The EMBO Journal (2006) 25, 2792–2801 | © 2006 European Molecular Biology Organization | All Rights Reserved 0261-4189/06
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THE EMBO JOURNAL

Musashi regulates the temporal order of mRNA translation during *Xenopus* oocyte maturation

Amanda Charlesworth[1], Anna Wilczynska[1], Prajitha Thampi[1], Linda L Cox[1] and Angus M MacNicol[1,2,*]

[1]Department of Neurobiology and Developmental Sciences, University of Arkansas for Medical Sciences, Little Rock, AR, USA and [2]The Arkansas Cancer Research Center, University of Arkansas for Medical Sciences, Little Rock, AR, USA

A strict temporal order of maternal mRNA translation is essential for meiotic cell cycle progression in oocytes of the frog *Xenopus laevis*. The molecular mechanisms controlling the ordered pattern of mRNA translational activation have not been elucidated. We report a novel role for the neural stem cell regulatory protein, Musashi, in controlling the translational activation of the mRNA encoding the Mos proto-oncogene during meiotic cell cycle progression. We demonstrate that Musashi interacts specifically with the polyadenylation response element in the 3′ untranslated region of the Mos mRNA and that this interaction is necessary for early Mos mRNA translational activation. A dominant inhibitory form of Musashi blocks maternal mRNA cytoplasmic polyadenylation and meiotic cell cycle progression. Our data suggest that Musashi is a target of the initiating progesterone signaling pathway and reveal that late cytoplasmic polyadenylation element-directed mRNA translation requires early, Musashi-dependent mRNA translation. These findings indicate that Musashi function is necessary to establish the temporal order of maternal mRNA translation during *Xenopus* meiotic cell cycle progression.

The EMBO Journal (2006) **25,** 2792–2801. doi:10.1038/sj.emboj.7601159; Published online 8 June 2006
Subject Categories: cell cycle; development
Keywords: cell cycle; cytoplasmic polyadenylation; Mos; *Xenopus*

Introduction

Regulated mRNA translation is emerging as a key mechanism to control temporal and spatial gene expression during numerous cellular and developmental processes including sex determination, anterior–posterior specification of the embryonic body axis, neuronal synaptic plasticity, neural stem cell self-renewal and control of vertebrate oocyte maturation (Kuersten and Goodwin, 2003). Regulatory elements within the 5′ and 3′ untranslated regions (UTRs) of target mRNAs are recognized by sequence-specific RNA protein complexes to mediate translational control (Wilkie *et al*, 2003; Colegrove-Otero *et al*, 2005; de Moor *et al*, 2005). Distinct mRNA translational regulatory pathways may function interdependently to coordinate and effect cellular and developmental decisions (Kuersten and Goodwin, 2003). The molecular mechanisms that effect these precise temporal and/or spatial changes in mRNA translation in response to cellular signal transduction pathways have not been well characterized.

Hormonally induced maturation in oocytes of the frog *Xenopus laevis* requires the temporally regulated translation of maternally derived mRNAs for meiotic cell cycle progression. The translational induction of the mRNA encoding the Mos proto-oncogene is necessary for meiotic cell cycle progression (Sagata *et al*, 1988, 1989; Furuno *et al*, 1994; Sheets *et al*, 1995; Dupre *et al*, 2002) and occurs soon (2–3 h) after exposure to the maturation stimulus, progesterone. In the absence of early Mos mRNA translation and subsequent activation of mitogen-activated protein (MAP) kinase signaling, oocytes exhibit delayed germinal vesicle breakdown (GVBD), meiosis I and fail to transition to meiosis II (Furuno *et al*, 1994; Gross *et al*, 2000; Dupre *et al*, 2002). By contrast, translational induction of the mRNA encoding the Wee1 protein tyrosine kinase occurs later in maturation, coincident with completion of GVBD (Kobayashi *et al*, 1991; Murakami and Vande Woude, 1998; Charlesworth *et al*, 2000). Inappropriate early translation of the Wee1 mRNA blocks activation of maturation promoting factor (MPF) and prevents meiotic cell cycle progression (Murakami and Vande Woude, 1998; Howard *et al*, 1999; Nakajo *et al*, 2000).

It has been recently recognized that the differential timing of *Xenopus* maternal mRNA translation is controlled through distinct 3′ UTR regulatory elements that direct the cytoplasmic polyadenylation of the target mRNAs (Charlesworth *et al*, 2002). The late polyadenylation and translational activation of the Wee1 mRNA is enforced by 3′ UTR cytoplasmic polyadenylation element (CPE) sequences (Charlesworth *et al*, 2000). By contrast, the early polyadenylation and translation of the Mos mRNA is controlled by a 3′ UTR polyadenylation response element (PRE) in a CPE-independent manner (Charlesworth *et al*, 2002). The PRE has been shown to be responsive to MAP kinase signaling (Charlesworth *et al*, 2002), whereas the CPE is responsive to MPF signaling (Paris *et al*, 1991; de Moor and Richter, 1997; Howard *et al*, 1999; Charlesworth *et al*, 2002). As progesterone-stimulated MAP kinase activation precedes MPF activation, the differential responsiveness of the 3′ UTR regulatory elements to these signaling pathways would effect temporal discrimination of maternal mRNA selection (Howard *et al*, 1999; Charlesworth *et al*, 2002).

In contrast to the MAP kinase-dependent feedback (Matten *et al*, 1996; Roy *et al*, 1996; Howard *et al*, 1999; Charlesworth *et al*, 2002), initial Mos mRNA translation is induced in a

*Corresponding author. Department of Neurobiology and Developmental Sciences, The Arkansas Cancer Research Center, Slot 814, University of Arkansas for Medical Sciences, 4301 West Markham, Little Rock, AR 72205, USA. Tel.: +1 501 686 8164; Fax: +1 501 686 6517; E-mail: Angus@UAMS.edu

Received: 1 December 2005; accepted: 28 April 2006; published online: 8 June 2006

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MAP kinase-independent manner (Gross *et al*, 2000) and is a prerequisite for progesterone-stimulated *Xenopus* p42 MAP kinase activation (Roy *et al*, 1996; Dupre *et al*, 2002). Progesterone stimulation leads to a rapid decrease in oocyte cAMP levels (reviewed in Smith, 1989) and PKA activity (Wang and Liu, 2004). Initiation of Mos mRNA translational activation has been shown to be downstream of progesterone-mediated PKA inhibition (Daar *et al*, 1993; Qian *et al*, 2001). The mediators of the progesterone 'trigger' signaling pathway leading to Mos mRNA translational activation have not been elucidated, but presumably regulate a *trans*-acting factor that binds to the PRE in the Mos 3′ UTR. The identity of the *trans*-acting factor controlling early PRE-dependent mRNA translational activation has not been determined.

