Spindle-localized CPE-mediated translation controls meiotic chromosome segregation

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Para vos, má.

Alicia sonrió: *“No tiene sentido que pruebe”*, dijo, *“uno no puede creer en cosas imposibles”*.
“Me atrevo a decir que no has intentado lo suficiente”, dijo la reina. *“cuando yo era joven, lo intentaba al menos media hora por día. Incluso, hubo días en que me creí hasta 6 cosas imposibles antes del desayuno.”*
“¿Por dónde tendría que empezar?”, preguntó.
“Empieza por el principio”, dijo el rey, *“y detente cuando llegues al final.”*

Alicia en el país de las maravillas, **Lewis Carrol** (1865)

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ABBREVIATIONS

aa-tRNA: aminoacyl-tRNA

APC/C: Anaphase-Promoting Complex/Cyclosome

ARE: AU-rich element

CaMKII: calcium-calmodulin-dependent protein kinase-II

CHX: cycloheximide

CPE: cytoplasmic polyadenylation element

CPEB: cytoplasmic polyadenylation element-binding protein

CPSF: cleavage and polyadenylation specificity factor

CSF: Cytostatic Factor

eIF: eukaryotic initiation factor

eEF: eukaryotic elongation factor

eRF: eukaryotic release factor

GV: germinal vesicle

GVBD: germinal vesicle breakdown

Hex: hexanucleotide

IRES: internal ribosome-entry sequence

MI: meiosis-I

MII: meiosis-II

m^7Gppp: 7-methyl-guanosine

MAP: microtubule-associated protein

Met-tRNAi: methionine-initiator tRNA

MPF: Maturing Promoting Factor

MT: microtubule

MTOC: microtubule organizing center

ORF: open reading frame

PI: prophase-I

PABP: poly(A)-binding protein

PARN: poly(A)-specific ribonuclease

PBE: Pumilio Binding Element

PKA: cyclic AMP-dependent protein kinase

RBP: RNA-binding protein

TACC: transforming acidic coiled-coil

TMA: MT-array

UTR: untranslated region

WT: wild type

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ABSTRACT

Meiotic progression and early development are programmed, at least in part, by the translational activation of maternally inherited mRNAs, such as the ones encoding for cyclin B1 or mos. These mRNAs are not translated en masse at any one time, or even at any one place; rather, their translation is specifically regulated by the cytoplasmic polyadenylation element (CPE) present in their 3'UTRs, which recruits the CPE-binding protein CPEB (Colegrove-Otero et al., 2005; de Moor et al., 2005; Mendez and Richter, 2001; Richter, 2007). This RNA-binding protein not only dictates the timing and extent of translational activation by cytoplasmic polyadenylation (Mendez et al., 2000a; Mendez et al., 2000b; Mendez et al., 2002) but also participates, together with the translational repressor Maskin, in the transport and localization, in a quiescent state, of its targets to the subcellular locations where their translation is going to take place (Huang et al., 2003; Huang and Richter, 2004). During *Xenopus* early development, CPEB localizes at the animal pole of oocytes and later on embryonic spindles and centrosomes (Groisman et al., 2000). Disruption of embryonic CPEB-mediated translational regulation results in abnormalities in the mitotic apparatus and inhibits embryonic mitotic divisions (Groisman et al., 2000).

In this thesis work we show that spindle-localized translational activation of CPE-regulated mRNAs, encoding for proteins with a well-known function in the structural aspects of the cell cycle namely spindle assembly and chromosome segregation, is essential to complete the first meiotic division and for chromosome segregation in *Xenopus* oocytes.

1. INTRODUCTION

1.1 Mechanisms of translational regulation.

1.1.1 The "RNA-regulons".

Reminiscent of transcription networks, translational control is achieved by the combination of regulatory *trans*-acting factors - primarily RNA-binding proteins (RBPs), but also non-coding RNAs - that recognize 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 (i.e., "RNA operons", (Keene, 2007; Keene and Tenenbaum, 2002)) 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)(Figure 1.1A). 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). As a consequence of this highly coordinated process, the proteins are efficiently synthesized to meet the needs of the cell (Keene, 2007; Keene and Tenenbaum, 2002) (Figure 1.1B).

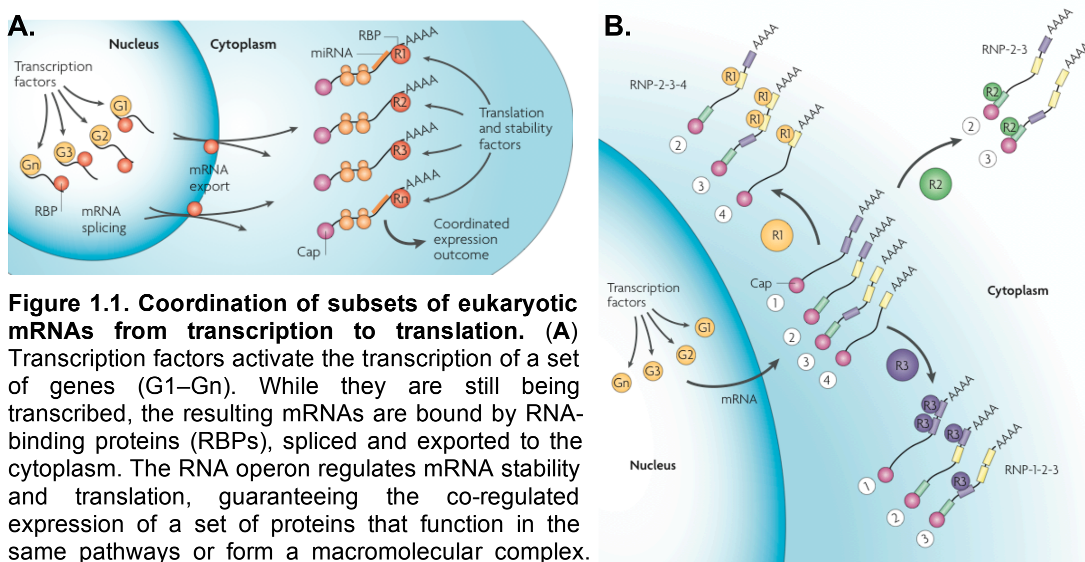


Figure 1.1. Coordination of subsets of eukaryotic mRNAs from transcription to translation. (**A**) Transcription factors activate the transcription of a set of genes (G1–Gn). While they are still being transcribed, the resulting mRNAs are bound by RNA-binding proteins (RBPs), spliced and exported to the cytoplasm. The RNA operon regulates mRNA stability and translation, guaranteeing the co-regulated expression of a set of proteins that function in the same pathways or form a macromolecular complex. RBPs and microRNAs (miRNA) can affect the stability of transcripts, activate or repress their translation or recruit the transcripts to ribosomes or processing bodies in the cytoplasm. (**B**) The four mRNAs shown (labeled with numbered white circles), when grouped in different combinations, form three different RNA operons, labeled as ribonucleoprotein (RNP)-2-3-4, RNP-2-3 and RNP-1-2-3. The make-up of each operon is determined by the binding of RBPs (labeled R1, R2 and R3) to specific sequence elements, which leads to both co-regulation within each RNA operon and overall coordination of all three operons as higher-order combinatorial regulons. The four transcripts contain different combinations of RBP-binding elements. Therefore, mRNAs that contain more than one binding element can be members of more than one RNP complex. Taken from (Keene, 2007).

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 neuronal plasticity, the translation of specific mRNAs is regulated, leaving most cellular transcripts unaffected (Abaza and Gebauer, 2008). The last decades witnessed an enormous progress in 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 (Abaza and Gebauer, 2008; Gebauer and Hentze, 2004; Krichevsky et al., 1999; Kuersten and Goodwin, 2003; 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, depending on the species (de Moor et al., 2005; Mendez and Richter, 2001; Sonenberg and Hinnebusch, 2007). Most vertebrate development is directed by maternally inherited mRNAs that are synthesized and stored during the very long period of oogenesis. These maternal mRNAs are dormant in oocytes, and their mobilization into polysomes does not occur until very specific times during meiosis and early developmental stages reviewed in (de Moor et al., 2005; Mendez and Richter, 2001; Richter, 2007).

The most extensively studied translational control mechanism of these maternal mRNAs during *Xenopus* meiotic resumption involves changes in their poly(A) tail length. During the last years, it has already characterized the *trans*-acting factors that mediate cytoplasmic polyadenylation-induced translation and their post-translational regulation (Colegrove-Otero et al., 2005; Mendez and Richter, 2001; Richter, 2007). However, the target mRNAs for this mechanism of gene expression control, its role during meiotic cell cycle progression and embryonic cell fate definition, the link between mRNA localization and cytoplasmic polyadenylation and, eventually, and more interestingly, the role of CPE-mediated localized-translational regulation in structural subcellular processes such as bipolar spindle assembly and chromosome segregation have not investigated.

In this introduction we will provide a description of the principal regulatory elements present in the mRNAs, follow by an overview of the molecular mechanics of how translation occurs in eukaryotes. We, then, emphasize the mechanisms that govern gene expression by RBPs recruited to regulatory sequences located at the 3'UTR of

maternal mRNAs during meiotic cell cycle progression and early embryonic development, focusing on the cytoplasmic polyadenylation-induced translation. The role of the key regulator CPEB (i.e., cytoplasmic polyadenylation element binding protein) in repression, activation and localization is discussed as well. Finally, we summarize the most important molecular events occurring during meiotic cell cycle progression as well as meiotic spindle assembly. The role of mRNA localization is also discussed.

1.2 Molecular mechanics of translation.

1.2.1 The mRNA.

An mRNA synthesized in the nucleus of a cell as a precursor-mRNA (pre-mRNA) acquires modifications that change dramatically their structure: the 7-methyl guanosine (m^7Gppp) cap is added to the 5' end, the introns are removed by the spliceosome and the 3' end is cleavaged and polyadenylated (Figure 1.2A).

The mature mRNA contains the following functional elements (Figure 1.2B and C):

m^7Gppp cap

The m^7Gppp cap is the structure that provides initial contact with the translational machinery and also plays an important role in pre-mRNA splicing, nuclear-cytoplasm transport as well as in message stability. This canonical modification critically determines the quality and quantity of mRNA translation (Banerjee, 1980; 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 (IRESs) present in the 5'UTR of mRNAs are able to recruit the small ribosomal subunit (reviewed in (Fraser and Doudna, 2007)).

Poly(A) tail

The poly(A) tail plays a key role in mRNA stability, export from the nucleus as well as in the 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 mRNAs do not contain poly(A) tail and include other motifs that recruit specific-factors, such as the Stem-loop Binding Protein (SLBP), which perform the same function than PABP (Marzluff, 2005).

Cap and poly(A) tail function synergistically

An interdependence exists between the cap and the poly(A) tail for efficient function at the level of regulating translational efficiency. Neither the cap structure nor the poly(A) tail alone is enough to drive efficient translation, but together they synergize and direct ribosome entry to the 5' end of the mRNA (Gallie, 1991; Preiss and Hentze, 1998).

Open Reading frame

The Open reading frame (ORF) is flanked by untranslated regions (5' and 3'UTRs) and is delimited by the AUG initiation codon in an optimal context (Kozak, 2002) and one of the three stop codons (UAA, UAG or UGA).

5'UTR

The 5'UTR of an mRNA can affect the translational rates including the length of the region, secondary structures or stem-loops, upstream AUGs (uAUGs) and uORFs (which normally reduce translation from the principal ORF). In addition, 5'UTRs can contain sequences that functions as a binding sites for regulatory factors (Mendez and Richter, 2001; Gebauer and Hentze, 2004; Wilkie et al., 2003) (Figure 1.2B).

3'UTR

The 3'UTRs contain numerous specific binding sites for regulatory factors. Usually these factors are proteins, but in some cases *trans*-acting RNAs (e.g., microRNAs, non-coding RNAs, small interfering RNAs) have been described. The length of the 3'UTR, and the *cis*-acting elements that are included, is regulated by the selection of the cleavage site defined by the choice of the polyadenylation signal or hexanucleotide (Hex) (usually AAUAAA or less frequently AUUAAA or AAUUAA). All the mRNAs contain at least one Hex that defines the nuclear polyadenylation, while some contain additional signals such as cytoplasmic polyadenylation elements (CPEs) that mediate cytoplasmic polyadenylation (Mendez and Richter, 2001; Gebauer and Hentze, 2004; Wilkie et al., 2003) (Figure 1.2C).

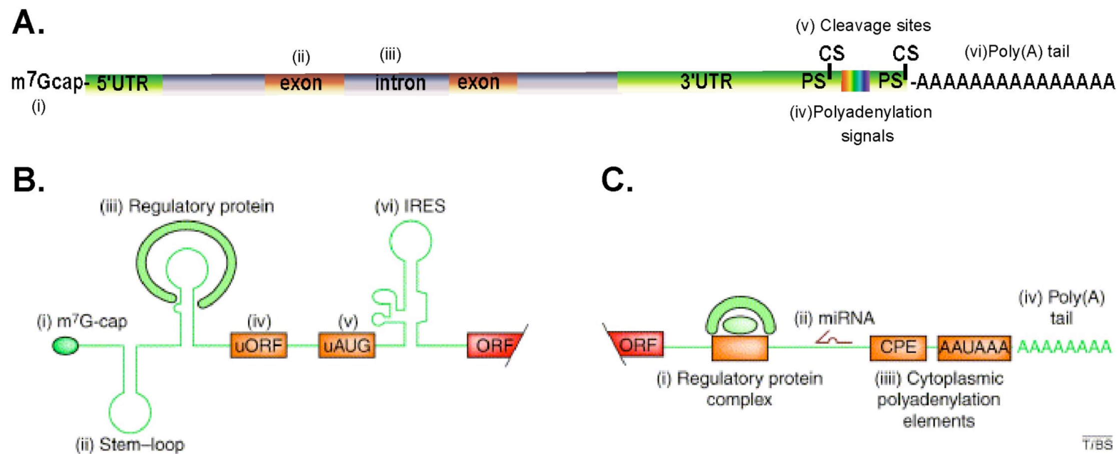


Figure 1.2. mRNA *cis*-acting elements. (**A**) Pre-mRNA. (i) m7G-cap structure added to the 5' end important in splicing, nuclear-cytoplasm transport and mRNA stability; (ii) exons (orange) separated from (iii) introns (grey) by splice junction sequences; (iv) polyadenylation signals and (v) cleavage sites. A large number of pre-mRNAs has alternative, mostly tandem, arrays of poly(A) sites within the 3'UTR. Specific regulatory factors can modulate pre-mRNA polyadenylation and cleavage site selection resulting in different mRNA variants; (vi) the poly(A) tail determinant for RNA stability and further translation stimulation. (**B, C**) Mature mRNA. The open reading frame (ORF, red) denotes the main ORF. (**B**) 5'UTR. (i) The modified cap structure determinant for translation efficiency because it is recognized by the cap-binding complex eIF4F; (ii) Stem-loops or secondary structures, negatively affect translation impeding the binding or migration of 40S ribosomal subunits; (iii) regulatory proteins interacts with specific elements present in the 5'UTR; (iv) upstream ORFs (uORFs) and (v) upstream AUGs (uAUGs) normally down-regulate translation at the main ORF by providing alternative start sites; (vi) internal ribosome entry sequences (IRES) promote cap-independent translation initiation. (**C**) 3'UTR. (i) Specific elements can act as a recognition sites for regulatory proteins (green). These elements can be structured or unstructured. Often, 3'UTR regulation requires a complex of multiple regulatory proteins rather than a single protein; (ii) short 21-nucleotide antisense microRNA (miRNA) repress translation by targeting complementary sequences within the 3'UTR; (iii) Cytoplasmic polyadenylation elements (CPEs) and the hexanucleotide (Hex) or polyadenylation signal are required to activate translation via poly(A) tail lengthening. In addition CPEs play a role in translational repression; (iv) the poly(A) tail stimulates translation by recruiting poly(A)-binding protein (PABP) molecules. (**B**, **C**) Adapted from (Wilkie et al., 2003).

1.2.2 The closed-loop model of translation.

Synergistic translational effect of the cap structure and the poly(A) tail strongly depend on its binding factors (Gallie, 1991). The cap and the poly(A) tail are recognized by the cap-binding initiator factor (eIF) eIF4E and multiple copies of the poly(A)-binding protein (PABP) respectively, and the eIF4G mediates the association between them (see also section 1.2.3) (Imataka et al., 1998; Tarun and Sachs, 1996; Wakiyama et al., 2000; Wells et al., 1998). In addition, the poly(A) tail-PABP complex recruits the 60S subunit (Sachs and Davis, 1990; Sachs and Davis, 1989). Thus, the 3' end certainly plays a pivotal role in the regulation of events occurring at the 5' end, through the "closed-loop model" where mRNA circularizes by a complex consisting of poly(A) tail-PABP-eIF4F-cap (Figure 1.3) (Christensen et al., 1987; Dubochet et al., 1973; Hsu and Coca-Prados, 1979; Ladhoff et al., 1981; Warner et al., 1962; Wells et al., 1998). These evidences could help understand in somehow reinitiation of translation (see section 1.2.3(iv)) and/or regulation of translation by factors binding to the 3' end of specific mRNAs (see section 1.3). Several lines of evidence also

suggest that PABP might affect translation of capped but nonpolyadenylated mRNAs (Horton et al., 2001; Khaleghpour et al., 2001a; Khaleghpour et al., 2001b; Wyers et al., 2000). Furthermore, this role appears to be independent of its binding to eIF4G (Otero et al., 1999).

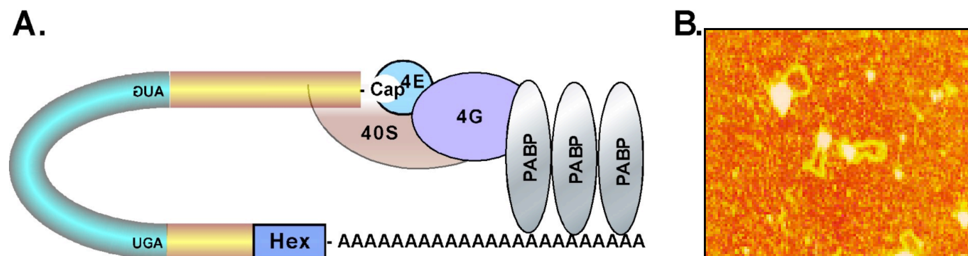


Figure 1.3. The closed-loop model. (**A**) Model of mRNA circularization and translational activation by poly(A) tail-PABP-eIF4G-eIF4E-cap interactions. eIF4G simultaneously binds eIF4E and PABP, thereby circularizing the mRNA and mediating synergistic stimulatory effect on translation of the cap and the poly(A) tail. (**B**) Visualization of circular RNA-protein complexes by atomic-force microscopy. Complexes formed on capped, polyadenylated double-stranded RNA in the presence of eIF4G, PABP and eIF4E. Picture taken from (Wells et al., 1998).

1.2.3 General Translation Factors.

The translation process can be subdivided in four main phases: (i) initiation, (ii) elongation, (iii) termination and (iv) recycling. Global regulation of translation principally occurs by modifications of general translation factors such as changes in their phosphorylation state, physical blockage or proteolytic cleavage reviewed in (Gebauer and Hentze, 2004; Sonenberg and Hinnebusch, 2007).

In this section we review the mechanics of translation in eukaryotes given more emphasis to initiation because it is the step in which most of the regulatory process known to date have been examined over the last years.

(i) Initiation.

Translation initiation in eukaryotes is a complex event requiring more than 30 polypeptides (not including the ribosomal proteins) that comprise 13 initiation factors (eIFs) (Sonenberg and Hinnebusch, 2007) (see Table 1: Eukaryotic initiation factors). Initiation starts with the assembly of a 43S pre-initiation complex that recognizes the 5’ end of an mRNA and scans it until identifies the initiation codon (AUG). The assembly of a 43S pre-initiation complex comprises the small ribosomal subunit (40S), together with the eukaryotic initiation factors eIF3, 1, 1A and 5, and the ternary complex (i.e., the methionine-initiator tRNA (Met-tRNAi) bound by the eIF2 coupled to GTP). The pre-initiation complex recognizes the mRNA by the interaction of the eIF3 with the eIF4G scaffold subunit of the cap-binding complex (eIF4F), which associates

with the 5' cap structure present in the mRNA. In addition, eIF4F also contains the eIF4E, which directly binds to both the cap structure and eIF4G, and the eIF4A, a Dead-box RNA helicase that unwinds secondary structure in the 5'UTR allowing mRNA binding and subsequent scanning by the 43S pre-initiation complex (Figure 1.4). eIF4G also contacts with the PABP mediating the circularization of the mRNA into the "closed-loop" complex (Gebauer and Hentze, 2004; Kapp and Lorsch, 2004) mentioned in section 1.2.2.

Table 1.1: 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**

The scanning model proposes that once a 43S complex is loaded onto the 5' end of an mRNA it moves linearly along the message toward the 3' end stopping when the first AUG codon is reached (Kozak, 2002). It has been established in mammals that an optimal context based on the sequences surrounding the AUG plays a role in specifying which codon is used as the initiation site of translation. This optimal context is GCC(A/G)CC**AUG**G (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 recognition of the proper AUG occurs through the formation of base pairs between the tRNA and the start codon and the processivity of the scanning critically depends on eIF1 and 1A (Pestova et al., 1998; Pestova and Kolupaeva, 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, the large ribosomal subunit (60S) joins 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).

Two GTP hydrolysis events are required during initiation. The first one is catalyzed by the stimulation of the GTPase activity of eIF2 by eIF5 upon start codon recognition allowing the 60S recruitment to the initiation complex and the release of most of the initiation factors. At the end of the initiation phase, the second step of GTP hydrolysis is stimulated by the ribosome and is required to release the eIF5B. Thus, the 80S become competent for polypeptide elongation (Lorsch and Herschlag, 1999; Pestova et al., 2000; Shin et al., 2002).

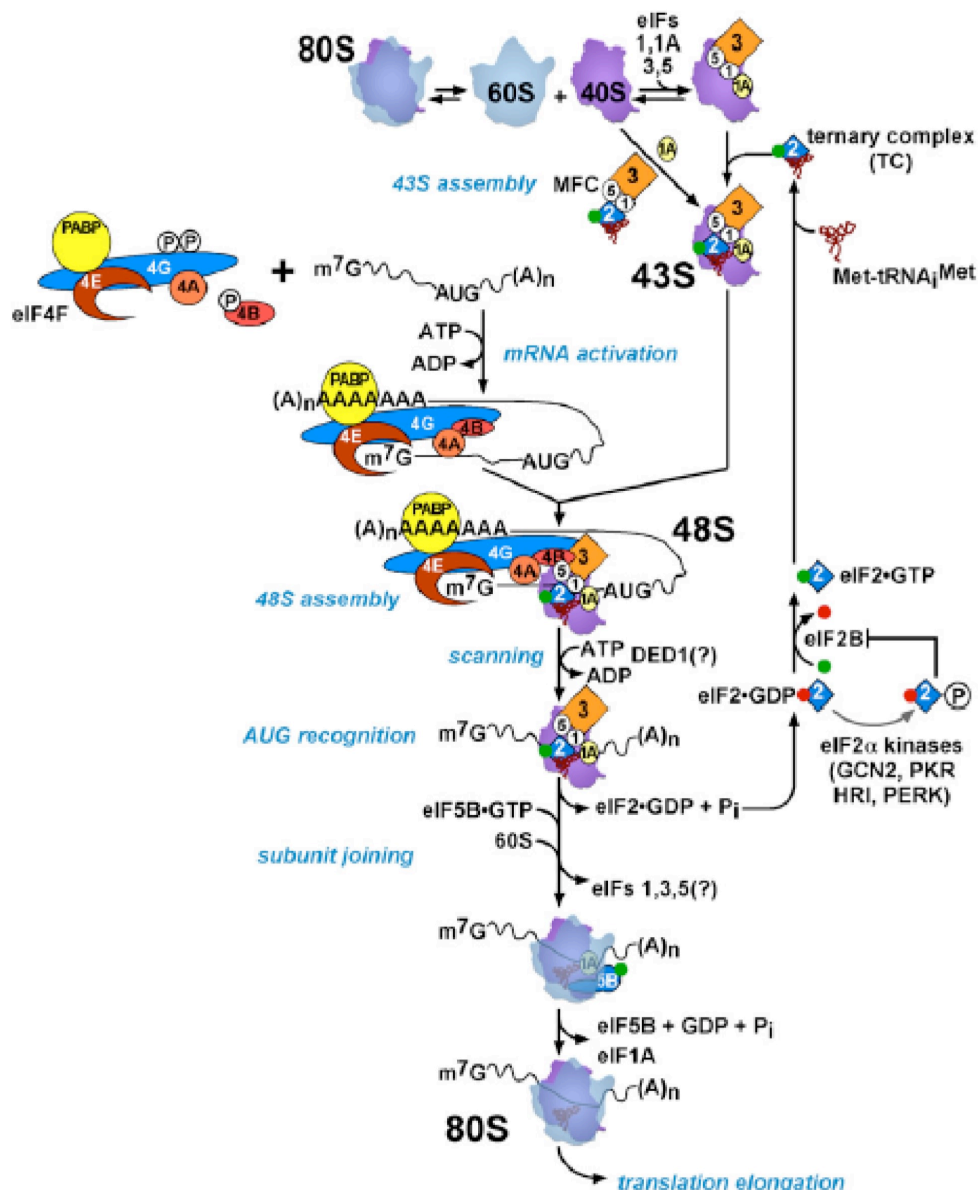


Figure 1.4. The initiation of translation. Current model for eukaryotic cap-dependent translation initiation. This process is mediated by initiation factors (eIFs) (see table 1.1 and text for details). Modified from (Sonenberg and Hinnebusch, 2007).

(ii) Elongation.

The ribosome contains three sites: the peptidyl site (P), where is hold the tRNA with the nascent polypeptide chain; the acceptor or aminoacyl site (A), where the incoming aminoacyl-tRNA (aa-tRNA) is brought (in); and the exit site (E), where the deacylated tRNA is awaiting to be discarded from the ribosome (Ramakrishnan, 2002).

Translation initiation ends with the Met-tRNAi in the P site (an unusual site for an aa-tRNA) and an empty ribosomal A site. Elongation starts with the recruitment of an aa-tRNA to the A site. Then, it continues with the formation of a new peptide bond between the incoming aa-tRNA and the former methionine carried by the initiator tRNA - or the possible nascent polypeptidyl chain-tRNA - and ends with the translocation of ribosome to the next codon of the mRNA (Spahn and Nierhaus, 1998).

The machinery of elongation includes three elongation factors (eEFs) that mediate all the process. An aa-tRNA is carried to the A site as part of a ternary complex with GTP and the elongation factor 1A (eEF1A). Correct codon-anticodon base pairing induces conformational changes in the decoding part of the small ribosomal subunit that stabilize tRNA binding and triggers hydrolysis of a molecule of GTP by eEF1A. The aa-tRNA is released into the A site in a form that can continue with the peptide bond formation. Then, the peptydil transferase center catalyzes the formation of a peptide bond between the incoming aa-tRNA and the peptidyl-tRNA. After translocation the empty tRNA occupies the E site, the nascent peptydil-tRNA is left in the P site and the next codon of the mRNA is placed into the vacant A site. This cycle is repeated until a stop codon appears and the process of termination starts. Hydrolysis of another molecule of GTP by the eEF2 facilitates translocation (Kapp and Lorsch, 2004; Ramakrishnan, 2002).

(iii) Termination.

To date termination is the less-studied step in translation regulation.

Termination occurs in response to the presence of any of the three stop codons (UAA, UAG, or UGA) in the ribosomal A site. At this point, instead of an aa-tRNA is loaded, release factors (eRFs) end this process by the liberation of the completed polypeptide following the hydrolysis of the ester bond linking the polypeptide chain to the tRNA in the P site. In eukaryotes, there are 2 release factors described to date, eRF1 and eRF3, which bind the ribosomal A site as a complex. After the finished peptide is released from the ribosome, one molecule of GTP is hydrolyzed by eRF3 (reviewed in (Kapp and Lorsch, 2004)).

(iv) Recycling.

Ribosome recycling in eukaryotes is largely mysterious. Some results have suggested a possible involvement of the ortholog of the bacterial IF3 in this process (Kapp and Lorsch, 2004). Indeed, Pisarev et al. have recently demonstrated that eIF3 is the only factor that could split post-termination ribosomes on its own and that this dissociating activity is strongly enhanced by eIF3j and less by eIF1 and 1A (Pisarev et al., 2007). An arising model proposes that once termination takes place and the completed peptide is released, one or both eRFs remain bound to the post-termination complexes. Then eIFs 3, 1, 1A, and 3j cooperatively dissociate such complexes into free 60S subunits and mRNA- and tRNA-bound 40S subunits. eIF1 then promotes dissociation of P site deacylated tRNA, after which eIF3j mediates the release of mRNA. eIF3 clearly initiates recycling, but the order in which other factors join the process is yet unknown. After dissociation, eIF3 (and likely other eIFs) plays a key role remained bound to recycled ribosomal small subunits, protecting them from re-association.

Although, it is generally believed that the function of eRF3 is solely to facilitate the release of completed peptides from ribosome, it has been found that eRF3 also associates with eIF4G through PABP (Hoshino et al., 1999; Uchida et al., 2002) suggesting a possible connection between the 5’ end of an mRNA close to the termination site. The association of [eRF1-eRF3]-PABP-eIF4F might promote tracking of terminating ribosomes to the 5’ end of the same mRNA (Uchida et al., 2002) (Figure 1.5). This mechanism could explain a missing link for the participation of recycled 40S subunits in new rounds of initiation on the same mRNA strongly suggesting a movement of small ribosomal subunits over the poly(A) tail back to the 5’ end of the mRNA - via the 5’-3’ end-associated factors - thus, allowing re-initiation of translation rather than (or in addition to) the first initiation event (Kapp and Lorsch, 2004; Uchida et al., 2002).

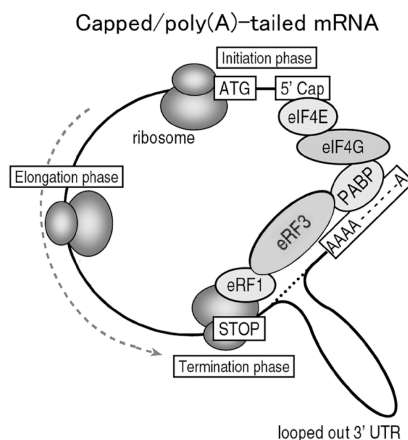


Figure 1.5. Re-initiation of translation. The circularization of the mRNA through a complex consisting of poly(A) tail-PABP-eIF4F-cap suggests the hypothetical model that a translation-terminating ribosome may be recruited to the next translation initiation. The fact that eRF3 interacts with eRF1 and PABP at the same time suggests that eRF3 may be the bridging protein to connect the stop codon with the poly(A) tail, thereby a 3’UTR which locates between a stop codon and a poly(A) tail, could looped out, and the terminating ribosome could passed to the 5’ cap structure allowing re-initiation of translation. Modified from (Uchida et al., 2002).

1.3 mRNA-specific translational regulation.

While translational control can occur in whichever of the steps that we have described in the previous section 1.2, this will affect the translation of all the cellular mRNAs, although not necessarily, to the same extent. A more specific mechanism to control translation is through the *cis*-acting elements present in the 3'UTRs of particular subpopulation of mRNAs. A hallmark of mRNA-specific translational control mechanisms is the participation of specific *trans*-acting factors that recognize and bind these *cis*-acting elements. They are the 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) 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 1.6A, B). Other mechanisms include regulation of ribosomal subunit binding, reviewed in (Colegrove-Otero et al., 2005; de Moor et al., 2005; Gebauer and Hentze, 2004) (Figure 1.6C, D) and an increasing number of studies indicate that mRNA translation is also regulated by small miRNAs (e.g., post-initiation repression) (Filipowicz et al., 2008; Jackson and Standart, 2007; Standart and Jackson, 2007), although it function in meiosis and early development is still unclear (Figure 1.6E).

The *cis*-acting elements also control a special and an extremely interesting case in the local regulation of translation that occurs in polarized cells allowing gene expression to be controlled in both spatial and temporal fashion (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 RNAs 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).

