Enforcing temporal control of maternal mRNA translation during oocyte cell-cycle progression

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Meiotic cell-cycle progression in progesterone-stimulated *Xenopus* oocytes requires that the translation of pre-existing maternal mRNAs occur in a strict temporal order. Timing of translation is regulated through elements within the mRNA 3' untranslated region (3' UTR), which respond to cell cycle-dependant signalling. One element that has been previously implicated in the temporal control of mRNA translation is the cytoplasmic polyadenylation element (CPE). In this study, we show that the CPE does not direct early mRNA translation. Rather, early translation is directed through specific early factors, including the Musashi-binding element (MBE) and the MBE-binding protein, Musashi. Our findings indicate that although the cyclin B5 3' UTR contains both CPEs and an MBE, the MBE is the critical regulator of early translation. The cyclin B2 3' UTR contains CPEs, but lacks an MBE and is translationally activated late in maturation. Finally, utilizing anti-sense oligonucleotides to attenuate endogenous Musashi synthesis, we show that Musashi is critical for the initiation of early class mRNA translation and for the subsequent activation of CPE-dependant mRNA translation.

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mRNAs encoding cell cycle-control proteins (Freeman *et al*, 1991; Roy *et al*, 1991; Sheets *et al*, 1995; Murakami and Vande Woude, 1998; Ferby *et al*, 1999; Howard *et al*, 1999; Nakajo *et al*, 2000). Translation of mRNAs is classed as early (i.e. occurring before cell division cycle 2 (*cdc2*) activation and oocyte germinal vesicle (nucleus) breakdown (GVBD) or late (i.e. coincident with or after *cdc2* activation and GVBD). The translation of the early class mRNA encoding the Mos proto-oncogene results in MAP kinase activation that contributes to *cdc2* activation and is necessary for the transition from metaphase I to metaphase II (Sagata *et al*, 1988, 1989; Furuno *et al*, 1994; Sheets *et al*, 1995; Gross *et al*, 2000; Dupre *et al*, 2002) (Kosako *et al*, 1994; Gotoh *et al*, 1995; Huang and Ferrell, 1996a; Palmer *et al*, 1998). By contrast, the mRNA encoding cyclin B1 is a late class mRNA, and translational activation requires previous *cdc2* activation (Ballantyne *et al*, 1997; de Moor and Richter, 1997; Howard *et al*, 1999; Barkoff *et al*, 2000).

The timing of translational activation of early and late class mRNAs is enforced by regulatory elements in their 3' untranslated regions (3' UTRs), which are bound by element-specific RNA binding proteins. Two regulatory elements that direct the timing of oocyte mRNA translation are the cytoplasmic polyadenylation element (CPE) and the Musashi/polyadenylation-response element (forthwith referred to as a Musashi-binding element, MBE) (Fox *et al*, 1989; McGrew *et al*, 1989; Charlesworth *et al*, 2002, 2006). The CPE sequence has general consensus U₅AU (Radford *et al*, 2008), although a number of variations have been documented (Charlesworth *et al*, 2004; Pique *et al*, 2008). The consensus MBE sequence is (G/A)U_{1–3}AGU (Imai *et al*, 2001).

The roles of CPE and MBE sequences in regulating the temporal order of mRNA translation in response to progesterone-induced signalling pathways have not been fully resolved. A recent study assigned roles for CPEs according to their responsiveness to signalling pathways, where *cdc2*-independent mRNA translation was defined as 'early' and *cdc2*-dependant mRNA translation was defined as 'late' (Pique *et al*, 2008). Using these temporal definitions, an analysis of signalling dependency for translational activation of B-type cyclin mRNAs led to the suggestion that position and arrangement of CPEs within the 3' UTR are the primary determinants of timing of translational activation (Pique *et al*, 2008). Specifically, CPE sequences positioned within 100 nucleotides of the polyadenylation hexanucleotide have been proposed to direct early translational activation of the cyclin B5 and cyclin B2 mRNAs, whereas a CPE which overlaps the polyadenylation hexanucleotide acts dominantly to direct late translation of the cyclin B1 and B4 mRNAs. These results were extrapolated to a generalized model for predicting the timing and extent of translation of CPE-containing mRNAs in *Xenopus* and potentially in other organisms (Pique *et al*, 2008; Richter, 2008). However, these conclusions are at odds with previous findings that indicate the early translational activation of multiple

Introduction

Meiotic cell-cycle progression in *Xenopus laevis* oocytes requires a strict temporal order of translation of pre-existing

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early class mRNAs may be independent of CPE sequences (Charlesworth *et al*, 2004, 2006).

Analysis of translation of the mRNA encoding the Mos proto-oncogene occupies the center of discussion on temporal control of maternal mRNA recruitment. Early translational activation of the Mos mRNA is essential for progression through the oocyte meiotic cell cycle (Sagata *et al*, 1988; Sheets *et al*, 1995; Dupre *et al*, 2002) and for the subsequent translation of late class mRNAs (Ballantyne *et al*, 1997; de Moor and Richter, 1997). As the Mos mRNA 3' UTR contains both a MBE and a CPE, initiation of Mos mRNA translation has been variously ascribed to both CPE-dependant and Musashi-dependant mechanisms. A role for CPE-directed control was proposed based on indirect evidence from experiments employing a dominant inhibitory form of the CPE-binding protein (Mendez *et al*, 2000) and inferred from computational predictions based on the CPE combinatorial code model (the Mos 3' UTR contains a single CPE in an 'early' configuration, (Pique *et al*, 2008)). However, mutational disruption of the Mos CPE does not affect the timing of Mos mRNA translational activation. Rather, the MBE in the Mos 3' UTR was shown to be critical for Mos mRNA translational activation (Charlesworth *et al*, 2002). Consistent with a Musashi-dependant regulatory mechanism, a dominant inhibitory form of Musashi prevents the translational induction of Mos and several additional early class mRNAs that contain both MBE and CPE sequences (Charlesworth *et al*, 2006). These findings suggest that although their 3' UTRs appear to satisfy the requirements of the proposed early CPE combinatorial code, the early activation of these mRNAs is actually directed through the MBE.