In this study, we demonstrate that *Xenopus* Musashi is a *trans*-acting PRE binding protein that is necessary for maternal mRNA translational activation in *Xenopus* oocytes. Musashi has been previously shown to be essential for asymmetric cell divisions of *Drosophila* neural progenitor cell populations and in mammalian neural stem cell self-renewal, where it appears to function as an mRNA translational repressor (Okano *et al*, 2002, 2005). Our findings reveal a novel role for Musashi in mediating mRNA translational activation during *Xenopus* oocyte maturation. Inhibition of Musashi function through expression of a dominant inhibitory form of Musashi specifically attenuated maternal mRNA translational activation and oocyte meiotic cell cycle progression in response to progesterone stimulation. We report that Musashi-mediated mRNA translational activation is a prerequisite for the subsequent translation of CPE-dependent mRNAs, indicating a critical role for Musashi in controlling the temporal order of maternal mRNA translational activation.

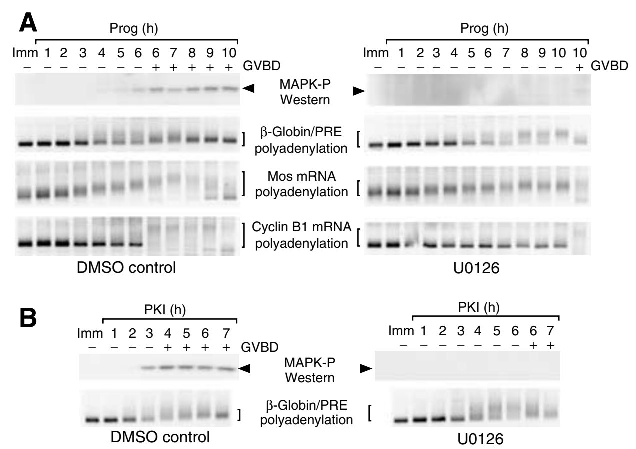

Figure 1 The Mos PRE is responsive to a MAP kinase-independent trigger pathway. (**A**) Immature oocytes were injected with RNA encoding the GST coding region fused to the β-globin 3′ UTR containing the Mos PRE (β-globin/PRE). Oocytes were pretreated where indicated with U0126 and then stimulated with progesterone. Protein and RNA were extracted from the same pools of oocytes at the specified times. Progression of maturation is indicated by whether the oocytes had (+) or had not (−) undergone GVBD. Top panel is a Western blot for active (phosphorylated) MAP kinase (arrowhead). Lysates from DMSO- and UO126-treated oocytes were processed and analyzed in parallel. Lower panels show polyadenylation (brackets) of the endogenous Mos and cyclin B1 mRNAs and the injected synthetic β-globin/PRE reporter mRNA, as assayed by RNA ligation-coupled RT–PCR from the same cDNA preparation. Retardation of the PCR products is indicative of polyadenylation. (**B**) Immature oocytes were injected with the β-globin/PRE reporter. Oocytes were pretreated where indicated with U0126 and then stimulated by injection of recombinant PKI protein. Top panel is a Western blot for active (phosphorylated) MAP kinase (arrowhead). Lysates from DMSO-and UO126-treated oocytes were processed and analyzed in parallel. Lower panels show polyadenylation of the injected synthetic β-globin/PRE reporter (brackets).

Results

The Mos PRE is a target of the progesterone 'trigger' signaling pathway

To determine if the Mos PRE was a direct target of the MAP kinase-independent progesterone trigger pathway, oocytes were treated with the MAP kinase pathway inhibitor U0126. U0126 significantly attenuated the rate of oocyte maturation (a 4 h delay to $GVBD_{50}$; Figure 1A) and abolished progesterone-stimulated MAP kinase activation, as previously reported (Gross *et al*, 2000). However, the initiation of endogenous Mos mRNA polyadenylation occurred with similar kinetics in the presence or absence of U0126, although the length of the poly[A] tail extension was reduced in U0126-treated oocytes. By contrast, CPE-directed polyadenylation of the endogenous cyclin B1 mRNA was significantly delayed in the U0126-treated oocytes, occurring coincident with GVBD. As the β-globin 3′ UTR does not undergo any significant polyadenylation (Charlesworth *et al*, 2002, 2004), the Mos PRE was inserted into the β-globin 3′ UTR to directly determine if the PRE was sufficient to direct MAP kinase-independent polyadenylation. Initiation of PRE-directed polyadenylation of the β-globin 3′ UTR occurred with similar kinetics in the presence or absence of U0126 (Figure 1A), although some differences in overall length of poly[A] tail extension can be seen between the two treatments. Injection of the PKA inhibitor (PKI) has been shown to induce progesterone-independent oocyte maturation (Huchon *et al*, 1981; Davidson, 1986). To determine if the PRE was downstream of PKA inhibition, we analyzed PRE-directed polyadenylation following injection of PKI. PKI induced PRE-directed polyadenylation of the β-globin 3′ UTR reporter mRNA, even in the presence of U0126 (Figure 1B). These findings indicate that the Mos PRE is downstream of PKA inhibition and is a target of the MAP kinase-independent progesterone trigger signaling pathway.

Xenopus Musashi is a Mos 3′ UTR interacting protein

We next sought to identify potential RNA sequence-specific binding proteins that control PRE function. The yeast three-hybrid system (SenGupta *et al*, 1996, 1999; Zhang *et al*, 1999; Bernstein *et al*, 2002) was used to screen a *X. laevis* pACT2 unfertilized egg cDNA library (Clontech) for proteins that specifically interact with a CPE-disrupted Mos UTR (Mos M1 48; Figure 2A). The CPE sequence was disrupted to prevent possible recovery of the CPE binding protein (CPEB1) in the library screen. In total, 1.3×10^7 initial yeast transformants were screened (Supplementary Table I) and two distinct cDNAs (designated A8 and C36) were recovered and shown to reconstitute specific RNA interaction with the Mos 3′ UTR (Figure 2B, M1 48). The protein encoded by the A8 cDNA interacts specifically with the Mos 3′ UTR but does not interact with the PRE sequence and will be described elsewhere (Charlesworth and MacNicol, in preparation).