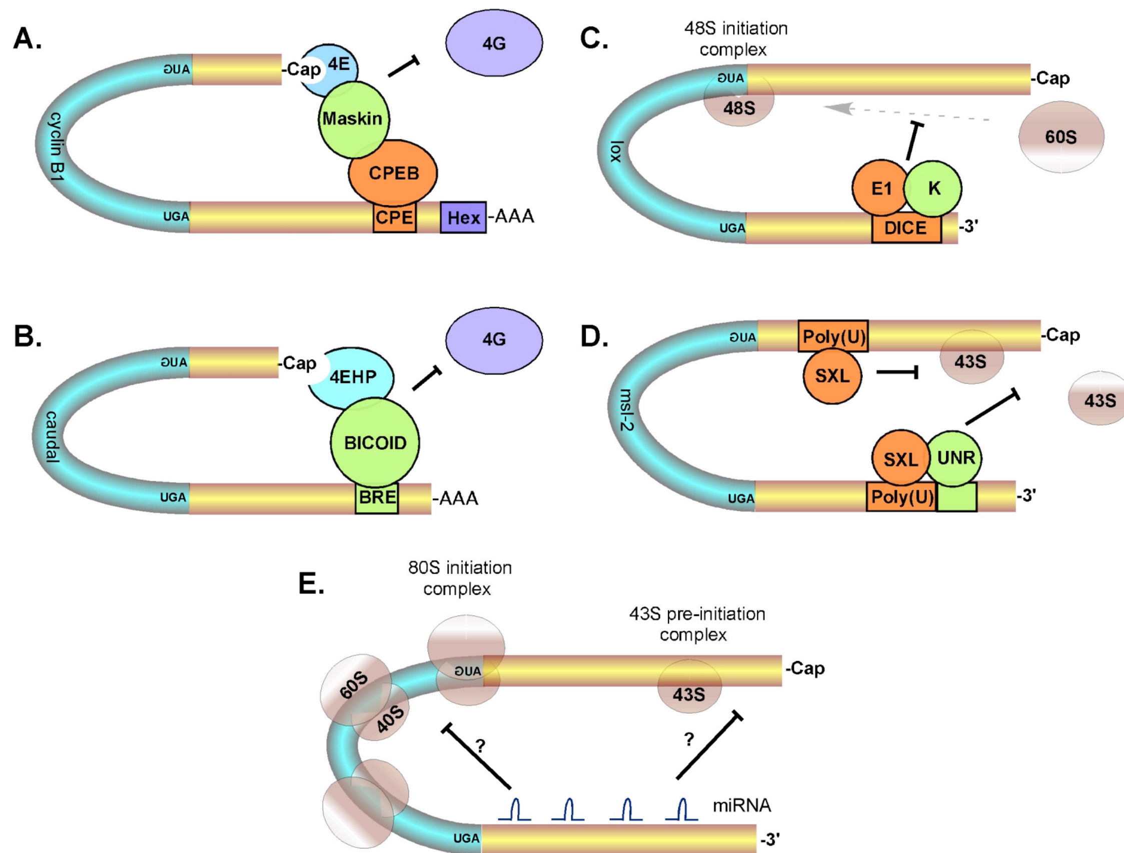


Figure 1.6. Mechanisms of mRNA-specific regulation. (**A**, **B**) **Interference with the eIF4F complex.** The eIF4E-binding proteins (**A**) Maskin and (**B**) Bicoid interact with eIF4E, thereby preventing its interaction with eIF4G. Whereas Maskin is targeted to the *Xenopus* cyclin B1 mRNA through the cytoplasmic polyadenylation element binding protein (CPEB) that recognizes the cytoplasmic polyadenylation element (CPE) in the 3'UTR, Bicoid directly binds to *Drosophila* caudal mRNA at the Bicoid response element (BRE) and recruits the translation incompetent cap-binding factor 4EHP. Adapted from (Gebauer and Hentze, 2004). (**C**, **D**) **Inhibition of ribosomal subunit binding.** (**C**) Binding of heterogeneous nuclear ribonucleoprotein K (hnRNP K) and hnRNP E1 to the differentiation-control element (DICE) in the 3'UTR of 15-lipoxygenase (*LOX*) mRNA prevents the 60S ribosomal subunit from joining the 48S initiation complex at the start AUG codon. (**D**) Binding of Sex-lethal (SXL) to uridine-rich sequences poly(U) at both the 5' and 3'UTR assists the recruitment of the Upstream of N-ras (UNR) to block the association of the 43S ribosomal complex with the 5'UTR of msl-2 mRNA, thereby inhibiting translation. The 5'UTR bound SXL blocks scanning that escaped the blockage mediated by the 3'UTR. Adapted from (Gebauer and Hentze, 2004) and (Abaza et al., 2006). (**E**) **miRNA-mediated post-initiation repression.** microRNA (miRNAs, blue) engage in imperfect base-pairing interactions with the 3'UTR and cause translational repression. At present, it is unclear which step of translation is affected by miRNAs. Some evidences indicate that this occurs in polysomal complexes after the initiation of the translation, however, miRNAs can also inhibit translation initiation. Adapted and modified from (Gebauer and Hentze, 2004).

More than 20 are known in *Drosophila* oocytes or embryos, and over 25 have been found in *Xenopus* oocytes (Bashirullah et al., 1998; Jansen, 2001). The number of localized mRNAs detected in somatic cells is increasing, with an estimated 400 mRNAs targeted to the dendrites of mammalian neurons (Eberwine et al., 2001). Finally, mRNA localization has been also detected in ascidian oocytes (Swalla and Jeffery, 1995, 1996a, b), equinoderm oocytes and embryos (Vlahou et al., 1996), mollusks oocytes (Alliegro et al., 2006; Lambert and Nagy, 2002), zebrafish embryos (Yoon et al., 1997) and even though in single-celled organisms such as yeast (Long et al., 1997; Takizawa et al., 1997) and protozoa (Han et al., 1997).

By restricting the distribution of proteins within the cytoplasm of a cell, mRNA localization can lead to functional cellular asymmetry that together with temporal regulation plays an essential role in axes formation, cell fate determination and body patterning during early stages of embryogenesis as well as in cell motility and neuronal synaptic plasticity in differentiated somatic cells (Bashirullah et al., 1998; Gavis, 1997; Jansen, 2001; Johnstone and Lasko, 2001; Lapidus et al., 2007; Palacios and St Johnston, 2001; St Johnston, 2005). Whatever the function of a localized mRNA, it must be targeted to the appropriate region of the cell by one of four basic mechanisms: (1) local synthesis; (2) local protection from degradation; (3) diffusion and anchoring by local trapping; or (4) active transport along the cytoskeleton by molecular motors such as myosins, dyneins and kinesins (St Johnston, 2005). The ability to localize a small subset of RNAs 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 of the cases, a combination between mRNA localization and translational control is also required. The mRNA usually has to be silenced until the proper destination is reached and/or the appropriate time arrives, at which point it is de-repressed and/or translationally activated (Huang and Richter, 2004; Mendez and Wells, 2002). Assembly of multiprotein complexes, even during pre-mRNA processing in the nucleus (Jansen, 2001), and most often in the 3'UTR of target mRNAs, ensure that translation is switched off until the time is right and the transcripts have reached the correct location within the cell or even though the embryo (Richter and Theurkauf, 2001). These complexes would include an adaptor that recognizes the *cis*-acting elements usually present in the 3'UTR, a motor that interacts with the cytoskeleton and transports the mRNA particle to the appropriate location within the cell and a repressor that maintains the mRNA translationally

inactive. Once at the appropriate destination, the mRNA would be translated through disassembly of the repressor and/or the recruitment of an activator (Jansen, 2001; Mendez and Wells, 2002; St Johnston, 2005).

1.3.1 3'UTR-regulatory binding proteins and poly(A) tail lengthening.

In this section we focus in the most broadly studied mechanism of translational control mediated by specific elements located at the 3'UTR and their binding proteins given more emphasis on changes in the poly(A) tail length of maternal mRNAs that takes place in *Xenopus* oocytes and embryos.

As we have mentioned in section 1.2, a long poly(A) tail is stimulatory to translation through the binding of multiple copies of cytoplasmic PABP, which interact with numerous translation factors including the eIF4A, eIF4G and the eRF3 circularizing the mRNA.

(i) Cytoplasmic polyadenylation.

Cytoplasmic polyadenylation is a highly regulated and conserved mechanism, first observed in clam oocytes (Rosenthal et al., 1983), that increases translation during meiotic maturation and after fertilization (Mendez and Richter, 2001; Richter, 1999, 2007). This phenomenon has been subsequently seen in a variety of other organisms including worms, starfish, flies, frogs, mice and somatic tissues (Fox et al., 1989; Groisman et al., 2002; Paris et al., 1988; Paris and Philippe, 1990; Rosenthal and Wilt, 1986; Salles et al., 1992; Salles et al., 1994; Standart et al., 1987; Vassalli et al., 1989).

Mos, cyclin B1, and several other dormant mRNAs in *Xenopus* oocytes contain short poly(A) tails (~20-40 nucleotides), and it is only when these tails are elongated (to ~100-250 nucleotides) that translation takes place (Mendez and Richter, 2001; Richter, 2007). The regulatory sequences that govern their translational activation and polyadenylation are primarily found in the 3'UTR of these maternal mRNAs. By the far the best characterized of these sequences is the cytoplasmic polyadenylation element (CPE), which is an U-rich element. In *Xenopus*, the CPE usually is $U_{4-5}A_{1-2}U$, although some variation may be tolerated in the context of specific mRNAs (Mendez and Richter, 2001; Pique et al., 2008; Richter, 2007; Stebbins-Boaz et al., 1996). Cytoplasmic polyadenylation requires two principal elements in the 3'UTRs of responding mRNAs, the Hex AAUAAA or AUUAAA, which is bound by a variant of the cleavage and polyadenylation specificity factor (CPSF) and the nearby CPE, which recruits the CPE-binding protein (CPEB) (Colegrove-Otero et al., 2005; de Moor et

al., 2005; Fox et al., 1989; Hake and Richter, 1994; McGrew et al., 1989; Mendez and Richter, 2001; Richter, 2007).

(ii) CPEB: the key regulator.

CPEB is the critical regulator for gene expression in early development. It was first cloned and characterized in *Xenopus* oocytes as a 62-kDa protein that bound specifically to the CPEs mediating cytoplasmic polyadenylation (Hake and Richter, 1994; Stebbins-Boaz et al., 1996). Later on, *Xenopus* CPEB has become the founding member of a large RNA-binding proteins family from *C. elegans* to humans (Bally-Cuif et al., 1998; Christerson and McKearin, 1994; Gebauer and Richter, 1996; Kurihara et al., 2003; Lantz et al., 1992; Liu and Schwartz, 2003; Luitjens et al., 2000; Theis et al., 2003; Walker et al., 1999).

Xenopus CPEB protein is composed of three regions (Figure 1.7): the amino-terminal regulatory portion, two RNA recognition motifs (RRMs), and a cysteine-histidine repeat similar to a metal-coordinating region or zinc-finger (Hake and Richter, 1994). The N-terminal half contains consensus 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), which mediates CPEB 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 and two unusual zinc-finger regions ($C_4C_2H_2$) required to binding CPE-bearing mRNAs *in vitro* (Hake et al., 1998).

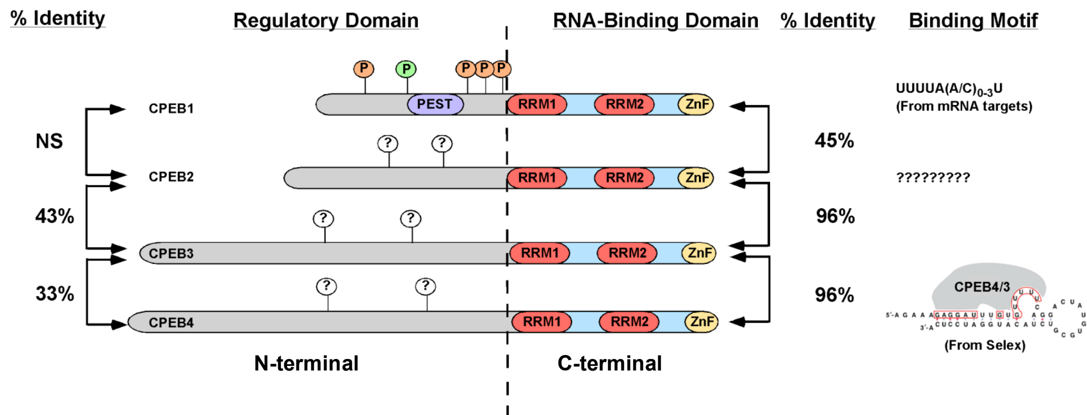


Figure 1.7. Comparison of CPEB family members. Schematic representation of the CPEB family of proteins. CPEB proteins contain an N-terminal regulatory domain and a C-terminal RNA-binding domain that consist of two RNA recognition motifs (RRM1, RRM2, red) and a Zinc-finger domain (ZnF, yellow). PEST box (blue), Aurora A-phosphorylation site (P, green) and Cdc2-phosphorylation sites (P, orange) are only present in CPEB1. Putative phosphorylation sites in the CPEB2-4 are shown (circled question marks). The percentage (%) of identity among the CPEB proteins as well the binding motifs in the targets mRNAs is also indicated. Whereas CPEB1 recognizes a primary sequence ($U_{4\text{-}5}A(A/C)_{0\text{-}3}U$), CPEB3-4 seems to recognize a secondary structure (U-rich loop within stem-loop structure) (Huang et al., 2006). CPEB2 binding motif is still unknown.

CPEB belongs to a family of proteins with four members (CPEB1, CPEB2, CPEB3 and CPEB4) (Figure 1.7). The founding member of the CPEB proteins is sometimes referred as ‘CPEB1’ (in this thesis, it is referred to ‘CPEB’). By sequence comparison within and across phyla, it has been shown that CPEB and CPEB2-4 constitute different branches of the CPEB family of proteins (Mendez and Richter, 2001). Mouse CPEB2 (mCPEB2), mCPEB3 and mCPEB4 isoforms are most similar between them and show less homology to mCPEB1. The four CPEBs are expressed in different tissues (Theis et al., 2003) and interact with distinct RNA motifs (Huang et al., 2006). The transcript of mCPEB1 is abundantly expressed in brain and weaker expressed in kidney, lung, heart and oocytes (Gebauer and Richter, 1996; Tay et al., 2000; Theis et al., 2003; Wu et al., 1998). mCPEB2 is abundantly expressed in testis and brain (Kurihara et al., 2003; Theis et al., 2003). mCPEB3 mRNA is strongly expressed in heart and brain, and finally, mCPEB4 mRNA in embryos and adult brain as well as kidney, lung and heart (Theis et al., 2003). Unlike mCPEB1, the other CPEB isoproteins lack PEST sequence and Aurora A kinase phosphorylation sites. However, alternative splice isoforms of CPEB2-4 posses putative phosphorylation sites for cyclic AMP-dependent protein kinase (PKA), calcium-calmodulin-dependent protein kinase-II (CaMKII) and p^{70}S6 kinase (Theis et al., 2003). Human homologues of all four mouse proteins have been also identified (Kurihara et al., 2003; Welk et al., 2001).

In mammals not only meiosis and development, but also cellular senescence, axon guidance, synaptic plasticity and long-term memory consolidation are also regulated by CPE/CPEB translational control, reviewed in (Richter, 2007).

(iii) CPEB-interacting proteins.

Xenopus CPEB performs a dual role – it represses cap-dependent translation in arrested oocytes and activates translation, via cytoplasmic polyadenylation, in meiotically maturing oocytes, eggs and early embryos (de Moor and Richter, 1999; Mendez and Richter, 2001; Richter, 2007). However, there is some controversy surrounding the CPEB-interacting partners belonging to the functional complex(es) involved in this regulation. Here, we summarize the CPEB-interacting proteins known to date.

Maskin

Maskin is member of the transforming acidic coiled-coil containing (TACC) family, which has important roles in cell division and cellular organization in both embryonic and somatic systems (reviewed in (Gergely, 2002)). Maskin not only associates with CPEB but also binds to the cap-binding initiation factor eIF4E. This configuration of factors precludes the interaction eIF4G-eIF4E and thereby inhibits translation by precluding 40S ribosomal subunit recruitment at the 5' end of the mRNA (Cao et al., 2006; Cao and Richter, 2002; Mendez and Richter, 2001; Richter, 2007; Stebbins-Boaz et al., 1999).

Pumilio

Xenopus Pumilio (Pum) is a RBP member of the Pumilio/Fem3-binding protein (PUF) family (Spassov and Jurecic, 2003; Wickens et al., 2002) that specific-associates to maternal mRNAs (Nakahata et al., 2001) as well as CPEB via its PUF domain, in a RNA-independent manner (Zamore et al., 1997; Nakahata et al., 2001; Nakahata et al., 2003). Pum possibly cooperates with CPEB repressing maternal mRNAs such as the one encoding for cyclin B1 (Nakahata et al., 2003).

RCK/Xp54

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 CPEB (Minshall and Standart, 2004) and seems to interact also with eIF4E (Minshall and Standart, 2004) suggesting a potential role in translation repression (Coller and Wickens, 2002; Minshall and Standart, 2004; Minshall et al., 2001).

4E-T

CPEB recruits the vertebrate Cup homolog 4E-T (4E-transporter), which in turns binds to an oocyte specific eIF4E isoform (4E1b) (Minshall et al., 2007) suggesting a CPEB-dependent repression complex. In this complex is also found the RNA helicase RCK/Xp54, and the P-body components P100 (Pat1) and Rap55 (Minshall et al., 2007).

PARN

The poly(A)-specific ribonuclease (PARN) is a cap-interacting and deadenylase enzyme (Balatsos et al., 2006; Copeland and Wormington, 2001; Gao et al., 2000) that also interacts to CPEB (Kim and Richter, 2006). The 64-kDa isoform is thought to be responsible for the short poly(A) tail of maternal CPE-containing mRNAs in *Xenopus* oocytes (Kim and Richter, 2006).

GLD-2

Xenopus germ-line-development factor 2 (GLD-2) is a divergent poly(A) polymerase belonging to the same large family of DNA polymerase β nucleotidyl transferases, but with a limited additional homology to the classical poly(A) polymerases and lacking the RNA-binding domain. Although GLD-2 directly binds to CPEB in both immature and mature oocytes, it is only required for cytoplasmic polyadenylation-induced translation (Barnard et al., 2004; Kim and Richter, 2007; Rouget et al., 2006; Rouhana et al., 2005; Rouhana and Wickens, 2007).

CPSF

The cleavage and polyadenylation specificity factor (CPSF) is a four subunits complex (30, 73, 100 and 160-kDa) that not only mediates RNA cleavage but also subsequent nuclear and cytoplasmic polyadenylation (Barnard et al., 2004; Bilger et al., 1994; Dickson et al., 1999; Kim and Richter, 2006; Mandel et al., 2006; Mendez et al., 2000b; Proudfoot et al., 2002; Rouhana et al., 2005; Wahle and Ruegsegger, 1999). CPSF directly interacts to CPEB 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).

Symplekin

Symplekin is a nuclear protein that are present in complexes containing processing factors involved in 3' end RNA processing. It possibly has a role as a scaffold protein upon which multicomponent complexes are assembled in the nucleus (Hofmann et al., 2002; Takagaki and Manley, 2000; Xing et al., 2004). In *Xenopus* oocytes, however, Symplekin is also found in cytoplasmic complexes with the cytoplasmic polyadenylation machinery such as CPEB, GLD-2 and CPSF (Barnard et al., 2004; Kim and Richter, 2007).

CstF77

CPEB associates with the subunit of the cleavage stimulatory factor CstF77 (Rouget et al., 2006), which is the subunit required for integrity of the CstF complex (77, 64 and 50-kDa proteins) (Takagaki and Manley, 1994; Takagaki et al., 1990) involved, together with CPSF, in pre-mRNA cleavage before nuclear polyadenylation. In addition to its nuclear function, CstF77 may have mRNA masking role in *Xenopus* oocytes although it probably has more general function than CPE-dependent mechanism (Rouget et al., 2006). CstF77 also interacts with GLD-2 and CPSF (Rouget et al., 2006).

Aurora A

Aurora A/Eg2 is member of the Aurora family of serine/threonine protein kinases, which has important roles in cell cycle progression, bipolar spindle formation and chromosome segregation (reviewed in (Crane et al., 2004; Ducat and Zheng, 2004; Marumoto et al., 2005)). Aurora A associates with and phosphorylates CPEB on Ser174 during early stages of oocyte maturation (Mendez et al., 2000a) increasing the affinity of CPEB for the CPSF (Mendez et al., 2000b). This phosphorylation is the crucial event in polyadenylation-dependent translation of specific maternal mRNAs (Barnard et al., 2005; Charlesworth et al., 2004; Mendez et al., 2000a; Mendez et al., 2000b; Sarkissian et al., 2004).

XGef

The Guanine Nucleotide Exchange Factor, xGef, is a member of the Rho family of GTPase proteins and it is a CPEB-interacting protein (Martinez et al., 2005; Reverte et al., 2003). xGef has been also proposed to stimulate early CPEB phosphorylation. Due to xGef immunoprecipitates seem to contain Mitogen-Activated Protein Kinase (MAPK), it may be required to bring CPEB to the signaling complexes involved in its phosphorylation (Keady et al., 2007).

APLP

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). Maskin, CPSF, GLD-2 and several other factors involved in polyadenylation are all detected by immuno-electron microscopy on membranes in the same fractions as APLP1, CPEB and CPE-containing mRNAs (Cao et al., 2005). While the association with amyeloid precursor proteins may have great significance for the role of CPEB 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.

In *Xenopus* oocytes, CPEB might reside in several ribonucleoprotein-complexes and accomplishes its dual role in translation regulation depending on its association with the above mentioned interacting factors. In arrested immature oocytes, these CPEB-interacting proteins may function in redundant repression mechanisms and, thus, at present is difficult to choose among the multitude of models proposed for translational repression mediated by CPEB. 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. Therefore, CPEB can assemble two functionally opposing complexes: one mediating translational repression by association with Maskin, Pumilio, RCK/XP54 and PARN; and other driving polyadenylation and translational activation in response to progesterone with CPSF, Symplekin and GLD-2 (Barkoff et al., 2000; Barnard et al., 2004; Cao and Richter, 2002; de Moor and Richter, 1999; Kim and Richter, 2006; Minshall and Standart, 2004; Minshall et al., 2001; Nakahata et al., 2001; Nakahata et al., 2003; Rouhana et al., 2005; Stebbins-Boaz et al., 1999) (Figure 1.8). The switch of the repression complex to the polyadenylation complex in response to progesterone is regulated by phosphorylation of their components. In addition to the above-described Aurora A phosphorylation of CPEB, all three CPEB, Maskin and Pum are regulated by other phosphorylation events (Barnard et al., 2005; Keady et al., 2007; Mendez et al., 2002; Nakahata et al., 2003; Sarkissian et al., 2004; Stebbins-Boaz et al., 1999).

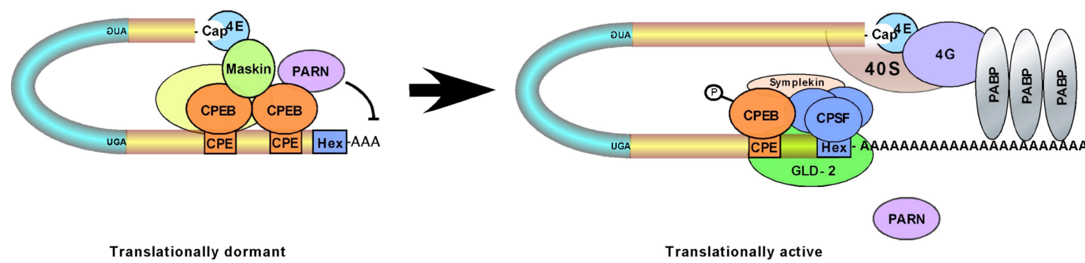


Figure 1.8. CPEB-mediated translational control. In immature oocytes, mRNAs containing cytoplasmic polyadenylation elements (CPEs) are translationally dormant (masked) and reside in a repression complex containing the CPEB, Maskin, eIF4E and PARN. (Note that other factors could be present (indicated in yellow)). Once maturation begins, newly phosphorylated CPEB (by Aurora A kinase) increases its affinity for the CPSF and expulses PARN from the complex resulting in GLD-2-catalized polyadenylation. The elongated poly(A) is then bound by multiple copies of PABP, which subsequently interacts with and helps eIF4G to displace Maskin from eIF4E allowing translation initiation.

(iv) Translational repression.

In dormant oocytes, mRNAs irrespective of whether they contain or not a CPE, might acquire a long poly(A) tail (~200-250 nucleotides) in the nucleus as a typical RNA maturation process (Huarte et al., 1992; Kim and Richter, 2006; Sachs and Wahle, 1993). Following nuclear export, only CPE-containing RNAs are able to interact with CPEB and its binding partners that, in turn, remove most of the poly(A) tail to ~20-40 nucleotides. Both PARN and GLD-2 are present and active, in immature oocytes, but due to PARN is more active in the complex, the poly(A) tail is removed as soon as it is added by GLD-2 (Kim and Richter, 2006). However, a short poly(A) tail by itself is not sufficient to fully repress translation. For this to occur, another factor, Maskin, is involved. Thus, CPEB through its bridging partner Maskin, repress translation precluding the association of eIF4G to eIF4E and therefore the 40S ribosomal subunit recruitment required for translation initiation at the 5’ end of the mRNA (de Moor and Richter, 1999; Stebbins-Boaz et al., 1999) (Figure 1.8).

Although CPEB can recruit PARN to the mRNA, deadenylation by PARN requires a 5’ cap structure (Balatsos et al., 2006; Copeland and Wormington, 2001; Gao et al., 2000; Kim and Richter, 2006) leading to a conflict surrounding the presence of Maskin and PARN in the same complex.

(v) Translational activation.

Progesterone-induced meiotic-resumption causes the translational activation of Ringo mRNA (Ferby et al., 1999; Gutierrez et al., 2006; Lenormand et al., 1999; Padmanabhan and Richter, 2006) and Glycogen Synthase Kinase (GSK-3β) inactivation leading to an activation of Aurora A kinase (Sarkissian et al., 2004). Thus, CPEB is activated by phosphorylation on Ser174 by Aurora A (Mendez et al., 2000a; Sarkissian et al., 2004). However, there is some discussion about the kinase

mediating this early phosphorylation and MAPK has been recently shown implicated in prime CPEB for Ser174 phosphorylation or even in activate the possible Ser174 kinase (Keady et al., 2007).

This CPEB phosphorylation on Ser174 as a result of progesterone stimulation increases its affinity for CPSF (Mendez et al., 2000b), which, in turn, recruits the cytoplasmic poly(A) polymerase GLD-2 (Barnard et al., 2004; Rouhana et al., 2005), and also induces the ejection of PARN from the complex (Kim and Richter, 2006). Concomitantly, Maskin is phosphorylated by Cdc2 (Barnard et al., 2005) or Aurora A kinase (Pascreau et al., 2005) and thus is dissociated from eIF4E (but not from CPEB) (Cao and Richter, 2002). In addition, embryonic poly(A)-binding protein (ePABP), is recruited to the 3' end of the mRNA by a transient association with the polyadenylation complex, promoting the recruitment of and help eIF4G to displace Maskin from eIF4E enabling initiation of translation (Cao and Richter, 2002; Kim and Richter, 2007; Wakiyama et al., 2000) (Figure 1.8).

1.4 Temporal control of translation.

1.4.1 Meiotic progression in *Xenopus* oocytes.

In most vertebrates, full-grown but immature oocytes are arrested at prophase of meiosis-I (prophase-I: PI; diplotene). During the long period of growth named oogenesis, these oocytes synthesize and store in theirs cytoplasm a complex population of mRNAs, which will drive oocyte re-entry in the meiotic cell cycle and later on early embryonic divisions (Mendez and Richter, 2001; Schmitt and Nebreda, 2002). Meiotic resumption, which includes germinal vesicle breakdown (GVBD), chromosome condensation and spindle formation marks the onset of oocyte or meiotic maturation and in *Xenopus* is stimulated by the hormone progesterone (Figure 1.9). Meiotic or oocyte maturation is comprised of two consecutive M-phases, meiosis-I and meiosis-II (MI and MII) without an intervening S-phase (Iwabuchi et al., 2000). At MII the oocytes become arrested for a second time, as the result of the synthesis and activation of the Cytostatic Factor (CSF), and await for fertilization (Sagata, 1996). Remarkably, in *Xenopus* oocytes these transitions occur in the absence of transcription, which does not resume until mid-blastula transition, and are fully dependent on the sequential translational activation of the maternal mRNAs accumulated during oogenesis (Mendez and Richter, 2001). Synthesis of the proto-oncogene c-mos, a serine/threonine kinase encoded by a cytoplasmically polyadenylated mRNA, leads to the activation of the MAPK, one of whose targets is $p90^{Rsk}$, reviewed in (Schmitt and Nebreda, 2002). Once fertilization takes place, meiosis finally ends and embryonic cell divisions start. Mitosis in the embryo is unlike

any other and consists on a rapid succession of M- and S-phases without intermediate G1 or G2 phases.

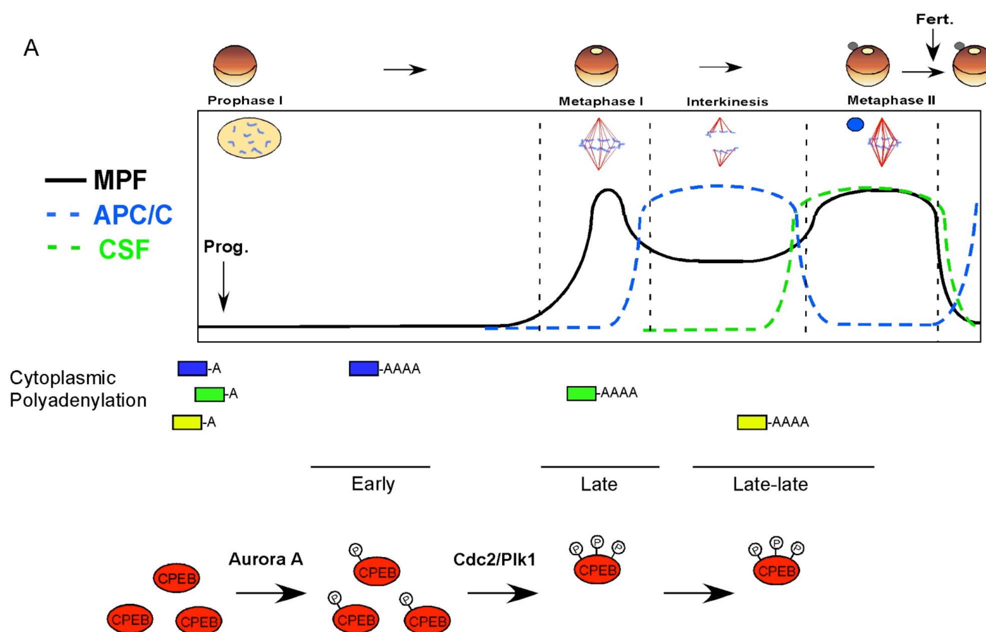


Figure 1.9. Meiotic cell cycle progression in *Xenopus* oocytes. Schematic representation of meiotic progression from PI-arrest to fertilization. Maturation-Promoting Factor (MPF), Anaphase-Promoting Complex/Cyclosome (APC/C) and Cytostatic Factor (CSF) activities are indicated. Cytoplasmic Polyadenylation Element Binding protein (CPEB) levels and regulation, and the three waves of cytoplasmic polyadenylation (early, late and late-late) are also depicted. (See text for details). Adapted from (Belloc and Mendez, 2008).

Three key activities control meiotic progression in *Xenopus* oocytes: (1) the Maturing Promoting Factor (MPF), (2) the Anaphase-Promoting Complex/Cyclosome (APC/C), and (3) the Cytostatic Factor (CSF). The MPF is an heterodimer of Cyclin B and its binding partner, Cdc2 kinase, that 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 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 APC/C, which induces the ubiquitination and posterior destruction of Cyclins B (Peters, 2006). However, during the interkinesis activation of APC/C is combined with the 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, 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/C by newly synthesized Emi2 and other components of the CSF, such as Cyclin E and high levels of Mos (Liu et al., 2007). But meiotic progression not only requires the translational activation of these 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.

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

Individual CPE-containing mRNAs display specific translational behavior during meiosis suggesting that individual features within their 3'UTRs determine their response to CPEB-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 CPEB-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. The results of these works allow the authors to postulate a set of rules or a “combinatorial code” that can be used to predict the translational behavior of CPE-containing mRNAs during meiosis (Figure 1.10).

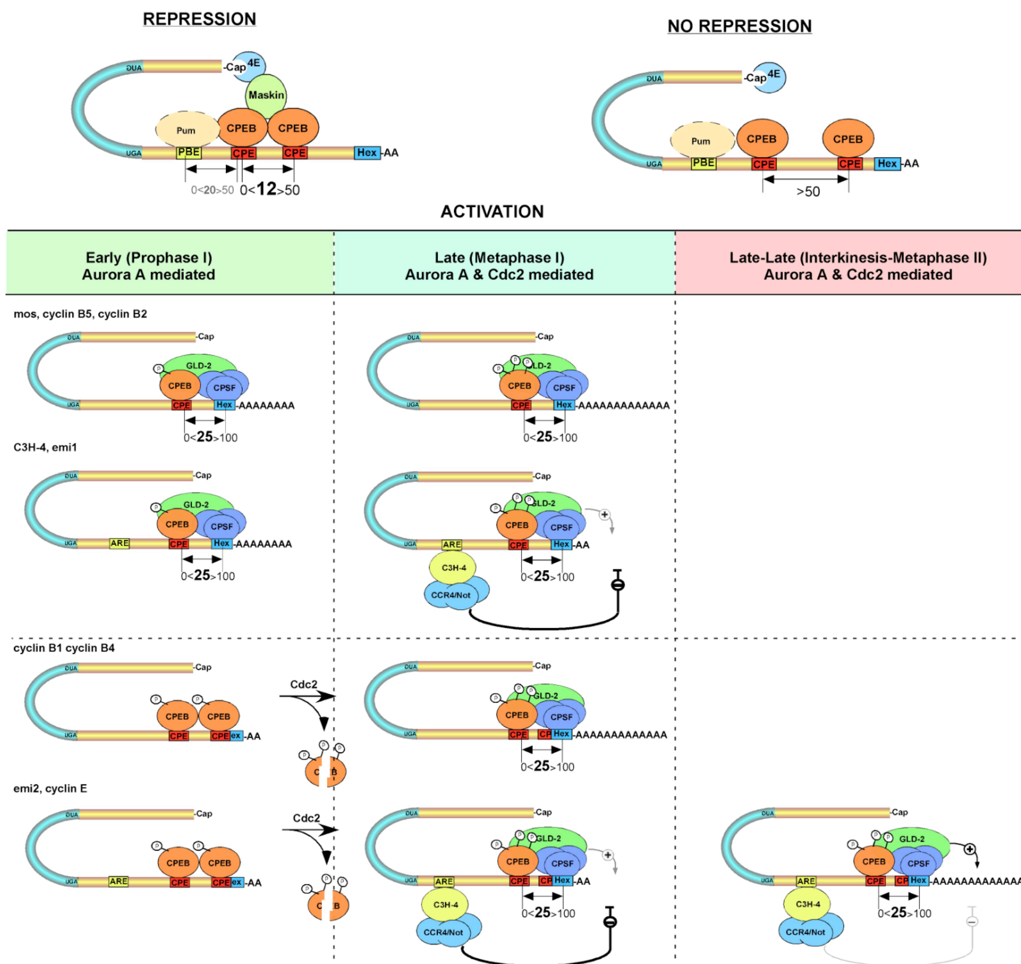


Figure 1.10. Model for CPE/ARE-mediated translational control. Schematic representation of the *cis*-elements and *trans*-acting factors recruited, with their covalent modifications. The distances required for translational repression and activation as well as the time of activation is indicated. Optional factors/elements are displayed with dotted lines. (See text for details). Taken from (Belloc et al., *in press*).