However, a role for Musashi in directing early class mRNA translational activation has come under question because the presence of a MBE is not *per se* an indicator of early mRNA translation as MBE sequences are also present in multiple late class mRNAs (Charlesworth *et al*, 2006). In this study we have re-evaluated the role and requirement for Musashi in controlling the timing of CPE-independent and CPE-dependant mRNA translation. Utilizing direct analyses of the timing of activation of endogenous B-type cyclin mRNAs, we have tested the prediction of the CPE combinatorial code model. We found that the CPE combinatorial code does not correctly predict the temporal translational activation of the cyclin B2 mRNA and although the cyclin B5 mRNA is translationally activated early as predicted by the CPE code, this activation is independent of the CPEs in the cyclin B5 3' UTR. Further, we demonstrate that Musashi is necessary to mediate the initial progesterone 'trigger' pathway independently of CPEB and that early Musashi-dependant mRNA translation is required for subsequent CPE-dependant mRNA translation. We propose a model in which oocyte cell-cycle progression is controlled through early MBE-directed mRNA translation followed by CPE-directed translation.

Results

The CPE combinatorial code does not predict the timing of cyclin B2 mRNA translational activation

A direct analysis of the timing of translational activation of mRNAs containing specific CPE 'code' sequences has not been carried out. We thus analyzed the timing of endogenous Mos, cyclin B5, cyclin B2 and cyclin B1 mRNA polyadenyl-

ation following progesterone stimulation (where extension of the 3' UTR polyadenylate tail is indicative of translational activation (Rosenthal *et al*, 1983; Dworkin *et al*, 1985; Vassalli *et al*, 1989)). The Mos and cyclin B5 mRNAs were polyadenylated early after progesterone stimulation (before GVBD), as predicted by the CPE-combinatorial code model (Figure 1A). Despite Mos and cdc2-independent regulation (Pique *et al*, 2008 and Supplementary Figure S1), the cyclin B2 mRNA exhibited late class translational activation that was indistinguishable from the established late class cyclin B1 mRNA profile (Figure 1A). The late timing of the cyclin B2 mRNA reported here is, thus, contrary to the CPE code prediction, but is consistent with an earlier independent study (Sheets *et al*, 1994).

We noted that the cyclin B5 mRNA, like the early class Mos mRNA, contained an MBE in addition to an 'early' CPE arrangement. In the case of the Mos mRNA, the MBE, rather than the CPE, has been previously demonstrated to be the critical determinant of early translational activation (Charlesworth *et al*, 2002). When examined in the context of a reporter mRNA, an MBE directs early polyadenylation (before GVBD), whereas a single CPE (in a predicted 'early' configuration, Pique *et al*, 2008) directs late polyadenylation coincident with the completion of GVBD (Figure 1B). We thus hypothesized that the MBE directed early translational activation of the cyclin B5 mRNA.

Early translational activation directed by cyclin B5 3' UTR requires the MBE

To test the role of the MBE in the early translation activation of cyclin B5 mRNA, we utilized reporter mRNAs fused to either the wild-type cyclin B5 mRNA 3' UTR (cyclin B5 wt) or a mutant cyclin B5 3' UTR (cyclin B5 mbm) containing a disruption in the MBE that attenuates Musashi binding (Charlesworth *et al*, 2006). It is important to note that the MBE mutation was engineered to leave the adjacent CPE intact within the cyclin B5 3' UTR (Figure 2A and Supplementary Figure S2). Functional integrity of the CPE sequences in the MBE-disrupted cyclin B5 3' UTR was demonstrated by showing that both the wild-type and MBE-disrupted cyclin B5 3' UTR were able to compete for interaction with the CPE-binding protein, CPEB, for a labelled CPE-containing cyclin B1 probe (Figure 2B). As expected, a CPE-disrupted cyclin B5 3' UTR was not able to compete for CPEB interaction. Incubation with the GST moiety alone (expressed at equivalent levels to GST-CPEB, Figure 2C) did not result in specific complex formation (Figure 2B).

When assessed directly in a polyadenylation assay, the wild-type cyclin B5 3' UTR directed robust polyadenylation (Figure 2D), with significant polyadenylation observed before the completion of GVBD (Figure 2D, 6 h time point, GVBD—lane). By contrast, disruption of the MBE in the cyclin B5 3' UTR dramatically attenuated progesterone-stimulated polyadenylation (Figure 2D). We confirmed that the progesterone-stimulated polyadenylation directed by the cyclin B5 3' UTR results in mRNA translational activation. When fused to a Firefly luciferase-coding region, wild-type cyclin B5 3' UTR exerted about a three-fold induction of translation in response to progesterone (Figure 2E). Disruption of the polyadenylation hexanucleotide in the cyclin B5 3' UTR mitigated the majority of the progesterone-dependant induction of translation (Figure 2E). Taken together, these results indicate