The pACT2 C36 cDNA was identical to amino acids 17–347 of nervous system-specific RNP protein 1b (Nrp-1b), more recently identified as the *Xenopus* homolog of the *Drosophila*

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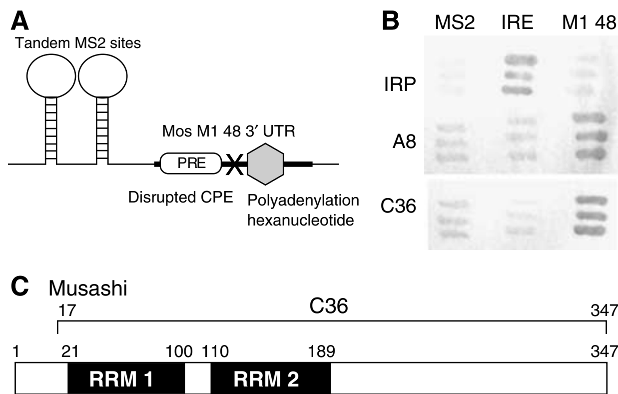

Figure 2 Identification of Mos UTR-specific binding proteins by yeast three-hybrid analyses. (**A**) Diagram of the RNA hybrid in the pIIIA/MS2 vector that was used to screen the *Xenopus* oocyte library. The last 48 nt of the Mos 3′ UTR (M1 48, bold line) containing the PRE (open rectangle), the canonical polyadenylation hexanucleotide (gray hexagon) and a disrupted CPE ('X', UUUUAU to UUUggU) were placed 3′ of the MS2 sites. (**B**) Clones A8 and C36 specifically bind to the Mos M1 48 hybrid RNA. pACT2 plasmids encoding A8 and C36 clones and IRP (iron response protein) hybrid proteins were co-transformed with pIIIA/MS2 plasmids specifying MS2 (empty vector), IRE/MS2 (IRE: iron response element) and MS2/M1 48 (M1 48) hybrid RNAs. Dark gray shows activation of the LacZ reporter, indicating interaction between protein and RNA. (**C**) Schematic of the *Xenopus* Musashi protein. The black boxes represent the RNA recognition motifs (RRM) of Musashi. Amino-acid position of the motifs and the fragment of Musashi (C36) that was recovered from the screen are indicated.

Musashi gene (Good *et al*, 1993, 1998). *Xenopus* Musashi has two RNA recognition motifs in the N-terminal region (Figure 2C) that are highly conserved (87% amino-acid identity) with the mammalian Musashi1 protein. The *Xenopus* Musashi protein localizes to the cytoplasm of the oocyte (Good *et al*, 1993), consistent with a potential role in the regulation of cytoplasmic mRNA translation.

Musashi binds specifically to the PRE in the Mos 3′ UTR

The 24-nt *Xenopus* Mos PRE (Charlesworth *et al*, 2002) contained a match to the SELEX-derived murine Musashi RNA binding consensus sequence (G/AU$_{1-3}$AGU) (Imai *et al*, 2001), and included a 3′ U residue essential for PRE function (Charlesworth *et al*, 2002) (Figure 3A, white nucleotides). Electrophoretic mobility shift assays (EMSAs) were performed to verify that Musashi interacts specifically with the Mos 3′ UTR. GST-tagged N-terminal Musashi (GST N-Msi), but not the GST moiety alone or unprogrammed reticulocyte lysate, formed specific complexes with the wild-type Mos UTR probe (Figure 3B, left panel). Musashi did not form a complex with the Mos UTR when the Musashi binding site was disrupted (AUAGU to AUccU; Figure 3B, right panel). Moreover, the Musashi-specific complexes formed with GST N-Msi could be supershifted with GST antisera, but not GFP antisera (Figure 3B, right panel). To delineate the Musashi target sequence within the PRE, we performed a competition experiment using 50-fold molar excess of unlabeled wild-type Mos UTR probe or unlabeled mutant Mos UTRs (Figure 3C). Whereas Musashi-specific binding to the wild-type Mos UTR probe was competed by unlabeled wild-type Mos UTR, Musashi-specific complex formation was not competed with a Mos UTR carrying a disruption in the Musashi binding site (Msi mut) or a Mos UTR lacking the 24-nt PRE (ΔPRE). Two additional RNAs encoding PRE mutations 5′ of the Musashi binding site effectively competed Musashi complex formation (Figure 3C). These findings indicate that Musashi does not bind to the 5′ region of the PRE, although our data do not exclude a role for the 5′ region of the PRE in facilitating Musashi function. Musashi-specific complex formation with the wild-type Mos UTR was not observed with the RNA binding-deficient form of the truncated Musashi protein (N-Msi-bm; Figure 3D), which encodes three amino-acid substitutions in the first RRM domain (Imai *et al*, 2001). Taken together, these results demonstrate that *Xenopus* Musashi interacts specifically with the consensus Musashi binding site within the Mos PRE sequence. As an additional demonstration of specific RNA binding, the full-length Musashi (Msi) and N-Msi proteins were able to interact with the endogenous Mos mRNA but not with the IPP2 mRNA that lacks a consensus Musashi binding site in the 3′ UTR (Figure 3E). No association of either Mos or IPP2 mRNAs was detected with the GST moiety alone.

Musashi interaction with the Mos 3′ UTR is necessary for early translational activation

To determine whether the Musashi consensus site was necessary for early, CPE-independent mRNA translational activation directed by the Mos PRE (Charlesworth *et al*, 2002, 2004), disruptive nucleotide substitutions were introduced into the Musashi consensus sequence (AUAGU to AUccU) and the polyadenylation and translation of reporter mRNAs analyzed. Insertion of the Mos PRE into the heterologous β-globin 3′ UTR confers progesterone-dependent polyadenylation and translational activation (two- to three-fold above β-globin alone; Figure 4A), whereas disruption of the Musashi binding site (PRE/msi mt) abolished this regulation (Figure 4A). These findings indicate that the integrity of the Musashi consensus site is critical for the translational activation directed by the Mos PRE. A role for the Musashi protein in mRNA translational activation in *Xenopus* oocytes was independently confirmed through the use of the MS2 tethered assay (Gray *et al*, 2000; Minshall *et al*, 2001; Collier *et al*, 2005). As can be seen in Figure 4B, fusion of Musashi to the MS2 RNA binding domain directed a two-fold stimulation of a firefly luciferase reporter mRNA containing 3′ UTR MS2 binding sites. No induction of the luciferase reporter mRNA was observed with the MS2 RNA binding domain alone, nor with a luciferase reporter mRNA lacking MS2 binding sites (ΔMS2; Figure 4B). In these experiments, the MS2-Dazl fusion protein serves as a positive control for translational induction (Collier *et al*, 2005). The firefly luciferase reporter mRNAs used in the tethered assay lack a consensus polyadenylation hexanucleotide, suggesting that tethering of Musashi can direct translation independently of polyadenylation. To test this directly, the polyadenylation hexanucleotide in the GST β-globin/PRE reporter mRNA was disrupted (AAUAAA to AAgAAA; Fox *et al*, 1989) to generate GST β-globin/PRE/hex mt. While eliminating progesterone-dependent polyadenylation, disruption of the polyadenylation hexanucleotide reduced, but did not eliminate, PRE-directed mRNA translation (approximately 1.5-fold above β-globin alone; Figure 4C). These findings suggest that the Musashi binding site in the Mos PRE directs both polyadenylation and translation in response to progesterone, but that polyadenylation is not obligatory for PRE-directed mRNA translation.

