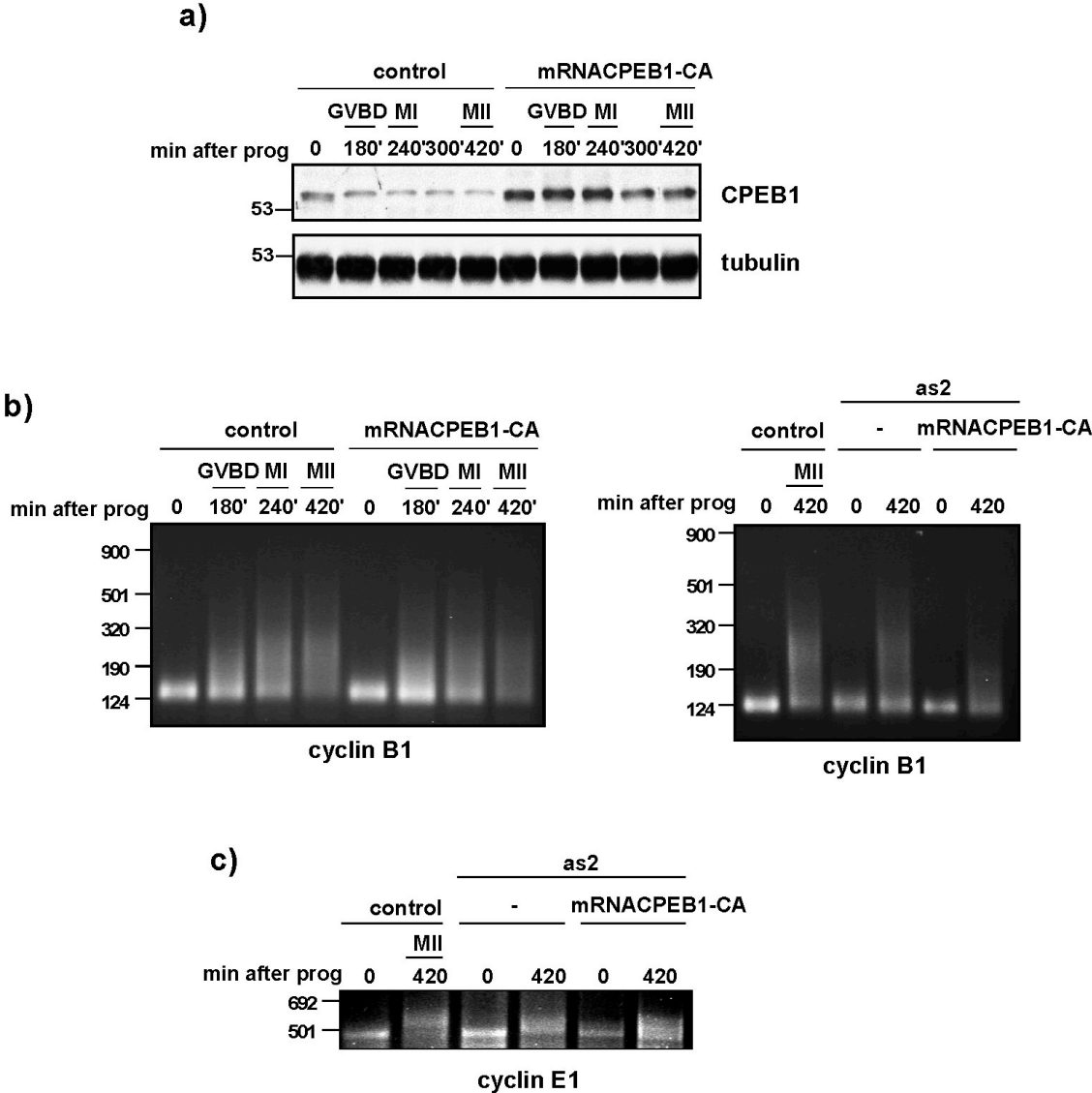
Figure 32. **Replacement of CPEB4 by a stable CPEB1 mutant affects polyadenylation in the second meiotic division.** *Xenopus* oocytes were injected with CPEB4 sense (control) or antisense (as2) oligonucleotides. After 16 hours, oocytes were microinjected with mRNAs encoding either CPEB4 or CPEB1-CA and incubated with progesterone. **(a)** Oocytes were collected at the indicated times and analyzed for CPEB1 levels by western blot using anti-CPEB1 and anti-Tubulin antibodies (1,5 oocyte equivalents were loaded per lane). **(b,c)** Total RNA from oocytes collected at the indicated times was extracted and polyadenylation status of cyclin B1and cyclin E mRNAs was measured by RNA-ligation-coupled RT-PCR.

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Discussion

Progression through the two meiotic divisions requires the sequential activation of maternal mRNAs encoding factors that drive cell cycle phase transitions. This sequential activation is achieved by a combination of successive phosphorylation events in CPEB1 with the combinatorial arrangement of CPEs and AREs in the CPEB-regulated mRNAs. First, Aurora A kinase activates CPEB1 and triggers the "early" wave of cytoplasmic polyadenylation required for the PI-MI transition (Mendez et al., 2000a; Mendez et al., 2000b; Pique et al., 2008). Then, in MI, Cdc2- and Plx-mediated phosphorylations target CPEB1 for SCF(beta-TrCP)-dependent degradation, thus lowering CPEB1 levels. Low CPEB1 levels are, in turn, necessary to trigger the second or "late" wave of polyadenylation required for MI-MII transition (Mendez et al., 2002; Pique et al., 2008; Reverte et al., 2001; Setoyama et al., 2007). These "late" mRNAs, such as cyclin B1 mRNA, contain at least two CPEs being the most distal one overlapping with the hexanucleotide, which becomes accessible to CPSF only upon CPEB1 degradation (Mendez et al., 2002). The drawback of the degradation of CPEB1 in MI is that the remaining levels of this factor are then very low for interkinesis and for the second meiotic division, when the third or "late-late" wave of cytoplasmic polyadenylation is required to mediate the MII-arrest by CSF (Belloc and Mendez, 2008). During interkinesis, APC activation is combined with increased synthesis of cyclins B1 and B4 (Hochegger et al.,

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2001; Pique et al., 2008) resulting in only a partial inactivation of MPF at anaphase-I and preventing entry into S-phase (Iwabuchi et al., 2000). Full reactivation of MPF for MII requires re-accumulation of high levels of cyclins B, as well as the inactivation of APC by newly synthesized Emi2 and other components of the CSF, such as cyclin E or high levels of Mos (Belloc and Mendez, 2008; Schmidt et al., 2006).

The recent discovery of other members of the CPEB family of proteins, together with the description of an autoregulatory loop of the CPEB-ortholog Orb (Tan et al., 2001), pointed us to explore the possibility that CPEB1 could activate the translation of other members of the CPEB family to compensate for its reduced levels after MI.

All CPEB-like proteins have a similar structure with most of the carboxy-terminal regions composed of two RNA recognition motifs (RRMs) and two zinc-fingers. On the other hand, the regulatory amino-terminal domains of the CPEB proteins show a small degree of identity; suggesting that they may be subjected to different regulation and recruit different co-factors. The most extensively studied member of the family, CPEB1, has dual functions as a translational repressor and activator, whereas CPEB3 and CPEB2 seem to act only as translational repressors (Hagele et al., 2009; Huang et al., 2006; Schmitt and Nebreda, 2002).

1. *Xenopus laevis* CPEB4 is encoded by a maternal mRNA regulated by CPEB1

We have identified *Xenopus laevis* CPEB4 protein, which shows 90% homology to other CPEB4 proteins in human, mouse and *Xenopus tropicalis* (Figure 19). Analysis of CPEB4 protein levels in a meiotic time course show very low levels (sometimes undetectable) in PI-arrested oocytes, gradual accumulation in response to progesterone and maximal levels in the second meiotic division

(Figure 22a). This expression pattern is complementary to CPEB1 expression during meiotic progression (Figure 22b). Contrary to CPEB1, CPEB4 is not degraded during meiotic progression and CPEB4 levels remain stable after fertilization and even after the mid-blastula transition (Figure 22c, d). This pattern of expression is the result of the translational regulation of CPEB4 mRNA by CPEB1 and C3H-4.

CPEB4 is encoded by a maternal transcript stored inactive with a short poly(A) tail in PI arrested oocytes, polyadenylated in the prophase I-metaphase I transition (PI-MI) by CPEB1, and partially deadenylated by C3H-4 from interkinesis to the second meiotic arrest in metaphase II (MII) by C3H-4 (Figure 23 and 24). Progesterone-induced polyadenylation mediated by CPEB4 3'UTR is abrogated when the CPEs or the hexanucleotide are inactivated by point mutations. Oocytes depleted of the ARE-binding protein C3H-4 show a partial prevention of CPEB4 deadenyation.

The translational control of CPEB4 mRNA is summarized in Figure 33. We postulate that in PI-arrested oocytes, CPEB4 mRNA is inactive with a short poly(A) tail and actively repressed by a dimmer of CPEBs, corresponding to CPEs 1 and 2 (Figure 25; constructs –C12, –C34 and –C1234) . The distance between adjacent CPEs defines the extent of repression with an optimal distance of 10-12 nucleotides. As the result of progesterone stimulation, CPEB1 is phosphorylated by Aurora A (Mendez et al., 2000a) inducing the first wave of early or cdc2-independent cytoplasmic polyadenylation of mRNAs. Early or cdc2-independent cytoplasmic polyadenylation requires, at least, a single consensus CPE (Figure 25; construct –C1234). The CPE must be closer than 100 nucleotides from the hexanucleotide, but not overlapping, such as in CPEB4 mRNA. The presence of AU-rich Elements (AREs) in CPEB4 mRNA, a feature of mRNAs regulated by deadenylation (Voeltz and Steitz, 1998), further defines the effect on polyadenylation dictated by the different arrangements of CPEs. In interkinesis, the AREs present in CPEB4 mRNA recruit a zinc-finger protein named C3H-4 that is encoded by a CPEB-regulated mRNA activated also during the early wave of cytoplasmic polyadenylation. In turn, C3H-4

recruits the CCR4/Not deadenylase complex to the ARE-containing mRNAs opposing CPEB activity on mRNAs containing both CPEs and AREs (Belloc and Mendez, 2008). The effect of C3H-4 mediated deadenylation on the target mRNA is defined by the arrangements of CPEs.
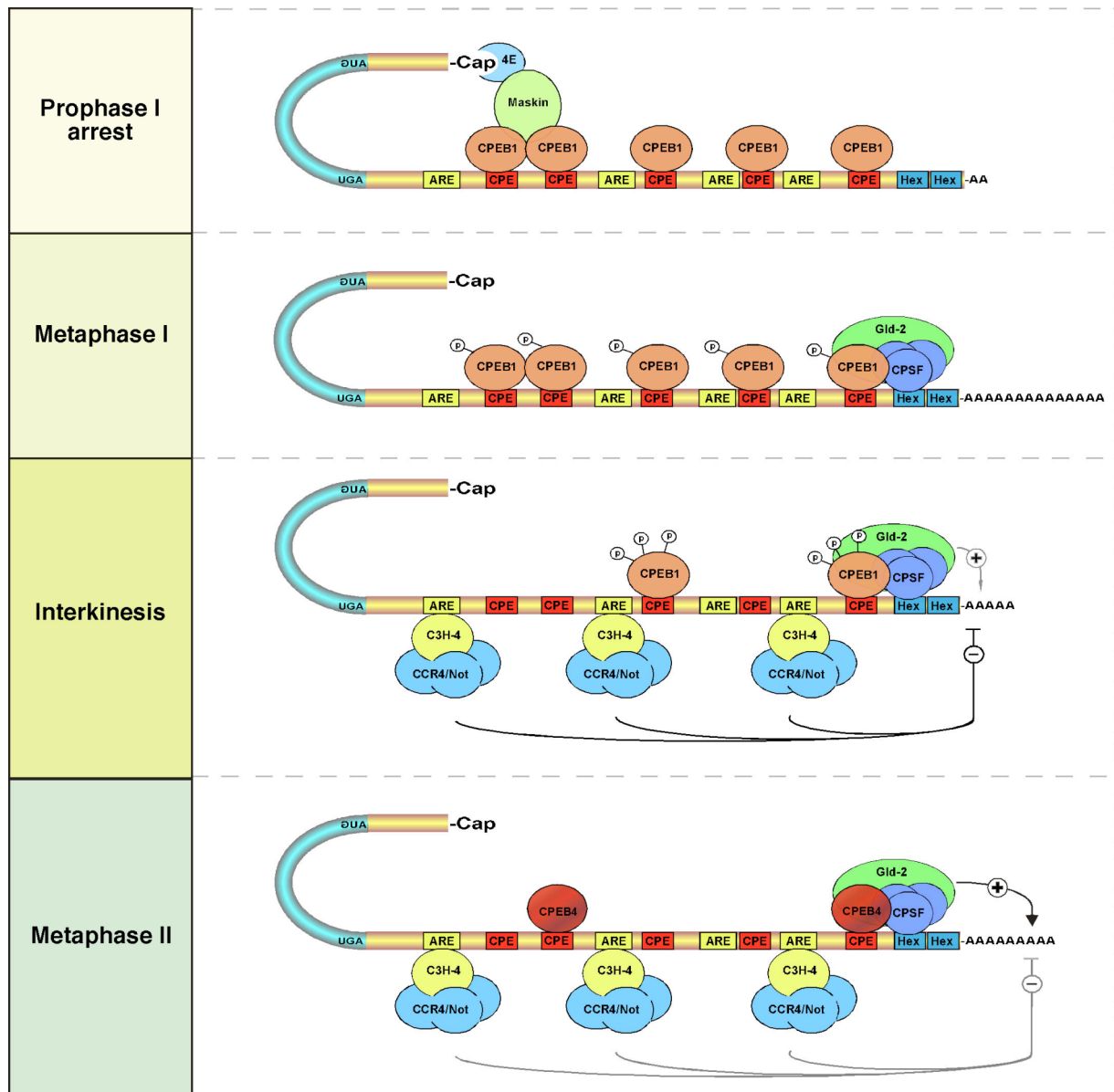
Figure 33. **Model for translational control of CPB4 mRNA.** Schematic representation of the *cis*-elements and *trans*-acting factors recruited to the 3'UTR of CPEB4 mRNA, with their covalent modifications.

Thus, for a strong polyadenylated mRNA, such as CPEB4 mRNA, with a consensus CPE at optimal distance from the hexanucleotide, the deadenylation cannot override completely the polyadenylation resulting in a partial deadenylation of the mRNA. Depletion of C3H-4 from the oocytes partially protects the deadenyation suffered by CPEB4 during interkinesis (Figure 24b). C3H-4 is not able to completely neutralize the polyadenylation but causes a decrease in CPEB4 protein synthesis delaying the accumulation of the protein to later meiotic phases, MII. This delay in CPEB4 protein accumulation could be a control mechanism of the oocyte to avoid high levels of CPEB4, similar to CPEB1 levels in PI-arrested oocytes, which could be deleterious for the correct meiotic progression or for the early stages of the embryonic development. We hypothesize that high CPEB4 levels during the meiotic progression could mediate the assembly of repression complexes by CPEB4, leading to translational silencing of some mRNAs essential to successfully complete meiosis. Alternatively, high levels of CPEB4 could occupy the CPE overlapping the Hex in "late" and "late-late" mRNAs, thus preventing their polyadenylation. Once high levels of CPEB4 are reached in MII, CPEB4 associates with its own mRNA (Figure 29), probably stabilizing a positive feed-back loop to maintain CPEB4 levels during the MII-arrest and the early embryonic development.