(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. This implies that the recruitment of Maskin must be mediated by a CPEB dimer and that the efficient repression mediated by multiple CPEs corresponds to the recruitment of this heterotrimer rather than multiple CPEB-Maskin heterodimers.

(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 CPEB-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-CPEB complex. Additional PBEs or CPEs have a positive effect except for an overlapping CPE, which has a negative effect.

(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 CPEB levels. Thus, during the PI to MI transition, where the levels of CPEB 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 CPEB is degraded (Mendez et al., 2002) and stochastically only one CPE would be occupied. Because the non-overlapping CPE has a higher affinity for CPEB than the overlapping CPE-Hex that would imply that now the single CPEB would be preferentially recruited to CPE and free to recruit CPSF to the Hex and promote polyadenylation.

(5) The presence of AU-Rich Elements (AREs), a feature of mRNAs regulated by deadenylation (Voeltz and Steitz, 1998), further defines the effect on polyadenylation dictated by the different arrangements of CPEs. During meiosis, these AREs recruit a zinc-finger protein named C3H-4 that is encoded by a CPEB-regulated mRNA activated 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. 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.

1.4.3 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 WT, 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. Three waves of exquisitely regulation are required (Figure 1.9 and Figure 1.11).

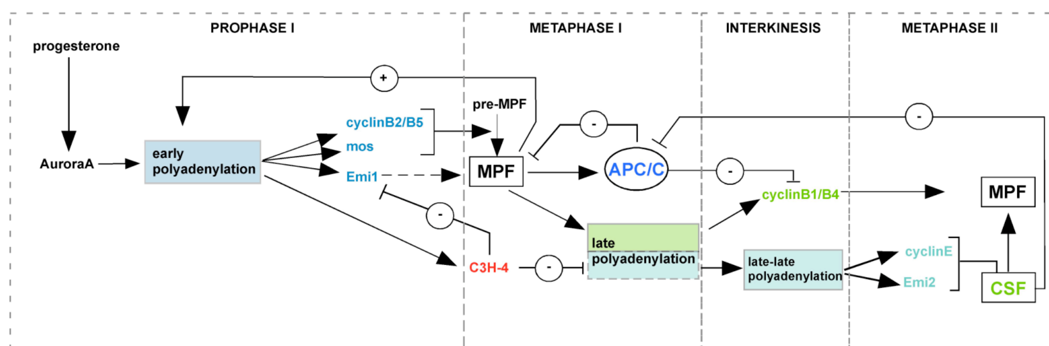


Figure 1.11. Sequential waves of polyadenylation and deadenylation drive meiosis. Schematic diagram showing the sequential waves of polyadenylation and deadenylation driving meiotic progression. Maturation-Promoting Factor (MPF), Anaphase-Promoting Complex/Cyclosome (APC/C) and Cytostatic Factor (CSF) activities are indicated. The three waves of cytoplasmic polyadenylation (early, late and late-late) and multiple positive and negative feedback loops are also depicted. (See text for details). 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 CPEBs. As the result of progesterone stimulation, CPEB 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/C inhibitor Emi1 (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 WT, 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 WT, 1996). At the same time, a negative feedback loop, which opposes CPEB 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/C and the transition to interkinesis. As the result of MPF activation in MI, CPEB is sequentially phosphorylated by Cdc2 and Plx1 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., 2000b; Pique et al., 2008; Setoyama et al., 2007). In addition, the partial destruction of CPEB 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/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 (Belloc and Mendez, 2008).

1.4.4 Meiotic spindle assembly during *Xenopus* oocyte maturation.

(i) Microtubule cytoskeleton reorganization during A-V axis establishment.

In the *Xenopus* oocytes, the animal-vegetal axis first becomes apparent during stage IV of oogenesis due to the asymmetric distribution of pigment granules between animal (A) and vegetal (V) cortex. Other features of polarization along this A-V axis during the last stages of oogenesis (i.e., stage IV-VI) include: the germinal vesicle (GV, the oocyte nucleus, 400μm) that moves from the center of the oocyte to its final position near the animal pole (Dumont, 1972), a distribution of yolk platelets that is established in the vegetal hemisphere (Danilchik and Gerhart, 1987) and an unequally distribution of specific maternal mRNAs at the animal and vegetal hemispheres (Capco and Jeffrey, 1982; Carpenter and Klein, 1982; Gard et al., 1995c; Gururajan et al., 1991; King et al., 2005; Melton, 1987; Weeks and Melton, 1987a, b; Yisraeli et al., 1990). In addition, confocal immunofluorescence microscopy has revealed that the microtubule (MT) cytoskeleton also becomes progressively more polarized during these stages of oogenesis (Gard, 1991). The proper establishment of this A-V axis determines the region in which the sperm will can penetrate the egg during fertilization (reviewed in (Minakhina and Steward, 2005)).

Dramatic episodes of MT reorganization take place during progesterone-induced maturation, ending in the assembly of the meiotic spindles. However, unlike most somatic cells, extensive networks of cytoplasmic microtubules exist throughout MI and MII. One of the earliest indicators of GVBD is the assembly of a disc-shaped

MTOC (Microtubule Organizing Center) near the basal (vegetal) surface of the GV. MTs extend from this MTOC forming a transient MT-array (TMA). This MTOC-TMA complex then collects and transports the dispersed condensed-meiotic chromosomes to the animal pole, where it serves as the precursor of the first meiotic spindle (Gard, 1992). Due to MTOC-TMA migrates to the nearest region of the cortex at the onset of GVBD, formation of meiotic spindles at animal pole is thus a direct consequence of the position of the GV in the animal hemisphere of stage VI oocytes. Moreover, the oocyte cortex also exhibits A-V polarization of its ability to support later spindle rotation and polar body formation (Gard, 1993). Finally, spindle rotation is mediated by interactions between astral MTs and cortical F-actin (Gard et al., 1995b)

(ii) Meiotic spindle assembly.

Four common stages characterize the assembly of both the first and the second meiotic spindle during the maturation of *Xenopus* oocytes (Figure 1.12): (1) formation of a compact aggregate of MTs and chromosomes (aggregation and compaction); (2) establishment of a short bipolar spindle axis; (3) prometaphase spindle elongation in an orientation parallel to the oocyte surface; and (4) rotation of the spindle into alignment with the A-V axis (Gard, 1992).

This pathway of spindle assembly during maturation of large oocytes such as those of *Xenopus* differs significantly from that observed in early *Xenopus* embryos (Gard et al., 1990), in mitotic extracts prepared from *Xenopus* eggs in vitro (Murray, 1991; Sawin and Mitchison, 1991) or even though in smaller eukaryotic cells undergoing mitotic division (Rieder and Khodjakov, 1997; Rieder et al., 1997). The enormous diversity in chromosome behavior in gametes (spermatocytes and oocytes), relative to mitotic cells, has been traditionally attributed to differences in chromosome structure (bivalents or homologous chromosomes Versus sister chromatids). Nevertheless, given the high importance of ensuring a proper distribution of replicated chromosomes, the more crucial aspects of spindle function would be greatly conserved between mitosis and meiosis. Thus, the most noteworthy difference between meiotic and mitotic spindles may result from the acentriolar nature of meiotic spindles such as in *Xenopus* oocytes (Huchon et al., 1981), which must then depend on other mechanisms for the establishment and organization of the spindle poles.

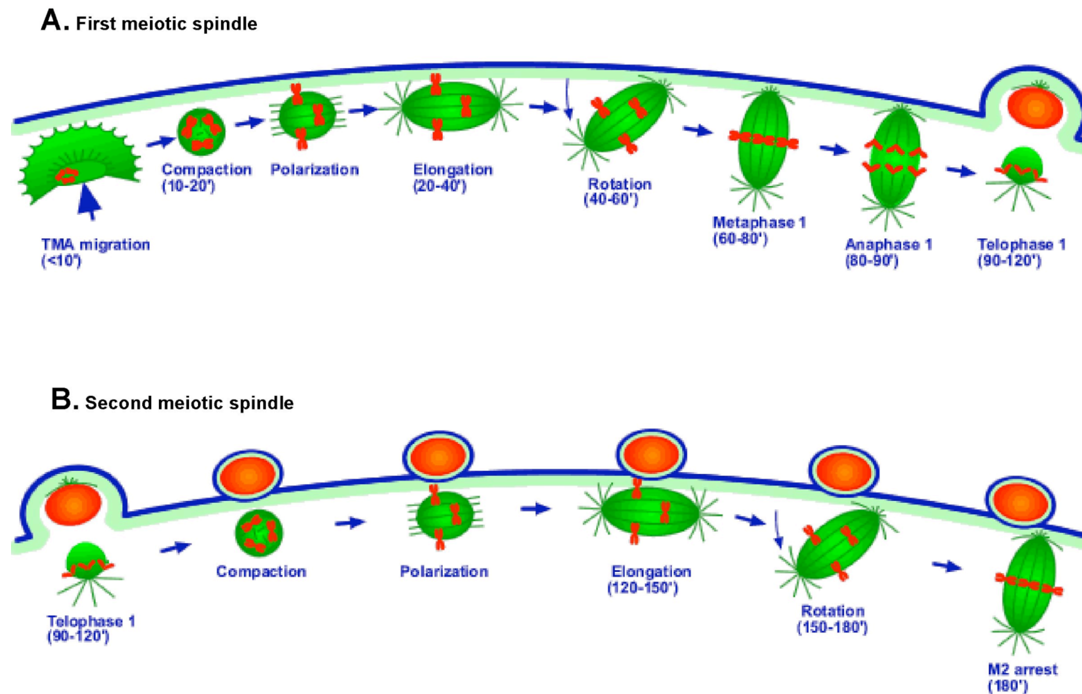


Figure 1.12. Assembly of the first and second meiotic spindle in *Xenopus* oocytes. (**A**) Assembly of the first meiotic spindle: (1) upon reaching the animal pole, the MTOC-TMA complex compacts to form a disordered aggregate of MTs (green) and chromosomes (red); (2) the bipolar axis of the MI spindle is formed (polarization); (3) the MI spindle elongates parallel to the oocyte surface during prometaphase; and (4) the MI spindle anchor to the oocyte cortex and rotates into alignment with the A-V axis. Following metaphase-I and anaphase-I, the first polar body is extruded (in red). (**B**) Assembly of the second meiotic spindle follows a pathway similar to that observed in MI. Unlike a typical interphase in a somatic cell, the condensed MI chromosomes never fully de-condense, and there is no evidence that a nuclear envelope forms around them. (1) Spindle assembly begins with formation of a compact, unpolarized aggregate of MTs and condensed meiotic chromosomes, which then (2) re-organize to form a short, bipolar spindle. (3) Much as in MI, the MII spindle elongates during prometaphase, (4) anchors to the cortex, and rotates into axial alignment. The meiotic cell cycle then arrests during second meiotic metaphase.
Taken from http://www.biology.utah.edu/gard/HTML/Cytoskeleton/MTs_frameset.htm.

Unlike mitotic spindle assembly, formation of meiotic spindle in maturing oocytes occurs in the absence of active centrosome. Numerous observations suggest that meiotic chromosomes play an important role in spindle formation during meiosis in *Xenopus* and other species (Church et al., 1986; Gard et al., 1995c; Karsenti et al., 1984; Sawin and Mitchison, 1991; Steffen et al., 1986; Theurkauf and Hawley, 1992). It has been shown that MTOC-TMA stains only weakly with antibodies against to γ-Tubulin. During the initial stages of spindle assembly, individual meiotic chromosomes are surrounded by brightly stained shells of γ-Tubulin and only after prometaphase elongation does γ-Tubulin become concentrated to the spindle poles suggesting the important role of chromosomes in the nucleation and assembly of spindle microtubules during meiosis. The kinetochores or the whole chromosomes are able to promote the nucleation and/or stabilization of MTs in their vicinity and then organize these MTs into a functional bipolar array by some kind of “cross-linking”

activities, reviewed by (Karsenti and Vernos, 2001; Rieder et al., 1993). Curiously, chromosomes as initiators of spindle organization are the responsible of the assembly of a complex structure that is induced by its one purpose: ensure the distribution of the duplicated genome to the daughter cell during cell division. Finally, despite lacking "classical" centrosome, oocytes and eggs contain a substantial maternal pool of centrosome components that possibly contribute to MT organization into a functional bipolar spindle (Gard et al., 1995a; Gard et al., 1995c).

1.5 Spatial control of translation.

1.5.1 The role of mRNAs on spindles.

RNA-cytoskeleton interactions have a crucial role and influence the transport, anchoring and even the translation of mRNA in most cell types (Bassell and Singer, 1997; Lopez de Heredia and Jansen, 2004). Although numerous cases of MT-associated mRNAs have been described so far, few cases of mRNA localization onto the bipolar spindle microtubules and/or centrosomes have been reported in the literature. Some studies indicate that RNA is on the spindle apparatus as a structural component both in *Xenopus laevis* and *Spisula solidissima* (i.e., surf clam) (Alliegro et al., 2006; Blower et al., 2005). Blower et al. have provided evidence for spindle-associated RNAs in *Xenopus* egg extract playing a direct and translational-independent role in spindle morphogenesis; and Alliegro et al. have shown that centrosomes are associated with specific RNAs (i.e., cnRNAs) in surf clam oocytes. Only one of these centrosome-associated RNAs, cnRNA11, looks like an mRNA predicted to encode a protein. However, the other four reported centrosome-associated RNAs seem to be non-coding RNAs with structural functions. In addition, the centrosome has been postulated as the site of assembly of molecules including translationally inactive mRNAs that are then delivered to the daughter cells during cell division (Lambert and Nagy, 2002). It is noteworthy that these mRNAs are recruited to only one of the two centrosomes, possibly via a minus-end directed microtubule motor, during early mollusk embryogenesis. Then, these centrosome-localized mRNAs are inherited exclusively by one daughter cell during cell division (i.e., asymmetrical inheritance) (Lambert and Nagy, 2002). In *Xenopus* and *Drosophila* developing embryos, an mRNA involved in mitotic cell cycle progression such as cyclin B1 mRNA appear to be concentrated in the regions surrounding chromosomes expected to be enrich in MTs (Groisman et al., 2000; Raff et al., 1990), and the mRNA for the checkpoint control protein Xbub3 has been detected recruited into each animal pole blastomeres in *Xenopus* embryos through a possible association with chromosomes (Goto and Kinoshita, 1999; Groisman et al., 2000), suggesting that

these regulators of cell division should be directed to the proximities of spindles and chromosomes. Accordingly, cytological and biochemical studies of spindles assembled from *Xenopus* egg extracts and sea urchin microtubules have demonstrated that ribosomes are tightly associated with spindle microtubules (Hamill et al., 1994; Liska et al., 2004; Mitchison et al., 2004; Suprenant, 1993; Suprenant et al., 1989). Indeed, abundance of ribosomes that appeared in clusters located around centrosomes and distributed along the length of the spindle microtubule has been shown by electron microscopy on spindles assembled from frog egg extract (Figure 1.13) suggesting that translation machinery may be spatially connected with the spindle during meiosis in *Xenopus* oocytes (Liska et al., 2004).

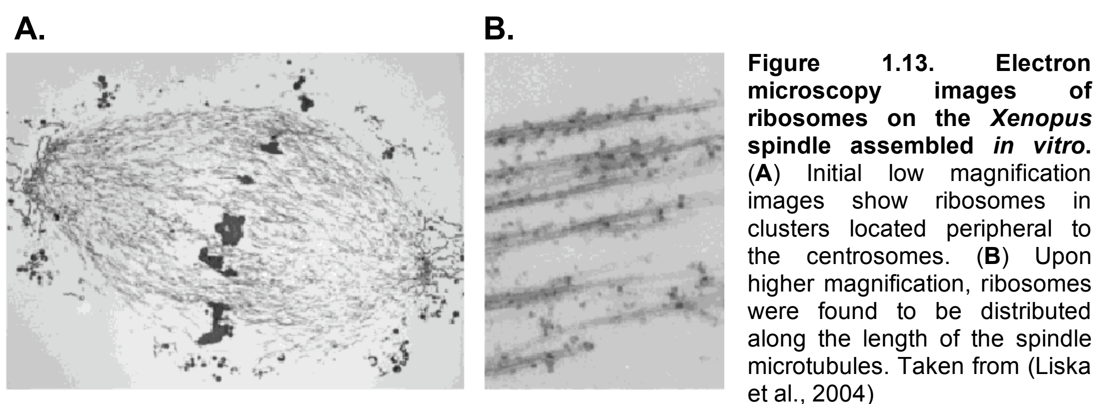


Figure 1.13. Electron microscopy images of ribosomes on the *Xenopus* spindle assembled *in vitro*. (**A**) Initial low magnification images show ribosomes in clusters located peripheral to the centrosomes. (**B**) Upon higher magnification, ribosomes were found to be distributed along the length of the spindle microtubules. Taken from (Liska et al., 2004)

Finally, the requirement of new protein synthesis to allow spindle morphogenesis in *Xenopus* oocytes and eggs is not fully understood. The controversy arises from different experiments performed in aster and/or spindle assembled in frog egg extract. It has been suggested that *Xenopus* egg extract contains all the RNAs and proteins required for the assembly of the mitotic spindles not requiring neither transcription nor translation for proper spindle assembly *in vitro* (Blower et al., 2005), however treatment with inhibitors of protein synthesis such as cycloheximide (CHX) has an effect on both aster size and spindle assembly in this system. The finding of that CHX-treated asters are slightly smaller than controls and that the number of normal spindles is consistently reduced (O'Brien et al., 2005) support the hypothesis that efficient spindle morphogenesis might require synthesis of one o more spindle assembly factor(s) that could be occur associated with polysomes-bound MT on spindles. Moreover, it has been shown that oocytes exposed to CHX shortly after GVBD do not complete MI and cause rapid entry into interphase and subsequent initiation of DNA replication (Furuno et al., 1994; Huchon et al., 1993; Wasserman and Masui, 1975). Cytological analysis revealed that under these conditions, a compact spindle is formed without properly aligned chromosomes. One hour after

GVBD this structure is disassembly completely indicating that protein synthesis is required for completion of MI and entry to MII (Hochegger et al., 2001).

1.5.2 CPEB-mediated mRNA localization.

CPEB orthologues from several species including *Drosophila*, zebrafish and mouse are involved in mRNA localization in oocytes, embryos, and neurons (Bally-Cuif et al., 1998; Christerson and McKearin, 1994; Huang et al., 2003; Lantz et al., 1994). In *Xenopus* oocytes, CPEB and Maskin are present in large multiprotein complexes containing mRNAs, at the animal pole (the pigmented portion of the oocyte in which the nucleus and the MTOC, which directs the meiotic spindle formation, are acentrically located). Once cytoplasmic polyadenylation takes place during oocyte maturation, most of the CPEB is destroyed (~80%) (Hake and Richter, 1994). Virtually all that remains stable is highly localized to the cortex of the animal pole, which in the developing embryo will give rise to the ectoderm (i.e., nervous system). After the fertilization, CPEB remains concentrated in the animal pole blastomeres and within these cells CPEB as well as Maskin localizes with the mitotic apparatus (Groisman et al., 2000). At metaphase, both proteins have been found along the length of the spindles, although there is a greater concentration of them towards the centrosomes (Groisman et al., 2000). Accordingly, both CPEB and Maskin have been shown onto *in vitro* assembled spindles from frog egg extract (Barnard et al., 2005; O'Brien et al., 2005; Peset et al., 2005). It is still not clear whether CPEB binds to MTs directly or rather by association of other(s) factor(s) linking CPE-containing mRNAs particles to the cytoskeleton. Recombinant CPEB can interact directly with *in vitro* polymerized microtubules (Groisman et al., 2000), however, in dendrites the association of CPEB with microtubules is not direct but rather probably mediated by the motors Kinesin and Dynein (Huang et al., 2003; Huang and Richter, 2004). In addition, although Maskin can bind directly to microtubules (O'Brien et al., 2005; Peset et al., 2005), it has been also found that Maskin associate with XMAP215, a microtubule-associated protein (MAP) that accumulates at the MTOC and has critical roles in the assembly and function of the meiotic/mitotic spindles and/or cell division (Gard et al., 2004; O'Brien et al., 2005; Peset et al., 2005). Accordingly, CPEB has been detected in XMAP215 immunoprecipitate as well (O'Brien et al., 2005). In addition, the association of Maskin with the mitotic spindle requires phosphorylation on Ser626 by both Kinase A and Aurora A (Barnard et al., 2005; Kinoshita et al., 2005; Peset et al., 2005). Other study has shown Maskin interacting to Rae1, an RNA-export factor involved in Ran-regulated spindle formation (Blower et al., 2005), suggesting that CPEB and Maskin

complex could directly anchor the Rae1-containing multiprotein complex on mRNAs to the mitotic apparatus. Interestingly, in dendrites CPEB and Maskin colocalize with CPE-bearing mRNAs (Huang et al., 2003; Huang and Richter, 2004), and even a translational control mechanism has been proposed in which these CPE-containing mRNAs can be transported to dendrites through the above mentioned MT-associated motors Kinesin and Dynein in a translationally dormant form, but activated at synapses in response to NMDA receptor stimulation (Huang et al., 2003). In *Xenopus* embryos, two CPE-containing mRNAs, cyclin B1 and Xbub3, appear to be concentrated in the region surrounding anaphase chromosomes exclusively in animal-half blastomeres (Goto and Kinoshita, 1999; Groisman et al., 2000). The CPEB-activating kinase Aurora A has been also found specifically on centrosomes, however, other proteins involved in polyadenylation-induced translation such as CPSF, eIF4E and the poly(A) polymerase, have been all detected throughout the blastomeres, although a slight concentrated coincident or near the mitotic spindle and/or centrosomes (Groisman et al., 2000).

Thus, the localization of the *trans*-acting factors associated with CPE-containing mRNAs suggests a possible function of CPEB-mediated translational control in the regulation of mitotic spindle dynamics. In agreement with this hypothesis, microinjection of agents that disrupt global cytoplasmic polyadenylation-induced translation (e.g., CPEB antibody, CPEB dominant negative mutant or the polyadenylation inhibitor cordycepin), block cell division and promote spindle and centrosome defects in embryos which include tripolar spindles, spindles detached from centrosomes or multiple centrosomes (Groisman et al., 2000; Mendez et al., 2002; Mendez et al., 2000a). However it is not clear whether this effect is a direct or indirect due to the block of the mitotic cell cycle (Groisman et al., 2001; Groisman et al., 2000). Finally, Maskin and Aurora A, have crucial CPEB-independent functions in centrosome maturation and spindle assembly (Albee et al., 2006; Dutertre et al., 2002; Goepfert and Brinkley, 2000; Kinoshita et al., 2005; O'Brien et al., 2005; Peset et al., 2005) that could be affected by the injection of α-Maskin or α-CPEB antibodies or by the overexpression of CPEB mutants.

Therefore, although the localization of CPEB, together with the requirement of cytoplasmic polyadenylation for embryonic cell division and spindle assembly, suggest a function for localized CPEB-mediated translational regulation in spindle formation and chromosome segregation, no direct evidence of this exist so far. Moreover, the spindle-specific potential targets of this regulation have not been yet identified.

2. OBJECTIVES

The main aims of this thesis are:

1. To determine whether there is CPEB-mediated spindle-localized translation.

2. To identify potential spindle-specific mRNA targets for localized CPEB-mediated translational regulation.

3. To define the biological function of spindle-localized CPEB-mediated translational control.

3. RESULTS

3.1 Identification of Xkid and TPX2 as putative target mRNAs of CPE/CPEB-mediated spindle-localized translational regulation in *Xenopus* oocytes.

To test directly whether there is localized CPEB-dependent translation, we started this thesis work identifying potential CPE-regulated mRNAs encoding for proteins with a well-known function in chromosome segregation and spindle formation, which could be locally translated during *Xenopus* oocyte maturation. We took advantage of a set of rules that we have recently developed in the laboratory to predict the translational behaviour of CPE-containing mRNAs (before mentioned in section 1.4.2 and (Pique et al., 2008). Since CPEB-mediated localization and repression are two linked events (Huang and Richter, 2004) and given that the candidate mRNAs should be activated prior to spindle assembly (i.e., PI-MI transition), we performed a genome-wide computational identification of mRNAs potentially repressed by CPEB and predicted to be cytoplasmically polyadenylated and translationally activated during the first prophase of meiosis. According to our predictions, this particular translational behaviour requires the presence of a cluster of at least two CPEs not-overlapping with the Hex, in their 3'UTRs. As a second stringency constrain we selected mRNAs where this arrangement of motifs was conserved between mouse, human and *Xenopus*. With these criteria, we identified the mRNAs encoding for TPX2, Bub1, Bub3, Eg5, Mad1, Mad2, BubR1, Xkid, Cenp-E, Nek2B, Aurora A and Aurora B as putative targets for CPEB-mediated localized translational regulation. From this list we selected two mRNAs encoding for proteins with a well-characterized structural function during cell division: **Xkid** and **TPX2**.

Xkid (*Xenopus* kinesin-like DNA binding protein) is a chromokinesin motor protein that has both MT- and DNA-binding domains and has been described to localize to both mitotic chromosomes and spindles during cell cycle (Antonio et al., 2000). Xkid is essential for the correct chromosome alignment on metaphase plate and the subsequent chromosome segregation (Antonio et al., 2000; Funabiki and Murray, 2000). In addition, Xkid is required for transition from MI to MII in *Xenopus* oocytes (Perez et al., 2002). TPX2 (Targeting Protein for *Xenopus* kinesin-like protein 2) is member of a novel family of vertebrate MAPs that additionally to its role in recruit Xklp2 to MT minus-ends, shows a dynamic localization during cell cycle, accumulating at spindle poles during mitosis in a dynein-dependent manner and playing a key role in MT nucleation and spindle pole organization (Gruss and Vernos, 2004; Wittmann et al., 2000).

3.1.1 The 3'UTR of Xkid and TPX2 mRNAs.

Xkid 3'UTR (Figure 3.1 and Figure 3.I in appendix I) contains 3 potential CPEs, two of them separated by 7 nucleotides and therefore with the potential of assembling a repression complex according to the combinatorial code of *cis*-elements (section 1.4.2 and (Pique et al., 2008)). The most 3' CPE and the Hex are flanked by, respectively, a GU-stretch and a UA-stretch, with the potential to form a stable secondary structure that places in close proximity the CPEs to the Hex. Human and mouse Kid 3'UTRs, although much shorter, also contain clusters of two CPEs not overlapping with the Hex and are, therefore, predicted to display the same translational behaviour. TPX2 3'UTR (Figure 3.1 and Figure 3.I in appendix I) contains multiple potential CPEs and two potential Hexs at positions 227 and 1207 of the 3'UTR. We have determined that only the shortest version of the 3'UTR, corresponding to the use of the upstream Hex during alternative nuclear cleavage reaction, can be detected in oocytes (Figure 3.2a). This short 3'UTR contains a cluster of 2 CPEs predicted to assemble a repression complex and another CPE at 10 nucleotides from the Hex, predicted to activate polyadenylation and translation in response to progesterone prior GVBD. Indeed, such behaviour was observed when constructs containing this 3'UTR were injected into oocytes, whereas the CPE arrangement of the long 3'UTR supported translational repression but not activation (Figure 3.2b). The CPE arrangement and the two potential polyadenylation sites are conserved in mouse and human TPX2 3'UTRs (Figure 3.1).

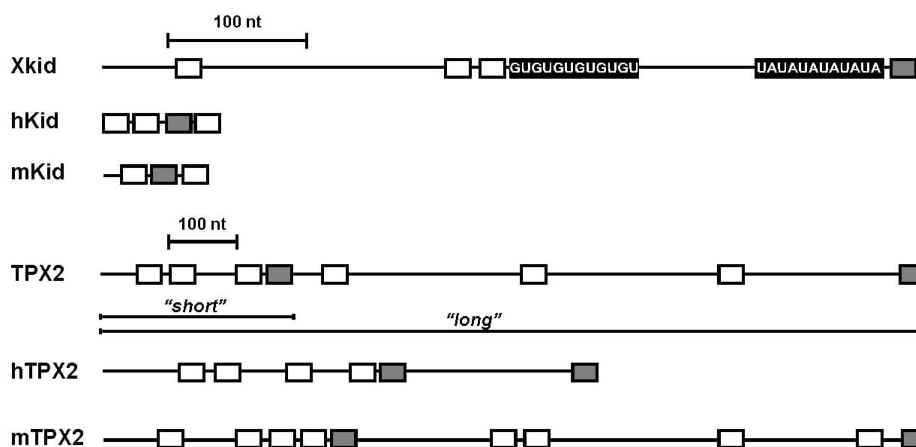


Figure 3.1. Schematic representation of the 3'UTRs from *Xenopus laevis*, human and mouse Kid and TPX2 mRNAs. The two possible 3'UTRs from TPX2 are shown as "short" and "long". CPEs (white boxes), polyadenylation signals (Hexanucleotide; grey boxes) and UGU/AUA repeats (black boxes) are indicated.

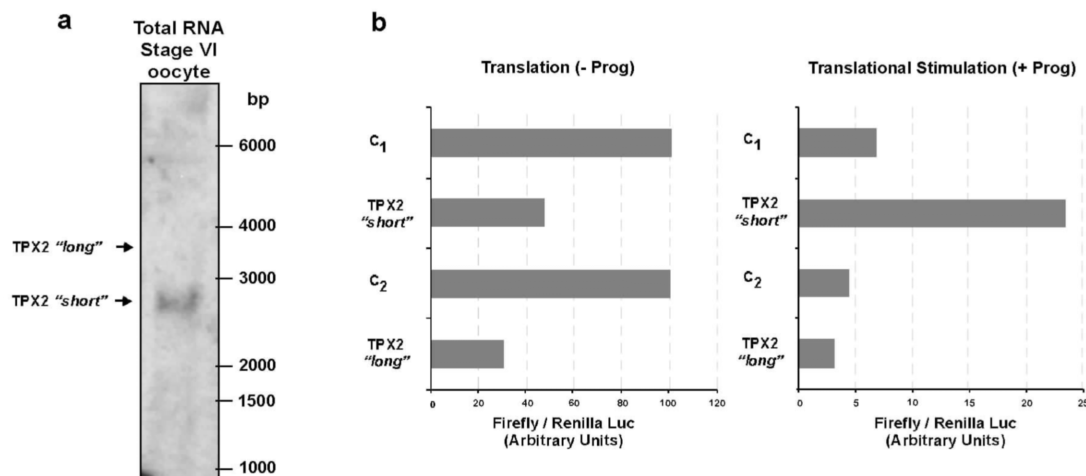


Figure 3.2. TPX2 mRNA in *Xenopus* oocytes. (**a**) Northern blot for TPX2 mRNA. Total RNA from 10 stage VI oocytes was extracted and analyzed by Northern blot with a labeled probe for TPX2 ORF. Molecular weight marker migrations are indicated. Arrows indicate the predicted migration position for both short and long TPX2 mRNAs. (**b**) *in vitro* transcribed chimerical mRNAs were coinjected into the oocytes as described in Figure 3.6. The mRNAs contained the Firefly Luciferase ORF fused to the following 3' UTRs: C_1 and C_2, control UTRs of 174 and 1139 nucleotides respectively; TPX2 "short" and TPX2 "long" 3'UTRs as shown in Figure 3.1 and in Figure 3.I in appendix I. Translational repression in the absence of progesterone (left panel) and translational stimulation in response to progesterone (right panel) were determined from the Luciferase activity in oocytes incubated with or without progesterone and collected 8 hours after injection of the reporters. The results shown are representative of 3 independent experiments.

3.1.2 Translational regulation of Xkid and TPX2 mRNAs by CPE/CPEB.

(i) Xkid and TPX2 mRNAs are translationally activated and cytoplasmically polyadenylated during oocyte maturation.

To validate the prediction above mentioned that Xkid and TPX2 are CPE-regulated mRNAs we decided to verify the functionality of the putative CPEs and to demonstrate that the endogenous mRNAs were indeed targets for cytoplasmic polyadenylation during oocyte maturation. First of all, we analyzed the expression of both Xkid and TPX2 proteins during progesterone-induced meiotic resumption by Western blot (Figure 3.3). In agreement with previous reports (Antonio et al., 2000; Perez et al., 2002), Xkid was not detectable in stage VI oocytes and was synthesized in response to progesterone, accumulating in MI to be then degraded in anaphase and re-accumulating in MII. TPX2 was present at low levels in stage VI oocytes but further accumulated in response to progesterone-induced meiotic resumption. This basal levels of TPX2 could be explained from the transient activation of CPEB that takes place in pachytene of PI oocytes (i.e., stage 0 in *Xenopus*) (Tay et al., 2003; Tay and Richter, 2001), combined with the fact that TPX2 localizes in the nucleus and is stable in PI arrested oocytes (Figure 3.4).

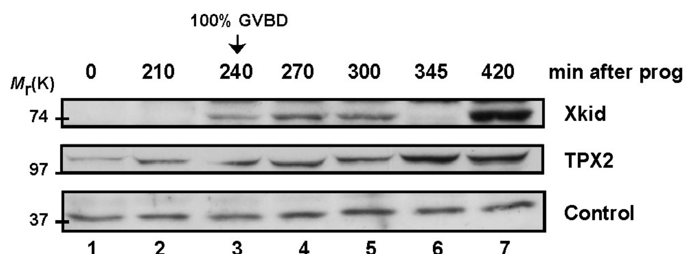


Figure 3.3 Xkid and TPX2 mRNAs are translationally activated in response to progesterone. *Xenopus* oocytes stimulated with progesterone were collected at the indicated times and analyzed by Western blot with either Xkid or TPX2 antibodies. The percentage of GVBD was determined by the appearance of the white spot at the animal pole of the oocytes. 1,5 oocyte equivalents were loaded per lane.

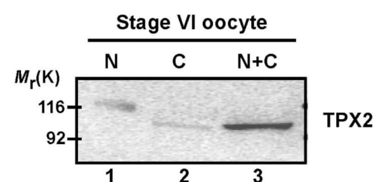


Figure 3.4 TPX2 is present in the nucleus of stage VI oocytes. *Xenopus* enucleated oocytes were analyzed by Western blot with TPX2 antibody. 4 nuclei and 2 oocyte equivalents were loaded per lane. N, nucleus; C, cytoplasm; N+C, stage VI oocyte used as a control.