Figure 1 The cytoplasmic polyadenylation element (CPE) combinatorial code does not correctly predict cyclin B2 mRNA polyadenylation. **(A)** Oocytes treated with or without progesterone for the indicated times were analyzed for initiation of polyadenylation of multiple endogenous mRNAs by RNA ligation-coupled PCR from the same cDNA preparation at each time point. Oocytes reached GVBD₅₀ (see Materials and methods) at 5.5 h and were segregated into those that had not (–) or had (+) completed GVBD. An increase in the size of the mRNA population is indicative of polyadenylation (bracketed). An asterisk indicates when polyadenylation of each mRNA initiated as evidenced by a shift in mRNA population size above the basal size in immature oocytes (indicated by a dotted line). On the right of the panel, a schematic representation of each mRNA 3′ untranslated region (UTR) shows the position of consensus Musashi-binding sites (solid black square), consensus CPEs (white circle), non-consensus CPEs (diagonal bar circle) and consensus polyadenylation hexanucleotides (grey hexagon). **(B)** β-globin reporter 3′ UTRs were generated by inserting a consensus Musashi-binding site (solid black square) or a consensus CPE (white circle) 5′ of the polyadenylation hexanucleotide (grey hexagon). Separate pools of oocytes were injected with the indicated mRNA reporter constructs, incubated for approximately 16 h and then left untreated or stimulated with progesterone. When 50% of the progesterone-treated oocyte population reached GVBD, oocytes were segregated based on whether they had (+ WS) or had not (–WS) completed GVBD and RNA isolated. RNA was also isolated from time matched immature oocyte samples. The polyadenylation status of the reporter mRNAs analyzed by RNA-ligation coupled PCR using appropriate forward primers. It should be noted that in the absence of inserted elements, the β-globin 3′ UTR does not undergo progesterone-dependant polyadenylation (Hyman and Wormington, 1988). The experiment was repeated three times with identical results.

that the MBE directs progesterone-dependant polyadenylation of the cyclin B5 3′ UTR and consequent progesterone-stimulated translational induction. Consistent with this interpretation, a direct comparison of reporter mRNAs fused to wild-type or MBE-disrupted mutant cyclin B5 3′ UTRs indicated that the MBE mediates translational activation in response to progesterone stimulation (Figure 2F). We note that the cyclin B5 mbm reporter did undergo limited polyadenylation and translation in response to progesterone (Figure 2D and F). A modest general progesterone-dependant increase in basal translation of reporter mRNAs lacking either CPE or MBE sequences has been previously observed (Charlesworth *et al*, 2002), which likely contributes to the residual regulation observed in the cyclin B5 mbm reporter. In addition, the MBE mutation may not completely inhibit Musashi regulation of cyclin B5 3′ UTR (see Figure 3C).

Antisense knockdown of Musashi blocks the early translational activation of endogenous cyclin B5 mRNA

To confirm that Musashi mediates the early translational activation of the endogenous cyclin B5 mRNA, we utilized

antisense oligonucleotide attenuation of endogenous Musashi mRNA translation. There are two isoforms of Musashi in *Xenopus*: Musashi1 (xMsi1, also known as Nrp1A/B) and Musashi2 (xMsi2, also known as Xrp1) (Good *et al*, 1993; Sakakibara *et al*, 2001). The Musashi1 mRNA appears to be the predominant form and is expressed at levels at least five-fold higher than the Musashi2 mRNA (Good *et al*, 1993). Injection of antisense oligonucleotides targeting both xMsi1 and xMsi2 mRNAs (Msi1/2 AS) inhibited progesterone-stimulated maturation of oocytes (Figure 3A). Musashi has been previously shown to control early translation of the Mos mRNA (Charlesworth *et al*, 2006). Consistent with loss of Mos mRNA translation, the downstream signalling pathways of MAP kinase and cdc2 were not activated in Msi1/2 AS injected oocytes (Figure 3B). It is interesting to note that the use of either Musashi1 or Musashi2 antisense oligonucleotides alone delayed rather than inhibiting oocyte maturation (Supplementary Figure S4), thus suggesting that like mammalian Musashi, each isoform can partially compensate for the loss of the other (Sakakibara *et al*, 2002). No inhibition of oocyte maturation or cellular