2. CPEB4 replaces CPEB1 to complete meiosis

Both, CPEB1 and CPEB4 recognize CPE elements in the 3'UTR of mRNAs, as seen by the co-immunoprecipitacion of cyclin B1 3'UTR wt but not of a construct with mutations in the CPEs (Figure 28). Gel retardation assays using the same cyclin B1 3'UTR constructs also showed interactions between CPEB1 and CPEB4 proteins with cyclin B1 3'UTR wt but not with the mutated construct, although CPEB1 displayed slightly higher affinity (Novoa et al., submited). Therefore, CPE-contaning mRNAs can be regulated by both CPEB1 and CPEB4, although they recognize these targets with different affinities. These findings contrast with a previous report that identified through SELEX analysis

the CPEB4 binding elements, distinct from CPEs and constituted by a secondary stem-loop structure rich in uridines (Huang et al., 2006). However, in some cases SELEX-identifyed motifs may fail to reflect physiological or functional interactions. Immunoprecipitation followed by RT-PCR of candidate genes suggest that CPEB1 and CPEB4 can recognize overlapping populations of mRNAs (Figure 29). In PI-arrested oocytes, CPEB1 binds to all CPE-containing mRNAs polyadenylated "early" (CPEB4, Emi1 and mos), "late" (cyclin B1) and "late-late" (Emi2, cyclin E). On the other hand, in MII CPEB4 binds to "late" and "late-late" mRNAs. CPEB4 binds also to the "early-strong" CPEB4 mRNA, but not to the ealy-weak 3'UTRs from Emi1 and mos mRNAs.

Moreover, CPEB1 and CPEB4 are able to recruit the polyadenylation machinery through the recruitment of the cytoplasmic poly(A) polymerase GLD-2 (Figure 30). Altogether, these observations indicate that CPEB4 would be the protein responsible of recruiting GLD-2 to the late and late-late mRNAs during interkinesis and in the MII arrest. No evidence was found suggesting that CPEB4 mediated deadenylation by recruiting PARN or CCR4, nor translational repression by recruiting maskin.

Depletion of CPEB4 causes external morphological changes and also partial decondensation of the chromosomes. In the absence of CPEB4 the characteristic metaphase II plate nor the first polar body are formed (Figure 26 b, c). Measurement of Cdc2 activity indicates correct meiotic resumption until anaphase I, indicating that CPEB4 depleted oocytes progress from PI to MI, but failed to transition between MI and MII (Figure 26d). This failure results in the exit from the meiotic progression to a S-like phase promoting DNA replication (Figure 27). This phenotype is rescued by overexpressing CPEB4 in the oocyte (Figure 26e). Because the CPE-regulated mRNAs are required for cdc2 reactivation and CSF activity in the second meiotic division are polyadenylated in the third or "late-late" meiotic wave (Belloc and Mendez, 2008), when CPEB1 levels are negligible, CPEB4 would bind these mRNAs recruiting GLD-2 and mediating their polyadenylation during the second meiotic division.

Although CPEB1 and CPEB4 recognize overlapping populations of mRNAs and recruit GLD2, they are not interchangeable because a stabilized CPEB1 cannot replace CPEB4 for the transition from MI to MII (Figure 31b) or for the polyadenylation the "late-late" mRNA encoding Cyclin E (Figure 32). The stable CPEB1 mutant mimics a situation in which CPEB1 levels are maintained high (similar to levels present in PI-arrested oocytes) during meiotic maturation (Figure 31a). We hypothesize that this could lead to the recruitment and reassembly of the deadenylation and the repression complexes by CPEB1, promoting deadenylaton of the target mRNAs in interkinesis and MII. The lower CPEB4 protein levels and the lower affinity of CPEB4 by CPEs, compared to CPEB1, will avoid the assembly of repression or deadenylation complexes.

In addition, CPEB1 and CPEB4 may recruit different cofactors (maskin and PARN) and be subjected to different post-translational regulation. Unlike CPEB1, CPEB4 does not contain Aurora A kinase phosphorylation sites and contains putative recognition sites for cyclic AMP-dependent protein kinase (PKA), CaMKII, and p70S6 kinase (a growth-factor-stimulated serine treonine kinase that acts on components of the translational apparatus) (Gingras et al., 2001b; Theis et al., 2003).

3. What controls the activity of different CPEB family members?

Besides the fact that CPEB1 contains a PEST box that mediates its degradation upon phosphorylation by Cdc2 and Plx, CPEB1 is the only member of the family that contains Aurora A Kinase phosphorylation sites (Mendez and Richter, 2001). Instead, CPEB4 contains putative recognition sites for PKA, CaMKII and S6 kinase (Theis et al., 2003), suggesting differential posttranslational regulation of both factors during meiotic progression. Accordingly, a slight mobility shift of endogenous CPEB4 protein was observed at later stages of the meioic progression, suggesting a possible posttranslational regulation by

phosphorylation (Figure 22a). Also, overexpressed myc-tagged-CPEB4 shows a mobility change in response to progesterone without any effect on its stability (Figure 22c), and hCPEB4 is phosphorylated when it is microinjected in oocytes stimulated with progesterone (Novoa). In addition, tethered CPEB4 requires progesterone to activate translation and polyadenylation of reporter mRNAs (Novoa et al., submited). These observations suggest that CPEB4 is not constitutively active, but, rather, it has to be posttranslationally modified to become active and, since CPEB4 does not have a consensus Aurora A Kinase phosphorylation site, it will most likely be activated by a different mechanism that takes place at later meiotic phases.

During Interkinesis, Aurora A is inactivated by rapid degradation mediated by the APC (Honda et al., 2000). Thus, we hypothesize that Aurora A inactivation could promote CPEB1 dephosphorylation during Interkinesis, mediating the re-assembly of repression complexes with Maskin and PARN, and repressing cyclin B1 and B5 mRNAs. On the other hand, CPEB4 would be regulated by another kinase(s) active during interkinesis and late meiosis, that would activate CPEB4. Moreover, as CPEB4 is not able to recruit Maskin or PARN, nor assemble repression complexes. Therefore, it seems plausible that degradation of CPEB1 and substitution by CPEB4 would be required to prevent deadenylation and repression of late mRNAs during interquinesis and to ensure the polyadenylation of "late-late" mRNAs before Aurora A is re-activated in MII.

Although the identity of the putative CPEB4 kinase(s) is far from clear some indirect observations point in the direction of P70(S6K). P70(S6K) plays a key role in translational control of cell proliferation in response to growth factors (Thomas and Hall, 1997). The p70(S6K) inhibitor Rapamycin, causes the oocyte to undergo GVBD earlier than control oocytes, and sensitivity to progesterone was increased (Schwab et al., 1999). Moreover the drug had no effect on the first meiotic division but affects the meiotic spindle formation and polar body (Schwab et al., 1999). This same phenotype is observed for CPEB4 depleted oocytes. This could indicate that this kinase could be responsible of CPEB4 activation. Interestingly, cyclins B and mos mRNAs are not affected by

rapamycin treatment (Lapasset et al., 2008), consistent with mRNAs being early polyadenylated by CPEB1 and not by CPEB4.

4. Sequential polyadenylation and deadenylation drives meiotic progression

Meiotic progression is a switch-like reversible process where the successive meiotic phases are discrete states sustained by multiple positive and negative feed-back loops that require protein synthesis (Abaza et al., 2006; Ferrell, 2002; Matten et al., 1996; Xiong and Ferrell, 2003) and keep the oocyte from sipping rapidly back and forth between cell cycle phases (Brandman et al., 2005; Ferrell, 2002). The hierarchical translation of specific subpopulations of mRNAs at each meiotic phase is regulated through sequential waves of polyadenylation and deadenylation (Figure 34).

Based on the findings presented in this study, we speculate that the maternal inherited mRNAs may be subjected to sequential translational regulation upon progesterone activation of the oocyte first by CPEB1 and from interkinesis on by CPEB4.

In PI arrested oocytes, the CPE-regulated mRNAs are either inactive with a short poly(A) tail or even actively repressed by a dimmer of CPEB1. As the result of progesterone stimulation, CPEB1 is phosphorylated by Aurora A (Mendez et al., 2000a) inducing the first wave of early or cdc2-independent cytoplasmic polyadenylation of mRNAs required for the PI-MI transition such as the ones encoding the MPF components Cyclins B2 and B5, the MPF activator c-mos, the APC/C inhibitor Emi1, and CPEB4. The switch-like activation of MPF is sustained by multiple positive feedback loops in the p42 MAPK/Cdc2 network (Ferrell, 2002; Matten et al., 1996), which require protein synthesis (Xiong and Ferrell, 2003) and that also target the re-activation of the early wave of

polyadenylation through the synthesis and activation of Aurora A (Frank-Vaillant et al., 2000; Howard et al., 1999; Ma et al., 2003; Matten et al., 1996).

At the same time, a negative feedback loop, which opposes CPEB1 activity on mRNAs containing both early-weak CPEs and AREs, is switched on through the early polyadenylation and translational activation of C3H-4 mRNA. C3H-4 generates a deadenylation wave that inactivates Emi1 translation in MI allowing for the activation of the APC/C and the transition to interkinesis (Belloc and Mendez, 2008). APC/C itself, by triggering protein degradation is a negative feedback loop, which has been shown to be required for the Cdc2 cell cycle oscillations (Pomerening et al., 2003). As a result of MPF activation in MI, CPEB1 is phosphorylated by Cdc2 and Plx1 triggering its partial destruction. The partial destruction of CPEB1 is necessary to allow polyadenylation of late mRNAs in MI and to prevent deadenylation in interkinesis and MII. Moreover, the C3H-4 deadenylation wave targets also CPEB4 mRNA, but because this 3'UTR contains strong CPEs, C3H-4 is only able to partially deadenylate CPEB4 mRNA. This partial deadenylation results in a decrease in CPEB4 protein synthesis delaying the accumulation of the protein until interkinesis. This delay in CPEB4 protein accumulation could be a control mechanism of the oocyte to avoid high levels of CPEB4, similar to CPEB1 levels in PI-arrested oocytes, which could be deleterious for the correct meiotic progression or for the early stages of the embryonic development.

The generation of the second wave of late or Cdc2-dependent polyadenylation of mRNAs such as the ones encoding cyclin B1 and cyclin B4, is required to sustain an intermediate MPF activity during interkinesis, and for the reactivation of MPF in MII (Mendez et al., 2002; Mendez et al., 2000b; Pique et al., 2008; Setoyama et al., 2007). The destruction of CPEB1 together with the synthesis of C3H-4 and CPEB4 generates, in turn, the third wave of late-late cytoplasmic polyadenylation. In addition, the destruction of CPEB1 would prevent the deadenylation of "late" mRNAs, such as cyclin B1 mRNA, during interkinesis.
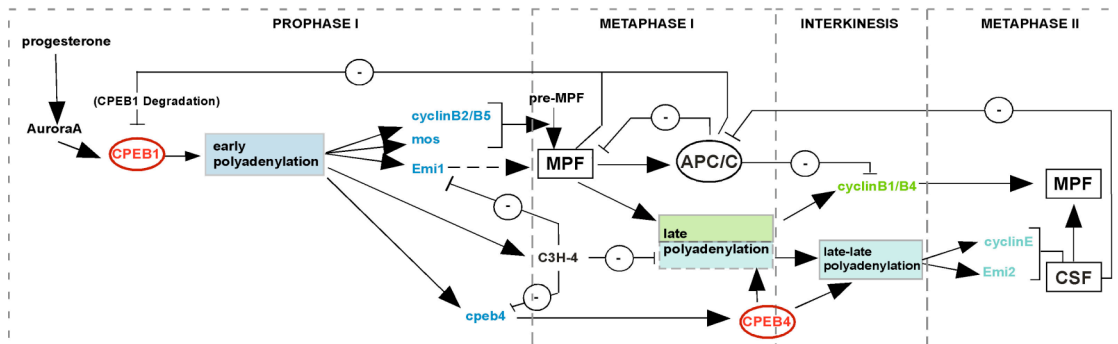
Figure 34. **Sequential waves of polyadenylation and deadenylation drive meiosis.** Schematic representation showing the sequential waves of polyadenylation and deadenylation driving meiotic progression. Cytoplasmic polyadenylation is driven by CPEB1 in the early stages of the meiotic progression and replaced by CPEB4 in interkinesis. The three waves of cytoplasmic polyadenylation are depicted with boxes (early, late, and late-late) and positive and negative feed-back loops are also indicated.

CPEB4 would target CPE-regulated mRNAs, which are activated by cytoplasmic polyadenylation during interkinesis and encode proteins required for the second meiotic division and to prevent DNA replication after MI (Belloc and Mendez, 2008; Eliscovich et al., 2008; Hochegger et al., 2001; Pique et al., 2008). This target mRNAs contain late-strong CPEs and AREs, such as the ones encoding the CPSF components Emi2 and cyclin E, which are synthesized during interkinesis. CPEB4 either activates late-late mRNAs or maintain the extended poly(A) tail and continued protein synthesis bringing Gld-2 to these CPE-regulated mRNAs. CPEB4 associates also with its own mRNA, probably stabilizing a positive feed-back loop to maintain CPEB4 levels during early embryonic development. CSF, in turn, inhibits the APC/C allowing the full reactivation of the MPF, now with cyclins B1 and B4, and maintaining the oocyte arrested in MII until fertilization takes place (Pique et al., 2008).

CPEB4 would only mediate polyadenylation of the target mRNAs and would not mediate formation of repression or deadenylation complexes. Consistent with this model, whereas depletion of CPEB4 did not block polyadenylation of "late" class mRNAs (e.g. cyclin B1), CPEB4 depletion did attenuate or block the

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polyadenylation of "late-late" mRNAs class (e.g. cyclin E). We propose that the sequential action of CPEB1 and CPEB4 allow for coordination of complex temporal patterns and extent of protein synthesis during vertebrate meiotic cell cycle progression.

The work presented in this thesis only provides some insights into the broad spectrum of CPEB family protein functions. Still more work needs to be done to further characterize the regulation of the proteins and to address the question if CPEB2-3 are also present in oocytes and have a role during oocyte maturation or early embryogenesis. Until now only a CPEB2 function in spermatogenesis has been described (Kurihara et al., 2003; Luitjens et al., 2000), whereas CPEB3 has been implicated in germ cell fates in *C.elegans* (Hasegawa et al., 2006; Stebbins-Boaz et al., 1996; Tay and Richter, 2001). However, both proteins have been described only as translation inhibitors (Huang et al., 2006; Kurihara et al., 2003), and is therefore unlikely that they functions will overlap with CPEB4.

Conclussions

1. CPEB4 mRNA is encoded by a repressed maternal mRNA, polyadenylated in metaphse I by CPEB1, and partially deadenylated in the second meiotic arrest in metaphase II by C3H-4.