These patterns of accumulation are consistent with these proteins being encoded by mRNAs translationally regulated by CPEB (i.e., repressed in arrested oocytes and stimulated upon meiotic resumption). Consequently, we observed that the endogenous mRNAs encoding for both proteins displayed a short poly(A) tail in arrested oocytes and were cytoplasmically polyadenylated in response to progesterone, as detected by RNA-ligation coupled RT-PCR technique in Figure 3.5.

To further define if this regulation was driven by the putative CPEs present in their 3'UTRs, we *in vitro* transcribed chimeric mRNAs containing the ORF of Firefly Luciferase followed by the 3'UTRs of either Xkid or TPX2 mRNAs, in their *Wild type* (WT) form or with all the putative CPEs inactivated and injected them into oocytes. We observed that in both cases the WT 3'UTRs mediated both translational repression in arrested stage VI oocytes and translational activation during meiotic resumption (Figure 3.6a). We found that these effects were CPE-dependent since the inactivation of the CPEs in the chimeric reporter constructs derepressed translation in arrested oocytes, if only partially for Xkid UTR, and inhibited the activation by progesterone treatment (Figure 3.6a). Furthermore, microinjected labeled reporter RNAs derived from both UTRs were also cytoplasmically polyadenylated in response to progesterone (Figure 3.6b).

Taken all together the results from section 3.1 demonstrate that Xkid and TPX2 are encoded by CPE-regulated maternal mRNAs stored with short poly(A) tails and translationally repressed in arrested oocytes, that are cytoplasmically polyadenylated and translationally activated as a result of progesterone-induced meiotic resumption.

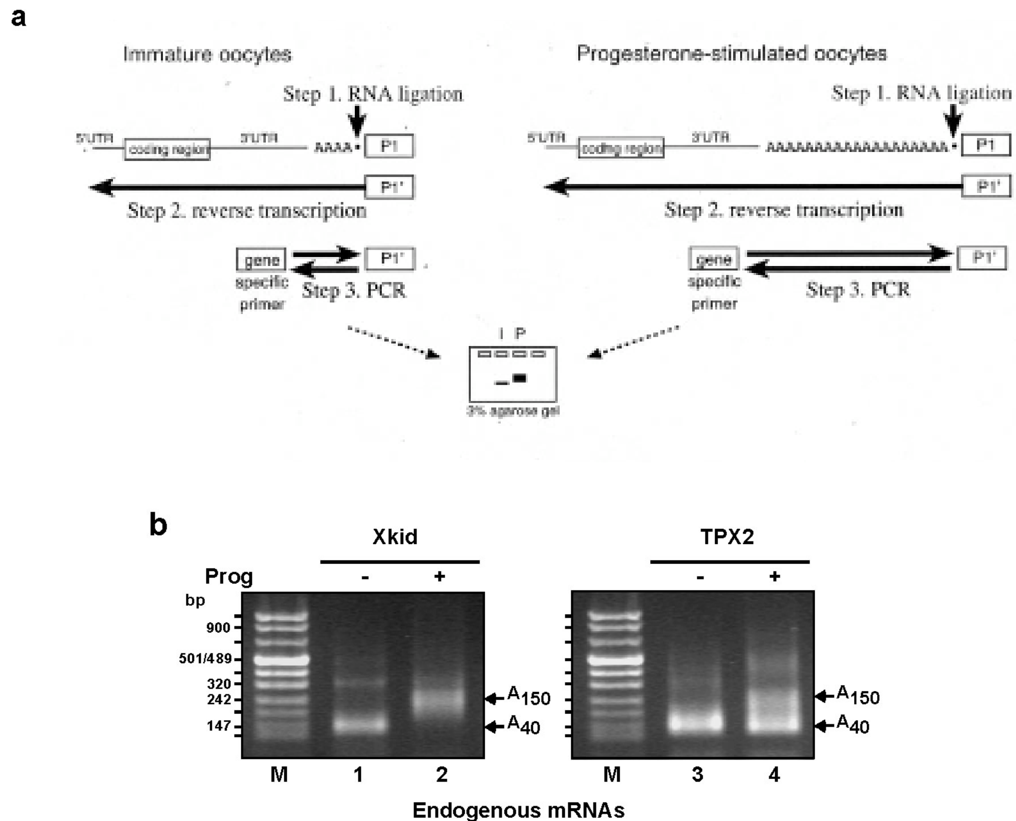
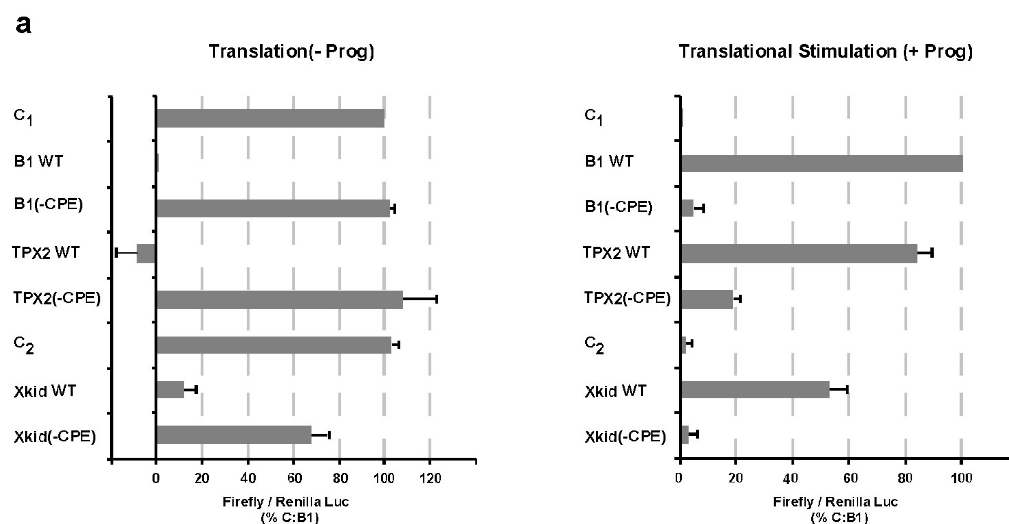


Figure 3.5. Xkid and TPX2 mRNAs are cytoplasmically polyadenylated in response to progesterone. (**a**) Schematic of the RNA-ligation coupled RT-PCR technique used in this study to see polyadenylation of endogenous mRNAs in response to progesterone. A DNA oligonucleotide (P1) is ligated directly onto the 3' end of all the RNAs in the sample preparation (step 1, RNA ligation). An antisense DNA oligonucleotide (P1'), complementary to the RNA-ligated P1 oligonucleotide, is then used as a primer for reverse transcription (step 2). From this pool of cDNAs, the polyadenylation status of individual mRNAs was assessed by PCR using a gene-specific forward primer and the P1' reverse primer (step 3). Retarded migration indicates polyadenylation. Adapted from (Charlesworth et al., 2004). (**b**) Total RNA extracted from oocytes incubated in the presence (+) or absence (-) of progesterone was analyzed by RNA-ligation coupled RT-PCR. The PCR products derived from the polyadenylated and non-polyadenylated Xkid and TPX2 mRNAs are indicated.



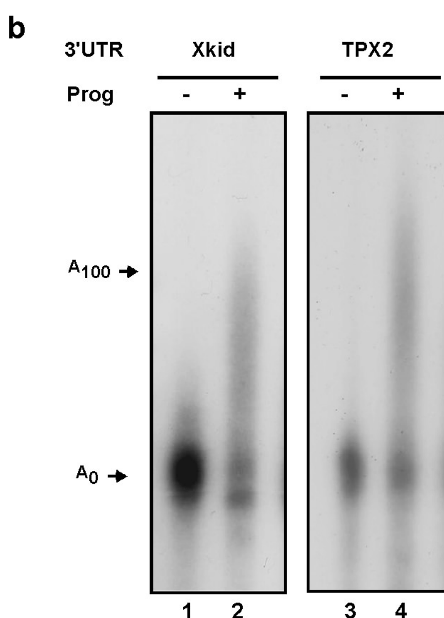


Figure 3.6. CPE-dependent translational regulation of Xkid and TPX2 3'UTRs. (**a**) *in vitro* transcribed chimeric mRNAs were coinjected into the oocytes together with Renilla Luciferase as a normalization control. The mRNAs contained the Firefly Luciferase ORF fused to the following 3'UTRs: C_1 and C_2, control UTRs of 174 and 470 nucleotides respectively; B1 WT and B1(-CPE), 3'UTRs derived from cyclin B1 mRNA with or without CPEs, respectively; TPX2 WT and TPX2(-CPE), 3'UTRs derived from TPX2 mRNA with or without CPEs, respectively; Xkid WT and Xkid(-CPE), 3'UTRs derived from Xkid mRNA with or without CPEs, respectively. The percentage of translational repression in the absence of progesterone (left panel) was normalized to C1 (100% translation) and to the fully repressed B1 (0% translation). Translational stimulation (right panel) was determined from the Luciferase activity in oocytes incubated with progesterone and collected 3 hours after GVBD. The percentage of translation stimulation was normalized to C1 (0% stimulation) and B1 (100% stimulation). Data are represented as mean ± s.d. (**b**) Oocytes were injected with either Xkid or TPX2 radiolabeled 3´ UTRs. Total RNA from oocytes incubated in the presence or absence of progesterone was extracted and analyzed by denaturing gel electrophoresis followed by autoradiography.

3.2 Localization of CPE-containing mRNAs on spindles.

Because Xkid and TPX2 are CPE-controlled mRNAs during oocyte maturation together with the fact that CPEB-mediated repression and localization are two functionally linked events (Huang and Richter, 2004), both mRNAs may therefore be good candidates to further explore the requirement for localized translation during the correct self-organization of microtubules and chromosomes into a functional bipolar spindle.

As a first approach we checked whether CPE-containing silenced RNAs showed enrichment in the animal hemisphere of the oocytes, where most of the CPEB and the MTOC, which directs the meiotic spindle formation, are present. We performed

Northern blots of RNAs extracted from either the animal or the vegetal halves of arrested oocytes and we observed that Xkid, TPX2, cyclin B1 and mos mRNAs, but not a control transcript without CPEs such as GAPDH mRNA, were highly enriched in the animal hemisphere (Figure 3.7).

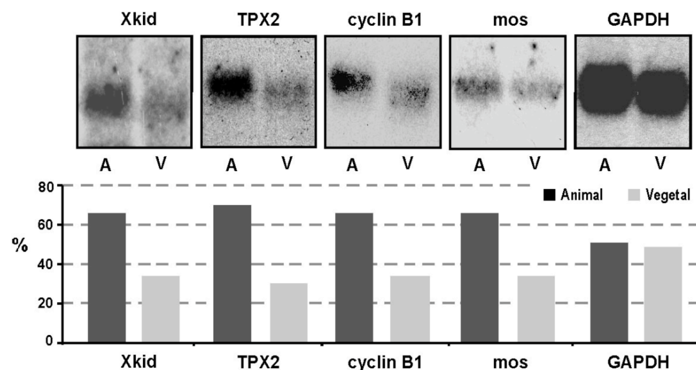


Figure 3.7. Enrichment of CPE-containing mRNAs at the animal hemisphere of *Xenopus* oocytes. Subcellular localization of endogenous Xkid, TPX2, cyclin B1 and mos mRNAs. Frozen immature oocytes were cut in half along the equator, and total RNA from animal (A) and vegetal (V) hemispheres was extracted and analyzed by Northern blot with labeled probes for Xkid, TPX2, cyclin B1, mos and GAPDH. Quantification of the relative Northern blot signals is shown.

3.2.1 The 3'UTRs of Xkid and TPX2 mRNAs direct their localization onto spindles.

To gain further insight into whether this animal-half enrichment was the result of the association of the mRNAs with microtubules, we performed *in situ* hybridization for Xkid mRNA in MI oocytes and on spindles assembled in egg extracts. Interestingly, we found that the endogenous Xkid mRNA colocalized with spindles and chromosomes in both systems suggesting that CPE-containing mRNAs associate with the bipolar spindle (Figure 3.8a, b). As previously has been described by (O'Brien et al., 2005; Barnard et al., 2005) immunofluorescence on spindles assembled in egg extract showed that CPEB localized onto the spindle and chromosomes (Figure 3.9), indicating that CPE-regulated mRNAs could be tethered to spindles by CPEB.

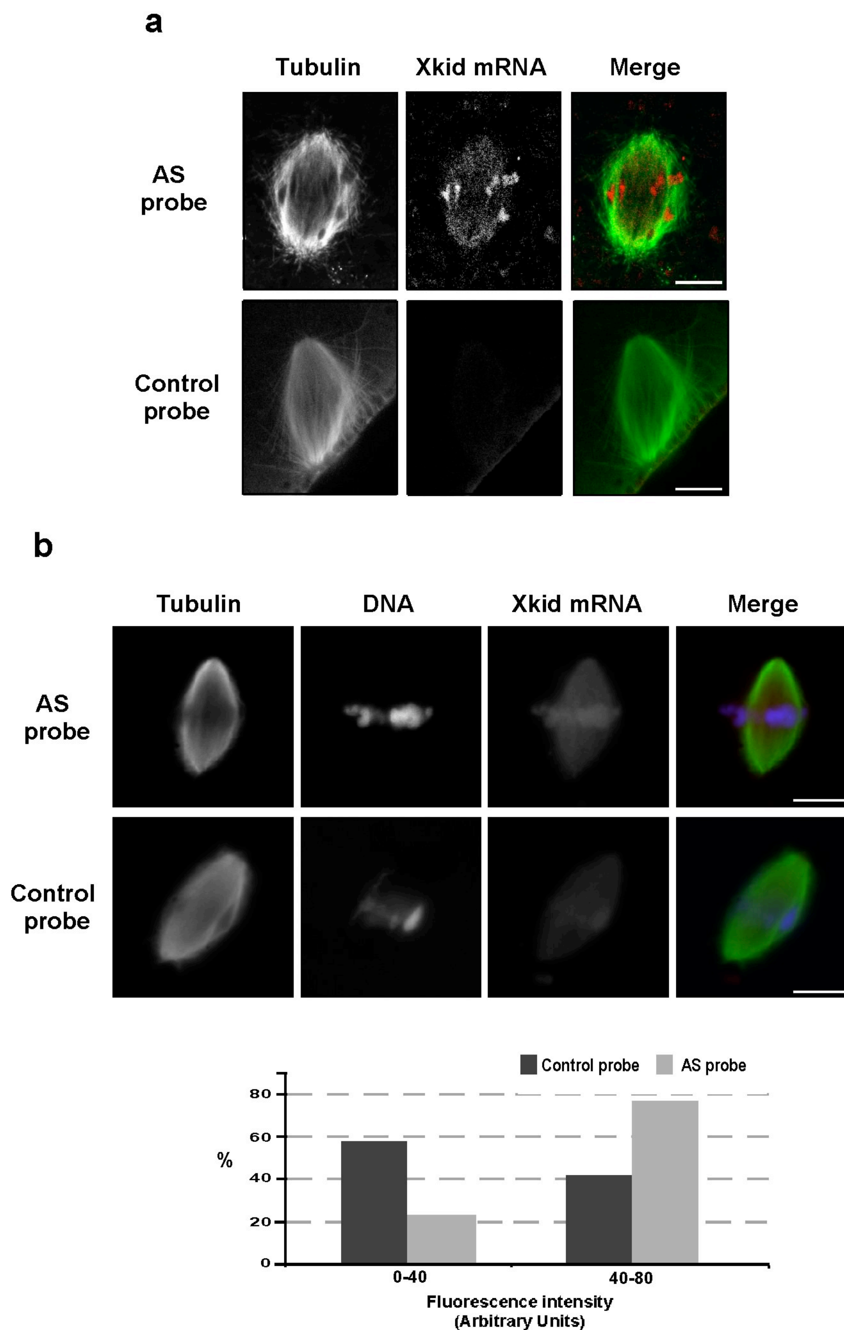


Figure 3.8. Endogenous Xkid mRNA localizes to the spindle apparatus. (**a**) Fluorescent *in situ* hybridization for Xkid mRNA in MI-spindles. Fixed mature oocytes were cut along A-V axis and hybridized with antisense digoxigenin-labeled (red) riboprobes for either Xkid mRNA or sense control, as indicated. Spindles were co-stained with antibodies against Tubulin (green). Scale bar 10 μm. (**b**) Fluorescent *in situ* hybridization for Xkid mRNA on *in vitro* assembled spindles. Spindles assembled in cycled egg extracts were hybridized with antisense digoxigenin-labeled (red) riboprobes for either Xkid mRNA or sense control, as indicated. Spindles were co-stained with antibodies against Tubulin (green) and Hoechst for DNA (blue). Scale bar 10 μm. Quantification of fluorescence intensity associated to microtubules in each case is also shown. (**b**) In collaboration with Isabel Peset and Isabelle Vernos.

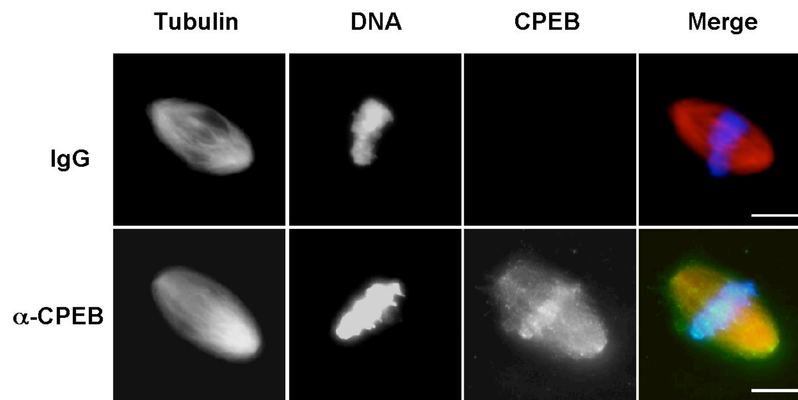


Figure 3.9. CPEB protein localizes to the spindle apparatus. CPEB immunostaining. Immunofluorescence of spindles assembled in egg extracts with the anti-CPEB antibody (green). Microtubules are visualized in red and DNA in blue. Scale bar 10 μm. In collaboration with Isabel Peset and Isabelle Vernos.

To obtain experimental evidence for the spindle-localization of CPE-bearing mRNAs we decided, in collaboration with Isabelle Vernos's group, to examine whether the association of mRNAs on spindles requires the CPEs present in their 3'UTRs. We therefore prepared reporter transcripts containing the 3'UTRs from either Xkid or TPX2 and a variant UTR with the CPEs inactivated, adding twelve MS2-binding sites for visualization with the recombinant fusion protein MS2-GFP (Bertrand et al., 1998). Chimeric RNAs and the MS2-GFP (Figure 3.10a) were added to CSF-arrested egg extracts used in cycled spindle assembly reactions. We observed that MS2-GFP protein localized onto the spindle and chromosomes in egg extracts containing reporter mRNAs with the 3'UTRs from either WT Xkid or TPX2 (Figure 3.10b). However, when we added to the extract the variant UTR with all the CPEs inactivated, we found that it did not localize to the spindles (Figure 3.10c). Thus, these results show that these transcripts associate with the spindles and chromosomes in a CPE-dependent manner, strongly suggesting that other CPE-repressed mRNAs may also associate with the spindle apparatus.

Altogether the results show in section 3.2 clearly demonstrate for the first time that two CPE-repressed mRNAs encoding for proteins with a well-characterized functions in spindle assembly and chromosome movements are enriched in the animal-half of the oocyte, where the meiotic spindle forms, and moreover, associate with the spindle microtubules and chromosomes in a CPE-dependent manner.

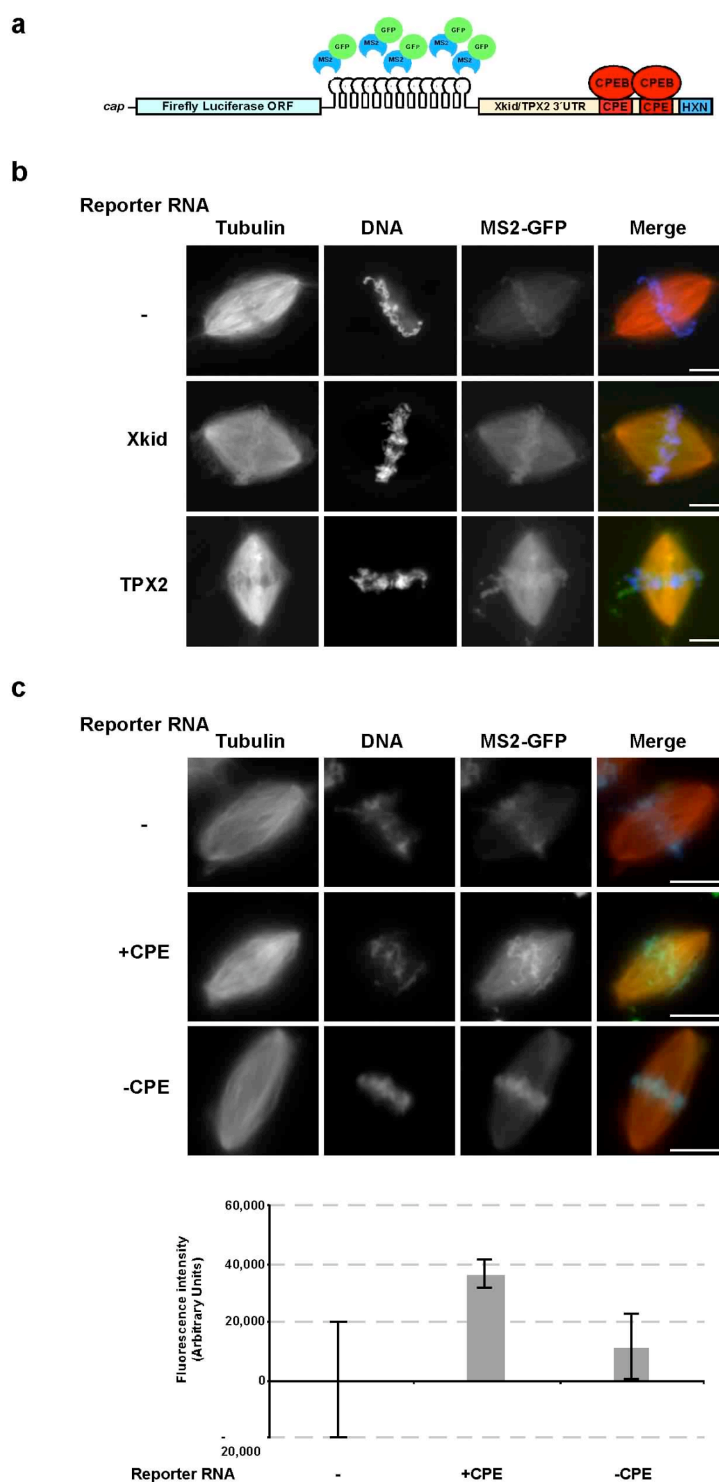


Figure 3.10. The 3'UTRs of Xkid and TPX2 mRNAs direct their localization to the spindles. RNA-mediated tethering of MS2-GFP to *in vitro* assembled spindles. (**a**) Schematic representation of the Luciferase reporter mRNAs followed by twelve MS2-binding sites and by the 3'UTR from either Xkid or TPX2 and the tethered MS2-GFP protein is shown. (**b**) Representative images of spindles assembled on *in vitro* cycled egg extracts supplemented, as indicated, with recombinant MS2-GFP protein and *in vitro* transcribed RNAs containing the Luciferase ORF followed by twelve MS2-binding sites and by the 3'UTR from either Xkid WT or TPX2 WT. (**c**) Representative images of spindles assembled on *in vitro* cycled egg extracts supplemented, as indicated, with recombinant MS2-GFP protein and *in vitro* transcribed RNAs containing the Luciferase ORF followed by twelve MS2-binding sites and by the 3'UTR from either TPX2 WT (+CPE) or a variant with all the CPEs inactivated (-CPE). Quantification of GFP-fluorescence intensity associated to microtubules is shown as mean ± s.d.. In collaboration with Isabel Peset and Isabelle Vernos. In (**b**) and (**c**), tethered MS2-GFP protein is detected as a green signal, rhodamine-labeled microtubules are detected in red and Hoechst-stained DNA in blue. Scale bars 10 μm.

3.3 Localized-translation activation of CPE-regulated mRNAs.

3.3.1 Localized-translational activation of Xkid mRNA is required for the accumulation of its protein.

Once we demonstrated that these maternal CPE-regulated mRNAs localize onto the spindle apparatus, we next aimed to determine whether, not only progesterone-induced protein synthesis, but also localized-translation of CPE-regulated mRNAs, was required for meiotic completion and MII arrest in *Xenopus* oocytes. In order to study the requirements for localized-translation, without interfering with general meiotic progression, we microinjected chimeric mRNAs while maintaining the translation of endogenous mRNAs unaffected, therefore allowing the oocytes to complete meiosis until the second metaphase arrest. We generated chimeric mRNAs containing the ORF of either Firefly Luciferase (FL) or Xkid (Xk), followed by artificial 3'UTRs containing one or two CPEs (UTR1cpe or UTR2cpe respectively) as we shown in Figure 3.11.

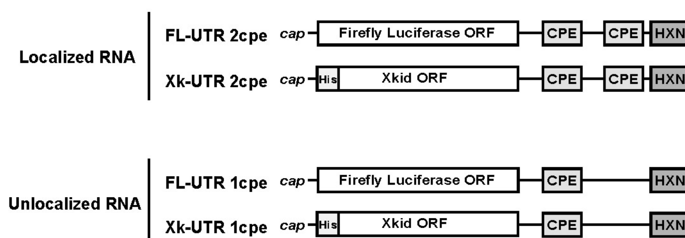


Figure 3.11. Design of chimeric mRNAs based on the rules for combinatorial CPE elements. Schematic representation of the chimeric mRNAs containing the ORF of either Firefly Luciferase (FL) or Xkid (Xk) fused to 3'UTRs containing one (UTR1cpe) or two (UTR2cpe) CPEs. The CPEs (light grey boxes) and the hexanucleotide (grey boxes) are indicated.

Based on the rules for combinatorial CPE-mediated translational regulation (section 1.4.2 and (Pique et al., 2008)) we hypothesized that the 3'UTR containing two CPEs (i.e., UTR2cpe) should mediate translational repression and mRNA localization in the absence of progesterone as well as cytoplasmic polyadenylation and translational activation upon meiotic resumption, whereas the 3'UTR with a single CPE (i.e., UTR1cpe) should not repress translation nor mediate localization but should still mediate polyadenylation in response to progesterone stimulation. These mRNAs were coinjected with a renilla mRNA containing a 3'UTR without CPEs as a control. As expected, we found that both artificial UTRs drove similar translational activation of the reporter Firefly Luciferase in response to progesterone, either alone or when coinjected with the equivalent Xkid mRNA (Figure 3.12a). Nevertheless, we observed that UTR2cpe, but not UTR1cpe, directed localization of the chimeric Xkid mRNA to the animal hemisphere of the oocyte, as we showed by RT-PCR technique (Fig 3.12b). Interestingly, when we analyzed the levels of the Xkid protein expressed either from the localized or unlocalized synthetic mRNAs we found that only the translational activation

of the localized mRNA was able to support accumulation of high levels of product (Figure 3.12c). We confirmed that these patterns of protein accumulation were not due to differences in the stability of the mRNAs, as shown by Northern blot of all chimeric mRNAs microinjected nor to translational repression of the Xk-UTR1cpe mRNA, which is incorporated into polysomes as efficiently as Xk-UTR2cpe mRNA (Figure 3.13a, b). Note that, due to the large stockpile of aminoacids, in oocytes it is not possible to perform pulse-chase experiments to directly measure the stability of the protein expressed from the localized and non-localized mRNAs. Therefore, taken together these results strongly suggest that localized-translation activation of Xkid mRNA results in higher stability of it protein product.

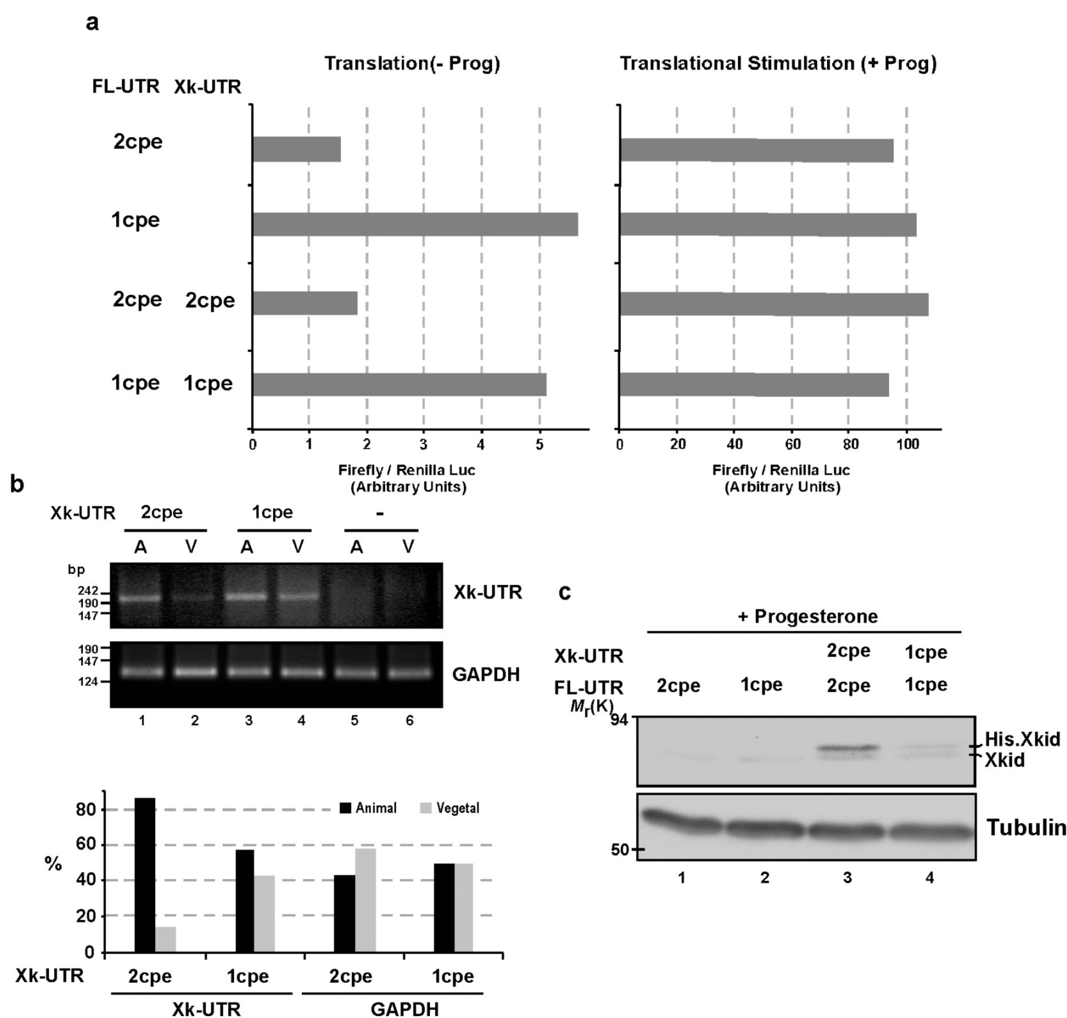


Figure 3.12. Localized translational activation of Xkid mRNA is required for the accumulation of Xkid protein. (**a**) Oocytes were injected with the indicated *in vitro* transcribed chimeric mRNAs and maintained for 3 hours, to allow proper mRNA localization, before oocytes were incubated in the presence or absence of progesterone for 7 hours. Then, the Luciferase activity was determined for pools of 5 oocytes. Translational repression in the absence of progesterone (left panel) and translational stimulation in the presence of progesterone (right panel) are indicated. Note the different scale in the X-axis for repression and activation. (**b**) Subcellular mRNA localization of injected mRNAs. Frozen oocytes injected with the indicated mRNAs were incubated in absence of progesterone and cut in half along the equator. Total RNA from animal (A) and vegetal (V) hemispheres was extracted and analyzed by RT-PCR using specific oligonucleotides for the injected RNAs and endogenous GAPDH mRNA. Quantification of the relative RT-PCR signals is shown. (**c**) The same injected-oocytes used in (**a**) were

analyzed for Xkid protein accumulation by Western blot with Xkid antibody. Equivalents of 1,5 oocytes were loaded onto each lane.

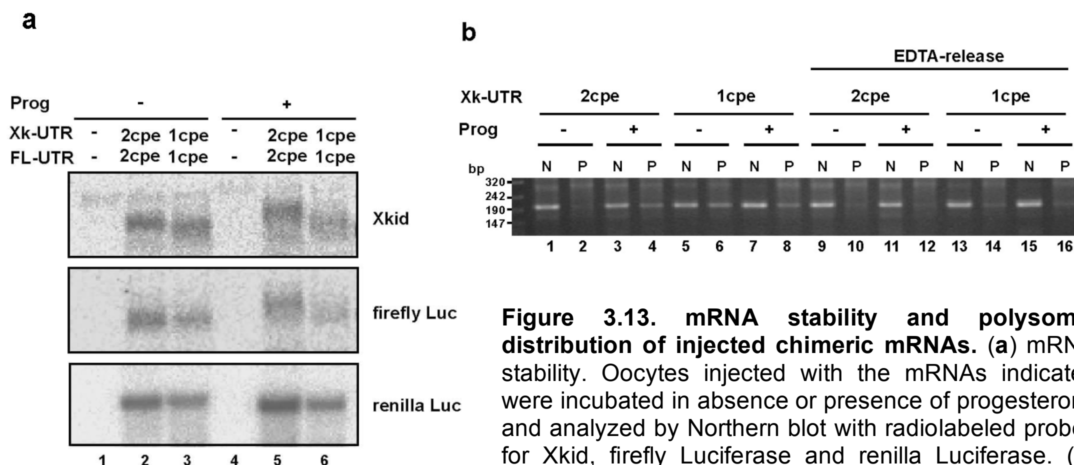


Figure 3.13. mRNA stability and polysomal distribution of injected chimeric mRNAs. (**a**) mRNA stability. Oocytes injected with the mRNAs indicated were incubated in absence or presence of progesterone and analyzed by Northern blot with radiolabeled probes for Xkid, firefly Luciferase and renilla Luciferase. (**b**) Polysomal distribution. Oocytes injected with the indicated mRNAs, were incubated in absence or presence of progesterone and fractionated by centrifugation over a sucrose cushion. Total RNA was extracted from both polysomal (P) and non-polysomal (N) fractions and analyzed by RT-PCR for the injected RNAs. In the indicated lanes, the extracts were treated with 50 mM EDTA to disrupt polysomes.