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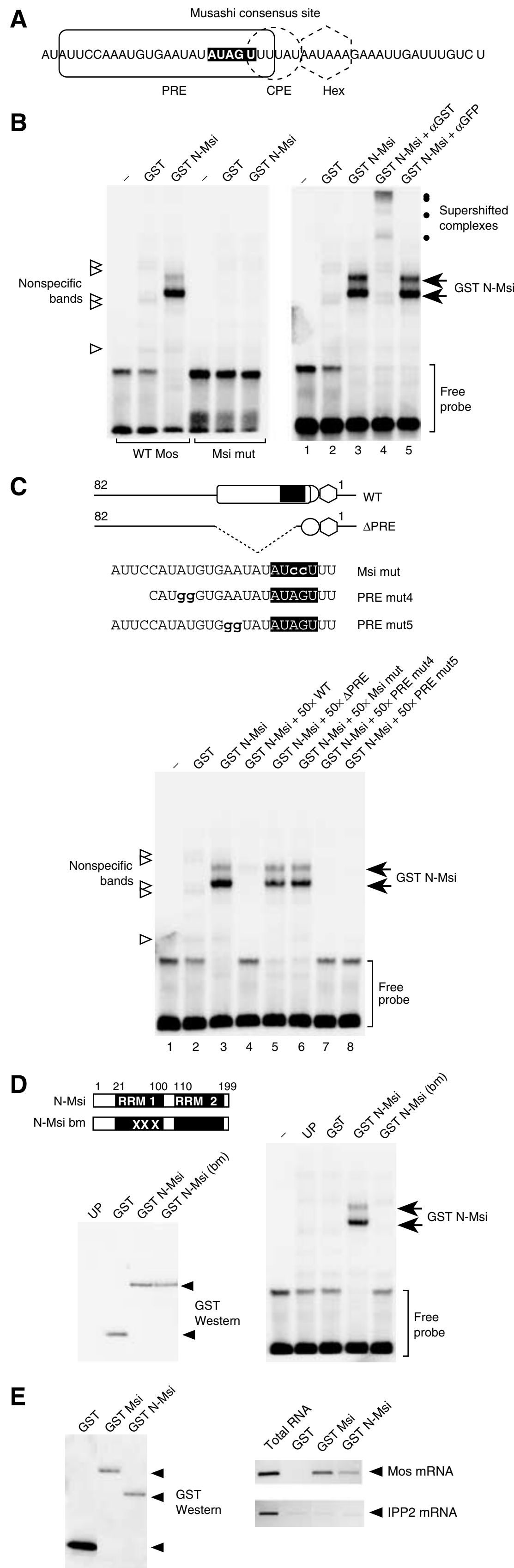

A truncated form of Musashi blocks Xenopus oocyte meiotic cell cycle progression

We reasoned that expression of a truncated form of Musashi encompassing only the RNA binding domain (N-Msi, aa 1–199) may function in a dominant inhibitory manner to block early Mos mRNA polyadenylation and translational activation in response to progesterone stimulation. Immature oocytes were injected with mRNA encoding either N-Msi or the RNA binding-deficient N-Msi-bm (Figure 3D). Compared to the N-Msi-bm-expressing oocytes, expression of N-Msi dramatically attenuated progesterone-stimulated oocyte maturation (Figure 5B, left panel). To determine the molecular basis for the block to oocyte maturation exerted by N-Msi, oocytes were analyzed for endogenous Mos and cyclin B1 mRNA polyadenylation. In contrast to the polyadenylation of the Mos mRNA in N-Msi-bm-expressing oocytes, Mos mRNA polyadenylation was attenuated in oocytes expressing N-Msi at all time points analyzed (Figure 5B, upper right panel). The polyadenylation of the cyclin B1 mRNA is a late event in progesterone-stimulated maturation and occurs coincident with oocyte GVBD (Sheets *et al*, 1994; Ballantyne *et al*, 1997; de Moor and Richter, 1997; Charlesworth *et al*, 2004). In N-Msi-bm-expressing oocytes, cyclin B1 mRNA polyadenylation occurred coincident with GVBD as expected, whereas expression of N-Msi protein prevented progesterone-stimulated GVBD and ablated cyclin B1 polyadenylation, even at late time points (Figure 5B, lower right panel). Two possible explanations could account for the inhibitory effect of N-Msi on cyclin B1 polyadenylation. First, N-Msi may exert an inhibitory effect by directly binding to the cyclin B1 3′ UTR and blocking CPE-dependent cytoplasmic polyadenylation. Alternatively, the effect of N-Msi may be indirect and occur through inhibition of Musashi-dependent mRNA translation events required for GVBD and/or the activation of

Figure 3 Musashi binds to the PRE in the Mos 3′ UTR. (**A**) Schematic showing regulatory element composition within the last 50 nt of the Mos 3′ UTR. The Musashi consensus binding site is indicated by white nucleotides. (**B**) Left panel: Wild-type (WT Mos) or Musashi binding site mutant (Msi mut) Mos UTR probes were analyzed for interaction with the GST-tagged, N-terminal domain of *Xenopus* Musashi (GST N-Msi) by RNA EMSA. Specific Musashi binding complexes were only detected with the wild-type Mos UTR probe. Right panel: Musashi-specific complex formation with the wild-type Mos UTR probe can be supershifted with antisera to the GST epitope tag and not with GFP antisera. (**C**) Musashi-specific complex formation with the wild-type Mos UTR probe can be competed with a 50-fold excess unlabeled wild-type, PRE mut4 and PRE mut5 Mos UTR RNA. No competition is observed with a Mos UTR lacking the entire PRE (ΔPRE) or a Musashi binding site mutant (Msi mut) Mos UTR. (**D**) Disruption of the first RRM in Musashi (GST N-Msi-bm) prevents Musashi-specific complex formation with the wild-type Mos UTR probe (right panel). Equivalent levels of each GST fusion protein were expressed in the reticulocyte lysates (left panel). UP, unprogrammed lysate. (**E**) The full-length (GST Msi) and N-terminal domain of Musashi (GST N-Msi) interact with the endogenous Mos mRNA. Left panel: GST Western blot showing recovery of exogenously expressed GST fusion proteins using glutathione Sepharose beads. Equivalent levels of GST Msi and GST N-Msi and higher levels of the GST moiety were recovered in the pulldown. Right panel: RT–PCR of Mos and IPP2 mRNA from RNA extracted from the indicated GST fusion proteins shown in the left panel. GST-Msi and GST-N-Msi interact with the Mos mRNA but do not interact with the IPP2 mRNA. The GST moiety alone fails to interact with either Mos or IPP2 mRNAs. RT–PCR from total oocyte RNA was used as a positive control to indicate the relative position of the expected PCR products in the pulldown lanes.