2. CPEB4 is present at very low levels in PI-arrested oocytes, and gradually accumulated in response to progesterone, reaching maximal levels in the second meiotic division. CPEB4 levels remain stable after fertilization and even after mid-blastula transition.

3. CEPB4 is required for the correct MI-MII transition and CPEB4-depleted oocytes exit meiosis replicating the DNA

4. CPEB1 and CPEB4 regulate an overlapping, but not identical, subpopulation of mRNAs in the first and the second meiotic division, respectively. CPEB1 is responsible for the polyadenylation of "early" and "late" mRNAs, while CPEB4 binds to "late-late" mRNAs and recruits the polyadenylation machinery (GLD-2).

5. A stable CPEB1 mutant is not able to compensate for CPEB4 depletion during meiotic progression. Degradation of CPEB1 and new synthesis of CPEB4 in late meiosis seems to be required to prevent deadenylation during interkinesis of "late" mRNAs polyadenylated by CPEB1 during PI-MI. Then, a third wave of "late-late" polyadenylation would be generated by CPEB4 in the oocyte.

Material and Methods

Xenopus oocytes preparation

Stage VI oocytes were obtained by surgical removal of ovaries from adult *Xenopus laevis* females. To remove follicular cells and extracellular connective tissue, ovary lobed were treated for 2 hours 30 minutes with 0,8 mg/ml collagenase and 0,48 mg/ml dispase II in Modified Bath's Saline media (1X MBS; 88mM NaCl, 1mM KCl, 1mM $MgSO_4$, 2,5 mM $NaHCO_3$, adjust pH to 7,8, add fresh 0,7 mM $CaCl_2$). Then, oocytes were thoroughly rinsed and then cultured in 1X MBS. Stage VI oocytes (~1,3 mm diameter) were sorted using a dissecting microscope. Microinjection of oocytes was performed using the Nanojoct II Drummond microinjector. Maturation was induced by incubating the oocytes in 1X MBS containing progesterone (10 μM).

Embryos obtention

Xenopus embryos were obtained from first priming Xenopus laevis females (injection of 50U of PMSG –pregnant mare serum gonadotropin- on day 1, 25U on day 3; injection of hCG –human chorionic gonadotropin- on day 8 to induce ovulation). The induced females were places in 1X MMr media (5mM Hepes, 100 mM NaCl, 2mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, 0.1 mM EDTA, pH 7.8) at

18ºC to lay the eggs. Testes were isolated from Xenopus laevis males, which were first sacrified by submerging in 0.05% benzocaine for 30 minutes and stored 4ºC. Prior to fertilization buffer was removed from the eggs and crush parts of the testis tissue in 1X MMr were poured over the eggs. After contact with sperm, the eggs were flooded with 0.1X MMR abd collected at the different embryonic stages.

Western blot analysis

Oocyte lysates, prepared by homogenizing 6-10 oocytes in histone H1Kinase buffer containing 0.5% NP-40 and centrifuged at 12,000g for 10 min, were resolved by 8% SDS-PAGE. Equivalents of 1-2 oocytes were loaded onto each lane. Antibodies used were rabbit antiserum affinity purified against CPEB4, rabbit antiserum against CPEB1, monoclonal antibody against α-tubulin (DM1A, Sigma).

Plasmid constructs

CPEB4 (bankit123762) cDNA was cloned by RT-PCR from total RNA of stage VI oocytes using primers 5'-CGGGATCCATGGGGGGATTACGGGTTTGGAG-3' and 5'-TCCCCCGGGTCAGTTCCAGCGGAATGAAATATGC-3', digested with Sma and BamHI and cloned in pGEX or pET30a expression vectors. CPEB4 3' UTR was amplified by RT-PCR from total RNA of stage VI oocytes using primers 5'-GAAGATCTTGAGCAACCCATGGCTTAGC-3' and 5'-TGCTTAATGCTTTTAATAGGCAACTGC-3', digested with Bgl-II and cloned in the pLucassette downstream the Firefly Luciferase ORF.

Hexanucleotide mutants of CPEB4 were obtained by PCR from the original plasmid with T3 standard primer as sense oligonucleotide and the following antisense oligonucleotides:

-H2as:

5'-TGCTTAATGCTTTTAATAGGCAACTGCTGACTTTTCCTTTTCAATAAAG-3';

-H3as:

5'-TGCTTAATGCTTTCCATTGGCAACTGCTGACTTTTTATTTTCAATAAAG-3';
-H23as:

5'-TGCTTAATGCTTTCCATAGGCAACTGCTGACTTTTCCTTTTCAATAAAG-3'.
CPE mutants were obtained with QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) following manufacture's instructions. The oligonucleotides used were:

C12: 5'-TATTATTTTTTTT<u>GG</u>TATATAATTT<u>GG</u>TCGGAGAGCAAAGC-3';

C3: 5'-CGAGAAATAGAGTATTTTTTTTT<u>GG</u>TTAAATTATTG-3';

C4: 5'- GGTTTGTTGAACAGATTTTTTTTT<u>GGG</u>ATATATATATATA-3';

C5: 5'-GTTTGTATTT<u>GGC</u>CAGACTTTATTGAAAATAAAAAG-3'.

RNA-ligation-coupled RT-PCR

Total oocyte RNA was isolated from 8-10 oocytes by Ultraspec RNA Isolation System (Biotecx Laboratories, Inc.), following manufacturer's instructions. Then, RNA-ligation coupled RT-PCR technique was performed as described previously (Charlesworth et al. 2004) with some modifications. Briefly, 5 µg of oocyte total RNA was ligated to 0.5 µg of a 3'-amino-modified DNA anchor primer (5´ -P-GGTCACCTCTGATCTGGAAGCGAC-NH2-3´) in a 10 µl reaction using T4 RNA ligase (New England Biolabs), according to the manufacturer's directions. RNA ligation reaction was used in a 50 µl reverse transcription reaction using RevertAid M-MuLV Reverse Transcriptase (Fermentas) and 0.5 µg of an oligo anti-sense to the anchor primer plus four thymidine residues on its 3'-end (5'- GTCGCTTCCAGATCAGAGGTGACCTTTTT-3'). The resulting reaction product was digested with 2 µg RNAse A (Fermentas) and two microliters of this cDNA preparation were used as a template for gene-specific PCR reaction. The specific oligos were: 5'-CCGAGGCATATTTCATTCCGCTGG-3' for CPEB4, 5'-GTCAAGGACATTTATGCTTACC-3' for cyclin B1, 5'-GTGCTTTAACTCTGTGCATCAC-3' for cyclin E. DNA products from the PCR reaction were analysed in a 2% agarose gel and visualized by ethidium bromide staining.

Southern Blot

DNA products from RNA-ligation coupled RT-PCR of the endogenous CPEB4 mRNA were analysed in a 2% agarose gel and transferred to Hybond-N+ nyclon memebrane (Amesham Pahrmacia Biotech) as described in the manufacturer's protocol. A speficic oligo targeting CPEB4 (5'-GGTGAAGGAGGGAGGAGATCG-3') was labeled with γ^{32}P-ATP. The membrane probed with the oligo was analyzed by autoradiography for visulaization.

Translational control by 3' UTR

Translation and polyadenylation of reporter mRNAs were assayed as described previously (Pique et al., 2006), with some modifications. 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 manufacturer's instructions.

Cytoplasmic polyadenylation by 3' UTR

The RNA probes were in-vitro transcribed with a T3 RNA polymerase (Fermentas) in the presence of α^{32}P-UTP. The RNA were microinjected into oocytes, which were incubated in the presence or absence of progesterone. Pools of 6-8 oocytes were collected at different time-points after maturation. Total RNA was isolated with Ultraspec Isolation System (Biotecx Laboratories, Inc.) and precipitated with isopropanol. The equivalent of 2 oocytes were analyzed by 6% polyacrimaldide/8M urea gel electrophoresis followed by autoradiography for visulaization.

Histone H1 kinase assay (Cdc2 Assay)

Oocyte lysates were prepared by homogenizing 3 oocytes in cold histone H1Kinase Buffer (80 mM Na β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 50 mM $NaVaO_4$) containing protease inhibitors (complete EDTA-free protease inhibition cocktail, ROCHE). Lysates were centrifuged at 12,000g for 10 minutes at 4°C. 10 µl of extract were incubated with 4 µg of histone H1 (sigma) and 2 µCi of $[\gamma\text{-}^{32}P]$ATP during 15 minutes at room temperature. The phosphorylation reaction was analyzed by 12% SDS-PAGE gel and autoradiography.

Chromosomes and polar body observation

Oocytes fixed for at least 1 h in 100% methanol were incubated overnight in presence of 20 µg/l Hoechst dye. Chromosomes and polar body of stained oocytes were viewed from animal pole under UV epifluorescence microscope (Leica DMR microscope, 63X magnification, Leica DFC300FX camera, Leica IM1000 Image Manager).

Antisense oligonucleotide and rescue experiment

To ablate the expression of CPEB4, oligonucleotides targeting either 5' UTR or the 3' UTR were designed; one complementary sequence was used as a control. In each oocyte, 99 ng of oligonucleotide was injected. After overnight (16 h) incubation at 18°C, progesterone was added as described. For rescue experiment, 0.06 pmol of in vitro transcribed RNA coding for the ORF of CPEB4 or 0.02 pmol of the non-degradable CPEB1 mutant were injected 1-2 h before progesterone incubation. Oligonucleotides used were: 19AS: 5'-GAGGAAATATATCTGGGTGAAG-3'; 20AS: 5'-GCAATGGGTTGCTCAGTTCCA-3'; 23S: 5'-CTTTGCAAGCATCCAAATAAG-3'.

Analysis of DNA synthesis

Oocytes were injected with 0.4 μCi [α-32P]dCTP and treated subsequently with progesterone to induce maturation. Mature oocytes were subjected to DNA extraction, as described by Wong et al 1998, and samples with equal number of total counts ($0,5 \times 10^6$ c.p.m.) were analyzed by 1% agarose gel electrophoresis and autoradiography, as described previously (Newport and Kirschner, 1984).

CLIP technique

Total extracts from stage VI and MII were obtained and the crosslinking and immunoprecipitation using antibodies against CPEB1 and CPEB4 were perform according (Ule et al., 2005).

Immunoprecipitation

CPEB4 antibody raised in rabbits against the CPEB4 71-85 peptide (DEILGSEKSKSQQQQ), and CPEB1 antibody were incubated with protein-A sepharose during 2 h at room temperature (RT) on wheel, washed with PBS and resuspended in sodium borat pH 9.0. 20mM dimethyl pimelimidate·2HCl (DMP) was added and incubated 30 min at RT on wheel. Reaction was stopped with two 5 min-washes at RT with 0.05 M glycine, and two extra washes with PBS. Fresh oocyte lysates from stage VI and MII (25 oocytes per condition) were added to the crosslinked antibody-beads and incubated 2 h at 4ºC on wheel. Immunoprecipitates were washed three times in lysis buffer (20 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5% NP-40, 1 mM $MgCl_2$, 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 analyzed by Western blotting.

Immunoprecipitations followed by RT-PCR were performed as described (Aoki et al., 2003) with fresh stage VI and MII oocyte lysates (25 oocytes per condition). CPEB4 antibody raised in rabbits against the CPEB4 71-85 peptide (DEILGSEKSKSQQQQ), and CPEB1 antibody. The protein-bound RNAs were purified by proteinase K digestion followed by phebol-chloroform extraction. Half

of the total RNA extracted was used for the retrotranscription, performed with the 3'Race primer (TAATACGACTCACTATAGGGCGGATCCTTTTTTTTTTTTTTTTTTTTTTTTVN) with the mMLuV reverse transcriptase from Fermentas following the manufacturer's instructions. A twentieth part of the cDNA was used for each specific PCR (EcoTaq polymerase, Ecogen) with following specific primers: 5'-for cyclin B1

GTCAAGGACATTTATGCTTACC-3' and 5'-CCATGTCCCGAATTTGAGCC-3';

for CPEB4

5'-TGAGCAACCCATGGCTTAGC-3' and

5'-TGCTTAATGCTTTTAATAGGCAACTGC-3';

for Emi1

5'-ACAGAATTTACGGAGGTTATAGTT-3' and

5'-CGGAATTCCGGGCAATAATTTATTTAGCACAAAAAAA;

for Emi2

5'-GCACAACATGGAGAAAACTGCTGCAG-3' and

5'-CTATAACCTCCGTAAATTCTGTTTGC-3';

for Cyclin E

5'-GCATCAATTTTGGACCTCGTGAACGC-3' and

5'-GCCTCTTTTTTAGGGATCCTCTTTGC-3';

for mos

5'-ATGTGTTGCATTGCTGTTTAAGTGG-3' and

5'-AGACAAATCAATTTCTTTATTATAAAAC-3';

for GAPDH

5'-GGCCGCCATTAAGACTGCATC-3' and 5'-GACTAGCAGGATGGGCGAC-3'.

Immunoprecipitacions of cyclin B1 injected mRNA were performed as previously described in stage VI and MII fresh oocytes injected with 0.02 pmol of cyclin B1 WT or a mutant lacking CPE elements.

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Appendices

Appendix I:

Sequences of the 3' UTRs from CPEB4 (bankit1237562) and cyclin B1 (BC041302) and its corresponding 3'UTR variants.

The name of each construct is indicated. The elements identified are indicated: CPE elements in bod red letters; polyadenylation signals (Hexanucleotide) in bold blue letters; overlapping sequences between CPE and hexanucleotide in bold purple letters; repeats and putative ARE sequences in yellow grey shadowed letters. The mutations in each 3' UTR are underlined and indicated with black bold letters.