3.3.2 Localized Xkid mRNA translation is required for MI to MII transition in *Xenopus* oocytes.

To address the functional relevance of the localized vs. non-localized CPE-regulated mRNA translation during oocyte maturation, we then decided to focus on Xkid since previous work has shown that its expression can be efficiently prevented by injection of either antisense oligonucleotides or morpholinos into stage VI oocytes that generates a phenotype that can be easily recorded (Perez et al., 2002). Microinjection of antisense oligonucleotides directed to the 5' and 3'UTRs of Xkid mRNA ablated efficiently endogenous Xkid synthesis (Figure 3.14a). As it has been reported before, low levels of Xkid synthesis did not alter the early events of MI entry, as shown by the activation of Cdc2, but prevented the progression to MII, as indicated by the failure to reactivate Cdc2 as compared with control oocytes (Figure 3.14b). Next, we proceed to analyze the meiotic effect of knocking-down the expression of Xkid from the oocytes. Thus, DNA staining of the Xkid-depleted oocytes, at the time at which the non-injected control oocytes had reached MII (indicated by the presence of the MII metaphase plate and the polar body), revealed misaligned chromosomes in late MI and no polar body (Figure 3.14c). Longer incubation of these Xkid knock-down oocytes resulted in partially decondensed and cleaved chromosomes suggesting exit from meiotic cell cycle followed by apoptosis as previously shown (Perez et al., 2002) (see Figure 3.II in appendix II). Furthermore, in order to test whether localized protein translation was functionally important we examined whether the chimerical Xkid mRNAs driving either

localized translation (Xk-UTR2cpe) or non-localized translation (Xk-UTR1cpe) of Xkid were able to rescue the lack of endogenous Xkid during oocyte maturation. It is noteworthy that both chimeric Xkid mRNAs (Xk-UTR1cpe and Xk-UTR2cpe) lack the regions targeted to the antisense oligonucleotides and thus are resistant to them. Interestingly, we found that only the localized Xk-UTR2cpe can rescue oocyte maturation as monitored by the presence in MII of the chromosomes aligned on the metaphase plate, polar body extrusion and high Cdc2 activity as opposed to the presence of misaligned chromosomes in late MI and no polar body in oocytes containing the unlocalized Xk-UTR1cpe (Figure 3.14a, c). Therefore our results undoubtedly demonstrate that localized-translation of Xkid mRNA is essential for the function of this protein in chromosome alignment and MI-MII transition, and suggest that localized-translation of CPE-regulated mRNAs is required to ensure the correct function of the encoded proteins in the structural aspects of the cell cycle namely spindle assembly and chromosome movements.

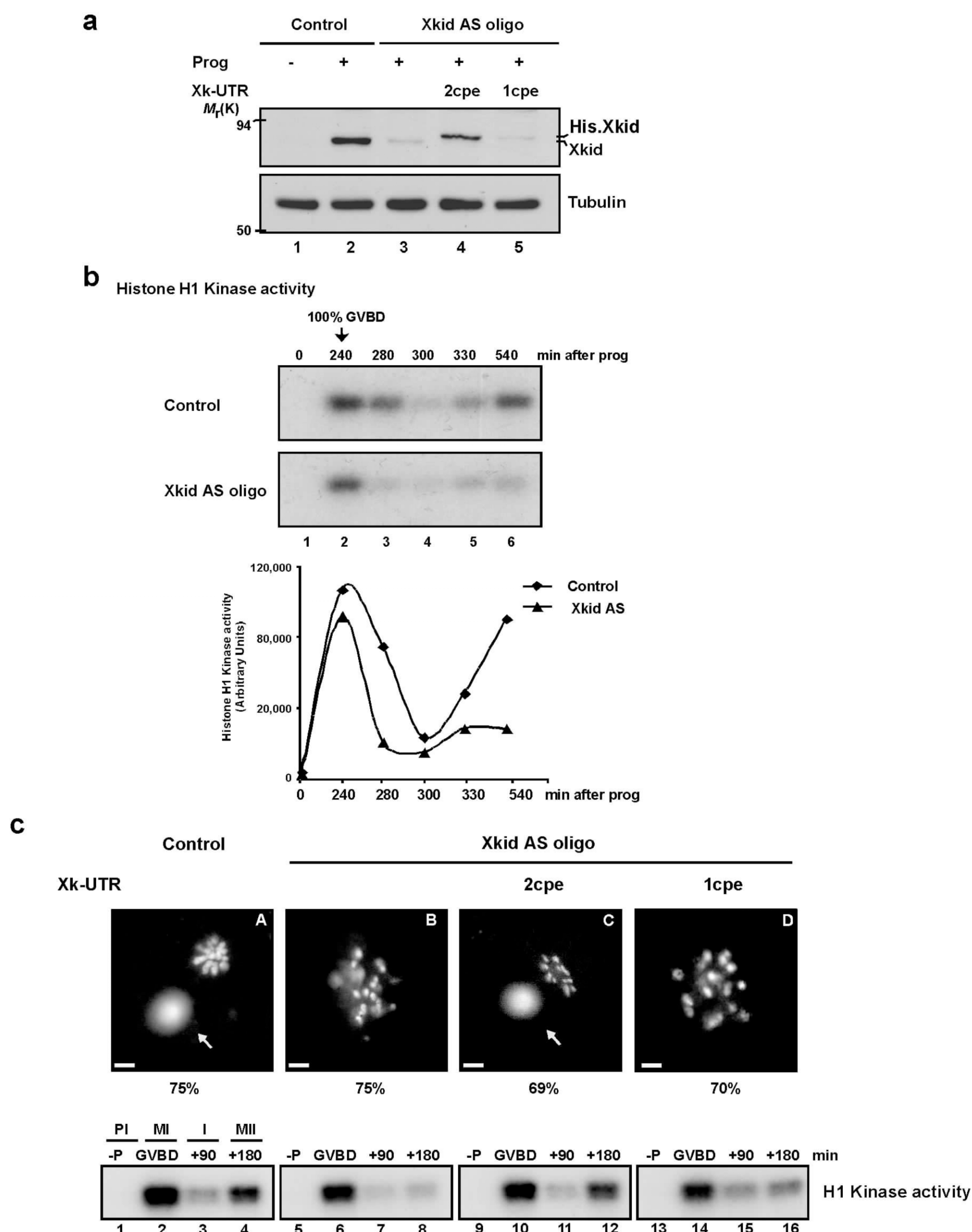


Figure 3.14. Localized Xkid mRNA translation is required for MI to MII transition. Oocytes were injected with Xkid sense or antisense oligonucleotides. After 12 hours both groups of oocytes were microinjected with 0,3 fmols of an mRNA encoding for Xkid ORF fused to 3'UTRs containing either one (UTR1cpe) or two (UTR2cpe) CPEs described in Figure 3.11. After 2 hours, the oocytes were incubated in the presence or absence of progesterone and collected 5 hours after the control, non-injected oocytes, displayed 100% GVBD. (**a**) One oocyte equivalent from each indicated treatment was analyzed by Western blot with Xkid and Tubulin antibodies. (**b**) Histone H1 Kinase activity in Xkid-depleted and control oocytes. Two oocytes collected at the indicated times after progesterone addition were lysed and the extract analyzed for Histone H1 Kinase activity. Quantification of the Histone H1 Kinase activity is shown**.** (**c**) Oocytes, treated as indicated, were fixed, stained with Hoechst and examined under the epifluorescence microscope. The percentage of oocytes displaying each phenotype (5 hours after GVBD) is shown (n>16). The arrow indicates the first polar body. Scale bar 10 μm. For each treatment, three oocytes were collected at the indicated times, lysated and the extracts analyzed for Histone H1 Kinase activity (-P, oocytes incubated in absence of progesterone). The meiotic phases in the control oocytes are indicated (PI, prophase-I; MI, metaphase-I; I, interkinesis; MII, metaphase-II).

3.3.3 Localized-translational activation of CPE-containing mRNAs is required for MI to MII transition.

Finally, to address whether the localized-translation of various CPE-regulated mRNAs was a general requirement for meiotic progression and normal chromosome dynamics, we aimed at delocalizing this type of transcripts by competition with a microinjected CPE-containing RNA that squelches the repression complexes (de Moor and Richter, 1999). We also microinjected the same RNA with the CPEs inactivated by point mutations as a control. The base of this approach is to out-compete CPEB protein by injecting high amounts of a small CPE-containing RNA in *Xenopus* oocytes. Under these conditions, CPEB-mediated repression and localization complex(es) should dissociate from their target mRNAs, but cytoplasmic polyadenylation and translational activation should not be affected upon progesterone stimulation (Figure 3.15).

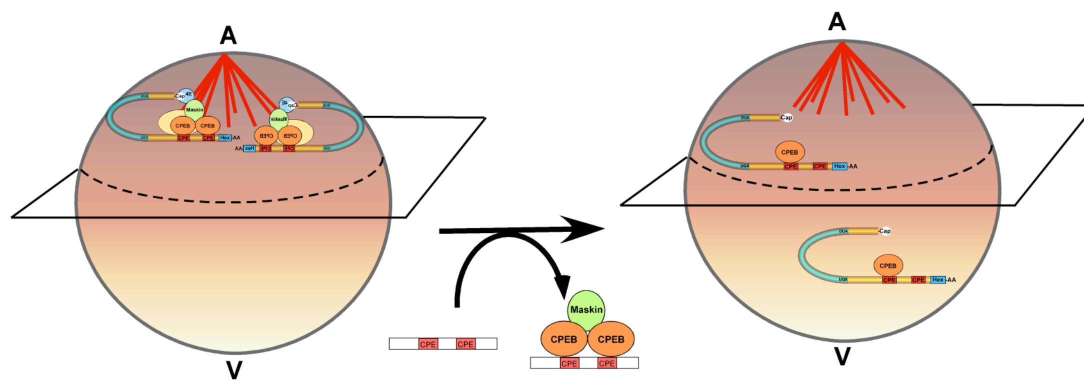


Figure 3.15. mRNA delocalization by competition with small CPE-containing RNA. CPE-containing mRNAs associated with microtubules (in red) at the animal-half of *Xenopus* oocytes are competed injecting high amounts of a small CPE-containing RNA that squelches the repression and localization functional linked complexes. After progesterone stimulation, cytoplasmic polyadenylation-induced translation takes place, but in a wrong place (i.e., in both hemispheres). A (animal half), V (vegetal half) is indicated. In the following Figures 3.16, 3.17 and 3.18, oocytes were injected with 1,5 pmol of 70 nucleotides long competitor RNAs containing (+CPE) or lacking (-CPE) multiple CPEs. After 2 hours, the microinjected oocytes were incubated with or without progesterone. Non-injected oocytes (-) were carried in parallel as a control.

As expected, we found that neither of these microinjected competitor RNAs blocked progesterone-induced cytoplasmic polyadenylation as shown for endogenous Xkid mRNA (Figure 3.16a) nor the synthesis of proteins in response to progesterone, as shown for Xkid and Cyclin B1 (Figure 3.16b), measured at the time when the control oocytes reached MII. We did not observe any effect on the kinetics of maturation (data not shown).

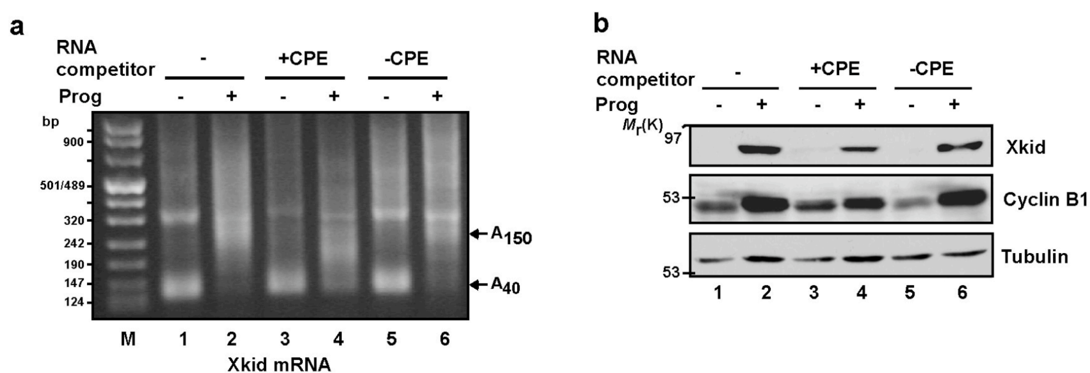


Figure 3.16. CPE-competition neither affects cytoplasmic polyadenylation nor translational activation in response to progesterone. (**a**) Cytoplasmic polyadenylation of Xkid mRNA. Samples were collected 5 hours after the non-injected oocytes reached 100% maturation (i.e. in MII). Total RNA from control and RNA-competed oocytes was extracted and polyadenylation of endogenous Xkid mRNA analyzed by RNA-ligation coupled RT-PCR as in Figure 3.5. (**b**) Protein expression in competed oocytes: control and RNA-competed oocytes were collected 5 hours after the non-injected oocytes reached 100% maturation and analyzed by Western blot with Xkid, Cyclin B1, and Tubulin antibodies. Equivalents of 2 oocytes were loaded onto each lane.

However, we observed a dramatic effect on the localization of the CPE-containing mRNAs and their newly translated encoded proteins within the MI oocyte (Figure 3.17a, b). Thus, while in the oocytes microinjected with a competitor RNA without CPEs (-CPE) both Cyclin B1 and Xkid accumulated mainly in the animal hemisphere, in the oocytes microinjected with a competitor RNA containing CPEs (+CPE), Cyclin B1 and Xkid were equally distributed in both halves. Moreover, although the total levels of Cyclin B1 were not affected by the delocalization of CPE-containing mRNAs, Xkid protein was not only delocalized but also the total levels were greatly reduced resulting in even lower levels of Xkid protein in the animal half of the oocyte. The competitor RNAs did not change either the localization of CPEB to the animal hemisphere or its phosphorylation by Cdc2 and its subsequent partial degradation in response to progesterone (Figure 3.17b). We conclude that competition with the CPE-containing RNA causes the delocalization of CPE-regulated mRNAs, like those of cyclin B1 and Xkid, inducing their translational activation all over the entire oocyte and the abnormal distribution of the encoded protein both in the animal and vegetal hemispheres.

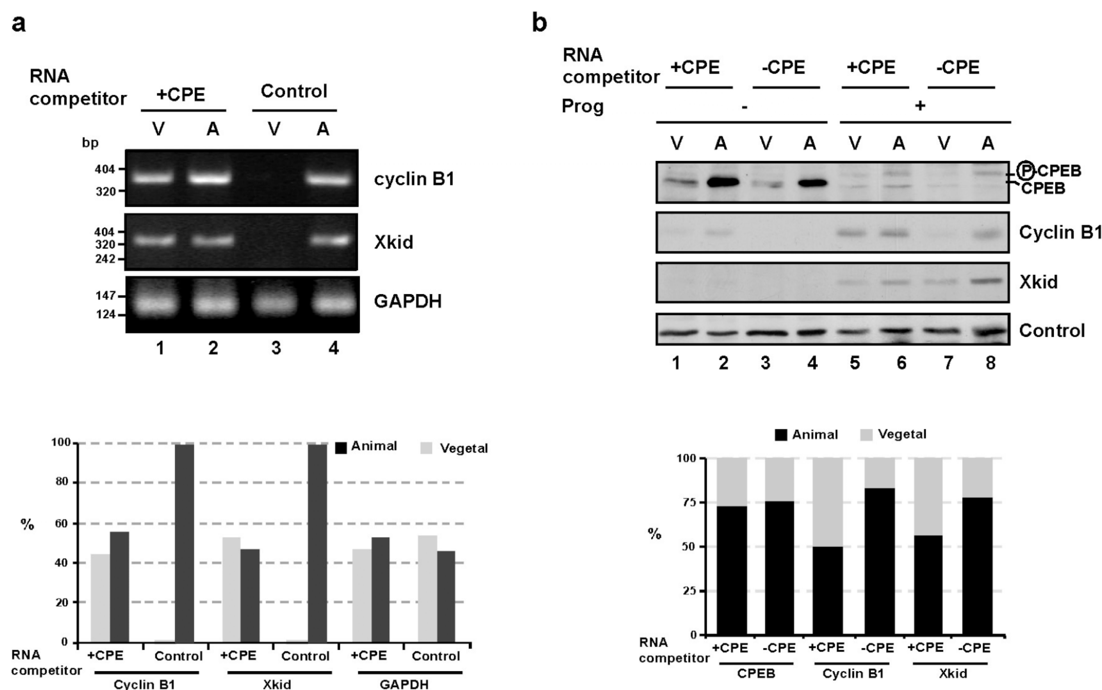


Figure 3.17. CPE-competition results in delocalization of CPE-containing mRNAs and theirs newly translated encoded proteins. (**a**) Subcellular localization of endogenous cyclin B1, Xkid and GAPDH mRNAs. Total RNA from animal (A) and vegetal (V) hemispheres was extracted and analyzed by RT-PCR. Quantification of the relative RT-PCR signals is shown. (**b**) Subcellular localization of CPEB, Cyclin B1 and Xkid proteins. Oocytes were treated as indicated and collected at GVBD. (A) Animal half, (V) vegetal half. Quantification of the Western blot signals is shown.

Then, in order to test the effects of the delocalized translational activation of CPE-containing mRNAs on meiotic progression, we stained the DNA of the competed oocytes at the time at which the non-injected control oocytes had reached MII, shown by the presence of the polar body and the chromosomes aligned at the metaphase plate (Figure 3.18a). We observed that in (+CPE)RNA competed oocytes the chromatin was no longer condensed and the polar body was absent, whereas the (-CPE)RNA competed oocytes displayed normal metaphase plates and extruded polar bodies. In Figure 3.18b, a time course of Histone H1 Kinase activity after progesterone stimulation of competed oocytes showed that in both (+CPE)RNA and (-CPE)RNA competed oocytes, activation of Cdc2 at MI entry was normal but Cdc2 activity decreased below normal levels after anaphase-I in (+CPE)RNA competed oocytes. Because a threshold level of Cdc2 activity is required to prevent DNA replication between the two meiotic divisions (Hochegger et al., 2001; Iwabuchi et al., 2000; Perez et al., 2002), this abnormal low levels of Cdc2 activity most likely causes DNA replication and the detected chromatin decondensation. At later times, H1 Kinase activity increases again probably due to the activation of Cdc2 and CdK2 that takes place in the early stages of apoptosis (Shi et al., 1994; Yao et al., 1996; Zhou et al., 1998). These results indicated that the transition between MI and MII was impaired in oocytes microinjected with the CPE-containing competitor RNA, causing a premature

termination of the meiotic cycle and exit to an interphase-like state, which eventually resulted in oocyte death (data not shown).

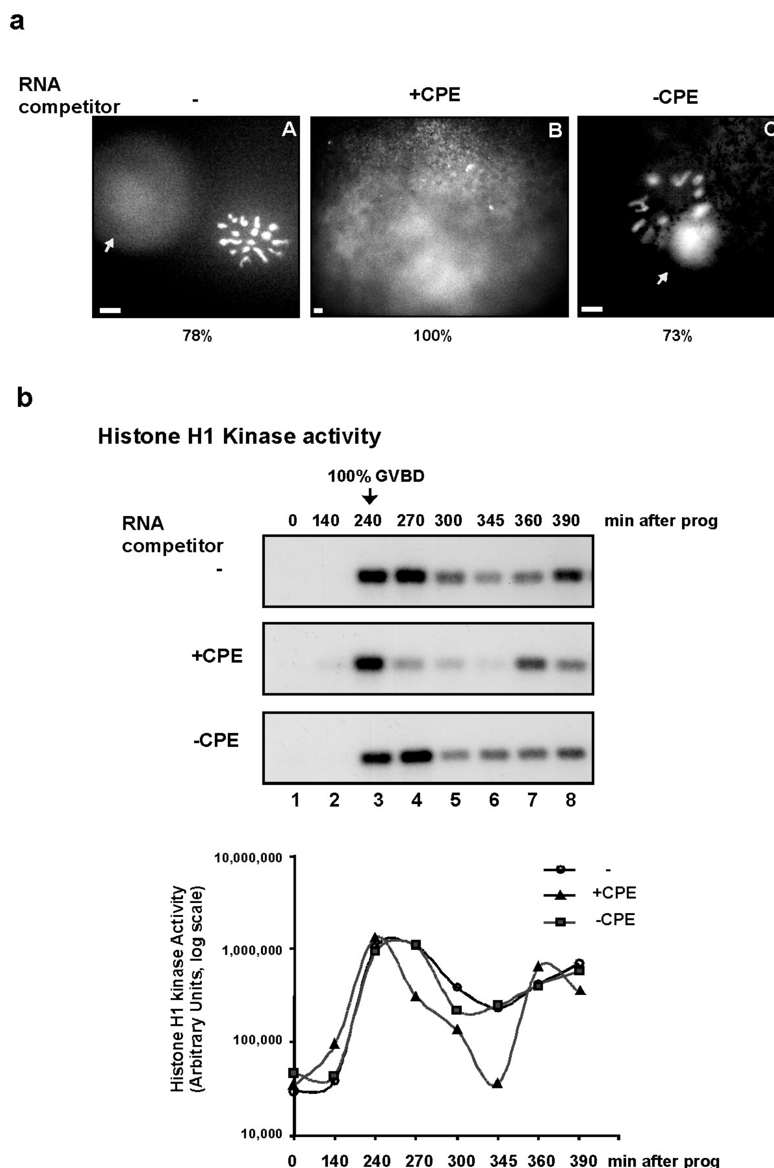


Figure 3.18 Localized-translational activation of CPE-containing mRNAs is required for MI to MII transition. (**a**) Control and RNA-competed oocytes were collected 5 hours after the non-injected oocytes reached 100% maturation and analyzed for meiotic structures by DNA staining as described for Figure 3.14c. The percentage of oocytes displaying each phenotype is shown (n>10). The arrow indicates the first polar body. Scale bar 10 μm. (**b**) Histone H1 Kinase activity in control and RNA-competed oocytes during meiotic maturation. Three oocytes from each RNA-competed and control populations were collected at the indicated time points and were analyzed for Histone H1 Kinase activity. Quantification of the Histone H1 Kinase activity is shown.

Finally, in order to test whether delocalized Xkid mRNA translation was the principal cause of the meiotic effects of the global delocalization of CPE-containing mRNAs we examined whether over-expression of Xkid mRNA was able to rescue this phenotype. We found that in (+CPE)RNA competed oocytes over-expressing Xkid from an injected mRNA, the chromatin was also no longer condensed and the polar body was absent

(Figure 3.19), suggesting that Xkid over-expression did not rescue the meiotic effects originated by delocalization of CPE-containing mRNAs.

Therefore, we conclude that not only localized-translation activation of Xkid mRNA, but also localized-translation of other CPE-containing mRNAs is crucially required to assemble the complex(es) that drive spindle assembly and chromosome segregation during meiotic cell cycle progression in *Xenopus* oocytes.

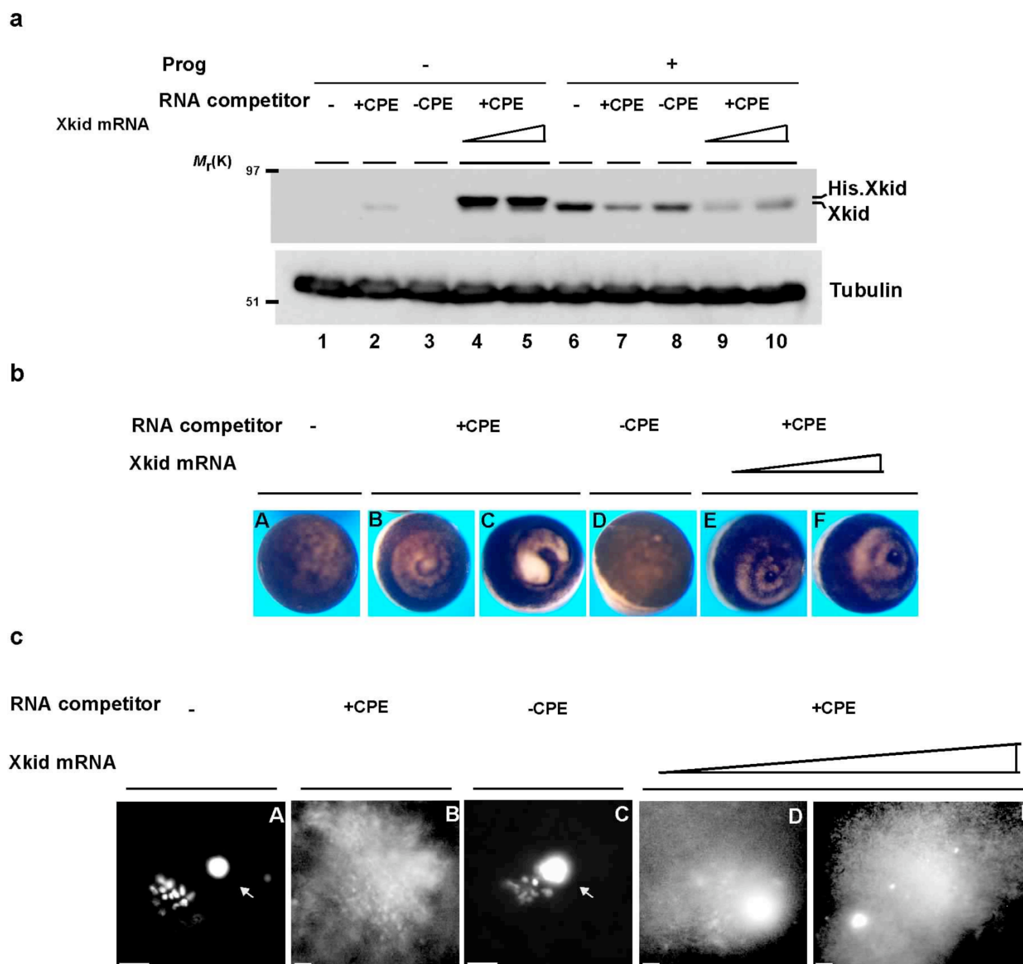


Figure 3.19 Xkid does not rescue global delocalization of CPE-containing mRNAs. Oocytes injected with 1,5 pmol of 70 nucleotides long competitor RNAs containing (+CPE) multiple CPEs were injected with 0,7 or 1,4 fmols of an mRNA encoding for Xkid. After 3 hours, the microinjected oocytes were incubated with or without progesterone. Non-injected oocytes (-) and oocytes injected with an RNA without CPEs (-CPE) were carried in parallel as a control. Samples were collected 4 hours after the control, non-injected oocytes, displayed 100% GVBD. (**a**) Oocytes were analyzed by Western blot with Xkid and Tubulin antibodies. Equivalents of 1,5 oocytes were loaded onto each lane. Note that Xkid is degraded during anaphase and when the oocytes exit meiosis into S-Phase (Perez et al., 2002). (**b**) Morphological appearance of (A) non-injected control oocytes, (B, C) (+CPE)RNA competed oocytes, (D) (-CPE)RNA competed oocytes and (E, F) (+CPE)RNA competed oocytes and over-expressing Xkid from an injected mRNA are shown. (**c**) Oocytes were analyzed for meiotic structures by DNA staining as described for Figure 3.14c. Representative images from (A) non-injected control oocytes, (B) (+CPE)RNA competed oocytes, (C) (-CPE)RNA competed oocytes and (D, E) (+CPE)RNA competed oocytes and over-expressing Xkid from an injected mRNA are shown. The arrow indicates the first polar body. Scale bar 10 μm.

3.4 APPENDICES

APPENDIX I

- Xkid 3´ UTR (598 nts)

ccuguaccaucaucaggcugcggcccgggucauacacgcuccaagggccacuga**uuuuau**uccucaccaacaacuug
aaaucccugagcuccuuauggcaaaacucugaaguguaaaucugaggcaaguuuggcuguuuuugcaccagcccccu
gcaaacacgaaccaaaugugccaaccggccgcuggauguuuuguuaaagugaguucaacgccgagacucugguuuaa
agguuuuuuuuuuugcauuuguaauguucaca**uuuuau**ucuacua**uuuuau**uaugagccugucugugugugugugugu
cuguguguguuugugugugugugugauaugugucucuugagugacugccccaguggggugaaugaguggcugcaaugugg
uuuggcagagaaguaacaguaguuuccaugcugugugaggcaccaacauugaaaccacuggcuccuguguaaauaua
uauauauauauauauauacuccuuaccccaugugcuucagcagcauuuacugcaucacauugcaggaagguucugcu
gguguuauuauauucuuccaaaucaaguccaugucuuucuguaa**aauaaa**cguuuuugg

- Xkid-CPE 3´ UTR (338 nts)

ca**uuuGGu**ucuacua**uuuGGu**uaugagccugucugugugugugugugucugugugugugu uugugugugugugauaugug
ucucuugagugacugccccaguggggugaaugaguggcugcaaugugguuuggcagagaaguaacaguaguuuccau
gcugugugaggcaccaacauugaaaccacuggcuccuguguaaauauauauauauauauauauauacuccuuacccc
augugcuucagcagcauuuacugcaucacauugcaggaagguucugcugguguuauuauauucuuccaaaucaaguc
caugucuuucuguaa**aauaaa**cguuuuugg

- TPX2 *"long"* 3´ UTR (1227 nts)

uucguuuuccugugucacagccaaagccag**uuuucu**gggugugguugccuguucaugcccuggaccauauagucuguu
gaacaaaacugugucc**uuuuaaau**aguggaguugacgcaggggcaagugucugcucauugggguuuuguaaauacuag
auauuaauggccuggaggggccuguuuuuaggucuuccaugugaauacguuaugc**uuuuau**uaugcccugu**aauaaa**c
uguguaaauguaaaguuugugccgaauucgggaaauauucggacguuuguaugaugauguugcacucugugacacugc
ga**uuuuau**uggcugucauguguaacuuccuuuucccuuucaugauuugcuugcaaguaauggcaauuacagauggucа
aauccugccuguaauuuaucagcguaaaaggagcagcaaacaccauuucaagaguugaauacagaccugcuuauauua
caaaauuaauaacuucuauguauaguugugaaacagaugcauaaaugacuguuccuggauccuauagcaacauuucug
uaagccacaccagcucacucacccaguucagccugucuggaauucauuuacaggagaauguggcuaagaugccaua**uu
uuau**auacugaacuuauugcaccagucuaaaguuucagcuucuucaaauuugucacgaggggucaccaucuuugaggg
agucugcgacacucacaugcucggugugcuuugagaagcuguugagaagcuaagauuggggucaucacaaauuuuca
agcagaaaaugagguuggccuguauuauaagcugaugcuacaaggcuaauucuuaaauucugaugcugauugcacugg
uuucugugcugcuauguaguaucuguauugguuacuaauuagccuuauauugugacauuaaga**uuuuau**guuuacugu
auauuuagucuaucccuggacccaguggguggcagcagcacagagcaugugcagugaguaagcggaggagaggauggg
gagcuacuggggcaucuuugagggcacggaucuuuacuguugaagguugugguugccuuggcugguacaguaaacuc
aaaacauaauguacaagauuucuggcccuucguugguuagacuuuaguucuccuuuaugccuugaugaagauaccgau
uuucaguagcugugcucuauaauugguuugaaaaccu**auuaaa**cuaaguccaccaac

- TPX2 *"short"* 3´ UTR (257 nts)

uucguuuuccugugucacagccaaagccag**uuuucu**gggugugguugccuguucaugcccuggaccauauagucuguu
gaacaaaacugugucc**uuuuaaau**aguggaguugacgcaggggcaagugucugcucauugggguuuuguaaauacuag
auauuaauggccuggaggggccuguuuuuaggucuuccaugugaauacguuaugc**uuuuau**uaugcccugu**aauaaa**c
uguguaaauguaaaguuugugcc

- TPX2-CPE 3´ UTR (257 nts)

uucguuuuccugugucacagccaaagccag**AAuucu**gggugugguugccuguucaugcccuggaccauauagucuguu
gaacaaaacugugucc**uuuGGaau**aguggaguugacgcaggggcaagugucugcucauugggguuuuguaaauacuag
auauuaauggccuggaggggccuguuuuuaggucuuccaugugaauacguuaugc**uuuGGu**uaugcccugu**aauaaa**c
uguguaaauguaaaguuugugcc

Figure 3.I. The 3'UTRs of Xkid and TPX2. Sequences of the 3'UTRs from Xkid and TPX2 mRNAs (accession numbers AJ49840 and AF244546, respectively). The two possible 3' UTRs from TPX2 are shown as "long" and "short". The corresponding UTR variants with the CPEs inactivated are shown. CPEs (underlined), polyadenylation signal (Hexanucleotide, boxes) and UA/GU repeats (filled boxes) are indicated.