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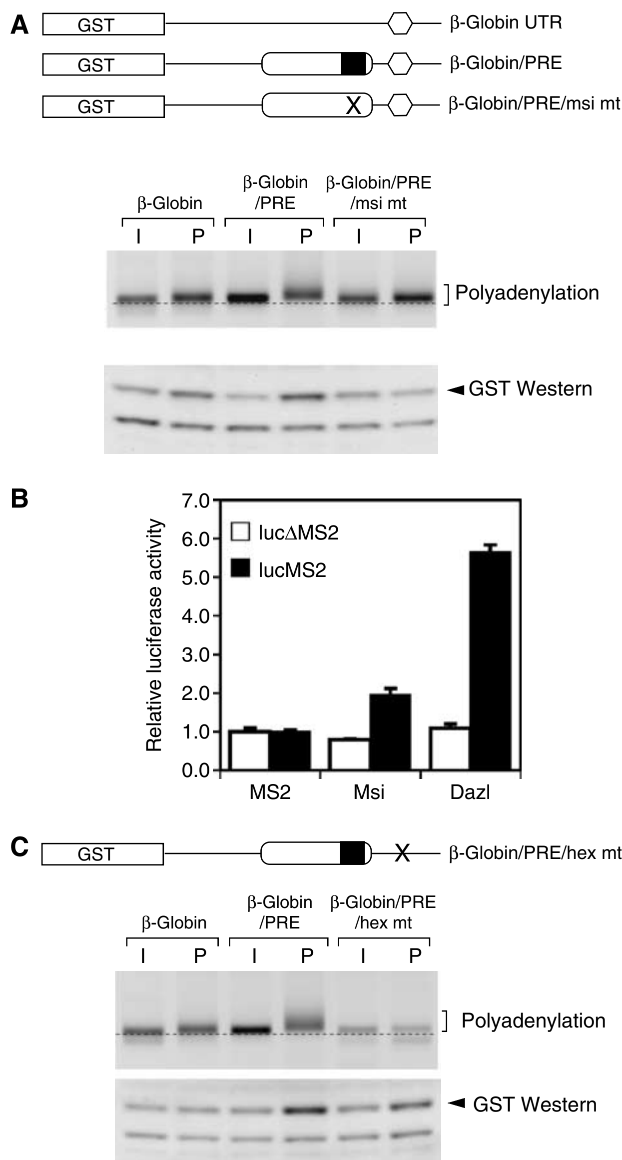

Figure 4 Musashi directs mRNA translational activation. (**A**) Upper panel: Schematic representation of the reporter mRNAs analyzed. The GST open reading frame (box) was fused to the β-globin 3′ UTR, or β-globin 3′ UTRs containing either the wild-type (PRE) or Musashi binding site mutant (PRE/msi mt) Mos PRE sequences. Middle panel: Polyadenylation of the indicated reporter mRNAs was assessed by RNA ligation-coupled PCR from time-matched immature (I) or progesterone-stimulated (P) oocytes taken at $GVBD_{50}$ from oocytes without white spots. Retarded migration of PCR products above the dotted line is indicative of polyadenylation. Lower panel: Western blot showing GST accumulation from time-matched immature or progesterone-stimulated oocytes taken at $GVBD_{100}$. (**B**) Tethered assay demonstrating the ability of MS2-Msi and MS2-Dazl fusion proteins to induce firefly luciferase expression. Open and solid bars represent luciferase reporters lacking or containing MS2 binding sites in the 3′ UTR, respectively. Luciferase activity was determined in triplicate and normalized to expression of the coinjected *Renilla* luciferase mRNA. Error bars represent standard deviation of the mean. The experiment was repeated three times with similar results. (**C**) Oocytes were injected with GST reporter mRNAs fused to the β-globin 3′ UTR, the β-globin 3′ UTR containing the Mos PRE (PRE) or the β-globin 3′ UTR containing the Mos PRE (PRE) and a disrupted polyadenylation hexanucleotide (hex mt). The polyadenylation and translation of GST reporter mRNAs were analyzed as described in (A).

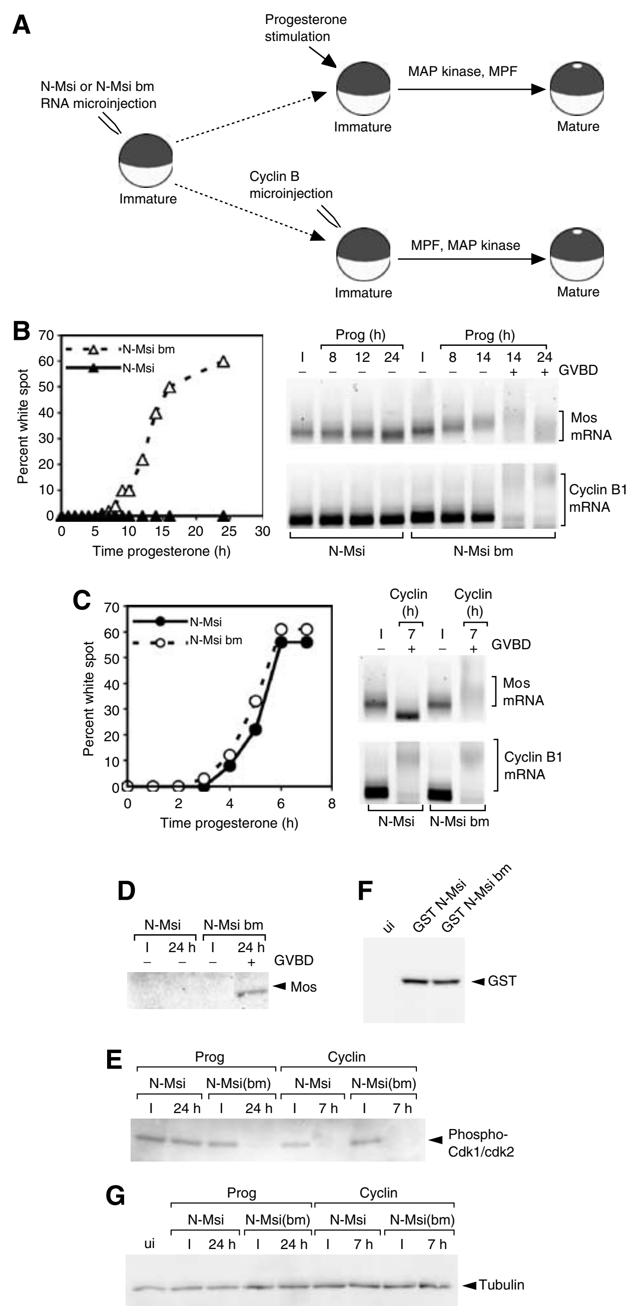

Figure 5 Dominant inhibitory Musashi blocks oocyte maturation and specifically prevents Mos polyadenylation and translation. (**A**) Schematic of experimental design. RNA encoding GST-tagged N-Msi or N-Msi-bm was injected into immature oocytes, which were left for 36 h to express the protein. Oocytes were then stimulated to mature either by addition of progesterone or injection of cyclin B protein. (**B**) Progesterone-stimulated maturation was scored by the appearance of a white spot at the animal pole and indicated by whether the oocytes had (+) or had not (−) undergone GVBD. The polyadenylation of endogenous Mos (upper panels) and cyclin B1 (lower panels) mRNAs was assessed by RNA ligation-coupled RT–PCR (brackets). In this experiment, progesterone-stimulated N-Msi-bm-expressing oocytes reached $GVBD_{50}$ at 14 h. (**C**) Oocytes were injected with cyclin B1 protein and the effect on maturation and mRNA polyadenylation was assessed as in (B). (**D**) The expression of Mos protein in immature (I) or progesterone-stimulated (24 h) oocytes from experiment B was determined by Western blot. (**E**) The activation status of MPF in lysates prepared from experiments B and C was assessed using antisera specific to inactive CDK1 and CDK2. (**F**) Western blot showing equivalent levels of GST-tagged N-Msi or N-Msi-bm expressed in oocytes used for experiments B and C. ui, uninjected lysate. (**G**) Tubulin Western blot showing equal protein loading in lysates analyzed in panels D–F.