CPEB4 3'UTR WT

GCAACCCAUUGCTTUUCUCACUUUGCAAGCAUCCAAAUAAGUGCACUCUUCUGU
UCUCUUAAUCUCCCUCCCUCCUACCAUCUUUAGGAACGCAUGUCCUCUUGUUG
UAGUCUGUAUUUUAACAAUAGUAUAAUGAAGAAUGGCCGACACCAUAGGUAUU
UUGUAGAGUCUUGUGUCAUUGAGAACUGUAUUGGAACGCCUCUUGUUCAUAAC
AAUAUCACUCUGAUGCAUGCAAGUUUCAUGCUGUCCUUUUCAAAUAGCAAGGGA
GCAGGGGGACUGCAAGCCUUAUUAUUAUUAUUAUUAUUAAUAUAUUAUUUUUUU
UUUAUAUAUAAUUUUUAUCGGAGAGCAAAGCUUAAAAGCAAGAAAACUAUUGAGCG
AGAAAUAGAGUAUUUUUUUUUUUAUUAAAUUAUUGUUAAAUAUAAAGUAACGUAA
GAAUACUGUUAAUGUAACCAUGCAACCACAAUAACACUAUGGGUUUGUUGAACA
GAUUUUUUUUUUAAAUAUAUAUAUAUAUAUUAUUAGAGGGGAUUUUUAGCAUCU
AUUUAUUGUUUGTAUUUUUCCAGACUUUAUUGAAAAUAAAAAGUCAGCAGUUG
CCUAUUAAAAGCAUUAAGCA

CPEB4 3'UTR –C12

GCAACCCAUUGCTTUUCUCACUUUGCAAGCAUCCAAAUAAGUGCACUCUUCUGU
UCUCUUAAUCUCCCUCCCUCCUACCAUCUUUAGGAACGCAUGUCCUCUUGUUG
UAGUCUGUAUUUUAACAAUAGUAUAAUGAAGAAUGGCCGACACCAUAGGUAUU
UUGUAGAGUCUUGUGUCAUUGAGAACUGUAUUGGAACGCCUCUUGUUCAUAAC
AAUAUCACUCUGAUGCAUGCAAGUUUCAUGCUGUCCUUUUCAAAUAGCAAGGGA
GCAGGGGGACUGCAAGCCUUAUUAUUAUUAUUAUUAUUAAUAUAUUAUUUUUUUUU
UGGUAUAUAAUUUGGUCGGAGAGCAAAGCUUAAAAGCAAGAAAACUAUUGAGCG
AGAAAUAGAGUAUUUUUUUUUUUAUUAAAUUAUUGUUAAAUAUAAAGUAACGUAA
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CCUAUUAAAAGCAUUAAGCA

CPEB4 3'UTR –C3

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AAUAUCACUCUGAUGCAUGCAAGUUUCAUGCUGUCCUUUUCAAAUAGCAAGGGA
GCAGGGGGACUGCAAGCCUUAUUAUUAUUAUUAUUAUUAAUAUAUUAUUUUUUUU

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CPEB4 3'UTR –C4

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CPEB4 3'UTR –C123

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CPEB4 3'UTR –C34

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AAUAUCACUCUGAUGCAUGCAAGUUUCAUGCUGUCCUUUUCAAAUAGCAAGGGA
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AGAAAUAGAGUAUUUUUU**UUUU**<u>**GG**</u>**UAAA**UUAUUGUUAAAUAUAAAGUAACGUAA
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GAUUUUUU**UUUU**<u>**GGG**</u>UAUAUAUAUAUAUAUUAUUAGAGGGGAUUUUUAGCAUC
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GCCU**AUUAAA**AGCAUUAAGCA

CPEB4 3'UTR –C1234

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GCAGGGGGACUGCAAGCCUUAUUAUUAUUAUUAUUAUUAAUAUAUUAUUUUU**UU**
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AGAAAUAGAGUAUUUUUU**UUUU**<u>**GG**</u>**UAAA**UUAUUGUUAAAUAUAAAGUAACGUAA
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GAUUUUUU**UUUU**<u>**GGG**</u>UAUAUAUAUAUAUAUUAUUAGAGGGGAUUUUUAGCAUC
UAUUUAUUGUUUGTAU**UUUUCCA**GACUUUAUUGAA**AAUAAA**AAGUCAGCAGUU
GCCU**AUUAAA**AGCAUUAAGCA

CPEB4 3'UTR –C5

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CPEB4 3'UTR –C12345

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GCCUAUUAAAAGCAUUAAGCA

CPEB4 3'UTR –H2

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CPEB4 3'UTR –H3

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CPEB4 3'UTR –H23

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AUUUAUUGUUUGTAUUUUUCCAGACUUUAUUGAAACCAAAAAGUCAGCAGUUGC
CUAUUCCAAAGCAUUAAGCA

Cyclin B1 3'UTR WT

UGUUGCACCAUGUGCUUCUGUAAAUAGUGUAUUGUGUUUUUUAAUGUUUUUACUG
GUUUUAAUAAAGC

Cyclin B1 3'UTR –C123

UGUUGCACCAUGUGCUUCUGUAAAUAGUGUAUUGUGUUUUGGGAUGUUGGACU
GGUUGGAAUAAAGC

Appendix II

The work presented in this thesis has resulted in the following article

submitted for publication to Molecular Cell

CPEB4 replaces CPEB1 to complete meiosis

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RUNNING TITLE: CPEB1 regulates the expression of CPEB4 in the second meiotic division.

Keywords: CPEB1, CPEB4, Cytoplasmic polyadenylation, Xenopus Oocytes, Meiosis, Translational regulation.

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Abstract

In vertebrate oocytes, meiotic progression is driven by the sequential translational activation of maternal messenger RNAs stored in the cytoplasm. This activation is mainly induced by the cytoplasmic elongation of their poly(A) tails, which is mediated by the cytoplasmic polyadenylation element (CPE) present in their 3' untranslated regions (3´ UTRs). Sequential, phase-specific translation of these maternal mRNAs is required to complete the two meiotic divisions. Although the earlier polyadenylation events in prophase I and metaphase I are driven by the CPE-binding protein 1 (CPEB1), 90% of this protein is degraded by the anaphase promoting complex in the first meiotic division. The low levels of CPEB1 during interkinesis and in metaphase II raise the question of how the cytoplasmic polyadenylation required for the second meiotic division is achieved. In this work, we demonstrate that CPEB1 activates the translation of the maternal mRNA encoding CPEB4, which, in turn, recruits the cytoplasmic poly(A) polymerase GLD2 to late CPE-regulated mRNAs driving the transition from metaphase I to metaphase II, and, therefore, replacing CPEB1 for late meiosis polyadenylation.

INTRODUCTION

Vertebrate immature oocytes are arrested at prophase of meiosis I (PI). During this growth period, the oocytes synthesize and store large quantities of dormant mRNAs, which will later drive the oocyte's re-entry into meiosis (Mendez and Richter, 2001; Radford et al., 2008). The resumption of meiosis in Xenopus is stimulated by progesterone, which carries the oocyte through two consecutive M-phases (MI and MII), without intervening S-phase (Iwabuchi et al., 2000), to a second arrest at MII. Remarkably, oocyte maturation occurs in the absence of transcription (Newport and Kirschner, 1982) and is fully dependent on the sequential translational activation of the maternal mRNAs accumulated during the PI arrest (reviewed in (Belloc et al., 2008). The most extensively studied mechanism to maintain repressed maternal mRNAs in arrested oocytes and to activate translation during meiotic resumption is mediated by the cytoplasmic polyadenylation element (CPE) present in the 3' UTRs of responding mRNAs. The CPE recruits the CPE-binding protein 1 (CPEB1), which assembles a translational repression complex in the absence of progesterone and mediates cytoplasmic polyadenylation and translational activation upon progesterone stimulation. Activated CPEB1 recruits the cleavage and polyadenylation specificity factor (CPSF) to the nearby polyadenylation hexanucleotide (Hex), and, together, they recruit the cytoplasmic poly(A) polymerase GLD2 (for reviews see (Mendez and Richter, 2001; Radford et al., 2008; Richter, 2007). Nevertheless, the activation of CPE-containing mRNAs does not occur in masse at any one time (Belloc et al., 2008). Instead, the polyadenylation of specific mRNAs is temporarily regulated (Ballantyne et al., 1997; de Moor and Richter, 1997) by two sequential phosphorylations of CPEB1. First, phosphorylation of CPEB1 by Aurora A Kinase at PI, which is required for the first or "early" wave of polyadenylation and the PI-MI transition ((Mendez et al., 2000a; Mendez et al., 2000b; Pique et al., 2008) although see (Keady et al., 2007)). Second, the phosphorylation by Cdc2 and Plx1 at MI, which targets CPEB1 for degradation and is necessary to activate the second or "late" wave of polyadenylation and the MI-MII transition (Mendez et al., 2002; Pique et al., 2008; Reverte et al., 2001; Setoyama et al., 2007). This CPEB1 degradation, however, results in very low levels of this protein for the second meiotic division, when a third or "late-late" wave of cytoplasmic polyadenylation is essential for MII entry and Cytostatic Factor (CSF)-arrest (Belloc and Mendez, 2008), raising the question of how the polyadenylation machinery is recruited to the mRNAs activated in the third wave. Recently, three additional genes encoding CPEB-like proteins have been identified in vertebrates (Kurihara et al., 2003; Mendez and Richter, 2001; Theis et al., 2003), thus opening the possibility that other members of the CPEB-family could compensate for the degradation of CPEB1 in the

first meiotic division. Because CPEB2 and CPEB3 have been shown not to mediate cytoplasmic polyadenylation or translational activation, but rather to act only as translational repressors ((Hagele et al., 2009; Huang et al., 2006), Novoa et al. subbmited), we focused our study in the expression and function of CPEB4. Here we show that CPEB4 is encoded by a maternal mRNA that is translationally activated by CPEB1 in response to progesterone, leading to the accumulation of the protein in the second meiotic division. CPEB4, in turn, recruits the cytoplasmic poly(A) polymerase GLD2 to late-polyadenylated CPE-regulated mRNAs and is required for MI-MII progression. Based on these findings, we propose that CPEB1 establishes a new meiotic circuit by activating the synthesis of CPEB4, which, in turn, compensates for the degradation of CPEB1 by mediating cytoplasmic polyadenylation in the second meiotic division.

RESULTS

CPEB4 is encoded by a maternal mRNA and accumulates in the second meiotic division.

To determine whether CPEB4 was expressed in oocytes, we first cloned the previously uncharacterized *Xenopus Laevis* CPEB4 (Supplementary Figure 1), raised antibodies against this protein and analyzed its expression in a meiotic time course. CPEB4 was present at very low levels PI and gradually accumulated in response to progesterone, reaching maximal levels in the second meiotic division (Figure 1A). Interestingly, CPEB4 followed an expression pattern complementary to that of CPEB1, which was highly expressed in PI-arrested oocytes and also in MI, but was degraded and virtually disappeared in MII-arrested oocytes (Figure 1A). Contrary to CPEB1 (Hake and Richter, 1994), CPEB4 levels remained stable after fertilization and even after the mid-blastula transition (Supplementary Figure 2). Because the expression pattern of CPEB4 was consistent with this factor being encoded by a maternal mRNA, i.e. silenced in PI-arrested oocytes and translationally activated by cytoplasmic polyadenylation in response to progesterone, we measured the poly(A) tail length of the endogenous CPEB4 mRNA (Figure 1B). The CPEB4 transcript, which contained a short-poly(A) tail in PI oocytes, was polyadenylated in metaphase I (MI) and partially deadenylated in the second meiotic arrest at metaphase II (MII).

To get further insight into the translational regulation of CPEB4 mRNA, the 3' UTR of the endogenous transcript was identified and cloned (Supplementary Figure 1C). CPEB4 3' UTR contains 3 potential Hexs, 5 potential CPEs and 3 long AU-rich stretches with potential AREs. To assess whether these elements mediate the

polyadenylation behavior observed for the endogenous CPEB4 mRNA, we in vitro transcribed and microinjected labeled probes corresponding to the WT or mutant variants of CPEB4 3' UTR (Figure 1C). The WT probe (CPEB4 3' UTR) displayed the same polyadenylation pattern than the endogenous CPEB4 mRNA, being polyadenylated in MI and then partially deadenylated during interkinesis and in MII. As control, we microinjected the 3' UTR of cyclin B1 (cyclin B1 3' UTR), which contained CPEs but not AREs (Belloc and Mendez, 2008), and was polyadenylated in MI remaining polyadenylated thereafter. These progesterone-induced polyadenylations were abrogated when the putative CPEs were inactivated by point mutations (CPEB4 3'UTR-CPEs and cyclin B1 3' UTR-CPEs; see Supplementary Figure 3 for sequence of the 3' UTR variants). When the same UTRs were microinjected with a long poly(A) tail [CPEB4 3' UTR-CPEs(polyA) and cyclin B1 3' UTR-CPEs(poly(A)], the CPEB4- but not the cyclin B1-derived probe was specifically deadenylated after MI. To test if this deadenylation was mediated by the recruitment of C3H-4, which is an ARE-binding protein that is synthesized from a maternal mRNA activated from the first wave of cytoplasmic polyadenylation and that modulates deadenylation of ARE-containing mRNAs after GVBD (Belloc and Mendez, 2008), we microinjected the CPEB4 3' UTR probe in oocytes depleted of C3H-4 (Figure 1D). The deadenylation of CPEB4 3' UTR was partially prevented in C3H-4 depleted oocytes, both when the UTR-probe was injected deadenylated, acquiring the poly(A) tail by cytoplasmic polyadenylation, or with a synthetic poly(A) tail (Figure 1D). Altogether, these results indicate that CPEB4 mRNA is a maternal transcript stored inactive in PI arrested oocytes, polyadenylated in MI by CPEB, and partially deadenylated in the second meiotic division by C3H-4.

To determine whether the observed changes in poly(A) tail length were also reproduced at a translational level, we microinjected chimaeric mRNAs with the luciferase ORF followed by WT or mutant CPEB4 3' UTRs (See Supplementary Figure 3 for sequences). In a meiotic time course (Figure 2A), both CPEB4 and cyclin B1 3' UTRs repressed translation in PI-arrested oocytes, compared with a control 3' UTR. After progesterone stimulation, both CPEB4 and cyclin B1 3' UTRs mediated translational activation. But, even though the accumulation of luciferase followed the same kinetics at early time points (MI), the increase in luciferase generated from the CPEB4 3' UTR chimerical construct slowed down during the second meiotic division, whereas the accumulation of luciferase from the cyclin B1 3' UTR chimerical mRNA continued to increase at a similar rate during the whole length of meiosis, until the MII arrest. These translational kinetics are in agreement with the fact that cyclin B1 3' UTR remains polyadenylated during the two meiotic divisions and CPEB4 3' UTR is partially deadenylated in the second meiotic division. The translational repression was

dependent on the CPE cluster of two consensus CPEs (CPEs 1 and 2), but the translational activation was sustained by either of the two more 3' CPEs (CPEs 4 or 5) and required the second Hex (Figure 2B). Thus, translational repression was most likely mediated by a CPEB dimer, as shown before for cyclins B UTRs (Pique et al., 2008), whereas the activation required the hexanucleotide and the nearby CPE, in agreement with being mediated by early cytoplasmic polyadenylation (Pique et al., 2008). We conclude from these data that CPEB1 mediates the early cytoplasmic polyadenylation of CPEB4 mRNA, activating its translation upon progesterone stimulation. CPEB4 mRNA is, however, partially deadenylated after C3H-4 accumulation in late MI (Belloc and Mendez, 2008), slowing down translation and leading to the gradual accumulation of CPEB4 that reach its highest levels only at the MII arrest.

CPEB4 is required for meiotic progression between MI and MII.