APPENDIX II

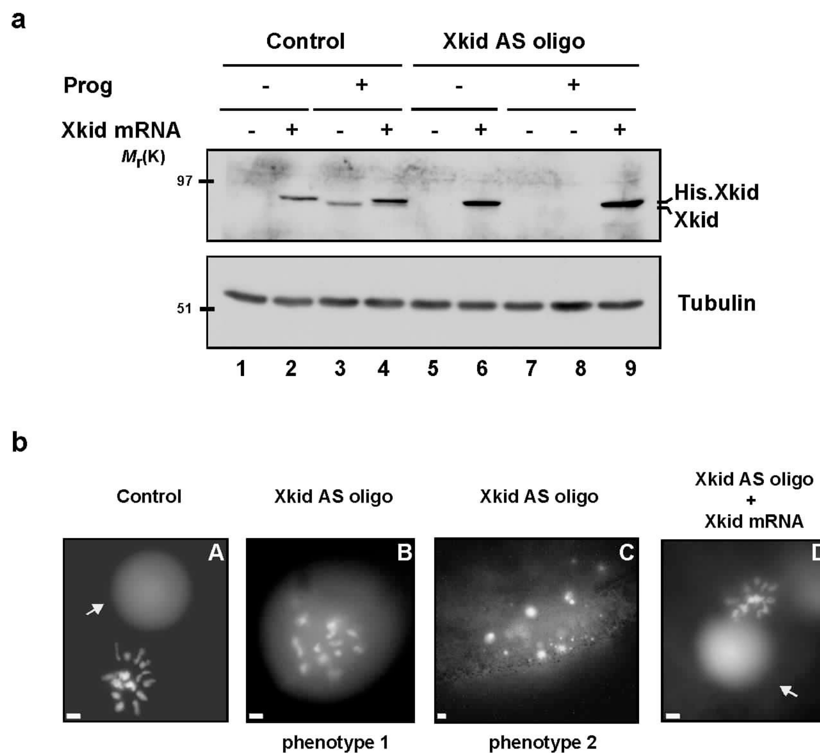


Figure 3.II. Xkid-depleted oocytes. Oocytes were injected with Xkid sense or antisense oligonucleotides. After 12 hrs both groups of oocytes were microinjected with 3 fmols of an mRNA encoding for Xkid and incubated in the presence and absence of progesterone. Oocytes were collected 5 hours after the control, non-injected oocytes, displayed 100% GVBD. (**a**) The oocytes were analyzed for Xkid levels by Western blot with Xkid and Tubulin antibodies. Equivalents of 2 oocytes were loaded onto each lane. (**b**) Oocytes were fixed, stained with Hoechst and examined under the epifluorescence microscope. Representative images from control oocytes, the two main phenotypes from Xkid depleted oocytes and oocytes depleted for the endogenous Xkid and over-expressing Xkid from an injected mRNA are shown. The arrow indicates the first polar body. Scale bar 10 μm.

Both phenotypes are consistent with previous reports for Xkid depletion (Perez et al., 2002), although the proportions are slightly different, maybe due to different levels of depletion or to the time at which the oocytes were analyzed. Indeed, at earlier times after GVBD (i.e. 5 hrs) phenotype 1 is detected more often whereas at later times (i.e. O/N) phenotype 2 is more abundant, suggesting that a defect in the chromosomal alignment in late MI results in meiotic exit followed by apoptosis. Interestingly, similar phenotypes are observed when total protein synthesis was inhibited after GVBD by microinjection of cycloheximide. Under these conditions a compact spindle formed without properly aligned chromosomes. This structure persisted for approximately 1 hour after GVBD and then chromosomes decondensed and microtubules disappeared (Hochegger et al., 2001).

4. DISCUSSION

Previous evidences suggested spindle-localized translation although no direct proof of this phenomena or its biological function existed so far.

4.1 Translation factors on spindles.

Previous to our work it was shown that CPEB and Maskin concentrate in large complexes at the animal pole cortex of *Xenopus* oocytes and eggs. Both proteins form a gradient along the spindle microtubules, with the greatest concentration nearest the centrosomes of embryonic animal pole blastomeres (Groisman et al., 2000). In addition, both proteins localize on *in vitro* assembled spindles from egg extracts (Barnard et al., 2005; O'Brien et al., 2005; Peset et al., 2005). The fact that recombinant CPEB (especially) and Maskin proteins can bind to *in vitro* polymerized microtubules led Groisman et al. to suggest that they probably interact directly with the mitotic apparatus. Accordingly, CPEB aminoacid residues 168-211, which contain a PEST box implicated in protein degradation (Rechsteiner and Rogers, 1996) as well as in protein-protein interaction (Niessing et al., 1999) has been found to mediate the interaction of this protein with *in vitro* polymerized microtubules and with centrosomes *in vivo* (Groisman et al., 2000). However, this direct interaction of CPEB with microtubules has been recently questioned (Huang et al., 2003). Other *trans*-acting factors associated with CPE-containing mRNAs (i.e., Aurora A, eIF4E, CPSF, poly(A) polymerase) also localize onto or in the vicinity of spindle microtubules or centrosomes (Goepfert and Brinkley, 2000; Groisman et al., 2000; O'Brien et al., 2005; Peset et al., 2005). Eventually, ribosomes are also associated with spindle microtubules (Liska et al., 2004; Blower et al., 2007) implying the possibility that CPEB could mediate translational control in the regulation of mitotic spindle dynamics in close proximity to the mitotic apparatus.

4.2 RNA on spindles.

Few reported cases directly show spindle-localized RNAs, which are limited to structural components of the spindle (Alliegro et al., 2006; Blower et al., 2005) and repressed centrosome-localized mRNAs for asymmetrical inheritance in embryonic divisions (Alliegro et al., 2006; Lambert and Nagy, 2002). Other evidences only show the localization of CPE-containing mRNAs, such as cyclin B1 and Xbub3 mRNAs, concentrated in the regions surrounding chromosomes expected to be enrich in MTs (Goto and Kinoshita, 1999; Groisman et al., 2000; Raff et al., 1990). The relative low resolution of these images did not allow determining unambiguously whether these mRNAs co-localize precisely with the spindle structure. Neither did they demonstrate

that this localization was mediated directly by CPE/CPEB nor that spindle-localized translation of CPE-bearing mRNAs was required. Here, we show that two particular mRNAs encoding for factors with a well-known function in spindle assembly and chromosome movements, such as Xkid and TPX2, associates with the spindle microtubules and chromosomes, and that this association occurs in a CPE-dependent manner (Figure 3.8, 3.9 and 3.10). Indeed, we demonstrate that their localized-translational activation is required for meiotic progression (Fig. 3.14). Accordingly, the previous observation of ribosomes associated with spindles assembled in frog egg extracts (Liska et al., 2004) has been recently strengthened by the finding of active mRNA translation sites all along the spindle microtubule but concentrate more near the spindle poles using fluorescent puromycin derivatives (Blower et al., 2007). This study also identified a subpopulation of MT-bound mRNAs in egg extract as regulators of mitosis, DNA metabolism and germ cell patterning, and a subset of these mRNAs has been found associated with polysomes bound to microtubules. Some of these mRNAs are found translated locally whereas others are translationally inactive (Blower et al., 2007), indicating that probably the latter group of mRNAs associates with the spindle as passive cargo, raising the possibility that targeting inactive mRNAs to the spindle may serve as a mechanism for their segregation during cell division. Examination of the 3'UTR of these MT-bound mRNAs revealed that the CPE is present in 5.7% of MT-bound mRNAs, which is an 4.1-fold enrichment compared with all the mRNAs (Blower et al., 2007). However the definition of CPEs in this work was too stringent and this percentage may be higher. These results are consistent with our findings that specific-mRNAs are targeted to spindle microtubules during cell cycle. However, it is noteworthy that due to the use of taxol to stabilize polymerized microtubules in egg extract, Blower et al. have found several regulated mRNAs targeted to the vegetal cortex as well (e.g., Vg1 mRNA) indicating that not only spindle-specific mRNAs but rather MT-bound mRNAs are identified. Finally, due to most MT-bound mRNAs do not contain CPEs, it is possible that additional *cis*-elements and/or another mechanism can also mediate mRNA targeting to the microtubule array.

4.3 Cytoplasmic polyadenylation and spindles.

The only suggestion that mRNA-specific localization on spindles was CPE/CPEB-mediated and/or that spindle-localized translation was required for chromosome dynamics and cell cycle progression was based on the use of the mutant variant CPEB lacking the PEST box domain (CPEB Δ4), which is capable of binding CPE-containing mRNAs but is defective for microtubule binding in an *in vitro* pull-down experiment with purified components (i.e., Tubulin and CPEB) (Groisman et al., 2000). This mutant

variant CPEB Δ4 blocked embryonic divisions after few cycles (allowing accumulation of Cyclin B1 protein for these few cycles) resulting in malformation of the mitotic apparatus including tripolar spindle, spindle detached from centrosomes and multiple centrosomes (Groisman et al., 2000). In addition, microinjection of reagents that disrupt global or mRNA-specific cytoplasmic polyadenylation (i.e., CPEB antibody, CPEB dominant negative (CPEB ΔN), cordycepin, eIF4E-blocking peptide or Maskin antibody) cause inhibition of cell division and, directly or indirectly, destroy the integrity of the mitotic apparatus in embryos (Groisman et al., 2001; Groisman et al., 2000; Mendez et al., 2002; Mendez et al., 2000a). It is noteworthy that Groisman et al. considered this CPEB mutant as a dominant negative variant for localization but not for cytoplasmic polyadenylation. However later findings have questioned this: (1) the association of CPEB with microtubules *in vivo* is not by direct binding but rather most likely mediated by the motors Kinesin and Dynein in dendrites (Huang et al., 2003), and/or by XMAP215 in *in vitro* assembled spindles (O'Brien et al., 2005; Peset et al., 2005); (2) CPEB Δ4 does not show any defect in *in vivo* localization nor in binding to Kinesin and Dynein (Huang et al., 2003); (3) non-degradable CPEB variants (such as CPEB Δ4) inhibit the "late" polyadenylation and translational activation, due to a high ratio CPEB/CPE, of mRNAs with multiple CPEs (such as cyclin B1) and when is over-expressed at sufficient levels mediate complete block of embryonic mitosis by inhibiting the synthesis of Cyclin B1 (Mendez et al., 2002). Thus, CPEB Δ4 can not be considered a dominant negative mutant for localization but rather for polyadenylation and therefore, previous to our thesis work, no direct evidence existed supporting the requirement for CPE-mediated mRNA localized translation in spindle assembly and chromosome segregation. Finally, the CPEB-associated factors, Maskin and Aurora A, have crucial CPEB-independent functions in centrosome maturation and spindle formation (Albee et al., 2006; Dutertre et al., 2002; Goepfert and Brinkley, 2000; Kinoshita et al., 2005; O'Brien et al., 2005; Peset et al., 2005) that could be affected by the injection of α-Maskin or α-CPEB antibodies or by the over-expression of CPEB mutants in embryos.

4.4 Spindle-localized CPE-mediated translation.

The results shown in this thesis clearly demonstrate for the first time that CPE-regulated mRNAs, encoding for proteins with a well-known structural function in spindle assembly and chromosome segregation, such as Xkid and TPX2, are enriched in the animal hemisphere of *Xenopus* oocytes and localize onto meiotic spindles as well as chromosomes (Figure 3.7, 3.8, 3.9 and 3.10). In addition, we demonstrate that this

localization is driven by the CPEs present in their 3'UTRs (Figure 3.10). Our results also show that a particular mRNA, such as the one encoding for Xkid protein, requires to be locally translated on spindles via cytoplasmic polyadenylation to guarantee accurate chromosome segregation and completion of meiotic cell cycle (Fig. 3.14). Only localized-translation activation of Xkid mRNA (Xk-UTR2cpe) results in higher stability of it protein product (Figure 3.12, 3.13 and 3.14) suggesting an attractive hypothesis in which Xkid partners would be also locally translated from a pool of CPEB-regulated mRNAs at the same time. This co-regulation of local protein synthesis could favour the assembly of functional complexes that prevent degradation of the mislocalized proteins, as shown here for Xkid. Future identification of the unknown Xkid partners and experiments to address this possibility will allow the validation of this hypothesis. Additionally, competition with high amount of small CPE-containing RNA causes the delocalization of CPE-regulated mRNAs, like those of cyclin B1 and Xkid, inducing their translational activation all over the entire oocyte and the abnormal distribution of the encoded protein in both hemispheres of the oocyte (Figure 3.17). In these (+CPE)RNA competed oocytes meiotic progression from MI to MII is inhibited showing the chromatin decondensed, no polar body extrusion and extremely low H1 kinase activity levels in anaphase-I causing a premature termination of the meiotic cycle and exit to an interphase-like state, which eventually resulted in oocyte death (Figure 3.18).

At early times, the phenotype for global CPE-containing mRNAs delocalized translation was more penetrant than the phenotype of Xkid ablation, with higher number of oocytes that decondensated the chromosomes and displayed signs of apoptosis after six hours of stimulation with progesterone (Figure 3.14c and 3.18a). At longer times, both phenotypes were equivalent (see also Figure 3.II in appendix II). These different penetrances suggest that the low levels of Xkid accumulation, due to the delocalized translation of its mRNA, are not the only component of the meiotic effects originated by the delocalization of CPE-containing mRNAs. Accordingly, the observation that Xkid over-expression does not rescue the meiotic effects of the global delocalization of CPE-containing mRNAs (Figure 3.19) may reflect that CPE-mediated translational control could co-regulate efficiently the local protein synthesis of other specific spindle-related factors that, together with Xkid, are required in the assembly of complexes specialized in drive spindle formation, chromosome movements and for MI-MII transition in the *Xenopus* oocyte. We speculate that mRNAs encoding for products critical for these functions including members of the spindle assembly checkpoint and chromosomal passenger complex, such as TPX2, Bub1, Bub3, Mad1, Mad2, BubR1, Cenp-E, Nek2B, Aurora A and Aurora B, that contain conserved CPE-arrangements in

their 3'UTRs could be co-regulated in time and space to ensure that theirs protein products are delivered to the sight at which they are needed. Thus, *Xenopus* meiotic spindle may be considered foci of mRNA localization that could serve to co-regulate the synthesis of particular proteins that are need to assemble or act together, thereby, facilitating efficient and rapid assembly and localization of the encoded proteins. In this direction, future functional screening for localized cytoplasmically polyadenylated mRNAs from purified mitotic spindles (Sauer et al., 2005; Sillje and Nigg, 2006) will allow us to systematically identify these target mRNAs and possible additional *cis*-acting elements and its *trans*-acting factors that, together with CPE/CPEB, may mediate mRNA localization to spindle microtubules. All this together will help us understand how translation of specific spindle-associated mRNAs is regulated in time and space, within the context of the spindle formation, chromosome segregation and cell cycle progression.

4.5 Open questions.

The large size (up to 1-2 mm in diameter) and the rapid cell cycles exhibited by amphibian oocytes, eggs and embryos require rates of MTs assembly and disassembly that are substantially faster (Gard et al., 1995) than those observed in cultured mammalian cells (Cassimeris et al., 1988) or even *in vitro* (Belmont et al., 1990; Walker et al., 1988). During oocyte maturation, the oocyte MT cytoskeleton is completely remodelled with a span of 30 min. (Gard, 1991, 1992). Moreover, the 20 or 30 min. cell cycles during early embryo cleavage require rapid cycling between the extensive cytoplasmic TMA of interphase and the characteristic bipolar organization of the mitotic spindles. These spectacular episodes of assembly and disassembly during oocyte maturation and early development may depend on the presence and regulation of factors and MAPs that are activated during M-phase to facilitate bipolar spindle morphogenesis with the aligned chromosomes through MT dynamics. Thus, in large cells such as *Xenopus* oocytes, the finely regulation of factors involved in the assembly of a functional spindle might be essential to accurately ensure chromosome segregation and meiotic cell cycle progression. Thus, CPE-mediated localized-translation of these spindle-related factors could directly influence spindle morphogenesis and its important role during cell cycle progression by providing locally elevated concentration of proteins that are needed in the assembly of protein complexes specialized for this particular function.

Although the pathway of spindle assembly during maturation of large cells such as those of *Xenopus* oocytes (Gard, 1992) differs significantly from that observed in smaller somatic cells undergoing mitotic divisions (Rieder and Khodjakov, 1997; Rieder

et al., 1997), the recently finding of a subpopulation of taxol-stabilized MT-associated mRNAs from mitotic human cell extracts (Blower et al., 2007) strongly suggests that localization of mRNAs encoding for mitotic cell cycle regulators should be a conserved and widely mechanism of regulation for locally enhance the concentration of proteins need to coordinate mitotic events and also delivering of translationally silenced mRNAs to the daughter cells. In addition, CPE-containing mRNAs have been also found in the fraction of MT-bound mRNAs (Blower et al., 2007) indicating that CPE-mediated localized translational regulation may occur in cultured somatic cells as well. However, further experiments to address this possibility will be required to complete the understanding of this regulation in the mitotic divisions.

The acentriolar spindle is a polarized structure with gradients forming from the chromosomes. The observation that microtubules can assembly around chromatin or chromosomes (Karsenti et al., 1984; Steffen et al., 1986) seems to indicate that chromosomes could locally modify the surrounding cytoplasm to favour microtubule growth. The notion of dynamic gradients within the cell governed by the state of the small Ran guanosine triphosphate (GTPase) (Hetzer et al., 2000) had helped understand the formation of a mitotic bipolar spindle (Carazo-Salas et al., 2001; Carazo-Salas et al., 1999). The induction of MT nucleation and stabilization by chromosomes requires the production of Ran-GTP. This nucleation is probably induced by local high levels of Ran-GTP around chromosomes that induce the dissociation of a complex composed by importins (α, β), TPX2 (Gruss et al., 2001), and other regulators (Dasso, 2001; Nachury et al., 2001). When released from this complex, TPX2 nucleates microtubules. These observations suggest that there is a spatial regulation of the local state of the cytoplasm defined by localized regulatory proteins that promote microtubule nucleation and stabilization around chromosomes ending in the formation of the bipolar spindle. In addition, mRNA localization and localized-translation are mechanisms often used to generate polarity, such as in the establishment of axis patterning in the *Drosophila* embryo (Bashirullah et al., 1998; Johnstone and Lasko, 2001; Palacios and St Johnston, 2001) or in neurons, with dendrites and axons (Black and Baas, 1989). Therefore, it seems conceivable that localized-translation could also contribute to generate polarity in the spindle dynamics. According to this hypothesis we found that CPE-regulated mRNAs and CPEB localize mainly to the chromosomes and to lesser extent with the spindle and spindle poles. On the other hand, active ribosomes localize mainly to spindle poles and to a lesser extent with the chromosomes and microtubules. These patterns may reflect a gradient where the repressed mRNAs are localized to the chromosomal plate and move towards the spindle poles once activated, thus generating a gradient. It even seems plausible that

the same mechanism that generates sequential waves of polyadenylation and deadenylation are used to generate mRNA-specific gradients. In support of this speculation, the APC/C, which degrades CPEB generating the "late" wave of polyadenylation during meiosis, is recruited to centromeres and mitotic kinetochores by members of the spindle assembly complex (Acquaviva et al., 2004; Vigneron et al., 2004) and activated from the chromosomal plate towards the spindle poles. In addition, the mRNA encoded C3H-4, which is responsible of the "late-late" wave of polyadenylation, has been found associated with microtubules (Blower et al., 2007).

5. CONCLUSIONS

1. We have identified a number of mRNAs encoding proteins with a well-known function in spindle formation and chromosome segregation and potentially regulated by CPEB. From these, we have experimentally validated that the mRNAs coding for Xkid and TPX2 are indeed regulated by CPEB-mediated cytoplasmic polyadenylation.

2. We have shown that CPEB mediates spindle-localization and localized translation of a subset of mRNAs with specific arrangements of CPEs.

3. We have shown that this CPEB-mediated spindle-localized translation is essential for chromosome segregation and meiotic progression.

6. MATERIALS & METHODS

6.1 *Xenopus* oocyte preparation.

Xenopus laevis females were injected in the dorsal lymph sac with pregnant mare serum gonadotropin (PMSG) (100 IU/frog) 5 to 15 days before oocyte retrieval. Frog ovarian lobes were isolated and treated with collagenase (8 mg ml^{-1}, Sigma)/dispase (4.8 mg ml^{-1}; Roche) for 2 hours at room temperature until the oocytes were free from follicular tissue (de Moor and Richter, 1999; Pique et al., 2006). Then stage VI oocytes were selected and cultured in modified Barth's saline solution (88 mM NaCl, 1 mM KCl, 1 mM $MgSO_4$, 5 mM HEPES, 2.5 mM $NaHCO_3$, 0,7 mM $CaCl_2$, pH 7,8 with NaOH,) for all procedures (Pique et al., 2006). To induce maturation, oocytes were incubated in presence of Progesterone (10 μM, Sigma). Germinal Vesicle Breakdown (GVBD) was used as an indicator of maturation and scored by the appearance of a white spot at the animal pole of the oocytes. After incubation, oocytes were collected at the indicated times, stored frozen and/or further processed as indicated.

6.2 Plasmid constructions.

The FTX5-Xkid full length was obtained from Angel Nebreda (Antonio et al., 2000); and the cDNA of TPX2 in pBSK was a gift of Isabelle Vernos (Wittmann et al., 2000). The complete cDNA sequences are available from GenBank under accession numbers AJ249840 and AF244546 respectively. The 3'UTRs of Xkid and TPX2 were amplified by PCR and were subcloned downstream of the Firefly Luciferase ORF into pLucassette (Pique et al., 2006). The oligonucleotides used were: Xkids, 5'GCGGATCCCCTGTACCATCATCAGGCTGCGGC3' and Xkidas 5' CCAAAAACGTTTATTTTACAGAAAGACATGG3' for Xkid 3'UTR; TPX2s, 5'GCAGATCTTTCGTTTTCCTGTGTCACAGCC3', TPX2cortoas, 5'GGGTTAACGGCACAAACTTTACATTTACACAG3' and TPX2largoas, 5'GGGTTAACGTTGGTGGACTTAGTTTAATAGG3' for TPX2 3'UTR short and long version respectively. pSL-MS2-6 and pPolII-MS2-eGFP were obtained from Edouard Bertrand and Robert H. Singer (Bertrand et al., 1998). The DNA corresponding to a 12 repeats of the MS2 binding site was digested with BamHI and subcloned downstream the Firefly Luciferase ORF in BglII site of the pLucassette followed either by Xkid or TPX2 3'UTR. The DNA corresponding to the MS2-eGFP was amplified by PCR and was subcloned into HindIII/XhoI sites in pET30a expression vector. The oligonucleotides used were: MS2orf–AUGs, 5'CCCAAGCTTTCGCTTCTAACTTTACTCAGTTCG3' and 3'GFPas, 5'CCGCTCGAGTTACTTGTACAGCTCGTCC3'.

6.3 *in vitro* transcription reactions.

Linearized DNA templates were transcribed with the mMessage mMachine Kit (Ambion) according manufacturer's directions. The mRNA obtained was purified and quantified by ethidium bromide stained 2% agarose gels.

6.4 Polyadenylation of endogenous mRNAs.

Total RNA was isolated from 5-8 oocytes by Ultraspec RNA Isolation System (Biotecx Laboratories, Inc.) following the manufacturer's instructions. Then, RNA-ligation coupled RT-PCR technique was performed as described (Charlesworth et al., 2002), with some modifications. Briefly, 4 µg of total oocyte RNA was ligated to 0.4 µg anchor 3' amino modified oligo (P1, 5'P-GGTCACCTTGATCTGAAGC-NH_2-3'; Sigma), using T4 RNA ligase (New England Biolabs). The RNA ligation reaction was used in a 50µl reverse transcription reaction using RevertAid M-MuLV Reverse Transcriptase (Fermentas), and 0.4 µg antisense oligo (P1', 5'GCTTCAGATCAAGGTGACCTTTTT3'; Sigma). The resulting reaction product was digested with 2 µg RNAse A (Fermentas) and then used as a template for gene-specific PCR reaction. The specific oligos used were: XkP2s, 5'CACATTGCAGGAAGGTTCTGC3' and TPXP2s, 5'CTAGATATTAATGGCCTGGAGGG3' designed to be at 65 and 80 nucleotides from the hexanucleotide of Xkid and TPX2 3'UTR, respectively. The resulting PCR reaction was analyzed on a 2% agarose gel and visualized by ethidium bromide staining.

6.5 Translational control and cytoplasmic polyadenylation by 3'UTR.

Translation and polyadenylation of reporter mRNAs were assayed as described (Pique et al., 2006), with some modifications. Briefly, oocytes were injected with 0,0125 fmol of each hybrid reporter mRNA together with 0,0125 fmol of renilla Luciferase normalizing RNA. Luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega). For polyadenylation assays, total RNA extracted from oocytes injected with radiolabeled 3'UTR RNAs was analyzed by 6% polyacrilamide/8M urea gel electrophoresis followed by autoradiography.

6.6 Histone H1 kinase assay.

Oocyte lysates prepared by homogenizing 3 oocytes in H1 kinase buffer (80 mM sodium β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 0.5 mM Na_2VaO_4) containing protease inhibitors (10 µg ml^{-1} each of leupeptin, pepstatin and chymostatin) and centrifuged at 12,000 g for 5 min at 4ºC were incubated with 4 µg Histone H1 (Sigma) and 2 µCi of [γ-^{32}P] ATP (3000 Ci $mmol^{-1}$) as described (Palmer et al., 1998;

Mendez et al., 2002). After 15 minutes of incubation at room temperature, the phosphorylation reaction was analyzed by 12% SDS-PAGE gel and autoradiography.

6.7 Western blot.

Oocyte lysates prepared by homogenizing 5-10 oocytes in H1 kinase buffer containing 0,5% NP-40 and centrifuged at 12,000g for 5-10 min were resolved by 10% SDS-PAGE. Equivalents of 1-2 oocytes were loaded onto each lane. Antibodies used: rabbit antiserum against Xkid (Antonio et al., 2000); rabbit antiserum against TPX2 (Wittmann et al., 2000); rabbit antiserum against CPEB (gift from J.D. Richter); goat antiserum against Cyclin B1; and monoclonal antibody against α-Tubulin (DM1A Sigma).

6.8 Oocyte enucleation.

Under a dissecting stereoscope, nuclei of stage VI oocytes were isolated using two pairs of forceps: one pair of bent and sharp tips that snip a small opening on the animal pole, and another pair of bent and blunt tips to hold and squeeze the nucleus out of the oocyte (Liu and Liu, 2006). The nuclei isolated were transferred to an eppendorf tube and immediately centrifuged for 30 seconds at 4 ºC. The supernatant was aspirated without disturbing the nuclei pellet and 2X sodium dodecyl sulfate sample buffer was added. The enucleated oocytes were further processed as described in Western blot procedures.

6.9 Egg-extracts, spindle assembly and RNA-mediated tethering assay.

Cytostatic factor arrested egg-extracts (CSF-extracts) were prepared as previously described (Murray, 1991). For cycled spindle assembly, 0.2 mg ml^{-1} rhodamine-labeled Tubulin and demembranated sperm nuclei (~500 nuclei μl^{-1}) were added to CSF-extracts on ice. The extracts were then released into interphase by the addition of 0.4 mM Ca^{2+} and incubated for 90 min at 20 ºC. These extracts were then cycled back into mitosis by addition of one volume of fresh CSF-arrested extracts. After 45 min, spindles were fixed in 1 ml BRB80 (80 mM K-Pipes, pH 6.8, 1 mM EGTA, 1 mM $MgCl_2$) containing 30% glycerol, 0.25% glutaraldehyde, and 0.1% Triton X-100 and centrifuged onto coverslips (Multifuge 3 L-R, Heraeus) through a 40% glycerol cushion in BRB80, as previously described (Wittmann et al., 2000).

To visualize mRNAs on spindles assembled in egg extracts, 20 ng μl^{-1} *in vitro* hybrid transcribed mRNAs and 10 μg ml^{-1} purified recombinant MS2-eGFP protein expressed in *E.coli* BL21 were added to the extracts before cycling them. Spindles assembled in

egg extracts were also processed for immunofluorescence as described (Wittmann et al., 2000) with the rabbit antiserum against CPEB in order to detect endogenous CPEB. Pictures were taken using the same camera settings (Leica DMI6000B microscope, 63X magnification, Leica DFC350FX camera, Leica Application Suite (LAS AF) advanced fluorescence 1.6.1 build 1057 software). For quantification ImageJ software was used.

6.10 Fluorescent mRNA *in situ* hybridization on the spindle and immunostaining.

Mature oocytes (GVBD) fixed overnight in HEPES solution containing 3.5% formaldehyde, 0.25% glutaraldehyde and 0.1% Triton X-100 were postfixed in 100% methanol for 16 hours at -20 ºC. After hemisection of the oocytes either in the plane of the equator or along the animal-vegetal axis, bleaching with 10% H_2O_2 in methanol and borohydride reduction were performed in order to eliminate oocyte pigmentation and reduce the autofluorescence generated during glutaraldehyde fixation (Becker and Gard, 2006). For the *in situ* hybridization, the oocytes were prehybridized for 6 hours at 60 ºC in Hyb Solution (50% formamide, SSC 1X, 0.1% tween-20, 9.2 mM citric acid, 50 $\mu g\ ml^{-1}$ heparin and 500 $\mu g\ ml^{-1}$ tRNA) and hybridized with 5 $\mu g\ ml^{-1}$ of digoxigenin-labeled riboprobe specific for Xkid ORF overnight at 60 ºC. For colocalization with the spindle, oocytes were incubated with monoclonal antibody against α-Tubulin (DM1A, Sigma) for 1 day at 4 ºC according to (Becker and Gard, 2006). The oocytes were sequentially incubated with anti-digoxigenin-rhodamine, Fab fragments from sheep, (50 $\mu g\ ml^{-1}$; Roche) and Alexa488-labeled antibody (Invitrogen) for 1 day at 4 ºC in PBS-T containing 2 mg ml^{-1} of BSA and 5% sheep serum. Images were acquired using Leica TCS SP5 confocal laser scanning microscope, 63X magnification, Leica Application Suite (LAS AF) advanced fluorescence.

Fluorescent mRNA *in situ* hybridization was also performed in spindles assembled in cycled egg extracts as described above, with some modifications according to (Wittmann et al., 2000). Images were acquired using Leica DMI6000B microscope, 63X magnification, Leica DFC350FX camera, Leica Application Suite (LAS AF) advanced fluorescence.

6.11 Chromosomes and polar body observation

Oocytes fixed for 1 hour in HEPES Buffer (100 mM KCl, 3 mM $MgCl_2$, and 10 mM HEPES, pH 7.8) containing 3.7% formaldehyde, 0.1% glutaraldehyde and 0.1% Triton X-100 (Castro et al., 2003) or 100% methanol were incubated overnight in presence of

20 μg l^{-1} 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 and/or (Leica DM6000B microscope, Leica DFC300FX camera, Leica Application Suite (LAS AF) Version 2.7.1.R).

6.12 Northern blot analysis.

Total RNA was isolated using Ultraspec RNA Isolation System (Biotecx Laboratories, Inc.) and analyzed by Northern blot as described (de Moor and Richter, 1999). Specific probes for *X. laevis* Xkid, TPX2, cyclin B1, c-mos and GAPDH were labeled by random priming (Megaprime DNA labeling Systems, Amersham Biosciences). The sequence of specific oligonucleotides used in RT-PCR analysis were: cycB1.iS, 5'GTCAAGGACATTTATGCTTACC3' and cycB1.iAS, 5'CCATGTCCCGAATTTGAGCC3' for cyclin B1; XkΔ1s 5'GCAGATCTCATTTTATTCTACTATTTTATTATGAGCC3' and Xkidas, 5' CCAAAAACGTTTATTTTACAGAAAGACATGG3' for Xkid; 5'CATCACGCAGCGGCCTGG3' and 5'GTGGTCGCTTTAGCTTCATCC3' that amplify both Xk-UTR1cpe and Xk-UTR2cpe; and xGAPDHs, 5'GTCGCCCATCCTGCTAGTC3' and T7AS, 5'GTAATACGACTCACTATAGGGC3' for GAPDH mRNAs.

6.13 Polysome pelleting.

Extracts from 10 oocytes were fractionated by centrifugation over sucrose cushions as described (Wormington, 1991). Pool of 10 oocytes were collected at the indicated times and homogenized in Polysomal Buffer (PB) (20 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 300 mM KCl, 4 μg ml^{-1} polyvinyl sulfate, 0.5% (v/v) NP-40) supplemented with 2 mM DTT (Sigma), 25 U ml^{-1} RNAse inhibitor (Fermentas) and 10 μg ml^{-1} cycloheximide (Sigma). For control EDTA-release experiments, cycloheximide was omitted and 50 mM EDTA was added to PB. The homogenate was centrifuged at 12,000 g for 15 minutes at 4 ºC and the supernatant was subsequently diluted in PB and layered over a 20% (w/v) sucrose cushion. The polysomes were pelleted by centrifugation at 149,000 g for 2 hours at 4 ºC in Beckman SW55Ti rotor. The polysomal pellet was treated with SDS-proteinase K (200 μg ml^{-1}, Stratagene) and the RNA extracted from polysomal and non-polysomal fractions was analyzed by RT-PCR with the following sense and antisense oligonucleotides: 5'CATCACGCAGCGGCCTGG3' and 5' GTGGTCGCTTTAGCTTCATCC3' that amplify both Xk-UTR1cpe and Xk-UTR2cpe.

6.14 Antisense experiments.

Oocytes injected with 120 ng of each Xkid antisense oligonucleotide were incubated O/N at 18 ºC to allow cleavage of Xkid mRNA and incubated in presence or absence of progesterone. For the rescue experiment, oocytes were also injected with 0.3 fmol of either Xk-UTR1cpe or Xk-UTR2cpe mRNA 2 hours before progesterone stimulation. Sense oligonucleotide was used as a control. The sequences of antisense oligonucleotides used were: 3XkAS, 5'GTTGGTGAGGAATAAAATC3' (based on the Xkid 3'UTR sequence); 5XkAS, 5'CATTCCCGCCTCGCTTCG3' (based on the Xkid 5'UTR sequence). The sequence of sense oligonucleotide used was, Xks, 5'GATTTTATTCCTCACCAAC3'. The sequence of antisense morpholino used was 5' GCCCAGTAAGAACCATTCCCGCCTC3' as described (Perez et al., 2002). Samples were collected 5 hours after the control, non-injected oocytes, displayed 100% GVBD, stored frozen and/or further processed as indicated.

6.15 CPE-competition Assays.

Oocytes were injected with 1.5 pmol of 70 nucleotides long competitor RNAs containing (+CPE) multiple CPEs as previously described (de Moor and Richter, 1999) and incubated for 3 hours to allow mRNA delocalization by CPEB-competition before progesterone treatment. Non-injected oocytes (-) and oocytes injected with an RNA without CPEs (-CPE) were carried in parallel as a control. For the rescue experiment, oocytes were also injected with 0,7 or 1,4 fmols of an mRNA encoding for Xkid. Samples were collected 5 hours after the control, non-injected oocytes, displayed 100% GVBD, stored frozen and/or further processed as indicated.

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8. ANNEX

The work presented in this thesis has resulted in the following article accepted for publication in Nature Cell Biology (*in press*).