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CPE-dependent mRNA polyadenylation. To distinguish between these possibilities, oocytes were microinjected with cyclin B1 protein to induce activation of MPF independently of progesterone stimulation (Freeman *et al*, 1991) and reverse the relative order of MAP kinase and MPF activation (Figure 5A; Charlesworth *et al*, 2002). N-Msi did not block cyclin B protein-induced maturation and the kinetics of maturation were indistinguishable between N-Msi- and N-Msi-bm-expressing oocytes (Figure 5C, left panel). Cyclin B protein induced the polyadenylation of the endogenous cyclin B1 mRNA in both N-Msi- and N-Msi-bm-expressing oocytes (Figure 5C, right panel). These results indicate that CPE-dependent polyadenylation of the cyclin B1 mRNA can occur in oocytes expressing dominant-negative Musashi if MPF is activated independently of progesterone-triggered early signaling effectors. By contrast, cyclin B-induced Mos mRNA polyadenylation was abrogated in N-Msi-, but not N-Msi-bm-, expressing oocytes (Figure 5C, right panel) demonstrating the specificity of N-Msi-mediated inhibition on PRE-regulated mRNAs irrespective of initiating signal. Mos mRNA deadenylation was observed in the N-Msi-expressing oocytes after GVBD. We conclude that the dominant-negative Musashi specifically blocks early progesterone effector pathways (including Mos mRNA translational activation) and the inhibition of CPE-dependent mRNA polyadenylation is an indirect consequence of a block to upstream Musashi-regulated mRNA translation events. Consistent with a block to Mos mRNA polyadenylation, progesterone-stimulated Mos protein accumulation was abrogated in N-Msi (but not N-Msi-bm)-expressing oocytes (Figure 5D). N-Msi, but not N-Msi-bm, also prevented progesterone-dependent activation of MPF (Figure 5E). In these experiments, the N-Msi and N-Msi-bm mutant proteins were expressed to comparable levels (Figure 5F). Although we cannot exclude additional effects of the truncated Musashi protein (N-Msi) on processes upstream of mRNA polyadenylation and translational activation, the inability of an RNA binding-deficient form of the truncated protein (N-Msi-bm) to block progesterone-stimulated cytoplasmic polyadenylation or cell cycle progression indicates that effect of the dominant inhibitory Musashi is primarily due to interaction with mRNA targets. Taken together, our data provide compelling evidence that Musashi regulates maternal mRNA translation and MPF activation during progesterone-stimulated meiotic cell cycle progression.

Musashi regulates polyadenylation of multiple Xenopus mRNAs

We next determined if Musashi could regulate additional *Xenopus* mRNAs. The mammalian Musashi consensus sequence (A/G)U_{1-3}AGU (Imai *et al*, 2001) was identified in 9.4% of *Xenopus* mRNA in the 3′ UTR database (Mignone *et al*, 2005). Musashi binding sites were found in the previously characterized early class mRNAs Mos, Aurora A/Eg2, D7 and FGF receptor 1 (Charlesworth *et al*, 2002, 2004), as well as in the late class cyclin B1 and cyclin A1 mRNAs. In addition, the Eg1/CDK2, Eg3, Xotch (*Xenopus* Notch), Bub3, and TATA binding protein 2 mRNAs were identified as having a Musashi site within the last 150 nt of the 3′ UTR. Consistent with Musashi- and PRE-directed regulation, the Eg1/CDK2, Eg3, Xotch (*Xenopus* Notch), Bub3 and TATA binding protein 2 mRNAs were polyadenylated in an early manner, before oocyte GVBD (Figure 6A). The length of the poly[A] tail extension varied between the different mRNAs but was not obviously correlated with position or sequence of the Musashi binding site within the 3′ UTRs. In contrast to the early mRNAs, polyadenylation of the CPE-dependent cyclin A1 mRNA occurred later, coincident with oocyte GVBD.

The progesterone-stimulated polyadenylation of the mRNAs analyzed in Figure 6A was ablated in oocytes expressing the dominant inhibitory N-Msi, but not the N-Msi-bm, protein (Figure 6B, left panels). As the PCR products of the early class mRNAs were reduced at late time points in progesterone-stimulated oocytes expressing N-Msi, our data suggest that these mRNAs become deadenylated. No apparent deadenylation of the late class cyclin A1 mRNA was observed in N-Msi-expressing oocytes. Active PRE-directed polyadenylation may normally preclude access of deadenylation factors or directly oppose deadenylation of early class mRNAs in maturing oocytes. The presence of a polyadenylation hexanucleotide-overlapping CPE, common to both the cyclin A1 and cyclin B1 3′ UTRs, may protect the late class mRNAs from deadenylation (see also Figure 5). With the exception of D7 and cyclin A1, mRNA polyadenylation was abrogated in N-Msi-, but not N-Msi-bm-, expressing oocytes injected with cyclin B protein. Cyclin B protein induced polyadenylation of the D7 and cyclin A1 mRNAs in the presence of N-Msi, suggesting that the CPE sequences in these 3′ UTRs were responsive to MPF signaling. These findings indicate that Musashi regulates the translational activation of multiple maternal mRNAs during progesterone-stimulated *Xenopus* oocyte meiotic cell cycle progression.

Discussion

Our previous studies have identified the *cis*-acting PRE element as a determinant of MAP kinase-dependent mRNA translational regulation during *Xenopus* meiotic cell cycle progression (Charlesworth *et al*, 2002, 2004). In this study, we show that the PRE is also a target of the MAP kinase-independent progesterone ‘trigger’ pathway. Furthermore, we have identified *Xenopus* Musashi as a regulator of PRE-directed Mos mRNA translational activation. Our results position Musashi as a mediator of the progesterone-stimulated ‘trigger’ signaling pathway that is necessary for initiation of maternal mRNA translational activation and cell cycle progression. The identification of Musashi as a regulator of PRE function was defined by a combination of yeast three-hybrid analyses, *in vitro* EMSA experiments and functional tests *in vivo*. In addition to blocking Mos mRNA translation, expression of a dominant-negative Musashi protein abolished CPE-dependent mRNA translational activation in response to progesterone stimulation. The dominant-negative form of Musashi did not block cytoplasmic polyadenylation of CPE-dependent mRNAs following cyclin B1 protein injection (Figure 5), indicating that Musashi-mediated mRNA translation normally precedes, and is necessary for, CPE-dependent mRNA translational activation in response to progesterone stimulation. These findings extend prior studies, which have demonstrated an inherent dependence of late class mRNA translational activation on prior translation of early class mRNAs (Ballantyne *et al*, 1997; de Moor and Richter, 1997).