We then proceeded to ask whether the CPEB1-induced synthesis of CPEB4 was required for meiotic progression. To this aim, CPEB4 mRNA was ablated by independent microinjection of 4 different antisense oligonucleotides, targeting either the ORF, the 3' or the 5' UTRs. These antisense oligonucleotides efficiently knocked down CPEB4 synthesis after progesterone stimulation and caused external morphological changes consistent with abnormal meiotic progression (Figure 3A and Supplementary Figure 4). The corresponding sense oligonucleotides were injected as control. To define the meiotic phenotype resulting from inhibiting CPEB4 synthesis, we monitorized the chromosome dynamics by direct visualization of stained DNA, and the H1-kinase (Cdc2) activity in oocyte extracts from a meiotic time course (Figure 3B). Control oocytes displayed the characteristic DNA staining with extruded polar body and oocyte chromosomes arranged in the methaphasic plate. In addition, cdc2 activity increased in response to progesterone, sharply decreased after MI and augmented again at MII. In CPEB4-depleted oocytes, the polar body was not detectable and the chromosomes were partially decondensed and not arranged in a methaphasic plate, indicating that these oocytes did not complete the first meiotic division. Cdc2 activity in depleted oocytes showed normal stimulation after progesterone and the subsequent partial inactivation, indicating correct meiotic resumption until anaphase-I (Figure 3B). At later times, H1 kinase was partially reactivated, most likely as a consequence of oocyte apoptosis, which stimulates cdc2 and cdk2 (Zhou et al., 1998). Accordingly, the reactivation of H1 kinase was more evident with the CPEB4as2, which show more apoptotic symptoms than the CPEB4as1 (Figure 3B and supplementary Figure S4). This phenotype was rescued by overexpressing CPEB4 from a microinjected mRNA

not targeted by the antisense oligonucleotides (Figures 3A and 3B). To further characterize the meiotic defect originated by preventing CPEB4 synthesis, we analyzed whether DNA replication was activated, denoting exit from meiosis between MI and MII (Figure 3C). Measurement of the incorporation of microinjected labeled dCTP into DNA demonstrated that, although control oocytes did not synthesize DNA in the course of a normal meiosis, new DNA was indeed generated in CPEB4-depleted oocytes. As a positive control, we depleted Xkid mRNA, which causes meiotic exit and DNA synthesis after MI (Perez et al., 2002). The incorporation of labeled dCTP was sensitive to aphydicoline, revealing that DNA replication, rather than DNA repair, was taking place (Furuno et al., 1994). Collectively, these data illustrate that CPEB4-depleted oocytes progress from PI to MI, but failed to transition between MI and MII and exit meiosis replicating the DNA.

CPEB1 and CPEB4 recruit the polyadenylation machinery to similar CPE-regulated mRNAs, but at different meiotic phases.

Because the CPE-regulated mRNAs required for cdc2 reactivation and CSF activity in the second meiotic division are polyadenylated in the third or late-late meiotic wave (Belloc and Mendez, 2008), when CPEB1 levels are negligible, we next sought to determine whether CPEB4 could also bind to these mRNAs, thus substituting CPEB1 function. We first tested if CPEB4 was able to bind cyclin B1 3' UTR in a CPE-dependent manner. Oocytes were microinjected with WT cyclin B1 3' UTR or with a variant in which the CPEs were inactivated by point mutations. Then, the association of CPEB1 and CPEB4 to these reporters was analyzed by IP followed by RT-PCR (Figure 4A). Both proteins co-immunoprecipitated the WT UTR, but not the mutant UTR. Interestingly, for CPEB1 the amount of immunoprecipitated probe was larger in the first meiotic division than in the second, whereas for CPEB4 the proportion was reverted with higher binding in MII (Figure 4A). Once determined that CPEB4 recognizes the same CPEs than CPEB1, we assessed the association of both proteins to "early" polyadenylated (CPEB4, Emi1 and mos), "late" polyadenylated (cyclin B1) and "late-late" polyadenylated (Emi2 and cyclin E) endogenous mRNAs (Figure 4B). We found that CPEB4 was bound to late and late-late mRNAs in MII, but not to the early mos and Emi1 mRNAs. CPEB1, however, was bound to all, early, late and late-late mRNAs in PI, but not in MII. As a negative control, GAPDH was not associated with CPEB1 or CPEB4. Thus, CPEB1 and CPEB4 regulate overlapping, but not identical, subpopulations of mRNAs in the first and second meiotic divisions, respectively. Interestingly CPEB4 was recruited to its own mRNA in MII, suggesting a positive feed back loop that may explain why CPEB4 mRNA is not completely deadenylated by

C3H-4. Intriguingly, both mos and Emi1 are not only "early" polyadenylated mRNAs but also weak-polyadenylated (Belloc and Mendez, 2008; Pique et al., 2008). This, together with the fact that CPEB4 has lowed affinity for the CPEs than CPEB1 (Novoa et al. submitted), may explain the differential associations of both proteins to CPE-regulated mRNAs.

To verify if endogenous CPEB4 was able to recruit the polyadenylation machinery, we immunoprecipitated both CPEB1 and CPEB4 and analyzed the co-immunoprecitates for the presence of cytoplasmic poly(A) polymerase GLD2. Both proteins were equally able to recruit GLD2 (Figure 4C). To rule out that the co-immunoprecipitation of GLD2 by CPEB4 was through association with CPEB1, we also analyzed the immunoprecipitates for the presence of CPEB1. CPEB4 did not co-immunoprecipitate CPEB1 in MII (Figure 4C), indicating that both CPEBs are not bound to the same mRNA, and that the association of GLD2 and CPEB4 is not indirectly mediated through a potential dimerization with CPEB1. Thus, both CPEB1 and CPEB4 recruit the polyadenylation machinery to CPE-regulated mRNAs, but at different meiotic phases.

CPEB1 and CPEB4 are functionally distinct.

We further determined whether CPEB1 and CPEB4 were functionally equivalent by substituting CPEB4 with CPEB1 in the second meiotic division. For this purpose, we overexpressed a non-degradable form of CPEB1. This CPEB1 had the cdc2-phosphorylated residues substituted by alanines (CPEB1-CA), but still contains the regulatory ser 174, targeted by Aurora A Kinase and required to activate CPEB1 (Mendez et al., 2000a). Phosphorylation of CPEB1 by Cdc2 is required for its degradation at anaphase I, (Mendez et al., 2002; Setoyama et al., 2007). To avoid the meiotic arrest caused by overexpressing high levels of non-degradable CPEB1 in PI (Mendez et al., 2002), we microinjected a deadenylated mRNA encoding CPEB1-CA, which drove the accumulation CPEB1-CA to similar levels than WT-CPEB in PI, but predominantly after GVBD (Figure 5A), and, therefore, without interfering with meiotic progression (Figure 5B). This pattern of overexpression of CPEB1-CA had no mayor effects in the polyadenylation of cyclin B1 mRNA (Figure 5C). Depletion of CPEB4 caused a meiotic blockage after MI (Figures 3B and 5B) and partially prevented the polyadenylation of the "late-late" mRNA encoding Cyclin E (Figure 5D), but did not affect the polyadenylation of cyclin B1 mRNA (Figure 5D), consistently with this transcript being polyadenylated by CPEB1 in MI, before CPEB4 accumulates. However, The non-degradable CPEB1 was not able to compensate for the lack of CPEB4 in the second meiotic division; if anything, the phenotype was even aggravated (Figure 5B). Accordingly, polyadenylation of cyclin E mRNAs was not rescued by

expressing CPEB1-CA (Figure 5D). Surprisingly, substitution of CPEB4 by CPEB1 resulted in a shortened poly(A) tail for cyclin B1 (figure 5D). Because this mRNA is polyadenylated by CPEB1 in MI, in the absence of CPEB4, and this polyadenylation was not affected by overexpressing CPEB1-CA (Figure 5C) we interpret this result as deadenylation at later meiotic times caused by the presence of non degradable CPEB1, in the absence of CPEB4, after anaphase I. Therefore, degradation of CPEB1 and new synthesis of CPEB4 in late meiosis seems to be required to prevent deadenylation during interkinesis of "early" and "late" mRNAs polyadenylated by CPEB1 during PI-MI, while maintaining the oocyte capability to generate the third wave of "late-late" polyadenylation.

Thus, we concluded that although both CPEB1 and CPEB4 were able to recruit the polyadenylation machinery to similar populations of mRNAs, they were not functionally exchangeable. This finding, together with the low homology of both factors in their regulatory N-terminal domains, suggests that CPEB1 and CPEB4 may be subjected to differential regulation during meiotic cell cycle beyond their complementary pattern of expression.

DISCUSSION

Progression through the two meiotic divisions requires the sequential activation of maternal mRNAs encoding factors that drive cell cycle phase transitions. This sequential activation is achieved by a combination of successive phosphorylation events in CPEB1 with a combinatorial arrangement of CPEs and AREs in the CPEB-regulated mRNAs. First, Aurora A kinase activates CPEB1 and triggers the "early" wave of cytoplasmic polyadenylation required for the PI-MI transition ((Mendez et al., 2000a; Mendez et al., 2000b; Pique et al., 2008) although see (Keady et al., 2007)). Then, in MI, Cdc2- and Plx-mediated phosphorylations target CPEB1 for SCF(beta-TrCP)-dependent degradation, thus lowering CPEB1 levels. Low CPEB1 levels are, in turn, necessary to trigger the second or "late" wave of polyadenylation required for MI-MII transition (Mendez et al., 2002; Pique et al., 2008; Reverte et al., 2001; Setoyama et al., 2007). These "late" mRNAs, such as cyclin B1 mRNA, contain at least two CPEs being the most distal one overlapping with the hexanucleotide, which becomes accessible to CPSF only upon CPEB1 degradation (Mendez et al., 2002). The drawback of the degradation of CPEB1 in MI is that the remaining levels of this factor are then very low for interkinesis and for the second meiotic division, when the third or "late-late" wave of cytoplasmic polyadenylation is required to mediate the MII-arrest by CSF (Belloc and Mendez, 2008). During interkinesis, APC activation is combined with

increased synthesis of cyclins B1 and B4 (Hochegger et al., 2001; Pique et al., 2008) resulting in only a partial inactivation of MPF at anaphase-I and preventing entry into S-phase (Iwabuchi et al., 2000). Full reactivation of MPF for MII requires re-accumulation of high levels of cyclins B, as well as the inactivation of APC by newly synthesized Emi2 and other components of the CSF, such as cyclin E or high levels of Mos (Belloc and Mendez, 2008; Schmidt et al., 2006).

The recent discovery of other members of the CPEB family of proteins, together with the description of an autoregulatory loop of the CPEB-ortholog Orb (Tan et al., 2001), pointed us to explore the possibility that CPEB1 could activate the translation of other members of the CPEB family to compensate for its reduced levels after MI. All CPEB-like proteins have a similar structure with most of the carboxy-terminal regions composed of two RNA recognition motifs (RRMs) and two zinc-fingers. On the other hand, the regulatory amino-terminal domains of the CPEB proteins show a small degree of identity. The most extensively studied member of the family, CPEB1, has dual functions as a translational repressor and activator, whereas CPEB3 and CPEB2 seem to act only as translational repressors (Hagele et al., 2009; Huang et al., 2006; Schmitt and Nebreda, 2002); Novoa et al. submitted).

In the present study, we have found that CPEB4 is encoded by a maternal mRNA activated by CPEB1 during the "early" wave of cytoplasmic polyadenylation, being then partially inactivated by C3H-4-mediated deadenylation. This translational regulation leads to the gradual accumulation of CPEB4 from MI to reach maximal levels in the MII arrest. In turn, CPEB4 is required for the MI-MII transition and recruits GLD-2 to "late" and "late-late" CPE-regulated mRNAs, which are activated by cytoplasmic polyadenylation during interkinesis and encode proteins required for the second meiotic division and to prevent DNA replication after MI (Belloc and Mendez, 2008; Eliscovich et al., 2008; Hochegger et al., 2001; Pique et al., 2008). Altogether, our work demonstrates that CPEB4 replaces CPEB1 for the second meiotic division by regulating CPE-containing mRNAs. Although CPEB1 and 4 recognize overlapping populations of mRNAs and recruit GLD2, they are not interchangeable because a stabilized CPEB1 can not replace CPEB4 for the transition from MI to MII nor for the polyadenylation the "late-late" mRNA encoding Cyclin E. This may reflect the differential regulation of both proteins. Besides the fact that CPEB1 contains a PEST box that mediates its degradation upon phosphorylation by Cdc2 and Plx, CPEB1 is the only member of the family that contains Aurora A Kinase phosphorylation sites (Mendez and Richter, 2001). Instead, CPEB4 contains putative recognition sites for PKA, CaMKII and S6 kinase (Theis et al., 2003), suggesting differential posttranslational regulation of both factors during meiotic progression. Accordingly,

overexpressed CPEB4 shows mobility changes in response to progesterone without any effect on its stability (Supplementary Figure 5). In addition, thethered CPEB4 requires progesterone to activate translation and polyadenylation of reporter mRNAs (Novoa et al submitted). These observations suggest that CPEB4 is not constitutively active, but, rather, it has to be posttranslationally modified to become active and, not having a consensus Aurora A Kinase phosphorylation site, it will most likely be activated by a different mechanism taking place at later meiotic phases. Furthermore, sustained levels of CPEB1 after MI, in the absence of CPEB4, result in the deadenylation of cyclin B1 mRNA, consistently with the described function of CPEB1 in deadenylation by recruiting PARN (Kim and Richter, 2006). Taken these findings together, we propose a model by which CPEB1 mediates the early wave of cytoplasmic polyadenylation required to enter the first meiotic metaphase. Then, CPEB1 degradation is necessary to allow the polyadenylation of "late" mRNAs in MI and to prevent deadenylation in interkinesis and MII. Concomitantly, CPEB1 activates the synthesis of CPEB4, which supports the third wave of "late-late" polyadenylation during interkinesis and MII.

MATERIAL AND METHODS

***Xenopus* oocytes preparation.** Stage VI oocytes were obtained from Xenopus females and induced to mature with progesterone (10 μM, Sigma), as described previously (de Moor and Richter 1994)

Plasmid constructs. CPEB4 (bankit123762) cDNA was cloned by RT-PCR from total RNA of stage VI oocytes using primers 5'-CGGGATCCATGGGGGGATTACGGGTTTGGAG-3' and 5'-TCCCCCGGGTCAGTTCCAGCGGAATGAAATATGC-3', digested with Sma and BamHI and cloned in pGEX or pET30a expression vectors. CPEB4 3' UTR was amplified by RT-PCR from total RNA of stage VI oocytes using primers 5'-GAAGATCTTGAGCAACCCATGGCTTAGC-3' and 5'-TGCTTAATGCTTTTAATAGGCAACTGC-3', digested with Bgl-II and cloned in the pLucassette downstream the Firefly Luciferase ORF.

Hexanucleotide mutants of CPEB4 were obtained by PCR from the original plasmid with T3 standard primer as sense oligonucleotide and the following antisense oligonucleotides: H2as: 5'-TGCTTAATGCTTTTAATAGGCAACTGCTGACTTTTCCTTTTCAATAAAG-3'; H3as: 5'-TGCTTAATGCTTTCCATTGGCAACTGCTGACTTTTTATTTTCAATAAAG-3'; H23as: 5'-TGCTTAATGCTTTCCATAGGCAACTGCTGACTTTTCCTTTTCAATAAAG-3'.

CPE mutants were obtained with QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) following manufacture's instructions. The oligonucleotides used were:

C12: 5'-TATTATTTTTTTT<u>GG</u>TATATAATTT<u>GG</u>TCGGAGAGCAAAGC-3';

C3: 5'-CGAGAAATAGAGTATTTTTTTTT<u>GG</u>TTAAATTATTG-3';

C4: 5'- GGTTTGTTGAACAGATTTTTTTTTT<u>GGG</u>ATATATATATATA-3';

C5: 5'-GTTTGTATTT<u>GGC</u>CAGACTTTATTGAAAATAAAAAG-3'.

Translational control and cytoplasmic polyadenylation by 3' UTR. Translation and polyadenylation of reporter mRNAs were assayed as described previously (Pique et al., 2006). 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 manufacturer's instructions.