Spindle-localized CPE-mediated translation controls meiotic chromosome segregation

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Meiotic progression requires the translational activation of stored maternal mRNAs, such as the ones encoding for cyclin B1 or mos. The translation of these mRNAs is regulated by the cytoplasmic polyadenylation element (CPE) present in their 3'UTRs, which recruits the CPE-binding protein CPEB[1]. This RNA-binding protein not only dictates the timing and extent of translational activation by cytoplasmic polyadenylation[2, 3] but also participates, together with the translational repressor Maskin, in the transport and localization, in a quiescent state, of its targets to the subcellular locations where their translation is going to take place[4]. During *Xenopus* early development, CPEB localizes at the animal pole of oocytes and later on embryonic spindles and centrosomes[5]. Disruption of embryonic CPEB-mediated translational regulation results in abnormalities in the mitotic apparatus and inhibits embryonic mitosis[5]. Here we show that spindle-localized translational activation of CPE-regulated mRNAs, encoding for proteins with a known function in spindle assembly and chromosome segregation, is essential to complete the first meiotic division and for chromosome segregation in *Xenopus* oocytes.

CPEB and Maskin localize at the animal pole of *Xenopus* oocytes and eggs and in embryonic centrosomes and mitotic spindles[5-8], most likely through the direct interaction with Kinesin and Dynein[4] and/or XMAP215[7, 8]. In addition, centrosomes and spindles contain RNAs, that serve structural functions[9] or that are carried along for asymmetric distribution during cell division[10, 11]. Ribosomes are also associated with spindles assembled in frog egg extracts[12, 13]. Moreover, inhibition of cytoplasmic polyadenylation blocks cell division and, directly or indirectly, promote spindle and centrosome defects in embryos[5], suggesting a function for localized CPEB-mediated translational regulation in chromosome segregation.

To test directly whether localized CPEB-dependent translation regulates meiotic progression and chromosome segregation, we aimed to identify potential CPE-regulated mRNAs encoding for proteins with a known function in chromosome segregation and spindle formation, which could be locally translated during oocyte maturation. Because CPEB-mediated localization and repression are two linked events[4] and given that the candidate mRNAs should be activated prior to spindle assembly, we performed a genome-wide computational identification of mRNAs with a CPE-arrangement conferring repression in prophase-I and polyadenylation and translationally activation during the prophase-I to metaphase-I transition[14]. With these criteria, we identified the mRNAs encoding for TPX2, Bub1, Bub3, Eg5, Mad1, Mad2, BubR1, Xkid, Cenp-E, Nek2B, Aurora A and Aurora B as putative targets for CPEB-mediated localized translational regulation, conserved between mouse, human and *Xenopus*. From this list we selected two mRNAs encoding for proteins with a well-characterized structural function during cell division: Xkid (*Xenopus* kinesin-like DNA binding protein) and TPX2 (Targeting Protein for *Xenopus* kinesin-like protein 2) (Fig. 1a and Supplementary Information, Fig. S1). Xkid localizes to mitotic chromosomes and spindles during cell division, and is required for metaphase chromosome alignment and for MI-MII transition[15, 16]. TPX2 localizes to the mitotic spindle poles in a dynein-dependent manner and plays a key role in microtubule nucleation and spindle pole organization[17].

To validate the predictions, we first analyzed the expression of Xkid and TPX2 during progesterone-induced meiotic resumption. Xkid was not detectable in stage VI oocytes and was synthesized in response to progesterone, accumulating in MI to be then degraded in anaphase

and re-accumulating in MII. TPX2 was present at low levels in stage VI oocytes but further accumulated in response to progesterone-induced meiotic resumption (Fig. 1b). These patterns of accumulation are consistent with these proteins being encoded by mRNAs translationally regulated by CPEB. Accordingly, the endogenous mRNAs encoding for both proteins displayed a short poly(A) tail in arrested oocytes and were cytoplasmically polyadenylated in response to progesterone (Fig. 1c). To further define if this regulation was driven by the putative CPEs present in their 3'UTRs, we *in vitro* transcribed chimeric mRNAs containing the ORF of Firefly Luciferase followed by the 3'UTRs of either Xkid or TPX2 mRNAs, in their *Wild type* (WT) form or with all the putative CPEs inactivated and injected them into oocytes. These WT 3'UTRs mediated both translational repression in arrested oocytes and translational activation during meiotic resumption, in a CPE-dependent manner (Fig. 1d). Furthermore, both UTRs were cytoplasmically polyadenylated in response to progesterone (Fig. 1e). Taken together, these data demonstrate that Xkid and TPX2 are encoded by CPE-regulated maternal mRNAs stored with short poly(A) tails and translationally repressed in arrested oocytes, that are polyadenylated and activated as a result of progesterone-induced meiotic resumption.

We next checked whether CPE-containing silenced RNAs were enriched in the animal hemisphere of the oocytes, where most of the CPEB and the Microtubule Organizing Center (MTOC) are present. Northern blots of the RNAs extracted from either the animal or the vegetal halves of the arrested oocytes showed that Xkid, TPX2, cyclin B1 and mos mRNAs, but not a control transcript without CPEs such as GAPDH mRNA, were highly enriched in the animal hemisphere (Fig. 2a). To determine whether this enrichment was the result of the association of the mRNAs with microtubules, we performed *in situ* hybridization for Xkid mRNA in MI oocytes and on spindles assembled in egg extracts. We found that the endogenous Xkid mRNA colocalized with spindles and chromosomes in both systems (Fig. 2b, c). As previously described, immunofluorescence of spindles assembled in egg extract showed that CPEB localized on the spindle and chromosomes (Fig. 2d). This suggested that CPE-regulated mRNAs could be tethered to spindles by CPEB. To obtain experimental support for this idea we examined whether this association required the CPEs present in the 3'UTRs. We therefore prepared reporter transcripts containing the 3'UTRs from either Xkid or TPX2 and a variant UTR with the CPEs inactivated, adding twelve MS2-binding sites for visualization with the

recombinant fusion protein MS2-GFP[18]. Chimeric RNAs and MS2-GFP were added to CSF-arrested egg extracts used in cycled spindle assembly reactions. Figure 2e shows that MS2-GFP localized onto the spindle and the chromosomes in extracts containing reporter mRNAs with the 3'UTRs from either WT Xkid or TPX2, but not with the variant without CPEs (Fig. 2f). Thus, these transcripts associate to the spindles and chromosomes in a CPE-dependent manner.

We next aimed to determine whether, not only progesterone-induced protein synthesis, but also localized translation of CPE-regulated mRNAs, was required for meiotic completion and MII arrest. To study the requirements for localized translation, without interfering with general meiotic progression until the second metaphase arrest, we microinjected chimeric mRNAs while maintaining the translation of endogenous mRNAs unaffected. We generated chimeric mRNAs containing the ORF of either Firefly Luciferase (FL) or Xkid (Xk), followed by artificial 3'UTRs containing one or two CPEs (Fig. 3a). Based on the rules for combinatorial CPE-mediated translational regulation[14] we hypothesized that the 3'UTR containing two CPEs (UTR2cpe) should mediate translational repression and mRNA localization in the absence of progesterone as well as cytoplasmic polyadenylation and translational activation upon meiotic resumption whereas the 3'UTR with a single CPE (UTR1cpe) should not repress translation nor mediate localization but should still mediate polyadenylation in response to progesterone stimulation. These mRNAs were coinjected with a renilla mRNA containing a 3'UTR without CPEs. As expected, both artificial UTRs drove similar translational activation of the reporter Firefly Luciferase in response to progesterone, either alone or when coinjected with the equivalent Xkid mRNA (Fig. 3b). However, UTR2cpe, but not UTR1cpe, directed localization of the chimeric Xkid mRNA to the animal hemisphere of the oocyte (Fig. 3c and see Supplementary Information, Fig. S3). Interestingly, when we analyzed the levels of the Xkid protein expressed either from the localized or unlocalized synthetic mRNAs we found that only the translational activation of the localized mRNA was able to support accumulation of high levels of product (Fig. 3d). These patterns of accumulation were not due to differences in the stability of the mRNAs nor to translational repression of the Xk-UTR1cpe mRNA, which is incorporated into polysomes as efficiently as Xk-UTR2cpe mRNA (see Supplementary Information, Fig. S3, and Fig. 3e). Taken together these results strongly suggest that localized translation of Xkid mRNA

results in higher stability of its protein product. Although, we can't rule out that CPEB itself, when associated with the spindle but not in its free form, may cause stabilization of the proteins encoded by CPE-regulated mRNAs by a mechanism not directly related to the localization of the mRNA.

To address the functional relevance of the localized vs. non-localized CPE-regulated mRNA translation during oocyte maturation, we ablated endogenous Xkid synthesis by microinjection of antisense oligonucleotides (Fig. 4a). As described before[16], low levels of Xkid synthesis did not alter the early events of MI entry, as shown by the activation of Cdc2, but prevented the progression to MII, as indicated by the failure to reactivate Cdc2 as compared with control oocytes (Fig. 4b). DNA staining of the Xkid-depleted oocytes, at the time at which the non-injected control oocytes had reached MII (defined by the MII metaphase plate and the polar body), revealed misaligned chromosomes in late MI and no polar body (Fig. 4c). Longer incubation of these Xkid knock-down oocytes resulted in partially decondensed and cleaved chromosomes suggesting exit from meiosis followed by apoptosis[16] (Supplementary Information, Fig. S4). We next tested whether the chimerical Xkid mRNAs driving either localized translation (Xk-UTR2cpe) or non-localized translation (Xk-UTR1cpe) of Xkid were able to rescue the lack of endogenous Xkid during oocyte maturation (Fig. 4a, c). Interestingly, only the localized Xk-UTR2cpe could rescue formation of the MII metaphase plate, polar body extrusion and high Cdc2 activity in MII. Thus, localized translation of Xkid mRNA is essential for the function of this protein in chromosome alignment and MI-MII transition.

To address whether the localized translation of various CPE-regulated mRNAs was a general requirement for meiotic progression and normal chromosome dynamics, we aimed at delocalizing this type of transcripts by competition with a microinjected CPE-containing RNA that squelches the repression complexes[19] or a control RNA without CPEs. Neither of these RNAs blocked maturation, progesterone-induced cytoplasmic polyadenylation nor the synthesis of proteins in response to progesterone (Fig. 5a, b). However, we observed a dramatic effect on the localization of CPE-containing mRNAs and their encoded proteins within the MI oocyte (Fig. 5c,d). Thus, while in the control oocytes (-CPE) both Cyclin B1 and Xkid accumulated mainly in the animal hemisphere, in the oocytes microinjected with a competitor RNA containing CPEs (+CPE), Cyclin B1 and Xkid were equally distributed in both halves and, Xkid levels were greatly

reduced. The competitor RNAs did not change either the levels or the localization of CPEB (Fig. 5d). We conclude that competition with the CPE-containing RNA causes the delocalization of CPE-regulated mRNAs and the abnormal distribution of the encoded proteins.

To test the effect of this translational delocalization on meiotic progression, we stained the DNA of the competed oocytes (Fig. 5e). In (+CPE)RNA competed oocytes the chromatin was no longer condensed and the polar body was absent, whereas the control oocytes displayed normal metaphase plates and extruded polar bodies.

A time course of Histone H1 Kinase activity showed that in both (+CPE)RNA and control oocytes, activation of Cdc2 at MI entry was normal but Cdc2 activity decreased below normal levels after anaphase-I in (+CPE)RNA competed oocytes (Fig. 5f), most likely causing DNA replication and chromatin decondensation[16, 20]. At later times, H1 Kinase activity increases again probably due to the activation of Cdc2 and Cdk2 that takes place in the early stages of apoptosis[21]. We concluded that localized translation of CPE-containing mRNAs is required to assemble the complex(es) that drive chromosome segregation and meiotic progression.

At early times, the phenotype for global CPE-containing mRNAs delocalized translation was more penetrant than the phenotype of Xkid ablation (Fig. 5e and Fig. 4c, see Supplementary Information, Fig. S4). These different penetrances together with the fact that Xkid overexpression did not rescue the meiotic effects of the global delocalization of CPE-containing mRNAs (see Supplementary Information, Fig. S5), suggest that localized translation of other CPE-containing mRNAs, such as Bub1, Bub3, Eg5, Mad1, Mad2, BubR1, Cenp-E, Nek2B, TPX2, Aurora A and Aurora B, is required for chromosome segregation and meiotic progression.

Altogether this work shows that spindle-localized translational activation of CPEB-regulated mRNAs, encoding for proteins with a known function in spindle assembly and chromosome segregation, such as Xkid mRNA, is essential for successful chromosome segregation during the first meiotic division and for the MI-MII transition.

METHODS

Xenopus oocyte preparation

Stage VI oocytes were obtained from *Xenopus laevis* females and induced to mature with Progesterone (10 μM, Sigma) as previously described[19].

Plasmid constructions

The FTX5-Xkid full length was obtained from Angel Nebreda[15]; cDNA of TPX2 in pBSK[22]. The complete cDNA sequences are available from GenBank under accession numbers AJ249840 and AF244546 respectively. The 3'UTRs of Xkid and TPX2 were amplified by PCR and were subcloned downstream of the Firefly Luciferase ORF into pLucassette[14]. pSL-MS2-6 and pPolII-MS2-eGFP were obtained from Edouard Bertrand and Robert H. Singer[18]. The DNA corresponding to a 12 repeats of the MS2-binding site was digested with BamHI and subcloned downstream the Firefly Luciferase ORF in BglII site of the pLucassette followed either by Xkid or TPX2 3'UTR. The DNA corresponding to the MS2-eGFP was amplified by PCR and was subcloned into HindIII/XhoI sites in pET30a expression vector. The sequences of antisense oligonucleotides used were: 3XkAS, 5´-GTTGGTGAGGAATAAAATC-3´ (based on the Xkid 3´UTR sequence); 5XkAS, 5´-CATTCCCGCCTCGCTTCG-3´ (based on the Xkid 5´UTR sequence). As a control, sense oligonucleotide was used, Xks, 5´-GATTTTATTCCTCACCAAC-3´. The sequence of antisense morpholino used was 5´-GCCCAGTAAGAACCATTCCCGCCTC-3´ as described[16].

Polyadenylation of endogenous mRNAs

Total RNA was isolated from 5-8 oocytes by Ultraspec RNA Isolation System (Biotecx Laboratories, Inc.) following the manufacturer's instructions. Then, RNA-ligation coupled RT-PCR technique was performed as described[23], with some modifications. Briefly, 4 μg of total oocyte RNA was ligated to 0.4 μg anchor 3´- amino modified oligo (Sigma), using T4 RNA ligase (New England Biolabs). The RNA ligation reaction was used in a 50 μl reverse transcription reaction using RevertAid M-MuLV Reverse Transcriptase (Fermentas), and 0.4 μg antisense oligo (Sigma). The resulting reaction product was digested with 2 μg RNAse A (Fermentas) and then used as a template for gene-specific PCR reaction. The specific oligos used were: XkP2s, 5´-CACATTGCAGGAAGGTTCTGC-3´ and TPXP2s, 5´-CTAGATATTAATGGCCTGGAGGG-3´ designed to be at 65 and 80 nucleotides from the

hexanucleotide of Xkid and TPX2 3´ UTR, respectively. The resulting PCR reaction was analyzed on a 2% agarose gel and visualized by ethidium bromide staining.

Translational control and cytoplasmic polyadenylation by 3´ UTR.

Translation and polyadenylation of reporter mRNAs were assayed as described[14], with some modifications. Briefly, oocytes were injected with 0,0125 fmol of each hybrid reporter mRNA together with 0,0125 fmol of renilla Luciferase normalizing RNA. Luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega).

Histone H1 Kinase assay

Oocyte lysates prepared by homogenizing 3 oocytes in H1 Kinase buffer and centrifuged at 12,000g for 5 min at 4 ºC were incubated with Histone H1 (Sigma) and [γ-^{32}P] ATP as described[3].

Western blot

Oocyte lysates prepared by homogenizing 5-10 oocytes in H1 Kinase buffer containing 0,5% NP-40 and centrifuged at 12,000g for 5-10 min were resolved by 10% SDS-PAGE. Equivalents of 1-2 oocytes were loaded onto each lane. Antibodies used: rabbit antiserum against Xkid[15]; rabbit antiserum against TPX2[22]; rabbit antiserum against CPEB (gift from J.D. Richter); goat antiserum against Cyclin B1; and monoclonal antibody against α-Tubulin (DM1A Sigma).

Egg-extracts, spindle assembly and mRNA localization assay

Cytostatic factor arrested egg-extracts (CSF-extracts) were prepared as previously described[24]. For cycled spindle assembly, 0.2 mg ml^{-1} rhodamine-labeled Tubulin and demembranated sperm nuclei (~500 nuclei μl^{-1}) were added to CSF-extracts on ice. The extracts were then released into interphase by the addition of 0.4 mM Ca^{2+} and incubated for 90 min at 20 ºC. These extracts were then cycled back into mitosis by addition of one volume of fresh CSF-arrested extracts. After 45 min, spindles were fixed in 1 ml BRB80 (80 mM K-Pipes, pH 6.8, 1 mM EGTA, 1 mM $MgCl_2$) containing 30% glycerol, 0.25% glutaraldehyde, and 0.1% Triton X-100 and centrifuged onto coverslips (Multifuge 3 L-R, Heraeus) through a 40% glycerol cushion in BRB80, as previously described[22].

To visualize mRNAs on spindles assembled in egg extracts, 20 ng μl^{-1} *in vitro* hybrid transcribed mRNAs and 10 μg ml^{-1} purified recombinant MS2-eGFP protein expressed in *E.coli* BL21 were added to the extracts before cycling them. Spindles assembled in egg extracts were

also processed for immunofluorescence as described[22] with the rabbit antiserum against CPEB in order to detect endogenous CPEB. Pictures were taken using the same camera settings (Leica DMI6000B microscope, 63X magnification, Leica DFC350FX camera, Leica Application Suite (LAS AF) advanced fluorescence 1.6.1 build 1057 software). For quantification ImageJ software was used.

Fluorescent mRNA *in situ* hybridization on the spindle and immunostaining

Mature oocytes (GVBD) fixed overnight in HEPES solution containing 3.5% formaldehyde, 0.25% glutaraldehyde and 0.1% Triton X-100 were postfixed in 100% methanol for 16 hours at -20 ºC. After hemisection of the oocytes either in the plane of the equator or along the animal-vegetal axis, bleaching with 10% H_2O_2 in methanol and borohydride reduction were performed in order to eliminate oocyte pigmentation and reduce the autofluorescence generated during glutaraldehyde fixation[25]. For the *in situ* hybridization, the oocytes were prehybridized for 6 hours at 60 ºC in Hyb Solution (50% formamide, SSC 1X, 0.1% tween-20, 9.2 mM citric acid, 50 $\mu g\ ml^{-1}$ heparin and 500 $\mu g\ ml^{-1}$ tRNA) and hybridized with 5 $\mu g\ ml^{-1}$ of digoxigenin-labeled riboprobe specific for Xkid ORF overnight at 60 ºC. For colocalization with the spindle, oocytes were incubated with monoclonal antibody against α-Tubulin (DM1A, Sigma) for 1 day at 4 ºC according to[25]. The oocytes were sequentially incubated with anti-digoxigenin-rhodamine, Fab fragments from sheep, (50 $\mu g\ ml^{-1}$; Roche) and Alexa488-labeled antibody (Invitrogen) for 1 day at 4 ºC in PBS-T containing 2 mg ml^{-1} of BSA and 5% sheep serum. Images were acquired using Leica TCS SP5 confocal laser scanning microscope, 63X magnification, Leica Application Suite (LAS AF) advanced fluorescence.

Fluorescent mRNA *in situ* hybridization was also performed in spindles assembled in cycled egg extracts as described above, with some modifications according to[22]. Images were acquired using Leica DMI6000B microscope, 63X magnification, Leica DFC350FX camera, Leica Application Suite (LAS AF) advanced fluorescence.

Chromosomes and polar body observation

Oocytes fixed for 1 h in HEPES buffer (100 mM KCl, 3 mM $MgCl_2$, and 10 mM HEPES, pH 7.8) containing 3.7% formaldehyde, 0.1% glutaraldehyde and 0.1% Triton X-100, or 100% methanol were incubated overnight in presence of 20 $\mu g\ l^{-1}$ 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).

Northern blot analysis

Total RNA was isolated using Ultraspec RNA Isolation System (Biotecx Laboratories, Inc.) and analyzed by Northern blot as described[19]. Specific probes for *X. laevis* Xkid, TPX2, cyclin B1, c-mos and GAPDH were labeled by random priming (Megaprime DNA labelling Systems, Amersham Biosciences). The sequence of specific oligonucleotides used in RT-PCR analysis were: cycB1.iS, 5´-GTCAAGGACATTTATGCTTACC-3´ and cycB1.iAS, 5´-CCATGTCCCGAATTTGAGCC-3´ for cyclin B1; XkΔ1s 5´-GCAGATCTCATTTTATTCTACTATTTTATTATGAGCC-3´ and Xkidas, 5´-CCAAAAACGTTTATTTTACAGAAAGACATGG-3´ for Xkid; 5´-CATCACGCAGCGGCCTGG-3' and 5'- GTGGTCGCTTTAGCTTCATCC-3' that amplify both Xk-UTR1cpe and Xk-UTR2cpe; and xGAPDHs, 5´-GTCGCCCATCCTGCTAGTC-3´ and T7AS, 5´-GTAATACGACTCACTATAGGGC-3´ for GAPDH mRNAs.

Polysome pelleting

Extracts from 10 oocytes were fractionated by centrifugation over sucrose cushions as described[26]. The RNA extracted from polysomal and non-polysomal fractions was analyzed by RT-PCR with the following sense and antisense oligonucleotides: 5´-CATCACGCAGCGGCCTGG-3' and 5'- GTGGTCGCTTTAGCTTCATCC-3' that amplify both Xk-UTR1cpe and Xk-UTR2cpe.

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FIGURE LEGENDS

Figure 1 Xkid and TPX2 mRNAs are cytoplasmically polyadenylated and translationally activated during meiotic maturation.

(**a**) Schematic representation of the 3'UTRs from *Xenopus laevis*, human and mouse Kid and TPX2 mRNAs. The two possible 3'UTRs from TPX2 are shown as short and long. CPEs (white boxes), polyadenylation signals (Hexanucleotide; grey boxes) and UGU/AUA repeats (black boxes) are indicated. (**b**) *Xenopus* oocytes stimulated with progesterone were collected at the indicated times and analyzed by Western blot with either Xkid or TPX2 antibodies. The percentage of GVBD (germinal vesicle breakdown) was determined by the appearance of the white spot at the animal pole of the oocytes. 1,5 oocyte equivalents were loaded per lane. (**c**) Total RNA extracted from oocytes incubated in the presence (+) or absence (-) of progesterone was analyzed by RNA-ligation coupled RT-PCR as indicated in methods. The PCR products derived from the polyadenylated and non-polyadenylated Xkid and TPX2 mRNAs are indicated. (**d**) *in vitro* transcribed chimeric mRNAs were coinjected into the oocytes together with Renilla Luciferase as a normalization control. The mRNAs contained the Firefly Luciferase ORF fused to the following 3'UTRs: C_1 and C_2, control UTRs of 174 and 470 nucleotides respectively; B1 WT and B1(-CPE), 3'UTRs derived from cyclin B1 mRNA with or without CPEs, respectively; TPX2 WT and TPX2(-CPE), 3'UTRs derived from TPX2 mRNA with or without CPEs, respectively; Xkid WT and Xkid(-CPE), 3'UTRs derived from Xkid mRNA with or without CPEs, respectively. The percentage of translational repression in the absence of progesterone (left panel) was normalized to C1 (100% translation) and to the fully repressed B1 (0% translation). Translational stimulation (right panel) was determined from the Luciferase activity in oocytes incubated with progesterone and collected 3 hours after GVBD. The percentage of translation stimulation was normalized to C1 (0% stimulation) and B1 (100% stimulation). Data are represented as mean ± s.d. (n = 4) (**e**) Oocytes were injected with either Xkid or TPX2 radiolabeled 3'UTRs. Total RNA from oocytes incubated in the presence or absence of progesterone was extracted and analyzed by denaturing gel electrophoresis followed by autoradiography.

Figure 2 The 3'UTRs of Xkid and TPX2 mRNAs direct their localization to the spindles.

(**a**) Subcellular localization of endogenous Xkid, TPX2, cyclin B1 and mos mRNAs. Total RNA from animal (A) and vegetal (V) hemispheres was extracted and analyzed by Northern blot with labeled probes for Xkid, TPX2, cyclin B1, mos and GAPDH. Quantification of the relative Northern blot signals is shown. Fluorescent *in situ* hybridization for Xkid mRNA on MI-spindles (**b**) or on *in vitro* assembled spindles (**c**). Samples were hybridized with antisense digoxigenin-labeled (red) riboprobes for either Xkid mRNA or sense control, as indicated. Scale bars 10 μm. Spindles were costained with antibodies against Tubulin (green). In (**c**) Hoechst-stained DNA (blue) and quantification of fluorescence intensity associated to microtubules in each case is also shown. Images are representative of 4 independent experiments, with over 15 spindles visualized in each experiment. (**d**) CPEB immunostaining. Immunofluorescence of spindles assembled in egg extracts with the anti-CPEB antibody (green). Microtubules are visualized in red and DNA in blue. Scale bar 10 μm. (**e**, **f**) RNA-mediated tethering of MS2-GFP to *in vitro* assembled spindles. A schematic representation of the Luciferase reporter mRNAs and the tethered MS2-GFP protein is shown. Representative images of spindles assembled *in vitro* on cycled egg extracts supplemented, as indicated, with recombinant MS2-GFP protein and RNAs containing the Luciferase ORF followed by twelve MS2-binding sites and by the 3'UTR from either Xkid WT or TPX2 WT (**e**), or by the 3'UTR from either TPX2 WT (+CPE) or a variant with all the CPEs inactivated (-CPE) (**f**). Tethered MS2-GFP protein is detected as a green signal, rhodamine-labeled microtubules are detected in red and Hoechst-stained DNA in blue. Images are representative of 3 independent experiments, with 25-30 spindles visualized in each one. Scale bars 10 μm. In (**f**) quantification of GFP-fluorescence intensity associated to microtubules is shown as mean ± s.d.

Figure 3 Localized translational activation of Xkid mRNA is required for the accumulation of Xkid protein.

(**a**) Schematic representation of the chimeric mRNAs containing the ORF of either Firefly Luciferase (FL) or Xkid (Xk) fused to 3'UTRs containing one (UTR1cpe) or two (UTR2cpe) CPEs. The CPEs and the hexanucleotide are indicated. (**b**) Oocytes were injected with the indicated *in vitro* transcribed chimeric mRNAs and maintained for 3 hours, to allow proper

mRNA localization, before oocytes were incubated in the presence or absence of progesterone for 7 hours. Then, the Luciferase activity was determined for pools of 5 oocytes. Translational repression in the absence of progesterone (left panel) and translational stimulation in the presence of progesterone (right panel) are indicated. Note the different scale in the X-axis for repression and activation. Representative of 4 independent experiments. (**c**) Subcellular mRNA localization of injected mRNAs. Frozen oocytes injected with the indicated mRNAs were incubated in absence of progesterone and cut in half along the equator. Total RNA from animal (A) and vegetal (V) hemispheres was extracted and analyzed by RT-PCR using specific oligonucleotides for the injected RNAs and endogenous GAPDH mRNA. Quantification of the relative RT-PCR signals is shown. (**d**) The same injected-oocytes used in (**b**) were analyzed for Xkid protein accumulation by Western blot with Xkid antibody. Equivalents of 1,5 oocytes were loaded onto each lane. Full scans are shown in Supplementary Information, Fig. S6. (**e**) Polysomal distribution. Oocytes injected with the indicated mRNAs, were incubated in absence or presence of progesterone and fractionated by centrifugation over a sucrose cushion. Total RNA was extracted from both polysomal (P) and non-polysomal (N) fractions and analyzed by RT-PCR for the injected RNAs. In the indicated lanes, the extracts were treated with 50 mM EDTA to disrupt polysomes.

Figure 4 Localized Xkid mRNA translation is required for MI to MII transition.

Oocytes were injected with Xkid sense or antisense oligonucleotides. After 12 hours both groups of oocytes were microinjected with 0,3 fmols of an mRNA encoding for Xkid ORF fused to 3'UTRs containing either one (UTR1cpe) or two (UTR2cpe) CPEs described in Fig. 3. After 2 hours, the oocytes were incubated in the presence or absence of progesterone and collected 5 hours after the control, non-injected oocytes, displayed 100% GVBD. (**a**) One oocyte equivalent from each indicated treatment was analyzed by Western blot with Xkid and Tubulin antibodies. Full scans are shown in Supplementary Information, Fig. S6. (**b**) Histone H1 Kinase activity in Xkid-depleted and control oocytes. Two oocytes collected at the indicated times after progesterone addition were lysed and the extract analyzed for Histone H1 Kinase activity. Quantification of the Histone H1 Kinase activity is shown. (**c**) Oocytes, treated as indicated, were fixed, stained with Hoechst and examined under the epifluorescence microscope. The

percentage of oocytes displaying each phenotype (5 hours after GVBD) is shown (n>16). The arrow indicates the first polar body. Scale bar 10 μm. For each treatment, three oocytes were collected at the indicated times, lysated and the extracts analyzed for Histone H1 Kinase activity (-P, oocytes incubated in absense of progesterone). Both chimerical Xkid mRNAs (Xk-UTR1cpe and Xk-UTR2cpe) lack the regions targeted to the antisense oligonucleotides. The meiotic phases in the control oocytes are indicated (PI, prophase-I; MI, metaphase-I; I, interkinesis; MII, metaphase-II).

Figure 5 Localized translational activation of CPE-containing mRNAs is required for MI to MII transition.

Oocytes were injected with 1,5 pmol of 70 nucleotides long competitor RNAs containing (+CPE) or lacking (-CPE) multiple CPEs. After 2 hours, the microinjected oocytes were incubated with or without progesterone. Non-injected oocytes (-) were carried in parallel as a control. (**a**) Cytoplasmic polyadenylation of Xkid mRNA. Samples were collected 5 hours after the non-injected oocytes reached 100% maturation (i.e. in MII). Total RNA from control and RNA-competed oocytes was extracted and polyadenylation of endogenous Xkid mRNA analyzed by RNA-ligation coupled RT-PCR as in Fig. 1c. (**b**) Protein expression in competed oocytes: control and RNA-competed oocytes were collected 5 hours after the non-injected oocytes reached 100% maturation and analyzed by Western blot with Xkid, Cyclin B1, and Tubulin antibodies. Equivalents of 2 oocytes were loaded onto each lane. (**c**) Subcellular localization of endogenous cyclin B1, Xkid and GAPDH mRNAs. Total RNA from animal (A) and vegetal (V) hemispheres was extracted and analyzed by RT-PCR. Quantification of the relative RT-PCR signals is shown. (**d**) Subcellular localization of CPEB, Cyclin B1 and Xkid proteins. Oocytes were treated as indicated and collected at GVBD. (A) Animal half, (V) vegetal half. Quantification of the Western blot signals is shown. Full scans are shown in Supplementary Information, Fig. S6. (**e**) Control and RNA-competed oocytes were collected 5 hours after the non-injected oocytes reached 100% maturation and analyzed for meiotic structures by DNA staining as described for Fig. 4c. The percentage of oocytes displaying each phenotype is shown (n>10). The arrow indicates the first polar body. Scale bar 10 μm. (**f**) Histone H1 Kinase activity in control and RNA-competed oocytes during meiotic maturation. Three oocytes from

each RNA-competed and control populations were collected at the indicated time points and were analyzed for Histone H1 Kinase activity. Quantification of the Histone H1 Kinase activity is shown.