Figure 2 Early translational activation of the cyclin B5 3' untranslated region (UTR) requires a Musashi-binding element (MBE). (A) Schematic showing the regulatory elements of cyclin B5 3' UTR fused to a Firefly luciferase reporter (see legend to Figure 1 for symbol definitions). The β -globin 3' UTR lacks progesterone-responsive elements and so serves as an unregulated control luciferase reporter mRNA. (B) RNA EMSA utilizing a biotin-labelled cyclin B1 3' UTR and reticulocyte expressing either the GST moiety alone or GST fused to the C-terminal RNA binding domain of CPEB (Δ N-CPEB). A specific cyclin B1 RNA binding complex is formed with Δ N-CPEB but not GST (arrowhead). This complex can be effectively competed with unlabelled wild-type (wt) cyclin B5 3' UTR and the Musashi-binding mutant form of the cyclin B5 3' UTR (mbm). By contrast, no competition for Δ N-CPEB binding is seen with a cytoplasmic polyadenylation element (CPE)-disrupted cyclin B5 3' UTR (CPE mut). (C) GST Western blot demonstrates equivalent levels amount of GST and GST- Δ N-CPEB protein expression in the reticulocyte lysates used in (B). (D) Wild-type (wt) or Musashi-binding mutant (mbm) cyclin B5 3' UTRs were fused to a GST open reading frame and the resulting transcribed RNA injected into immature oocytes. The polyadenylation status of the reporter RNAs was assessed by RNA ligation PCR where an increase in the size of the mRNA population is indicative of polyadenylation (bracketed). Imm, immature oocytes. Whether oocytes had (+) or had not (–) completed GVBD is indicated above each time point. (E) Oocytes were co-injected with a Renilla luciferase mRNA and a Firefly luciferase RNA under the control of either the wild-type (wt) or polyadenylation hexanucleotide mutant cyclin B5 UTR and incubated for 16 h before progesterone treatment. When the oocytes reached GVBD₅₀, oocytes were segregated based on whether they had or had not completed GVBD. For these experiments, three pools of five oocytes were harvested for each analysis from the oocytes, which had not yet completed GVBD (and so are considered to be in the early phases of maturation). In addition, three pools of five oocytes were harvested from time matched immature oocyte samples. The triplicate sets of Firefly:Renilla ratios of the cyclin B5 UTR constructs from progesterone-stimulated oocytes were normalized to the Firefly:Renilla ratios for the same 3' UTR constructs in immature oocytes (ratio P:I). The data presented are the mean $\pm$ SEM from three independent experiments. (F) Oocytes were co-injected with a Renilla luciferase mRNA and a Firefly luciferase RNA under the control of either the wild-type (wt) or Musashi binding mutant (mbm) cyclin B5 UTR. Luciferase analyses were performed as described in (E) and the data are presented from two independent experiments with the SEM shown for each individual experiment. We confirmed that the reporter mRNAs were expressed to similar levels (Supplementary Figure S3).

signalling was observed using an equivalent level of control antisense oligonucleotides that do not target Musashi1 or Musashi2 mRNAs (Figure 3A and B).

To address the point of action of the antisense Msi1/2 oligonucleotides during progesterone stimulated oocyte maturation we examined additional early and late signalling

events. In response to progesterone, Ringo mRNA translation occurs very rapidly, with endogenous Ringo protein accumulation detectable 30–60 min after stimulation (Gutierrez *et al*, 2006; Padmanabhan and Richter, 2006). We found that the accumulation of the endogenous Ringo protein was not affected in Msi1/2 antisense oligonucleotide injected oocytes

Figure 3 Antisense oligonucleotides targeting endogenous Musashi1 and Musashi2 mRNAs inhibit oocyte maturation. (A) Immature oocytes were injected with water, 200 ng control antisense oligonucleotide (Control AS) or a combination of 100 ng xMsi1 and 100 ng xMsi2 antisense oligonucleotides (xMsi1 + xMsi2 AS) and incubated for 16 h. The oocytes were then stimulated with progesterone and GVBD scored over the course of the experiment. (B) Pools of 10 oocytes from each experimental condition in panel (A) were harvested when control AS-injected oocytes reached GVBD₅₀. Time-matched immature and progesterone-treated samples were analyzed for MAP kinase and cdc2 activation. In these analyses, phosphorylated MAP kinase (pMAPK) indicates activation and loss of phosphorylated (and inhibited) cdc2 (pcdc2) indicates activation. Ringo and CPEB protein levels were also assessed in the same samples by western blotting, with tubulin serving as an internal control for protein loading. The data are representative of three separate experiments. (C) Oocytes were injected with water, 200 ng control antisense oligonucleotide (AS), or a combination of 100 ng xMsi1 and 100 ng xMsi2 AS and incubated for 16 h before progesterone stimulation. Pools of 10 oocytes were harvested for each condition when control AS-injected oocytes reached GVBD₅₀. Similar to (A), the xMsi1/2 AS-injected oocytes failed to mature in response to progesterone stimulation. The polyadenylation of endogenous cyclin B5, Mos, TATA BP2, cyclin B2 and cyclin B1 mRNAs was determined using RNA ligation-coupled PCR from the same cDNA preparations at each time point. Progesterone-dependant polyadenylation of each mRNA is indicated (bracket). The experiment was repeated three times with similar results.

(Figure 3B). We conclude that progesterone-stimulated Ringo mRNA translation is independent of Musashi function. Further, this finding indicates that Musashi antisense oligonucleotide injections did not adversely affect general mRNA translation in the oocyte. We also assessed the levels of CPEB in the antisense oligonucleotide injected oocytes. CPEB protein is expressed in immature oocytes, but undergoes cdc2-dependant phosphorylation at GVBD which targets a proportion of the protein population for proteosomal degradation (Reverte *et al*, 2001; Mendez *et al*, 2002). This CPEB degradation is linked to the activation of late class CPE-dependant mRNAs that contain a CPE overlapping the polyadenylation hexanucleotide (e.g. cyclin B1; Mendez *et al*, 2002). In contrast to water and control antisense oligonucleotide injected oocytes that demonstrated CPEB destruction at GVBD (Figure 3B), the injection of Msi1/2 antisense oligonucleotides prevented CPEB destruction and CPEB levels

were equivalent irrespective of whether the oocytes were or were not stimulated with progesterone.

Our findings suggest that the CPEB protein present in the Musashi antisense oligonucleotide injected oocytes is unable to mediate oocyte maturation or CPE-dependant mRNA translational activation when Musashi function is attenuated. Consistent with this hypothesis, endogenous polyadenylation of the late class cyclin B1 and B2 mRNAs was blocked in the Msi1/2 antisense injected oocytes (Figure 3C). Similarly, the polyadenylation of the MBE-regulated early class mRNAs Mos and cyclin B5 (Figure 2) was blocked as expected, as was the polyadenylation of the MBE-containing early class TATA BP2 mRNA which lacks a functional CPE (Charlesworth *et al*, 2006). The effective inhibition of endogenous cyclin B5 mRNA polyadenylation in Musashi antisense oligonucleotide injected oocytes suggests that the residual polyadenylation and translation in the cyclin B5 mbm reporter (Figure 2) may

be a consequence of incomplete attenuation of Musashi function by the introduced 3' UTR MBE mutation.