Western blot analysis. Oocyte lysates, prepared by homogenizing 6-10 oocytes in histone H1Kinase buffer containing 0.5% NP-40 and centrifuged at 12,000g for 10 min, were resolved by 8% SDS-PAGE. Equivalents of 1-2 oocytes were loaded onto each lane. Antibodies used were rabbit antiserum affinity purified against CPEB4, rabbit antiserum against CPEB1, monoclonal antibody against α-tubulin (DM1A, Sigma).

RNA-ligation-coupled RT-PCR. Total oocyte RNA was isolated from 8-10 oocytes by Ultraspec RNA Isolation System (Biotecx Laboratories, Inc.), following manufacturer's instructions. Then, RNA-ligation coupled RT-PCR technique was performed as described previously (Charlesworth et al. 2004) with some modifications. Briefly, 5 μg of oocyte total RNA was ligated to 0.5 μg of a 3'-amino-modified DNA anchor primer (5´ -P-GGTCACCTCTGATCTGGAAGCGAC-NH2-3´) in a 10 μl reaction using T4 RNA ligase (New England Biolabs), according to the manufacturer's directions. RNA ligation reaction was used in a 50 μl reverse transcription reaction using RevertAid M-MuLV Reverse Transcriptase (Fermentas) and 0.5 μg of an oligo anti-sense to the anchor primer plus four thymidine residues on its 3'-end (5'- GTCGCTTCCAGATCAGAGGTGACCTTTTT-3'). The resulting reaction product was digested with 2 μg RNAse A (Fermentas) and two microliters of this cDNA preparation were used as a template for gene-specific PCR reaction. The specific oligos were: 5'-CCGAGGCATATTTCATTCCGCTGG-3' for CPEB4, 5'-GTCAAGGACATTTATGCTTACC-3' for cyclin B1, 5'-GTGCTTTAACTCTGTGCATCAC-3' for cyclin E. DNA products from the PCR reaction were analysed in a 2% agarose gel and visualized by ethidium bromide staining.

Southern Blot. DNA products from RNA-ligation coupled RT-PCR of the endogenous CPEB4 mRNA were analysed in a 2% agarose gel and transferred to Hybond-N+ nyclon memebrane (Amesham Pahrmacia Biotech) as described in the manufacturer's protocol. The membrane was probed using 5'-GGTGAAGGAGGGAGGAGATCG-3' oligo targeting the 3'UTR of CPEB4.

Cytoplasmic polyadenylation by 3' UTR. Total RNA was isolated from 6-8 oocytes injected with radiolabeled 3' UTR by Ultraspec Isolation System (Biotecx Laboratories, Inc.). RNAs were analyzed by 6% polyacrimaldide/8M urea gel electrophoresis followed by autoradiography, as previously described in (Pique et al., 2006).

Histone H1 kinase assay (Cdc2 Assay). Oocyte lysates prepared by homogenizing 3 oocytes in histone H1Kinase Buffer (80 mM Na β-glycerophosphate, 20 mM EGTA, 15 mM MgCl$_2$, 50

mM NaVaO$_4$) and centrifuged at 12,000g for 10 minutes at 4ºC were incubated with histone H1 (sigma) and [γ-^{32}P]ATP (3000 Ci/mmol) as described previously (Mendez et al., 2000a). The phosphorylation reaction was analyzed by 12% SDS-PAGE gel and autoradiography.

Chromosomes and polar body observation. Oocytes fixed for at least 1 h in 100% methanol were incubated overnight in presence of 20 μg/l Hoechst dye. Chromosomes and polar body of stained oocytes were viewed from animal pole under UV epifluorescence microscope (Leica DMR microscope, 63X magnification, Leica DFC300FX camera, Leica IM1000 Image Manager).

Antisense oligonucleotide and rescue experiment. To ablate the expression of CPEB4, oligonucleotides targeting either 5' UTR or the 3' UTR were designed; one complementary sequence was used as a control. In each oocyte, 99 ng of oligonucleotide was injected. After overnight (16 h) incubation at 18ºC, progesterone was added as described. For rescue experiment, 0.06 pmol of in vitro transcribed RNA coding for the ORF of CPEB4 or 0.02 pmol of the non-degradable CPEB1 mutant were injected 1-2 h before progesterone incubation. Oligonucleotides used were: 19AS: 5'-GAGGAAATATATCTGGGTGAAG-3'; 20AS: 5'-GCAATGGGTTGCTCAGTTCCA-3'; 23S: 5'-CTTTGCAAGCATCCAAATAAG-3'.

Analysis of DNA synthesis. Oocytes were injected with 0.4 μCi [α-32P]dCTP and treated subsequently with progesterone to induce maturation. Mature oocytes were subjected to DNA extraction, as described by Wong et al 1998, and samples with equal number of total counts (0,5x10^6 c.p.m.) were analyzed by 1% agarose gel electrophoresis and autoradiography, as described previously (Newport and Kirschner, 1984).

Immunoprecipitation. CPEB4 antibody raised in rabbits against the CPEB4 71-85 peptide (DEILGSEKSKSQQQQ), and CPEB1 antibody were incubated with protein-A sepharose during 2 h at room temperature (RT) on wheel, washed with PBS and resuspended in sodium borat pH 9.0. 20mM dimethyl pimelimidate·2HCl (DMP) was added and incubated 30 min at RT on wheel. Reaction was stopped with two 5 min-washes at RT with 0.05 M glycine, and two extra washes with PBS. Fresh oocyte lysates from stage VI and MII (25 oocytes per condition) were added to the crosslinked antibody-beads and incubated 2 h at 4ºC on wheel. Immunoprecipitates were washed three times in lysis buffer (20 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.5% NP-40, 1 mM MgCl$_2$, 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 analyzed by Western blotting.

Immunoprecipitations followed by RT-PCR were performed as described (Aoki et al., 2003) with fresh stage VI and MII oocyte lysates (25 oocytes per condition). CPEB4 antibody raised in rabbits against the CPEB4 71-85 peptide (DEILGSEKSKSQQQQ), and CPEB1 antibody. The protein-bound RNAs were purified by proteinase K digestion followed by phebol-chloroform extraction. Half of the total RNA extracted was used for the retrotranscription, performed with the 3'Race primer (TAATACGACTCACTATAGGGCGGATCCTTTTTTTTTTTTTTTTTTTTTTTTTTTVN) with the mMLuV reverse transcriptase from Fermentas following the manufacturer's instructions. A twentieth part of the cDNA was used for each specific PCR (EcoTaq polymerase, Ecogen) with

following specific primers: 5'-GTCAAGGACATTTATGCTTACC-3' and 5'-CCATGTCCCGAATTTGAGCC-3' for cyclin B1; 5'-TGAGCAACCCATGGCTTAGC-3' and 5'-TGCTTAATGCTTTTAATAGGCAACTGC-3' for CPEB4; 5'-ACAGAATTTACGGAGGTTATAGTT-3' and 5'-CGGAATTCCGGGCAATAATTTATTTAGCACAAAAAAA for Emi1; 5'-gcacaacatggagaaaactgctgcag-3' and 5'-CTATAACCTCCGTAAATTCTGTTTGC-3' for Emi2; 5'-GCATCAATTTTGGACCTCGTGAACGC-3' and 5'-GCCTCTTTTTTAGGGATCCTCTTTGC-3' for Cyclin E; 5'-ATGTGTTGCATTGCTGTTTAAGTGG-3' and 5'-AGACAAATCAATTTCTTTATTATAAAAC-3' for mos; 5'-GGCCGCCATTAAGACTGCATC-3' and 5'-GACTAGCAGGATGGGCGAC-3' for GAPDH.

Immunoprecipitacions of cyclin B1 injected mRNA were performed as previously described in stage VI and MII fresh oocytes injected with 0.02 pmol of cyclin B1 WT or a mutant lacking CPE elements.

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FIGURE LEGENDS

Figure 1. CPEB4 mRNA polyadenylation results in CPEB4 accumulation during the second meiotic division. (A) Xenopus oocytes stimulated with progesterone (prog) were collected at the indicated times and analysed by western blotting using anti-CPEB4, anti-CPEB1 or anti-Tubulin antibodies. The meiotic phases of the oocyte are indicated (PI, prophase-I; GVBD, germinal vesicle breakdown; MI, metaphase-I; I, interkinesis; MII, metaphase-II). GVBD was determined by the appearance of the white spot at the animal pole of the oocyte. (B) Total RNA extracted from oocytes untreated (-P) or incubated with progesterone and collected at metaphase-I (MI) and metaphase-II (MII) were analysed by RNA-ligation-coupled RT-PCR followed by southern blot hybridization with a labelled probe for the 3'UTR of CPEB4 mRNA. The PCR products derived from the polyadenylated and non-polyadenylated CPEB4 mRNA are indicated. (C) Oocytes were injected with the indicated radiolabeled 3'UTRs. Total RNA was extracted from oocytes collected at the indicated times after progesterone stimulation and analyzed by gel electrophoresis followed by autoradiography. Schematic representation of the 3' UTRs is shown: CPEs as dark grey hexagons, Hexanuclotide as grey boxes, PBEs as rhombus, putative AREs elements as light grey ovals. CPE point mutations are indicated as a cross. (D) Oocytes were injected with C3H-4 antisense oligonucleotide (asC3H-4) or C3H-4 sense oligonucleoitde (control). After 16h, oocytes were injected with the indicated radiolabeled 3'UTRs. Total RNA was extracted from oocytes collected at the indicated times after progesterone stimulation and analyzed by gel electrophoresis followed by autoradiography.

Figure 2. CPEB4 is translationally activated by CPEB1 during meiotic maturation. (A,B) The indicated in vitro transcribed Firefly luciferase chimerical mRNAs were co-injected into oocytes together with Renilla luciferase as a normalization control. (A) Firefly luciferase ORF fused to a control 3'UTR of 470 nucleotides (control); cyclin B1 3'UTR wild-type (cyclin B1 3'UTR) and CPEB4 3'UTR wild-type (CPEB4). Oocytes were stimulated with progesterone, collected at the indicated times and the luciferase activities were measured. Data are mean±s.d. (n=4). (B) The indicated Firefly luciferase-3' UTR variants were injected in oocytes. Oocytes were then incubated in the absence (Repression) or presence (Activation) of progesterone and the luciferase activities determined after six hours. The percentage of translational repression in the absence of progesterone (left panel) was normalized to control (100% translation) and to the fully repressed B1 (0% translation). The percentage of translation stimulation

was normalized to control (0% stimulation) and B1 (100% simulation). Data are mean±s.d. (n=5). A schematic representation of the 3' UTR, as in Figure 1, is shown.

Figure 3. CPEB4 synthesis is required for the MI to MII transition.

(A,B) Xenopus oocytes were injected with CPEB4 sense (s) or antinsense (as1, as2) oligonucleotides as indicated and incubated for 16 hours. Then, the oocytes were microinjected with CPEB4-enconding mRNA and incubated in the presence or absence of progesterone (prog) as indicated. All the oocytes were collected 4 hours after the control, non-injected oocytes, displayed 100% GVBD and analyzed as follows. (A) The oocytes were anlyzed for CPEB4 levels by western blot using anti-CPEB4 and anti-Tubulin antibodies (2 oocyte equivalents were loaded per lane). (B) Oocytes were fixed, stained with Hoechst and examined under epifluorescence microscope. Representative images and the percentage of appearance for each phenotype are shown. The arrow indicates the first polar body. Scale bar 10 μm. Oocytes collected at the indicated times after progesterone stimulation were analyzed for H1 Kinase activity as described in materials and methods. (C) Oocytes injected with CPEB4 antisense oligonucleotide (as2), CPEB4 sense oligonucleotide (control) and Xkid antisense oligonucleotide (asXkid) were incubated for 16 hours and then injected with 0.4 μCi [α-32P]dCTP. Then, the oocytes were stimulated with progesterone and incubated in the presence or absence of Aphydicolin (Aph) as indicated. Oocytes were collected 5 hours after control oocytes displayed 100% GVBD, DNA was extracted and analyzed by agarose gel electrophoresis followed by autoradiography.

Figure 4. CPEB1 and CPEB4 are sequentially associated with CPE-containing mRNAs.

(A) Xenopus oocytes were microinjected with in vitro transcribed RNAs derived from WT cyclin B1 3'UTR (cyclin B1) or the corresponding variant with the CPEs inactivated by point mutations (cyclin B1-CPE). Then, the oocytes were incubated for 8 hours in the presence (MII) or absence (-P) of progesterone and subjected to immunoprecipitation with anti-CPEB1, anti-CPEB4 and control IgG antibodies followed by RT-PCR for the microinjected RNAs. The PCR products derived from the microinjected (imput) and coimmunoprecipitated (IP) RNAs were visualized by stained agarose gel electrophoresis. (B,C) Cytoplasmic extracts from oocytes untreated (-P) or incubated with progesterone for 8h (MII) were subjected to immunoprecipitation with anti-CPEB1, anti-CPEB4 and control IgG antibodies. The coimmunoprecipitates were analyzed by RT-PCR for the presence of the indicated mRNAs, as in d (B) or by western blotting for the presence of GLD-2 and CPEB1 proteins (C).

Figure 5. A stable CPEB1 mutant cannot replace for CPEB4 in the second meiotic division. Xenopus oocytes were injected with CPEB4 sense (control) or antisense (as2) oligonucleotides. After 16 hours, oocytes were microinjected with mRNAs encoding either CPEB4 or CPEB1-CA and incubated with progesterone. (A) Oocytes were collected at the indicated times and analyzed for CPEB1 levels by western blot using anti-CPEB1 and anti-Tubulin antibodies (1,5 oocyte equivalents were loaded per lane) (B) Oocytes were collected 4 hours after control oocytes display 100% GVBD and treated as fig 3b. (C,D) Total RNA from oocytes collected at the indicated times was extracted and polyadenylation status of cyclin B1and cyclin E mRNAs was measured by RNA-ligation-coupled RT-PCR.