Expression of the dominant inhibitory form of Musashi abolished progesterone-stimulated MPF activation and meiotic cell cycle progression (Figure 5). This catastrophic block

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to cell cycle progression is unlikely to simply reflect inhibition of Mos mRNA translation. Indeed, the inhibition of cell cycle progression exerted by the dominant-negative form of Musashi is different from the effects observed as a result of the inhibition of Mos mRNA translation by antisense Mos morpholino oligonucleotides (Dupre *et al*, 2002) or pharmacological inhibition of Mos-mediated MAP kinase signaling (Gross *et al*, 2000), which result in a delay, rather than a block, to GVBD. Our findings suggest that Musashi-dependent translational activation regulates a more comprehensive range of mRNA targets that contribute to induction of MPF and progression to GVBD in response to progesterone stimulation.

The identity of all potential Musashi-regulated mRNAs that contribute to oocyte cell cycle progression has not been established. We have utilized the mammalian Musashi SELEX-derived consensus binding sequence to identify new early class maternal mRNAs that display Musashi-dependent cytoplasmic polyadenylation (Figure 6). However, some Musashi consensus site-containing mRNAs do not exhibit early class polyadenylation (e.g. cyclin B1 and cyclin A1; Figures 5 and 6). Several possibilities may explain these observations including mRNA secondary structure considerations (Imai *et al*, 2001) or potential inhibitory influences exerted by other regulatory elements in the same 3′ UTR. Consistent with the latter possibility, the cyclin B1 and cyclin A1 3′ UTRs have a CPE that overlaps the polyadenylation hexanucleotide, which may prevent cytoplasmic polyadenylation until the MPF-dependent degradation of bound CPEB1 at GVBD (Mendez *et al*, 2002).

PRE-like sequences have been identified in the early class mRNAs encoding D7, G10, histone-like B4, Eg2/Aurora A and FGF receptor 1 mRNA 3′ UTRs (Charlesworth *et al*, 2004). It has not been determined if these PRE-like sequences are equivalent to the Mos PRE or if they represent distinct regulatory elements with similar functional properties. Unlike the Mos PRE, the identified PRE-like sequences lack a consensus Musashi binding site. Nonetheless, Musashi function is necessary for polyadenylation of the PRE-like sequences-containing mRNAs (Figure 6), suggesting that either PRE-like sequences represent additional non-consensus Musashi binding sites or PRE-like function is indirectly controlled through upstream Musashi-dependent mRNA translation events. It is interesting to note that although full Musashi-directed mRNA translation requires polyadenylation in response to progesterone stimulation, Musashi can nonetheless direct a lower level of polyadenylation-independent translation (Figure 4). We are continuing to examine the requirements for Musashi RNA binding, 3′ UTR regulatory element composition and secondary structure to further elucidate the determinants of Musashi-dependent mRNA translational activation.

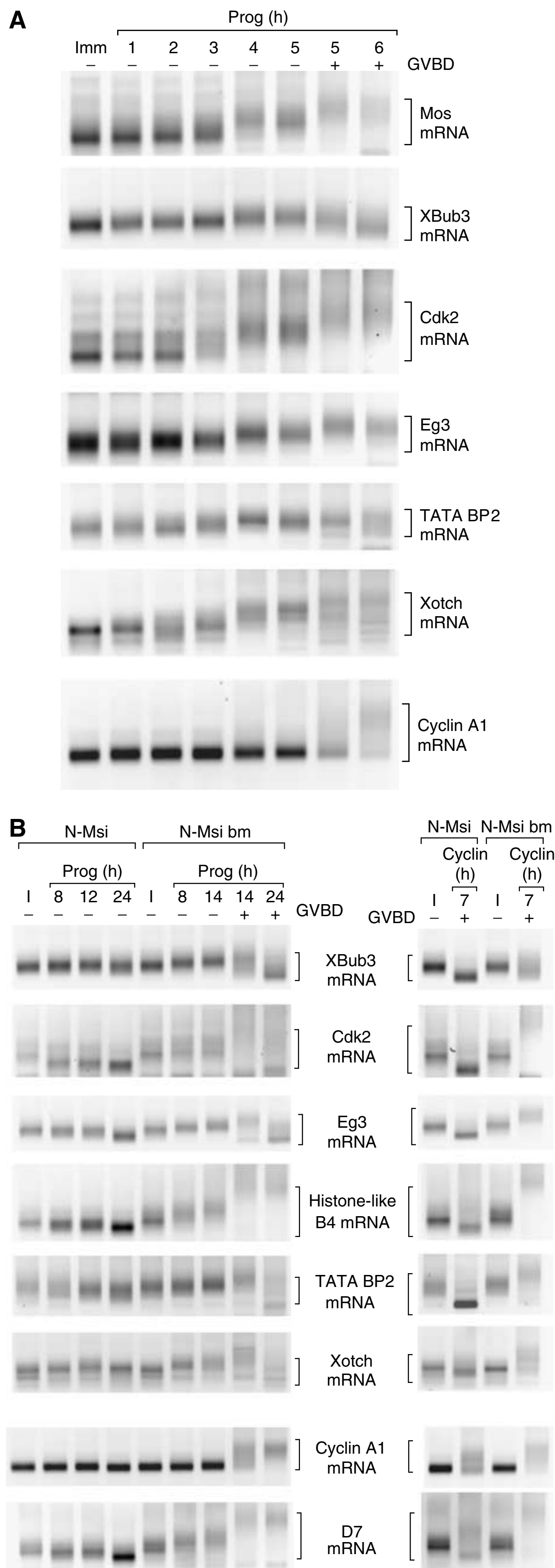

Figure 6 Musashi regulates progesterone-dependent polyadenylation of multiple mRNAs. (**A**) Time course of polyadenylation of endogenous mRNAs was assayed from the same cDNA preparation using appropriate gene-specific forward primers. Brackets indicate the extent of mRNA polyadenylation. Progression of maturation is indicated by whether the oocytes have (+) or have not (−) undergone GVBD. In this experiment, progesterone-stimulated N-Msi-bm-expressing oocytes reached $GVBD_{50}$ at 14 h. (**B**) Polyadenylation of the indicated endogenous mRNAs was assessed following progesterone stimulation (left panel) or cyclin B1 protein injection (right panel) as described in Figure 5. Cyclin B1 protein-stimulated polyadenylation of the mRNAs in the six upper panels is prevented by N-Msi. Cyclin B1 protein-stimulated polyadenylation of the mRNAs in the lower two panels is not prevented by N-Msi. Brackets indicate the extent of mRNA polyadenylation or deadenylation.