To address the specificity of the Msi1/2 antisense inhibition of oocyte maturation, we attempted to rescue the inhibition of GVBD and cellular signalling events by injecting an mRNA encoding wild-type *Xenopus* Musashi fused to an N-terminal GST moiety (GST-Msi). As can be seen in Figure 4A, injection of GST-Msi1 efficiently rescued the ability of Msi1/2 antisense injected oocytes to undergo GVBD in response to progesterone. In addition to the extent of rescue, wild-type Musashi1 also rescued normal kinetics of maturation (Supplementary Figure S5). By contrast, an RNA-binding deficient form of *Xenopus* Musashi1 (GST-Msi bm; Charlesworth *et al*, 2006) was unable to effect a rescue of

oocyte maturation (Figure 4A, Msi1/2 + Msi bm) indicating that Musashi must be able to interact with target mRNAs to mediate cell-cycle progression to GVBD. Similarly, overexpression of a GST-CPEB protein was unable to rescue the maturation defect (Figure 4A). Likewise, only the wild-type GST-Musashi protein was able to generate high MAP kinase activation and downstream cdc2 activation in Msi1/2 injected oocytes (Figure 4B). The lower level of Msi1/2 antisense oligonucleotides used in this experiment was less effective at suppressing MAP kinase signalling (compared with Figure 3B). Nonetheless, the lower levels of Msi1/2 antisense oligonucleotides did still exert efficient inhibition of oocyte maturation (Figure 4A). These findings are in agreement with previous studies that have observed a threshold of

Figure 4 Musashi, but CPEB, controls the activation of early class mRNAs and enforces temporal regulation of maternal mRNA polyadenylation and translation. (A) Oocytes were separately injected with 100 ng control antisense oligonucleotide (AS) or a combination of 50 ng xMsi1 and 50 ng xMsi2 AS and incubated for 16 h before progesterone stimulation. The injected oocytes were then split into pools and left untreated (Con AS and Msi AS) or re-injected with the indicated GST fusion proteins: wild-type Musashi1 (GST-Msi); RNA binding deficient mutant Musashi (GST-Msi bm) or CPEB (GST-CPEB). After addition of progesterone, oocyte GVBD was assessed after 7 h. The data represent three independent experiments with the SEM indicated. UI, uninjected oocytes treated with progesterone. (B) Lysates from the experiment in (A) were assessed for expression of the GST fusion proteins (upper panel). The anticipated full length 70 kD wild-type (wt) and RNA binding deficient mutant (bm) Musashi and 90 kD CPEB proteins were detected at similar levels. Minor CPEB breakdown products were also observed in these samples. Active MAPK kinase (pMAPK) and inactive cdc2 (pcdc2) were assessed as described in the legend to Figure 3. In these experiments, some active MAPK was detected in Msi AS injected oocytes, but the levels were below the enhanced level seen in control that had completed GVBD. No activation of cdc2 was observed in Msi AS or Msi AS oocytes expressing Msi bm or CPEB. A tubulin western blot of the same lysates illustrates equivalent loading of protein in all lanes. Whether the oocytes did (+) or did not (-) complete GVBD after progesterone stimulation is indicated. (C) Total RNA was prepared from pools of five oocytes harvested from immature or progesterone-stimulated samples described in (A) above when control AS-injected oocytes reached GVBD₅₀. Progesterone-stimulated oocytes were segregated based on whether they had or had not completed GVBD (+ and -, respectively). The polyadenylation of endogenous cyclin B5 and cyclin B1 mRNAs was determined using RNA ligation-coupled PCR from the same cDNA preparations at each time point.

progesterone-stimulated Mos accumulation and MAP kinase activation necessary for cell-cycle progression (Huang and Ferrell, 1996b; Chen and Cooper, 1997; Chen *et al*, 1997) and indicate that the Musashi1/2 antisense oligonucleotides function in a dose-dependant manner to suppress Mos mRNA translation and subsequent MAP kinase activation. The Musashi1/2 antisense oligonucleotides only reduced cellular Musashi1 protein levels by about 20% ($\pm 4.24\%$ s.d.; $n = 2$, Supplementary Figure S6), in progesterone stimulated oocytes. We have not been able to definitively ascertain the effect of the antisense oligonucleotides on the minor Musashi2 protein isoform because of the inadequacy of available antibodies. Our findings suggest that Musashi function and oocyte cell-cycle progression is sensitive to a critical threshold of Musashi, akin to Mos and MAP kinase activation described above. Alternatively, newly synthesized Musashi rather than pre-existing Musashi protein may be critical for early translational activation in response to progesterone stimulation. Further experiments will be required to address these issues.