Figure 6. **Schematic diagram showing the sequential activities of CPEB1 and CPEB4 mediating the three waves of polyadenylation driving meiotic progression.**

Igea & Mendez Fig 1
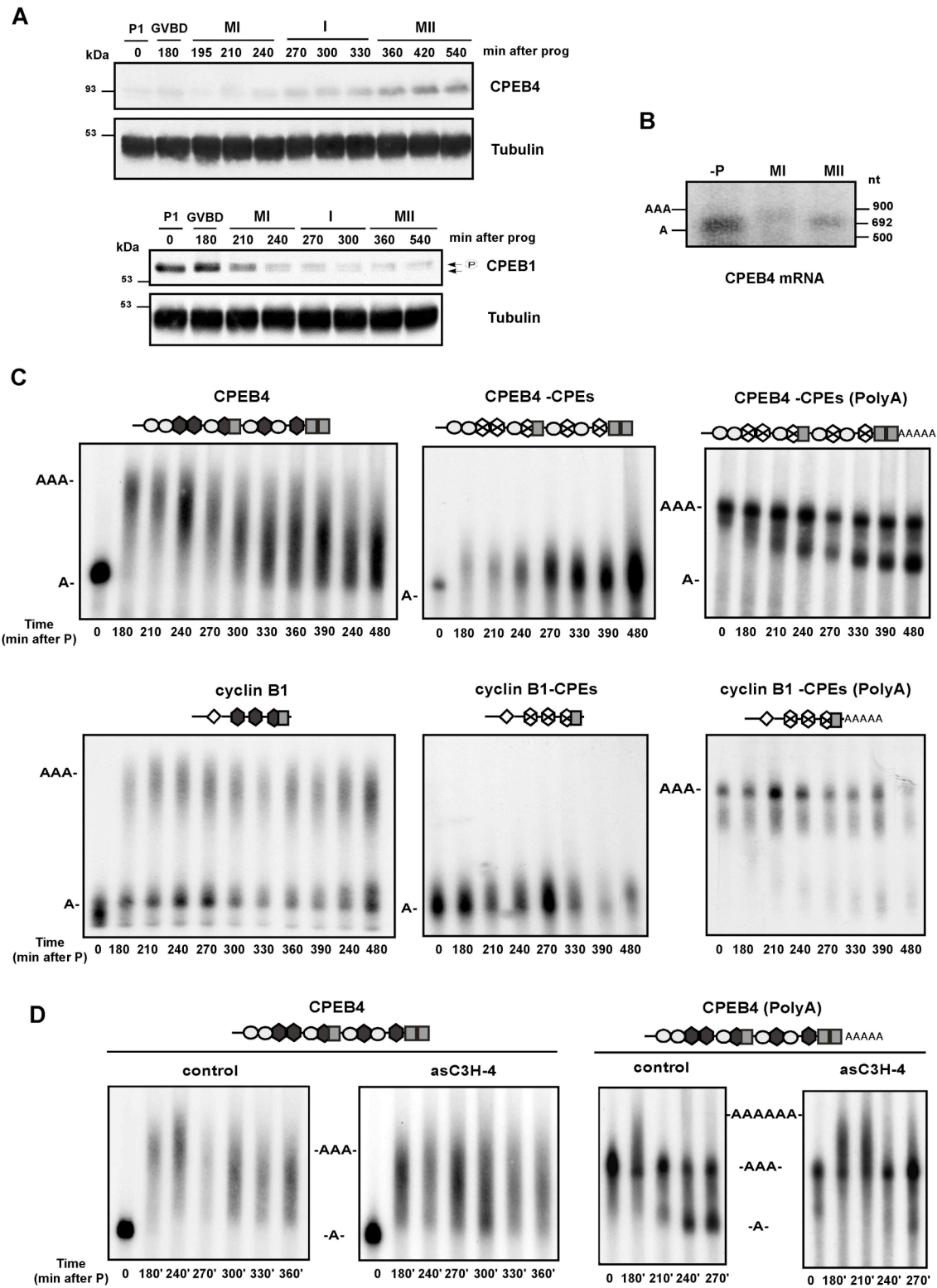
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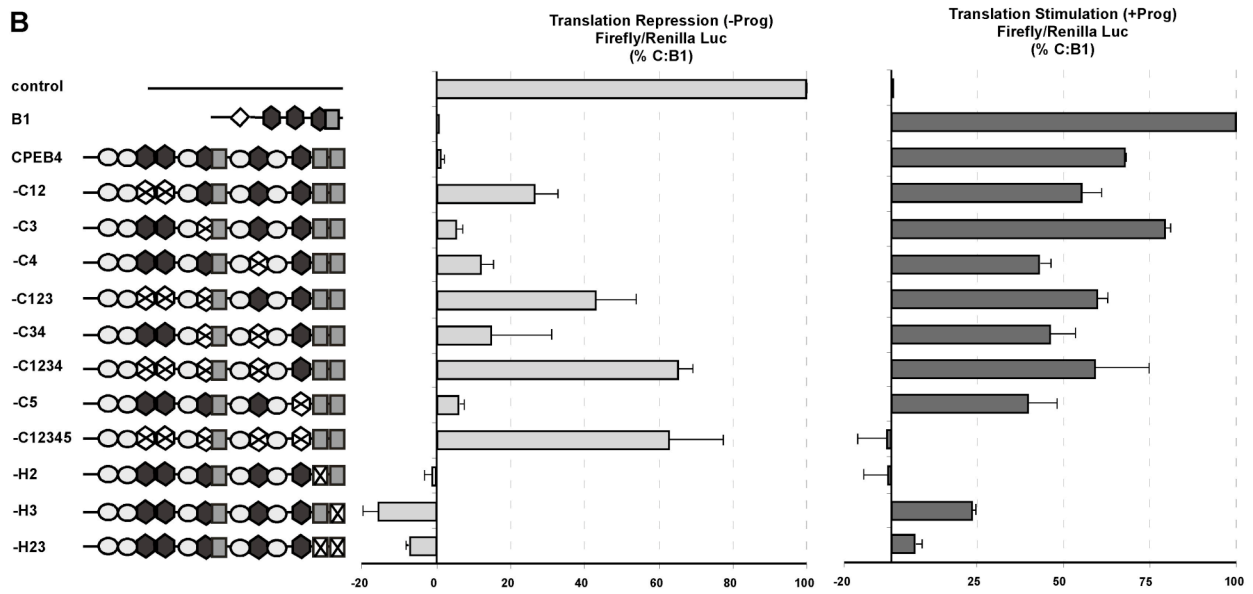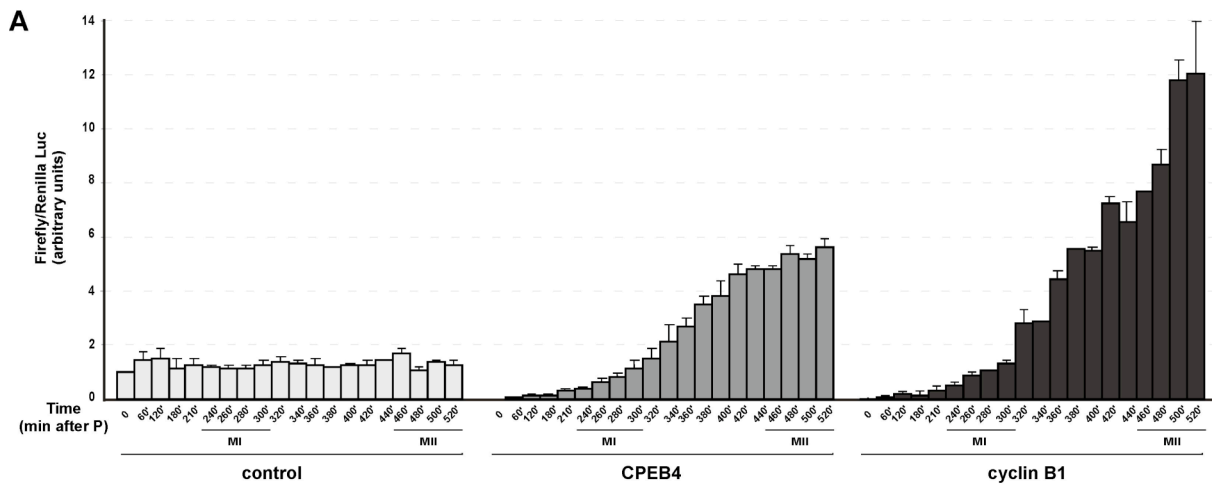
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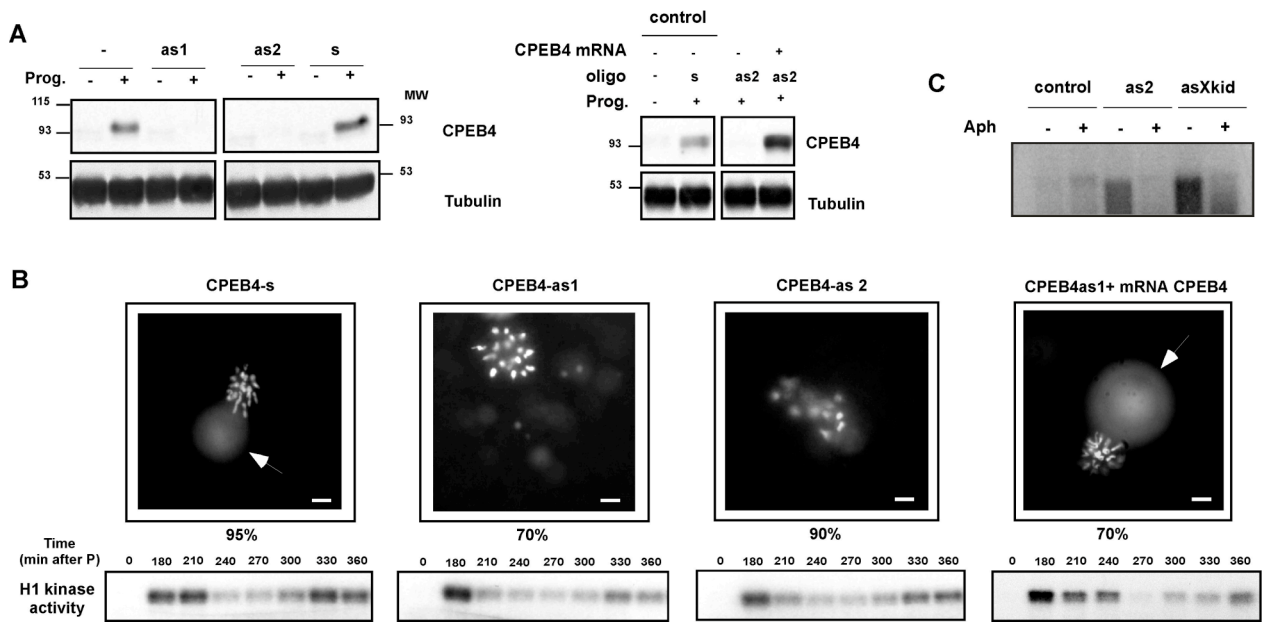
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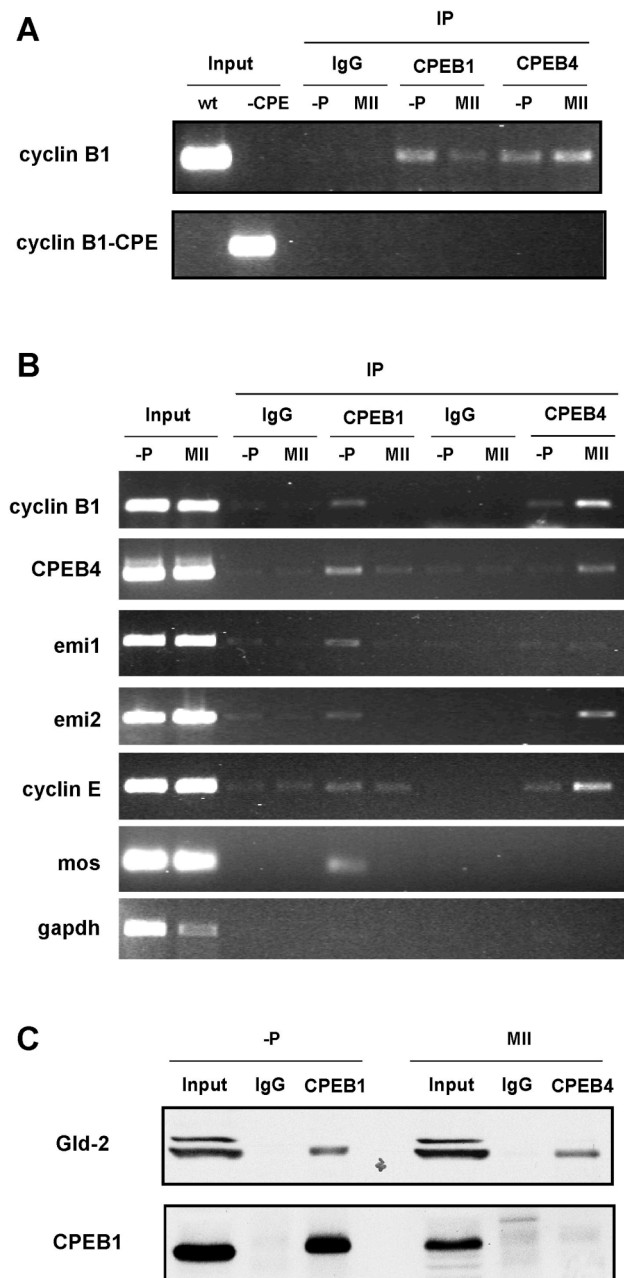
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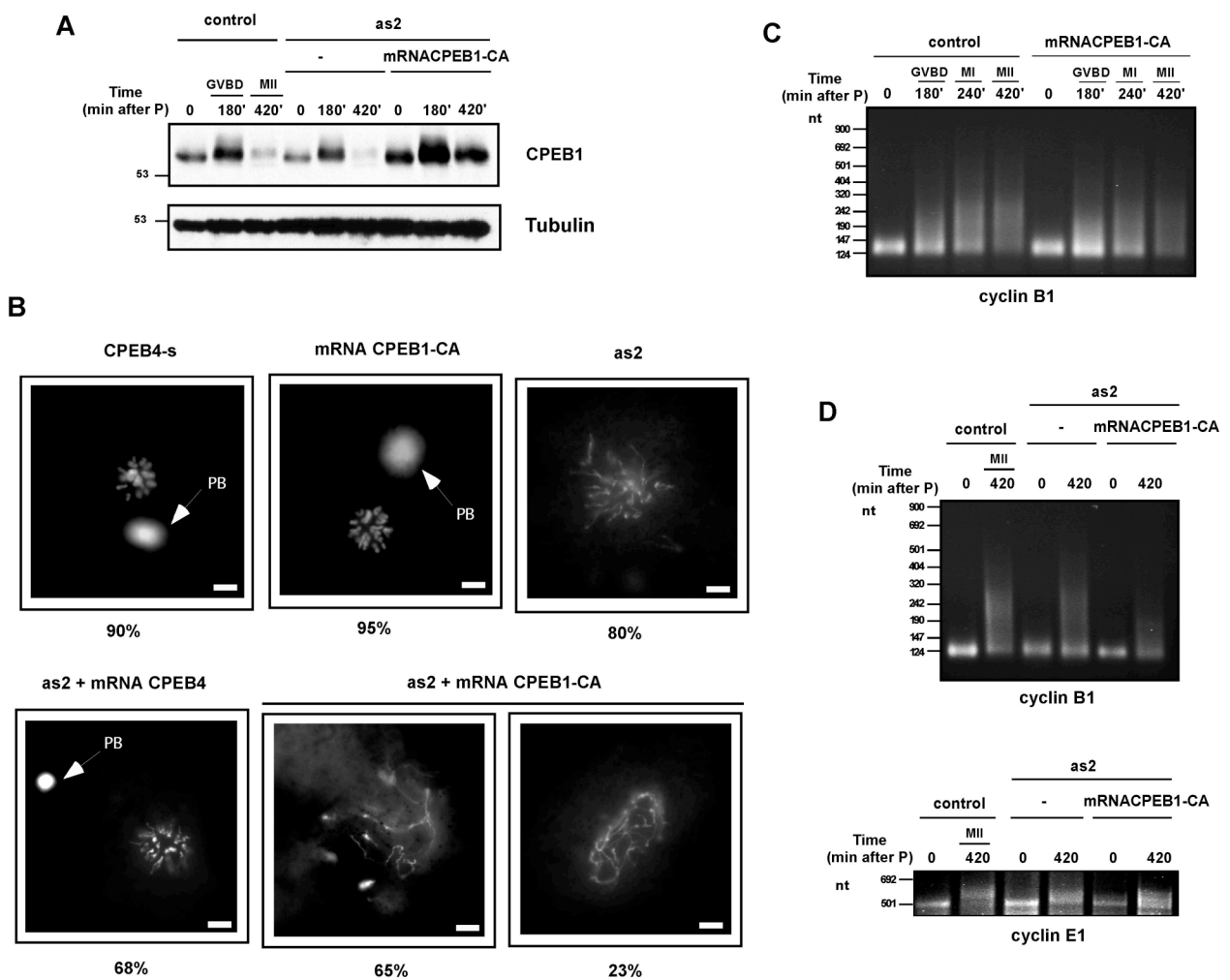
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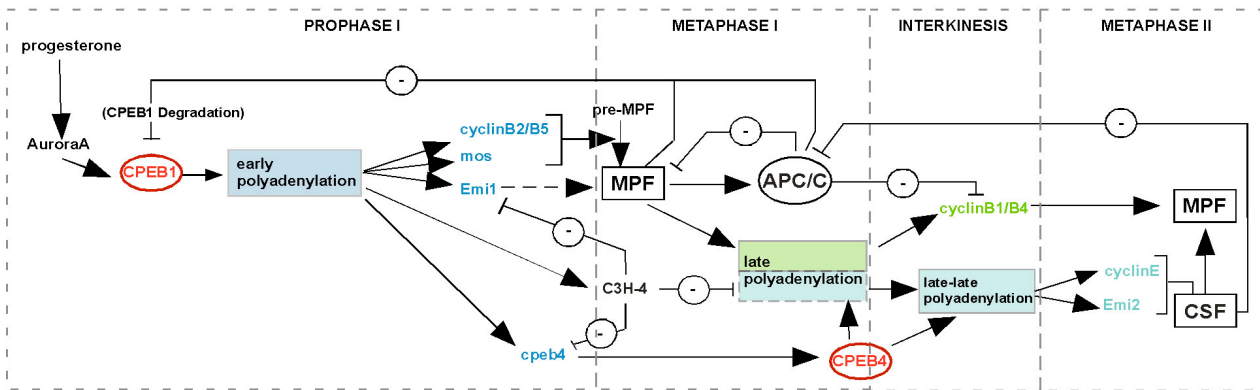
Supplementary information
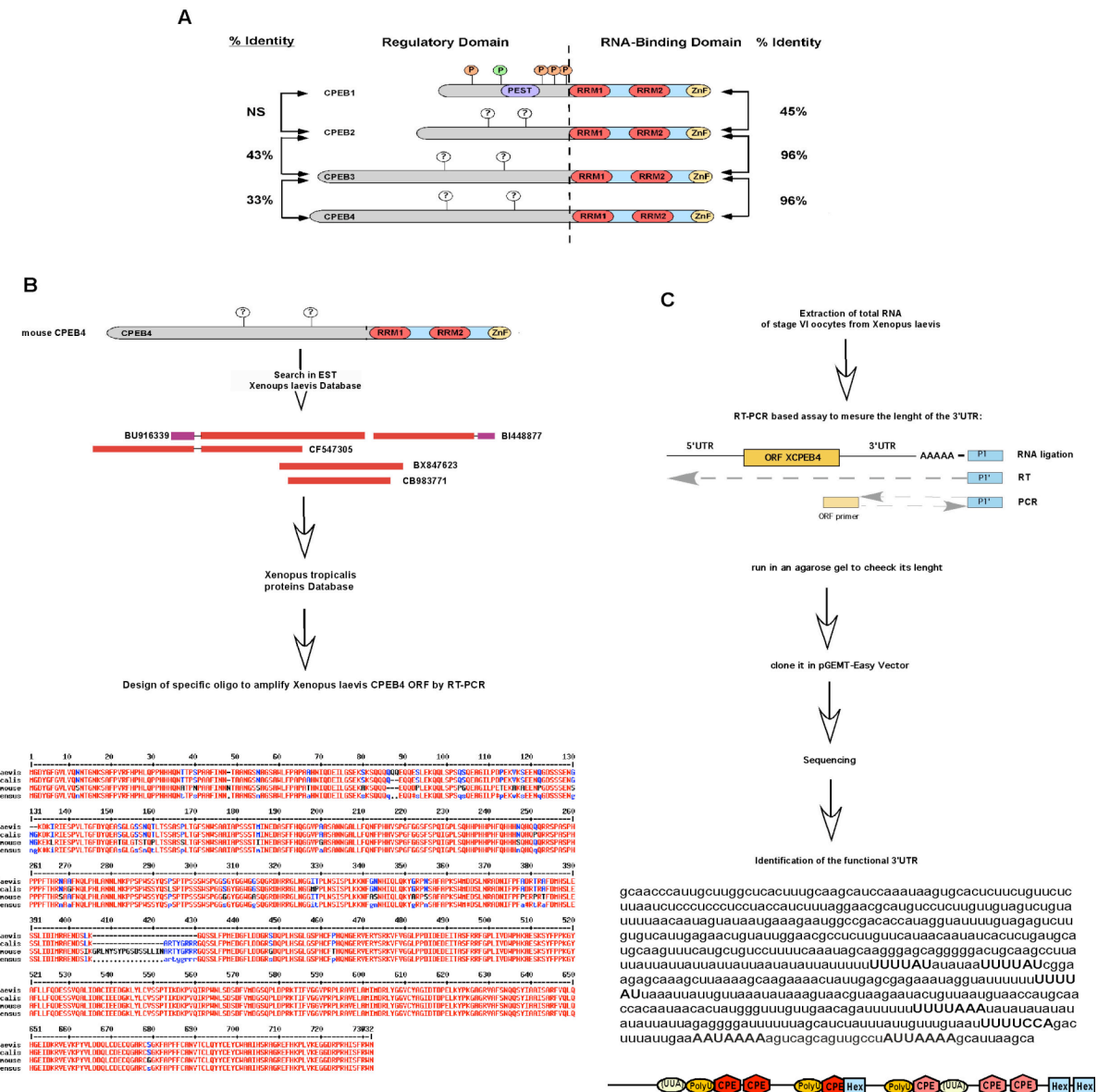
Supplementary 1. Identification and cloning of the Xenopus laevis CPEB4 ORF and 3' UTR. (A) Schematic diagram of the mouse CPEB family members. The RRMs (red), the Zinc Finger Domain (ZnF, yellow) and the PEST box (purple, present only in CPEB1) are indicated. The circled Ps indicate the phosphorylation sites by Cdc2 (orange), and by Aurora A Kinase (green). Percentages of identity of the different