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Musashi has been previously proposed to repress translation of target mRNAs in *Drosophila* neural progenitor cells, as well as mammalian neural stem cell populations (Imai *et al*, 2001; Okabe *et al*, 2001; Battelli *et al*, 2006). We now report that Musashi is required for *Xenopus* maternal mRNA translational activation and meiotic cell cycle progression (Figures 4 and 5). The role of Musashi in these different cell types appears to be contradictory. However, despite exerting opposing effects on target mRNA translational regulation, the outcome of Musashi action in each situation is to promote cell cycle progression. It is possible that the differential regulation of target mRNA translation in oocytes and neural stem cells may be exerted through the expression of specific partner proteins or regulators that can differentially enforce Musashi-mediated mRNA repression or translational activation. Given the importance of Musashi function in stem cell self-renewal (Okano *et al*, 2005) and the possible implications of aberrant Musashi expression in tumors (Kanemura *et al*, 2001; Toda *et al*, 2001; Hemmati *et al*, 2003; Potten *et al*, 2003; Yokota *et al*, 2004) and neurodegenerative disorders (Lovell and Markesbery, 2005), it is now critical to understand the molecular mechanisms that control Musashi-mediated mRNA translation. A characterization of the role and regulation of Musashi function during *Xenopus* oocyte maturation will not only enhance our understanding of this key aspect of reproductive biology but may also provide insight into the mechanisms contributing to stem cell self-renewal.

Materials and methods

Plasmid construction

A detailed methodology of construction of all plasmids used in this study is provided as Supplementary data.

Yeast three-hybrid screen

The yeast three-hybrid screen was performed as described (SenGupta *et al*, 1996) in the yeast strain YBZ-1 (Bernstein *et al*, 2002) using the *X. laevis* oocyte Matchmaker cDNA Library (Clontech). Initial transformants were plated in the presence of 0.5 mM 3-AT to reduce false positives. A summary of the screen is provided in Supplementary Table 1. To investigate binding specificity by mating, the R40 coat strain, transformed with an empty vector (pIIIA MS2-2.1), a negative control plasmid (pIIIA IRE (iron response element)) or pIIIA MS2-2.1 M1 48 was used. The orientation of the Mos UTR relative to the MS2 sites in the hybrid RNA was critical for the success of the screen.

RNA electrophoretic mobility shift assays

GST fusion proteins were *in vitro* transcribed/translated using TNT SP6-coupled Reticulocyte Lysate System (Promega). 5′ biotin-labeled RNA oligonucleotide probes were synthesized by Integrated DNA Technologies. An 80 fmol portion of labeled probe was incubated with 1 μl of reticulocyte lysate in binding buffer (50 mM Tris pH 7.5, 20 mM KCl, 150 mM NaCl, 2 mM EGTA, 0.05% NP-40, 6 mM DTT, 8 U RNase OUT; Okabe *et al*, 2001) in a final volume of 20 μl. Unlabeled competitor RNA was added to 4 pmol (50-fold molar excess). The binding reaction was incubated at room temperature for 20 min and then 0.5 μl of 200 mg/ml heparin was added and incubated for a further 20 min. For supershift analysis, 1 μl of anti-GST antibody (Santa Cruz) or anti-GFP antibody (Molecular Probes) was added 10 min before the end of the incubation. A 5 μl volume of the binding reaction was run on a 6% DNA retardation gel (Invitrogen) and transferred to Biodyne 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, with the modification that incubation with the streptavidin-HRP conjugate was for 40 min. Image collection was performed using an AlphaInnotech ChemiImager.

Tethered assay

Oocytes were injected with 23 ng of appropriate MS2 fusion protein mRNA, 0.1 ng of firefly luciferase mRNA, 0.35 pg of *Renilla* luciferase control mRNA and incubated for 20 h. Three pools of five oocytes were harvested for each experimental point and lysed in 50 μl of Passive Lysis Buffer (Promega) per oocyte. A 10 μl 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. Mean values and standard deviation were determined for each experimental point, with the ratio of firefly to *Renilla* luciferase normalized to 1.0 for the MS2 protein. MS2 fusion protein expression was comparable as assessed by Western blot (MS2 antibodies generously provided by Mike Kiledjian).

Oocyte isolation, culture, microinjection and lysate preparation

Xenopus oocyte isolation and culture has been described (Machaca and Haun, 2002). Where indicated, oocytes were pretreated with 50 μM U0126 (Promega) and 0.5% DMSO for 1 h before stimulation. Oocytes were induced to mature with 2 μg/ml progesterone, cyclin B protein (Howard *et al*, 1999) or by injection of approximately 0.04 U of recombinant rabbit PKI-α (Calbiochem). GVBD was used as an indicator of maturation and assessed by the presence of a white spot on the animal pole. Where indicated, oocytes were segregated at the time when 50% of the oocyte population had reached GVBD ($GVBD_{50}$) based on whether they had (+) or had not (−) completed GVBD. A 250 ng portion of RNA encoding GST N-Msi or GST N-Msi-bm was injected per oocyte and left for 36 h for protein expression and equilibration with endogenous Musashi. The oocyte culture medium was changed after about 18 h. RNA and protein were extracted from the same pool of oocytes as previously described (Charlesworth *et al*, 2002). MPF activation was assessed using phospho-CDK1/CDK2 antisera (Cell Signaling). MAP kinase activation as well as Mos and GST protein accumulation was assessed as previously described (Howard *et al*, 1999).

Polyadenylation assays

RNA ligation-coupled RT–PCR was performed as described previously (Charlesworth *et al*, 2004). To analyze polyadenylation of reporter constructs, primers to the GST region were used (Charlesworth *et al*, 2004). The primers used to analyze the novel mRNAs are described in Supplementary data.

mRNA co-association assay

A 25 ng portion of RNA encoding the indicated GST fusion protein was injected into immature oocytes and cultured for 24 h to allow GST fusion protein expression. Pools of 25 oocytes were lysed in 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40, 6 mM DTT, protease inhibitor cocktail (Sigma), PMSF, 10 mM ribonucleoside vanadyl complex (NEB) and 400 U/ml RNase OUT (Invitrogen) and centrifuged to remove cell debris. GST-tagged proteins were affinity purified with glutathione Sepharose for 30 min and washed 5 × 1 ml with the same buffer. RNA was extracted using STAT-60 and analyzed by RNA ligation-coupled RT–PCR.

GST reporter mRNA translation assays

Analyses of wild-type and mutant 3′ UTRs on GST reporter mRNA translation were performed using 0.2 ng reporter RNA as previously described (Charlesworth *et al*, 2000, 2002).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We gratefully acknowledge Marv Wickens for providing the yeast strains and the empty and control plasmids for the three-hybrid screen; Niki Gray for the MS2 fusion expression plasmid and plasmids encoding firefly luciferase reporter mRNAs; and Nancy Standart for the plasmid encoding the *Renilla* luciferase reporter mRNA. We thank Melanie MacNicol for critical reading of the manuscript. This work was supported by a grant from the National Institutes of Health (HD35688) to AMM.

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