Expression of the wild-type form of Musashi (but not the RNA binding mutant or the CPEB protein) also rescued endogenous cyclin B5 and cyclin B1 polyadenylation (Figure 4C). Of particular importance, the wild-type Musashi protein was specifically able to re-establish the correct temporal order of endogenous mRNA polyadenylation in Musashi antisense oligonucleotide injected oocytes. With the expression of wild-type Musashi, cyclin B5 mRNA polyadenylation occurred before GVBD (Figure 4C, Msi AS + Msi WT GVBD–), whereas late class cyclin B1 mRNA polyadenylation only occurred after GVBD (Figure 4C, Msi AS + Msi WT GVBD+). By contrast, overexpression of CPEB in Musashi antisense oligonucleotide injected oocytes failed to rescue either early (cyclin B5) or late (cyclin B1) mRNA polyadenylation. The expression of wild-type Musashi was also able to rescue the early polyadenylation of the Mos mRNA in Msi AS treated oocytes (Supplementary Figure S7). We conclude that Musashi is the critical mediator of early class mRNA translational activation in response to progesterone, and that Musashi function is necessary for the subsequent CPE-dependant mRNA translation.

A distinct hierarchy of mRNA regulatory pathways control temporal activation during oocyte maturation

The profound effect of attenuation of Musashi function upon GVBD (Figures 3 and 4), suggests that Musashi has a fundamental role in mediating the cell-cycle progression during oocyte maturation. Towards an understanding of the mechanism by which Musashi regulates cell-cycle progression we noted that in addition to attenuating early MBE-regulated mRNAs, Musashi antisense oligonucleotide injection also blocked late, CPE-dependant mRNA polyadenylation (e.g. cyclin B1). To rescue CPE function in Musashi antisense oligonucleotide injected oocytes, we expressed cyclin B $\Delta 87$ protein (a non-degradable form of human cyclin B1 (Kumagai and Dunphy, 1995)) to activate cdc2 independently of upstream progesterone-dependant processes. Activation of cdc2, through injection of cyclin B1 protein, has been shown to stimulate CPE-directed mRNA translational activation (de Moor and Richter, 1997). Injection of cyclin B $\Delta 87$ rescued both GVBD and cdc2 activation (Figure 5A), thus indicating that the Msi1/2 AS acts at a point upstream of cdc2

activation. Furthermore, CPEB degradation, an event linked to late class CPE-dependant mRNA activation (Mendez *et al*, 2002), occurred in cyclin B $\Delta 87$ -injected, Msi1/2 AS treated oocytes (Figure 5A). Consistent with this observation, cyclin B $\Delta 87$ was able to induce polyadenylation of the late class cyclin B1 mRNA in Msi1/2 AS oocytes (Figure 5B) although the extent of this polyadenylation was less than that observed in control oocytes. By contrast, injection of cyclin B $\Delta 87$ protein did not rescue MBE-regulated Mos or cyclin B5 mRNA polyadenylation, nor late class cyclin B2 mRNA polyadenylation (Figure 5B).

Discussion

Our analysis indicates that a complex mechanism regulates the temporal order of mRNA translation during oocyte cell-cycle progression. We report that both 'early' cdc2-independent and late cdc2-dependant CPE-mediated mRNA translation require previous Musashi-dependant mRNA translation. Utilizing timing of translation of B-type cyclins, we demonstrate that neither of the two cyclin mRNAs used to form the basis for defining 'early' CPE-directed mRNA translational activation fulfills this role (Pique *et al*, 2008). Although the cyclin B2 mRNA contains an 'early' CPE configuration it is not actually activated early but rather shows kinetics of activation that are indistinguishable from the established late class cyclin B1 mRNA, which occurs coincidentally with GVBD ((Sheets *et al*, 1994; de Moor and Richter, 1997; Barkoff *et al*, 2000; Charlesworth *et al*, 2002), see Figure 1B). The cyclin B5 mRNA also contains an 'early' CPE configuration and would apparently fit with the prediction of the CPE combinatorial code. However, mutational analysis of cyclin B5 mRNA 3'UTR and use of Musashi antisense oligonucleotides indicates that Musashi and the MBE, rather than the CPEs, directs early cyclin B5 translational activation (see Figures 2–4).

Previous studies have established that the temporal activation of the Mos mRNA serves as an exemplar of early class mRNA regulation. The Mos mRNA 3' UTR, like the cyclin B5 UTR, contains both a CPE and MBE. Similar to the behaviour of the CPEs within the cyclin B5 mRNA demonstrated in this study, the CPE has been shown to be irrelevant to the initiation of Mos mRNA translation which is instead directed through the MBE (Charlesworth *et al*, 2002, 2006). CPEB function is required subsequently for enhanced Mos protein accumulation as oocytes complete GVBD (Charlesworth *et al*, 2002). In addition to the Mos mRNA, a number of other early class mRNAs have also been shown to undergo CPE-independent regulation (Charlesworth *et al*, 2004). Although the 'early' CPE combinatorial code appears to correlate with the translation of these early class mRNAs, the underlying control of translational initiation is nonetheless CPE independent (Charlesworth *et al*, 2004, 2006). Taken together, we propose that the early CPE combinatorial code is *permissive* for early translational activation but is not instructive for this process.

Pique *et al* (2008) proposed that a 'late' CPE arrangement (a CPE overlapping the 3' UTR polyadenylation hexanucleotide) predicts both dependancy on Mos/cdc2 signalling and late translational activation. The late translational activation of mRNAs containing a CPE overlapping the polyadenylation hexanucleotide is consistent with earlier studies that suggested this arrangement defined late class mRNAs

Figure 5 A regulatory hierarchy governs activation of early and late class mRNAs. Oocytes were injected with water or antisense oligonucleotides (AS) as indicated and incubated for 16 h before Cyclin B Δ87 protein injection. Injection of cyclin B Δ87 induced cdc2 activation and GVBD in water, control AS- and Musashi AS-injected oocytes. In these experiments, GVBD₅₀ occurred 2–3 h after injection. Cyclin B Δ87 injected oocytes were harvested when all the oocytes had completed GVBD and compared with samples prepared from time matched, mock-injected oocytes (cyclin B Δ87 + or – as indicated). **(A)** Lysates were analyzed for cdc2 and CPEB activation status by western blotting (see Figure 3 legend). A tubulin western blot of the same samples serves as an internal loading control. **(B)** Polyadenylation of endogenous mRNAs (brackets) was assessed by RNA ligation-coupled PCR of the same oocyte samples used in **(A)**. An increase in size of the PCR products is indicative of polyadenylation. **(C)** A model illustrating the regulatory roles of the Musashi-binding element (MBE) and the cytoplasmic polyadenylation element (CPE) in establishing the temporal order of maternal mRNA translation. Musashi is necessary for both early class, CPE-independent mRNA translational activation as well as for subsequent CPE-dependant mRNA translation. See text for details.