proteins are indicated. (B) Mouse CPEB4 was used to screen Xenopus laevis ESTs database. 5 ESTs (BU916339, BI448877, CF547305, BX847623, CB983771) were found to contain putative open reading frame (ORF) for Xenopus Laevis CPEB4. Specific oligonucleotides were designed to amplify by RT-PCR the endogenous CPEB4 mRNA (see matherials ans methods for sequences of the oligonucleotides). Alignment of Xenopus laevis, Xenopus tropicalis and Mus musculus CPEB4 proteins is shown. (C) Total RNA was extracted from stage VI oocytes and 3' UTR length was measured by RNA-ligation-coupled RT-PCR (see matherials ans methods for sequences of the oligonucleotides). The obtained fragment was cloned in pGEMT-Easy vector (Promega, Cat. No. A1360) and sequenced to obtain CPEB4 3' UTR sequence. A schematic representation is also shown: CPEs as red hexagons, Hexanuclotide as light blue boxes, putative AREs elements as yellow ovals and UUA repeats as light yellow ovals.

Supplementary information

Igea & Mendez Suppl 2
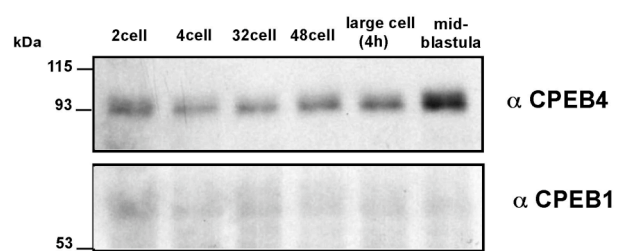
Supplementary 2. CPEB4 levels are maintained constant after fertilization. Fertilized eggs were collected at 2 cells, 4 cells, 32 cells, 48 cells, large cell (4 h) and mid-blastula stages and analyzed by western blotting using anti-CPEB4 and anti-CPEB1 antibodies.

Supplementary information

Igea & Mendez Suppl 3

CPEB4 3'UTR wt

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CPEB4 3'UTR -C12

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CPEB4 3'UTR -C3

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CPEB4 3'UTR -C4

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CPEB4 3'UTR -C123

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CPEB4 3'UTR -C34

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CPEB4 3'UTR -C1234

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CPEB4 3'UTR -C5

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CPEB4 3'UTR -C12345

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CPEB4 3'UTR -H2

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CPEB4 3'UTR -H3

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CPEB4 3'UTR -H23

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cyclinB1 wt

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cyclinB1 -C123

uguugcaccaugugcuucuguaaauaguguauugug**UUUGGGAU**g**UUGGACU**gg**UUGGAAU**aaagc

Supplementary 3. CPEB4 and cyclin B1 3' UTR sequences. Sequences of the 3' UTRs from CPEB4 (bankit1237562) and cyclin B1 (BC041302) and its corresponding 3'UTR variants. CPEs (uppercase) and polyadenylation signal (Hexanucleotide, bold) are indicated and mutations in each 3' UTR are underlined.

Supplementary information

Igea & Mendez Suppl 4

sense

antisense

CPEB4-as1   CPEB4-as2   CPEB4-as3   CPEB4-as4

See figure 3B   See figure 3B
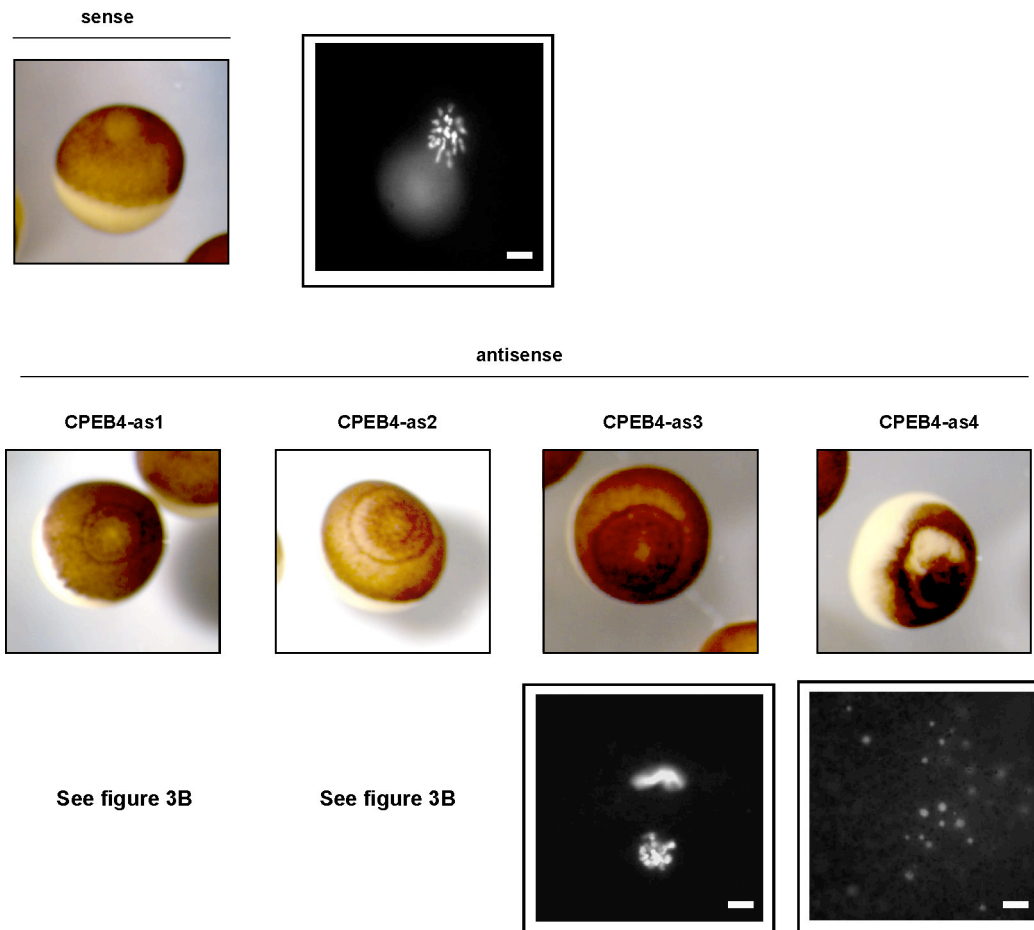
Supplementary 4. CPEB4 expression is needed to complete meiosis. Xenopus oocytes were injected with CPEB4 sense (sense) or antisense (as1, as2, as3, as4) oligonucleotides and treated with progesterone. 4 hours after the control oocytes displayed 100% GVBDAt oocytes were analysed for their external morphology, fixed and analysed as in Fig 3B.

Supplementary information

Igea & Mendez Suppl 5
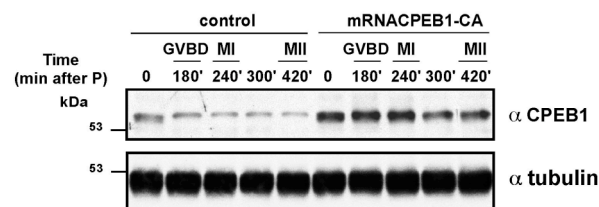
Supplementary 5. The CPEB1-CA mutant is not degraded during meiotic progression. Control oocytes or oocytes injected with an in vitro transcribed mRNA encoding non-CPEB1-CA were treated with progesterone, collected at the indicated times and analyzed for CPEB1 levels by western blot using anti-CPEB1 and anti-Tubulin antibodies (1 oocyte equivalents were loaded per lane).

Supplementary information

Igea & Mendez Suppl 6
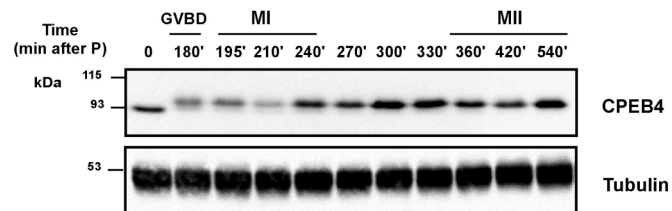
Supplementary 6. Overexpressed Myc-tagged-CPEB4 changes mobility during meiotic progression. In vitro transcribed and polyadenylated mRNA encoding myc-tagged-CPEB4 mRNA was injected in Xenopus oocytes. Oocytes were treated with progesterone (prog) and collected at the indicated times. CPEB4 levels were analysed by western blotting using anti-CPEB4 and anti-Tubulin antibodies GVBD was determined by the appearance of the white spot at the animal pole of the oocytes (2 oocyte equivalents were loaded per lane).