Figure-1

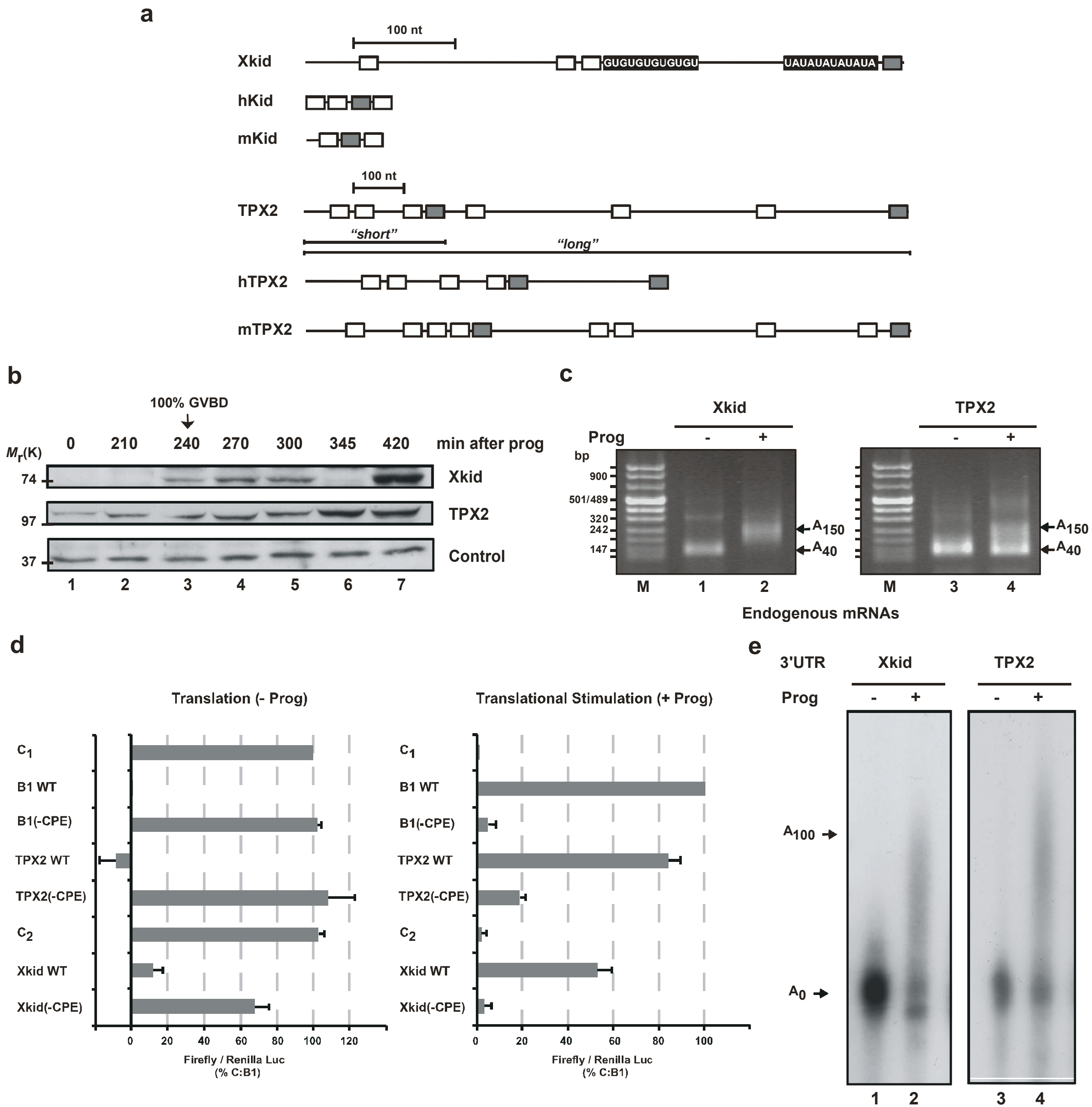


Figure-2

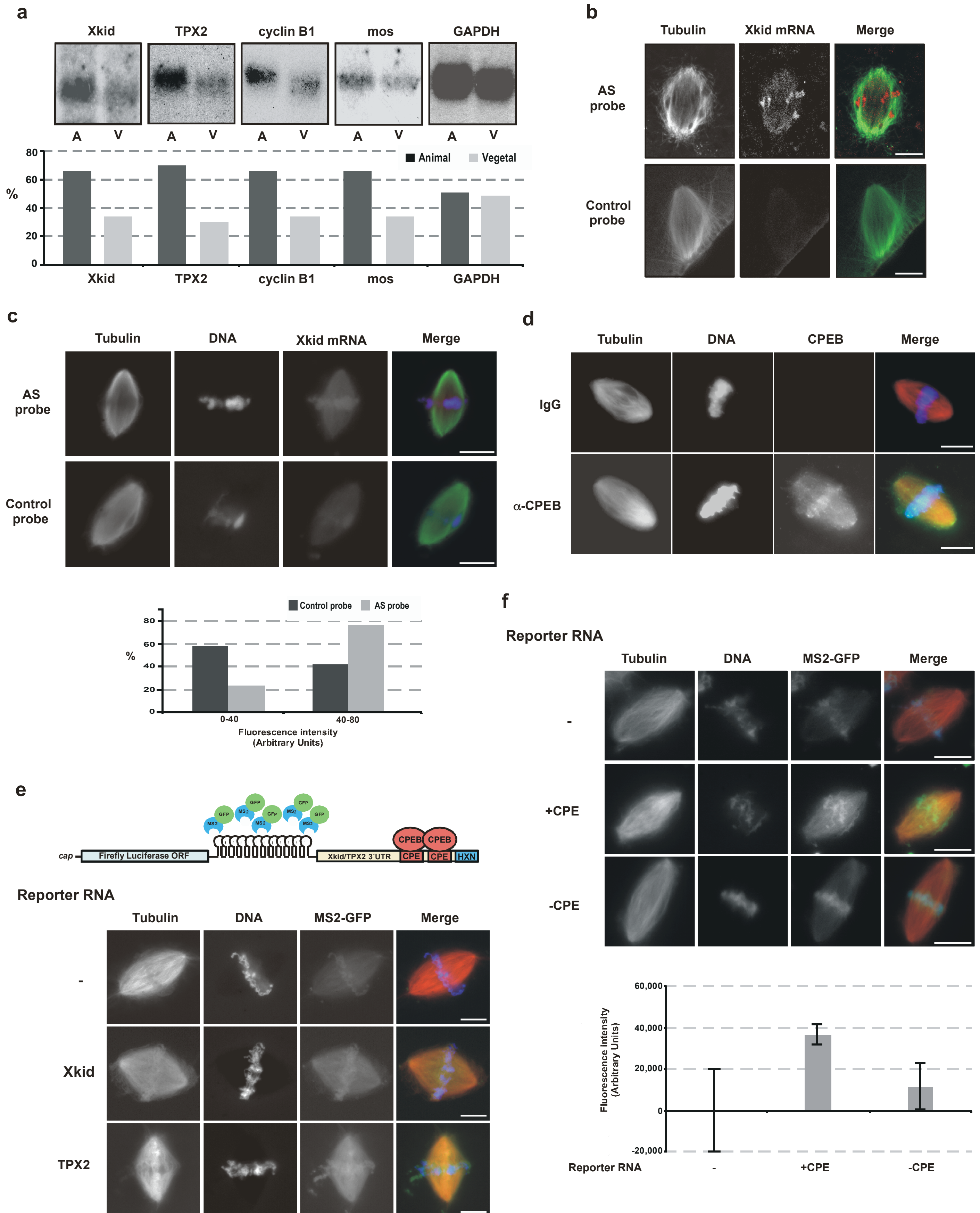


Figure-3

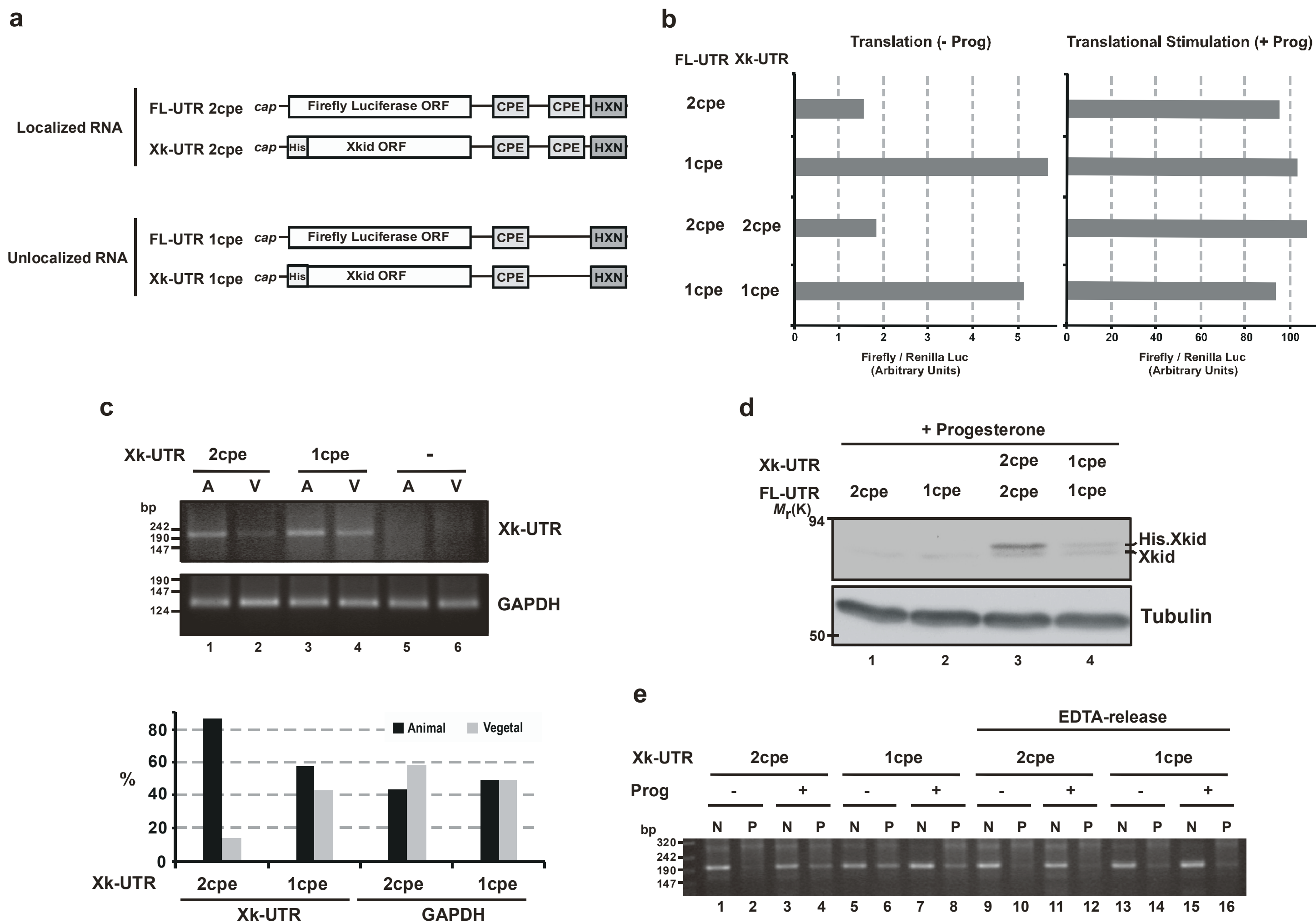


Figure-4

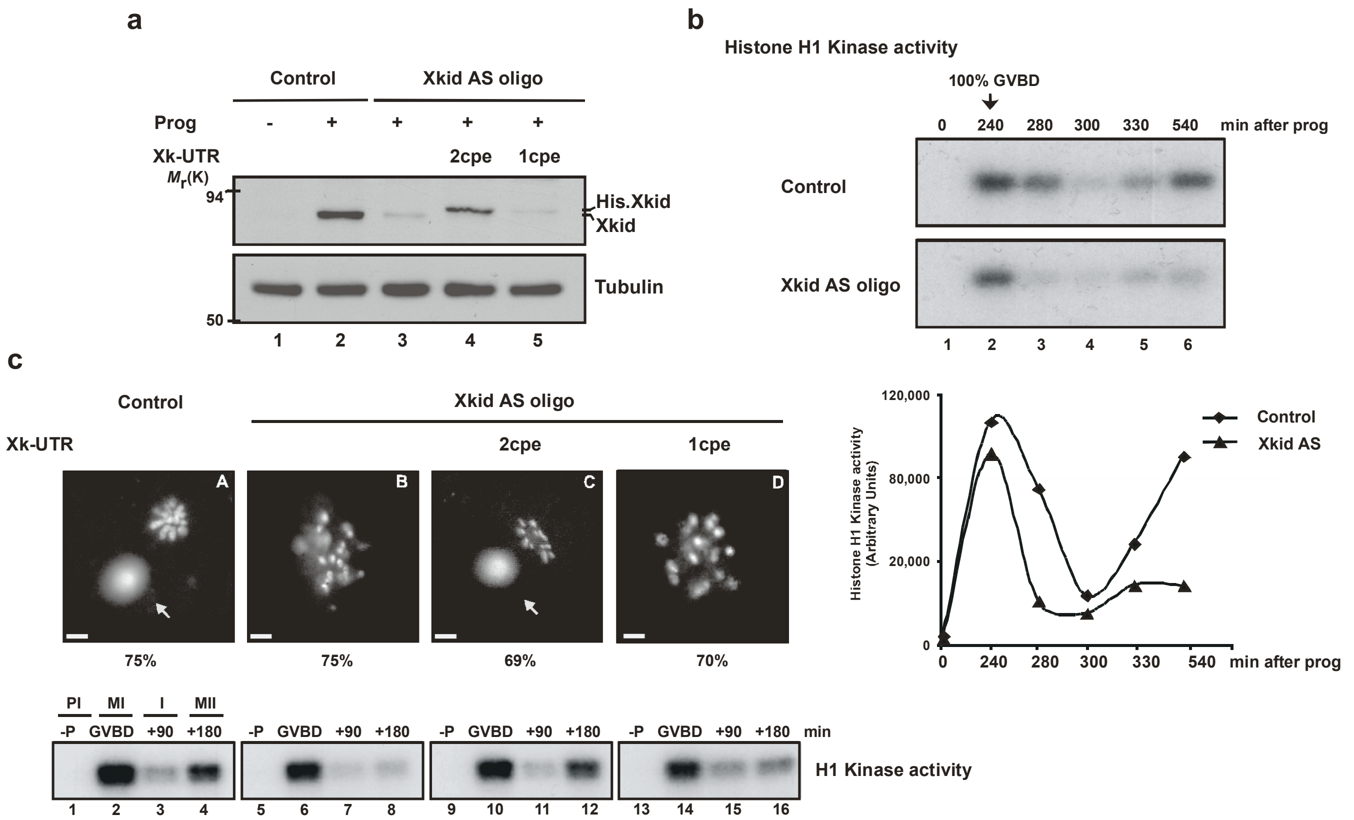
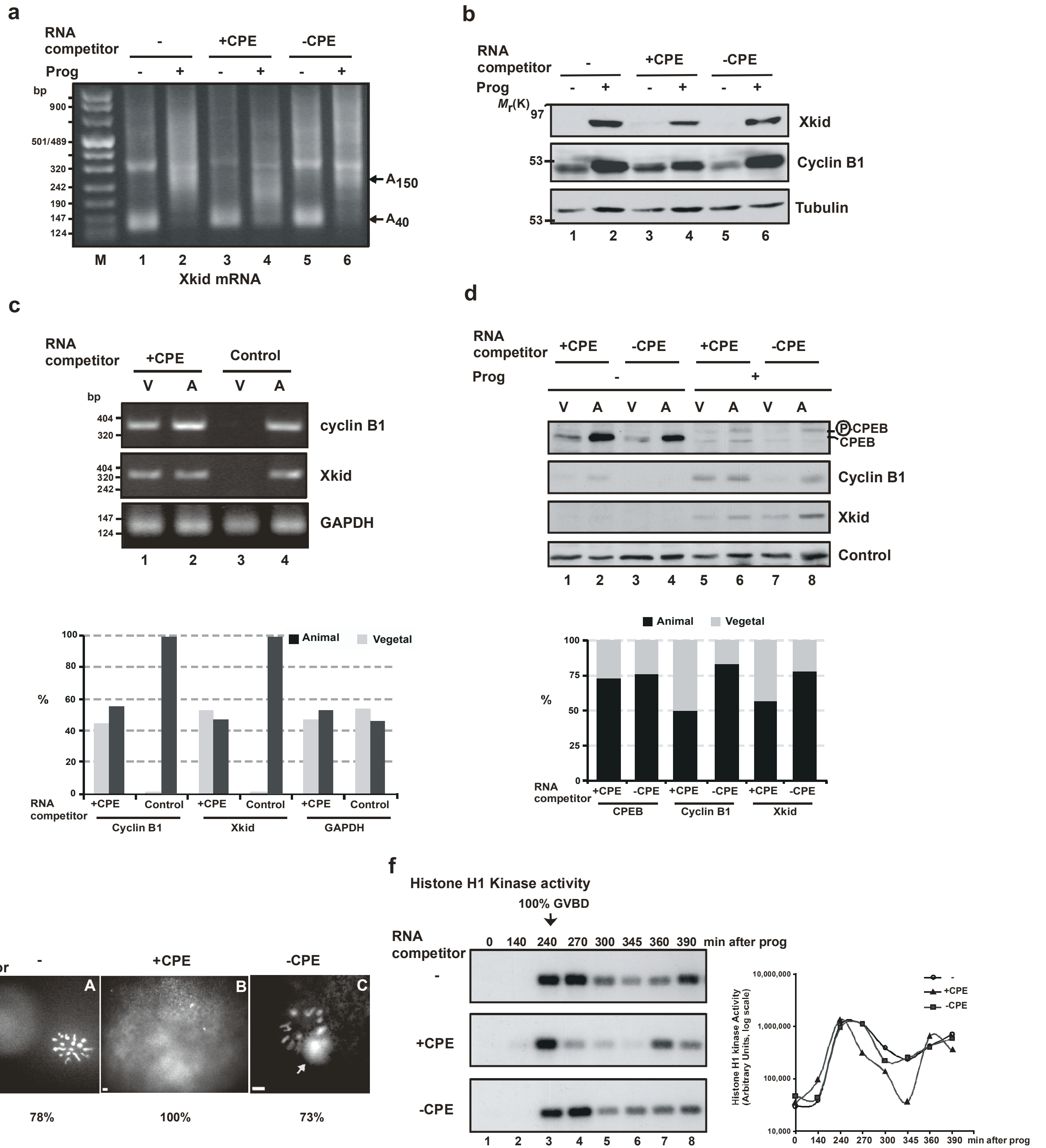


Figure-5



Supplementary Information

- Xkid 3´ UTR (598 nts)

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gcaaacacgaaccaaaugugccaaccggccgcuggauguuuuguuaaagugaguucaacgccgagacucugguuuaa
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- Xkid-CPE 3´ UTR (338 nts)

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ucucuugagugacugccccaguggggugaaugaguggcugcaaugugguuuggcagagaaguaacaguaguuuccau
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augugcuucagcagcauuuacugcaucacauugcaggaagguucugcugguguuauuauauucuuccaaaucaaguc
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- TPX2 *"long"* 3´ UTR (1227 nts)

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- TPX2 *"short"* 3´ UTR (257 nts)

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auauuaauggccuggaggggccuguuuuuaggucuuccaugugaauacguuaugc**uuuuau**uaugcccugu**aauaaa**c
uguguaaauguaaaguuugugcc

- TPX2-CPE 3´ UTR (257 nts)

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auauuaauggccuggaggggccuguuuuuaggucuuccaugugaauacguuaugc**uuuGGu**uaugcccugu**aauaaa**c
uguguaaauguaaaguuugugcc

Figure S1 Xkid and TPX2 3'UTRs.

Sequences of the 3' UTRs from Xkid and TPX2 mRNAs (accession numbers AJ49840 and AF244546, respectively). The two possible 3' UTRs from TPX2 are shown as "long" and "short". The corresponding UTR variants with the CPEs inactivated are shown. CPEs (underlined), polyadenylation signal (Hexanucleotide, boxes) and UA/GU repeats (filled boxes) are indicated. Xkid 3'UTR contains 3 potential CPEs, two of them separated by 7 nucleotides and therefore with the potential of assembling a repression complex[14]. The most 3' CPE and the Hexanucleotide (Hex)

are flanked by, respectively, a GU-stretch and a UA-stretch, with the potential to form a stable secondary structure. Human and mouse Kid 3'UTRs, although much shorter, also contain clusters of two CPEs not overlapping with the Hex and are, therefore, predicted to display the same translational behavior (Fig. 1a). TPX2 3' UTR contains multiple potential CPEs and two potential Hexs at positions 227 and 1207 of the 3' UTR. We have determined that only the shortest version of the 3' UTR, corresponding to the use of the upstream Hex during alternative nuclear cleavage reaction, can be detected in oocytes (see Supplementary Information, Fig. S2). This short 3' UTR contains a cluster of 2 CPEs predicted to assemble a repression complex and another CPE at 10 nucleotides from the Hex, predicted to activate polyadenylation and translation in response to progesterone prior to germinal vesicle breakdown (GVBD). Indeed, such translational behavior was observed when constructs containing this 3'UTR were injected into oocytes, whereas the CPE-arrangement of the long 3'UTR supported translational repression but not activation (see Supplementary Information, Fig. S2). The CPE-arrangement and the two potential polyadenylation sites are conserved in mouse and human TPX2 3' UTRs (Fig. 1a).

Supplementary Information

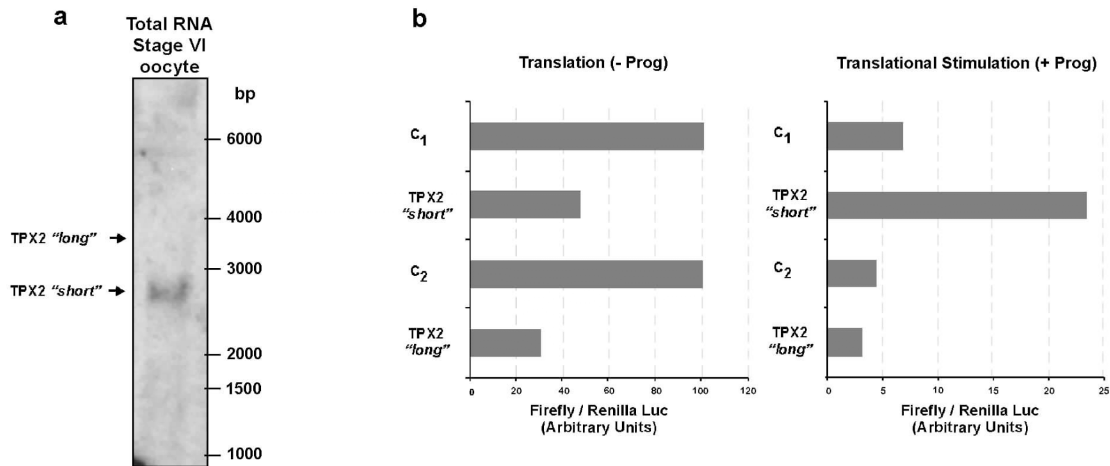

Figure S2 TPX2 mRNA in *Xenopus* oocytes.

(**a**) Northern blot for TPX2 mRNA. Total RNA from 10 Stage VI oocytes was extracted and analyzed by Northern blot with a labeled probe for TPX2 ORF. Molecular weight marker migrations are indicated. Arrows indicate the predicted migration position for both short and long TPX2 mRNAs. (**b**) *in vitro* transcribed chimeric mRNAs were coinjected into the oocytes as described in Fig. 1d. The mRNAs contained the Firefly Luciferase ORF fused to the following 3' UTRs: C_1 and C_2, control UTRs of 174 and 1139 nucleotides respectively; TPX2 "short" and TPX2 "long" 3'UTRs as shown in Supplementary Information, Fig. S1. Translational repression in the absence of progesterone (left panel) and translational stimulation in response to progesterone (right panel) were determined from the Luciferase activity in oocytes incubated with or without progesterone and collected 8 hours after injection of the reporters. The results shown are representative of 3 independent experiments.

Supplementary Information

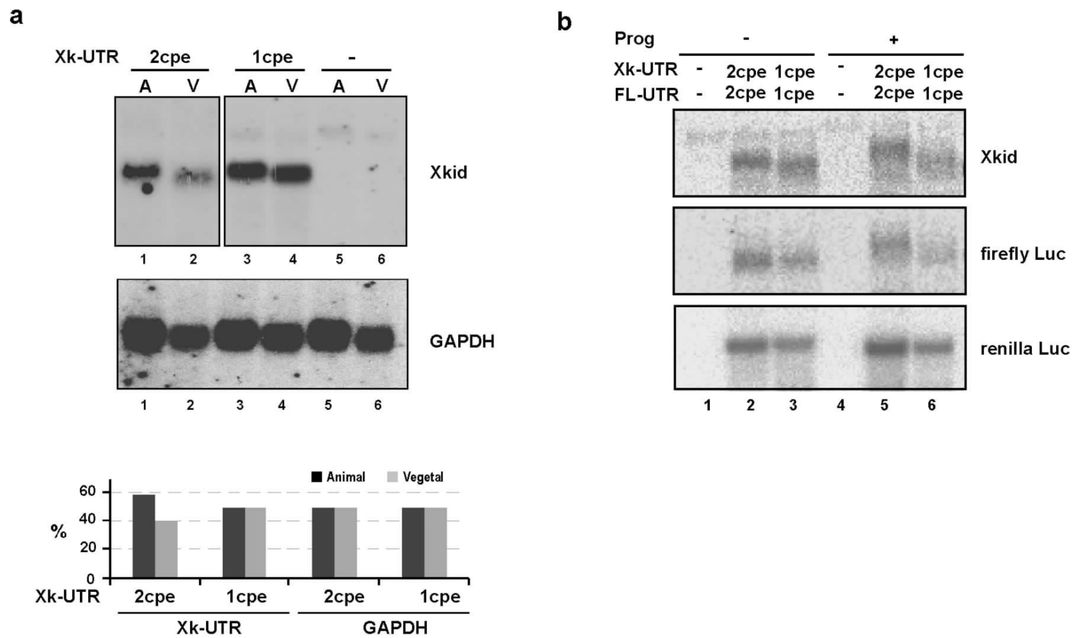

Figure S3 Localization and stability of injected mRNAs.

(**a**) Subcellular mRNA localization of injected mRNAs. Frozen oocytes injected with the indicated mRNAs were incubated in absence of progesterone and cut in half along the equator. Total RNA from animal (A) and vegetal (V) hemispheres was extracted and analyzed by Northern blot with Xkid and GAPDH radiolabeled probes. Quantification of the relative Northern blot signals is shown. (**b**) mRNA stability. Oocytes injected with the mRNAs indicated were incubated in absence or presence of progesterone and analyzed by Northern blot with radiolabeled probes for Xkid, firefly Luciferase and renilla Luciferase.

Supplementary Information

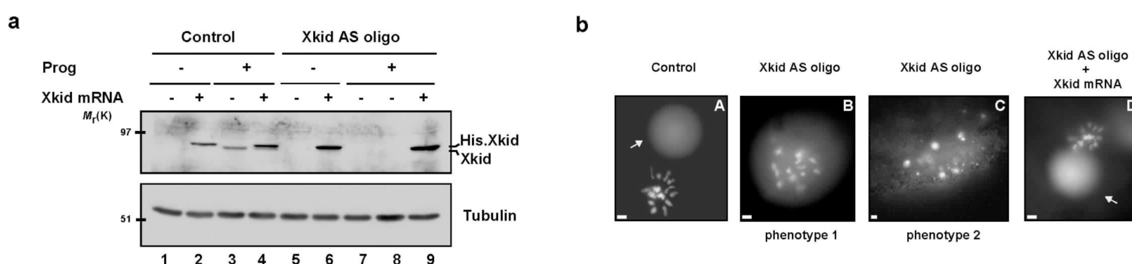

Figure S4 Xkid is required for MI to MII transition.

Oocytes were injected with Xkid sense or antisense oligonucleotides. After 12 hrs both groups of oocytes were microinjected with 3 fmols of an mRNA encoding for Xkid and incubated in the presence and absence of progesterone. Oocytes were collected 5 hours after the control, non-injected oocytes, displayed 100% GVBD. (**a**) The oocytes were analyzed for Xkid levels by Western blot with Xkid and Tubulin antibodies. Equivalents of 2 oocytes were loaded onto each lane. (**b**) Oocytes were fixed, stained with Hoechst and examined under the epifluorescence microscope. Representative images from control oocytes, the two main phenotypes from Xkid depleted oocytes and oocytes depleted for the endogenous Xkid and overexpressing Xkid from an injected mRNA are shown. The arrow indicates the first polar body. Scale bar 10 μm.

Both phenotypes are consistent with previous reports for Xkid depletion[16], although the proportions are slightly different, maybe due to different levels of depletion or to the time at which the oocytes were analyzed. Indeed, at earlier times after GVBD (i.e. 5 hrs) phenotype 1 is detected more often whereas at later times (i.e. O/N) phenotype 2 is more abundant, suggesting that a defect in the chromosomal alignment in late MI results in meiotic exit followed by apoptosis. Interestingly, similar phenotypes are observed when total protein synthesis was inhibited after GVBD by microinjection of cycloheximide. Under these conditions a compact spindle formed without properly aligned chromosomes. This structure persisted for approximately 1 hour after GVBD and then chromosomes decondensed and microtubules disappeared[27].

27. Hochegger, H. et al. New B-type cyclin synthesis is required between meiosis I and II during Xenopus oocyte maturation. *Development* **128**, 3795-807 (2001).

Supplementary Information

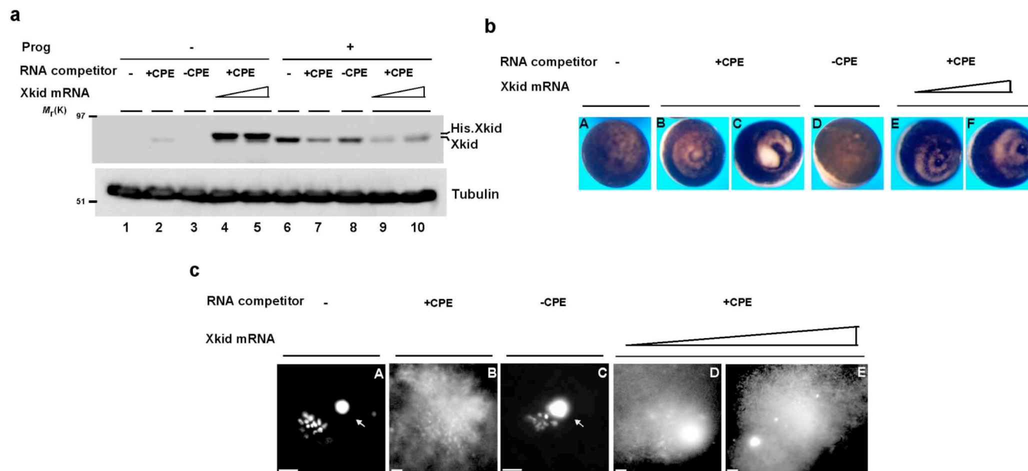

Figure S5 Xkid does not rescue global delocalization of CPE-containing mRNAs.

Oocytes injected with 1,5 pmol of 70 nucleotides long competitor RNAs containing (+CPE) multiple CPEs were injected with 0,7 or 1,4 fmols of an mRNA encoding for Xkid. After 3 hours, the microinjected oocytes were incubated with or without progesterone. Non-injected oocytes (-) and oocytes injected with an RNA without CPEs (-CPE) were carried in parallel as a control. Samples were collected 4 hours after the control, non-injected oocytes, displayed 100% GVBD. (**a**) Oocytes were analyzed by Western blot with Xkid and Tubulin antibodies. Equivalents of 1,5 oocytes were loaded onto each lane. Note that Xkid is degraded during anaphase and when the oocytes exit meiosis into S-Phase[16]. (**b**) Morphological appearance of (A) non-injected control oocytes, (B, C) (+CPE)RNA competed oocytes, (D) (-CPE)RNA competed oocytes and (E, F) (+CPE)RNA competed oocytes and overexpressing Xkid from an injected mRNA are shown. (**c**) Oocytes were analyzed for meiotic structures by DNA staining as described for Fig. 4c. Representative images from (A) non-injected control oocytes, (B) (+CPE)RNA competed oocytes, (C) (-CPE)RNA competed oocytes and (D, E) (+CPE)RNA competed oocytes and overexpressing Xkid from an injected mRNA are shown. The arrow indicates the first polar body. Scale bar 10 μm. (Leica DM6000B microscope, Leica DFC300FX camera, Leica Application Suite (LAS AF) Version 2.7.1.R).

Supplementary Information

Figure 3d

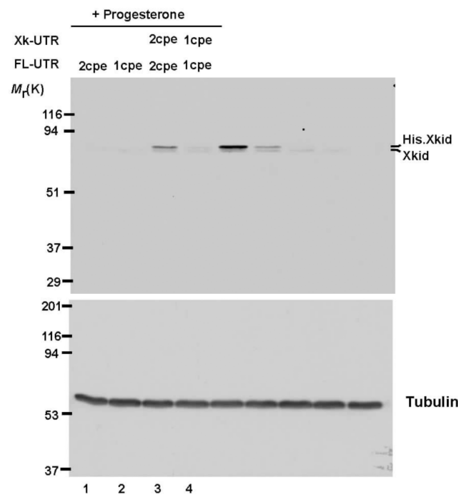

Figure 5d

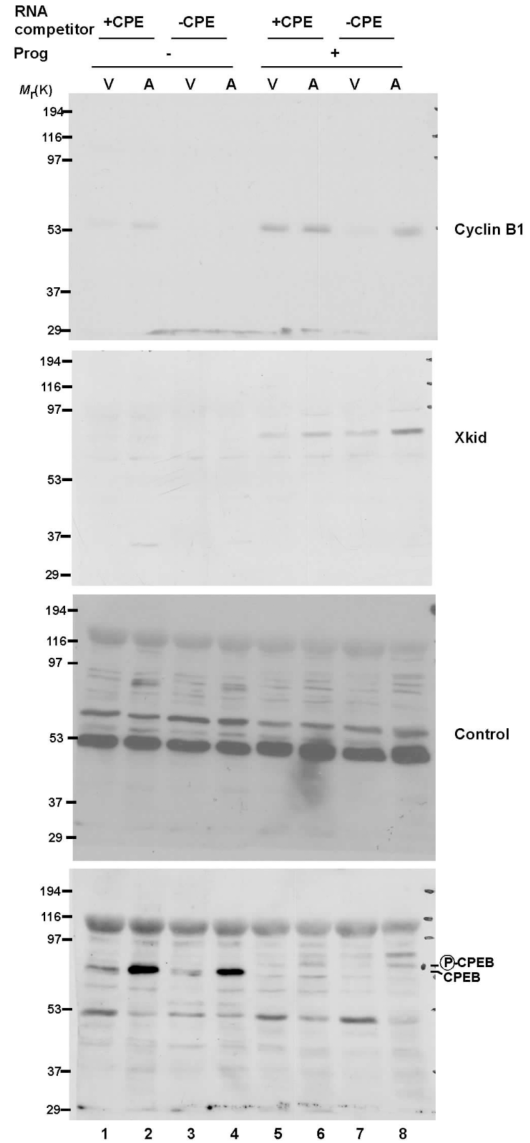

Figure 4a

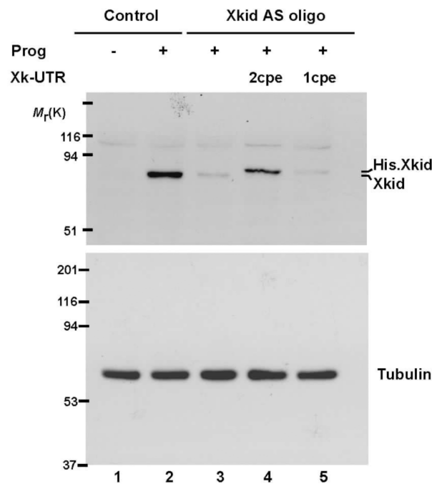

Figure S6 Full scans of Figure 3d, 4a and 5d.

Full scan of western blot shown in Figure 3d analyzed with Xkid antibody (upper panel) and Tubulin antibody (lower panel). Full scan of western blot shown in Figure 4a analyzed with Xkid antibody (upper panel) and Tubulin antibody (lower panel). Full scan of western blot shown in Figure 5d analyzed with Cyclin B1, Xkid and CPEB antibodies.