(Charlesworth *et al*, 2004; Tung *et al*, 2007). This ‘late’ CPE configuration is non-permissive for early translational activation, even in the presence of the early acting MBE, as demonstrated by the insertion of a ‘late’ CPE configuration in the cyclin B5 3’ UTR that resulted in late (rather than early) translational activation (Pique *et al*, 2008). Precedence for a dominant inhibitory effect of CPE sequences that overlap the polyadenylation hexanucleotide has been observed with the attenuation in *cis* of the early class translational control sequence (TCS) regulatory element in the Wee1 mRNA 3’ UTR (Wang *et al*, 2008). This dominant inhibitory effect of ‘late’ configuration CPEs is presumably mediated through the CPEB protein blocking access of the multisubunit cytoplasmic polyadenylation specificity factor (CPSF) complex or other factors to the polyadenylation hexanucleotide before cdc2-mediated degradation of CPEB (Mendez *et al*, 2002). The presence of a CPE overlapping the polyadenylation hexanucleotide and a consequent dominant inhibitory effect of CPEB, may explain why other mRNAs that contain MBE sequences (e.g. cyclin A1 and cyclin B1 mRNAs) are activated late in maturation. Although, repositioning of the CPE that overlaps the polyadenylation nucleotide in the cyclin B1 mRNA 3’ UTR to change it from a late to an ‘early’ config-

uration did indeed result in cdc2-independent translational activation (Pique *et al*, 2008), our analyses of the Mos and cyclin B5 mRNAs would suggest that the MBE in the cyclin B1 3’ UTR directs the early translational activation of the modified cyclin B1 mRNA. As discussed above, the new ‘early’ CPE configuration in the mutant cyclin B1 UTR would be permissive for MBE-directed early translational activation.

We propose a fundamentally different model to predict the timing of mRNA translational activation during oocyte cell-cycle progression. This model defines a cdc2-independent CPE configuration as permissive for early activation and incorporates our current findings for MBE-dependant regulation of early class mRNA translation (Figure 5C). In this model, progesterone triggers an initiating signalling pathway that leads to Musashi activation. Our observation that the early progesterone-dependant translational activation of the endogenous Ringo mRNA was not inhibited in Musashi antisense oligonucleotide-injected oocytes (Figure 3) indicates that the control of Ringo mRNA translation and Ringo proteolytic processing (Gutierrez *et al*, 2006; Padmanabhan and Richter, 2006) are independent of Musashi-dependant mRNA translation. Given the timing of Ringo translation it is conceivable that rather than acting in parallel to Musashi

Once activated, Musashi leads to the translational activation of MBE-regulated early class mRNAs, including *Mos* and cyclin B5, independent of *cdc2* and CPE-directed mRNA translational activation. The CPEs present in these early class mRNAs appear to be unable to initiate translational activation in the absence of Musashi function. Indeed, activation of *cdc2* by injection of cyclin B $\Delta 87$ was not able to rescue *Mos* or cyclin B5 polyadenylation in the presence of Musashi antisense oligonucleotides (Figure 5). It is likely that these CPEs function primarily to mediate repression of the early class mRNAs in immature oocytes. The translation of Musashi-dependant early class mRNAs then leads to the subsequent activation of CPE-dependant late class mRNAs (Figure 5C).

Repression of mRNA translation in immature oocytes is mediated, to a large extent, by CPEs (Radford *et al*, 2008). However, we propose a model wherein the subsequent progression of progesterone-stimulated events resulting in selective mRNA translational activation (Figure 5C) are quite distinct from a commonly accepted pathway of signal transduction leading from progesterone stimulation to Aurora A activation to CPEB activation (by S174 phosphorylation) to Mos mRNA translation and then MAP kinase and cdc2 activation (Mendez *et al*, 2000). Although Aurora A activation was initially proposed to be an early event during oocyte cell-cycle progression (Andrsson and Ruderman, 1998; Mendez *et al*, 2000), a number of studies have questioned this assessment. Indeed, Aurora A activation may instead occur at or after GVBD and as such preclude a role in the activation of CPEB before GVBD (Frank-Vaillant *et al*, 2000; Castro *et al*, 2003; Maton *et al*, 2003). Consistent with the late activation of Aurora A, the inhibition of Aurora A did not delay the early oocyte cell-cycle progression to GVBD (Castro *et al*, 2003; Keady *et al*, 2007). Rather than requiring Aurora A activity, the early CPEB activation-mediated phosphorylation on S174 has been shown to be MAP kinase-dependant (Keady *et al*, 2007). By incorporating the findings described in this study, we propose that progesterone induces Musashi-dependant

Materials and methods

Detailed methodologies employed are described in the Supplemental Materials that accompany this paper.

Xenopus oocytes were isolated and cultured as described previously (Machaca and Haun, 2002). Oocytes were induced to mature with 2 µg/ml progesterone, or by cyclin B Δ87 protein injection as described previously (Howard *et al*, 1999). The rate of GVBD was scored morphologically by observing the appearance of a white spot on the animal pole. Animal protocols were approved by the UAMS Institutional Animal Care and Use committee, in accordance with Federal regulations.

Oocytes were injected with 0.1 ng of Firefly luciferase mRNA and 0.35 μ g of Renilla luciferase control mRNA, and incubated at 18°C for 18 h. Three pools of five oocytes were lysed for each experimental point in 50 μ l of passive lysis buffer (Promega). A 10 μ l portion of lysate was analyzed for Renilla and Firefly luciferase activity using the Dual-Luciferase Assay System (Promega) on a TD-20/20 Turner Designs luminometer. Luciferase activity was measured as a ratio of Firefly/Renilla activity.

Glutathione *S*-transferase fusion proteins were *in vitro* transcribed/translated using TNT SP6-coupled Reticulocyte Lysate System (Promega). 5' biotin-labelled cyclin B1 RNA oligonucleotide probe (5'-Biotin-cuguaaaaguguaauuguguuuuaauguuuacugguuuaaagc-3') was synthesized by Integrated DNA Technologies. Unlabelled competitor mRNAs were transcribed *in vitro*. An 80 fmol portion of labelled probe was incubated with 1 µl of reticulocyte lysate and 50-fold molar excess of unlabelled mRNA in binding buffer (10 mM HEPES pH 7.7, 100 mM KCl, 1 mM MgCl₂, 10 mM DTT, 20 mM ZnCl₂, 50 g/ml tRNA, 0.1% BSA, 5% glycerol, 0.25% NP40 (Charlesworth *et al*, 2000)) in a final volume of 20 µl. The binding reaction was incubated at room temperature for 20 min and then 1 µl of 20 mg/ml heparin was added and incubated for a further 20 min. A 5 µl volume of the binding reaction was run on a 6% DNA retardation gel (Invitrogen) and transferred to Biotodyne B membranes (Pierce) according to the manufacturer's instructions. Biotinylated RNA was detected using Chemiluminescent Nucleic Acid Detection Module (Pierce) according to the manufacturer's directions. Image collection was carried out using an AlphaInnotech Chemilmager.

cDNAs for polyadenylation assays were synthesized using RNA ligation-coupled PCR as described previously (Charlesworth *et al.*, 2004). The increase in PCR product length is specifically due to extension of the poly[A] tail (Charlesworth *et al.*, 2002, 2004). A detailed description of primers employed and experimental conditions is provided in the Supplemental data.

cDNAs for semi-quantitative PCR analysis were synthesized using RNA ligation-coupled RT-PCR as described previously (Charlesworth *et al*, 2004). For the PCR reaction, 2 μ l of cDNA was used with 3 mM MgCl₂, 1 mM Firefly luciferase primers

(forward: 5'-GAGTACTTCGAGATGAGC-3', reverse: 5'-CACGAAGTC GTACTCGTT-3') and 0.25 mM cyclin B1 primers (forward: 5'-GGCTT GAGACCTCGTACAGC-3', reverse: 5'-CAGGGAGGCAACCAGATG-3').

Antisense oligodeoxynucleotide injections

Antisense oligodeoxynucleotides 5'-GCGCTTCTGTCTCCATTCGGTC TCT-3' and 5'-CCCATCTGCCTCCATAGCCTTCTC-3' were designed to target endogenous xMsi1 and xMsi2 mRNAs, respectively. For double-knockdown experiments oocytes were injected with both anti-xMsi1 and anti-xMsi2 oligodeoxynucleotides. Oocytes injected with a level of randomized oligonucleotide sequence 5'-TAGAGAAG ATAATCGTCATCTTA-3' (Ferby *et al*, 1999) equivalent to the combined levels of Msi1 and Msi2 oligonucleotides were used as a specificity control. Oocytes were incubated at 18°C for 16 h followed by injection of mRNA encoding GST rescue proteins as indicated and/or progesterone treatment.

Antibodies

Abcam antibodies to Msi1 were used at 1:1000. Sigma Tubulin antibodies were used at 1:20 000. The phospho-specific Cdc2 antibody (Cell Signaling) was used at 1:1000 and detects the inhibitory Tyr15 phosphorylation. The phospho-specific MAP kinase antibody (Cell Signaling) was used at 1:1000 and detects the activating phosphorylations at Thr202/Tyr204. The human CPEB monoclonal antibody and the *Xenopus* Ringo antibody were obtained as generous gifts from Dr Dominique Weil and Dr Angel Nebreda, respectively.

Western blotting

Oocytes were lysed in NP40 lysis buffer containing sodium vanadate and a protease inhibitor cocktail (Sigma). A portion of

the lysate was transferred to STAT-60 for RNA extraction. The lysate was then spun, clarified and transferred immediately to 1 × sample buffer (Nupage). The lysates were run on a 10% Nupage gel and transferred to a 0.2 µm-pore-size nitrocellulose filter (Protran; Midwest Scientific). The membrane was blocked with 1% bovine serum albumin (Sigma) in TBST for 60 min at room temperature. Following incubation with primary antibody, filters were incubated with horseradish peroxidase-conjugated secondary antibody using enhanced chemiluminescence in a Fluorchem 8000 Advanced Imager (Alpha Innotech). Except where indicated, oocyte lysates were utilized both for western blots and polyadenylation assays.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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