The RNA-binding protein Mex3b has a fine-tuning system for mRNA regulation in early Xenopus development

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Post-transcriptional control by RNA-binding proteins is a precise way to assure appropriate levels of gene expression. Here, we identify a novel mRNA regulatory system involving Mex3b (RKHD3) and demonstrate its role in FGF signaling. Mex3b mRNA has a 3’ long conserved UTR, named 3’ LCU, which contains multiple elements for both mRNA destabilization and translational enhancement. Notably, Mex3b promotes destabilization of its own mRNA by binding to the 3’ LCU, thereby forming a negative autoregulatory loop. The combination of positive regulation and negative autoregulation constitutes a fine-tuning system for post-transcriptional control. In early embryogenesis, Mex3b is involved in anteroposterior patterning of the neural plate. Consistent with this, Mex3b can attenuate FGF signaling and destabilize mRNAs for the FGF signaling components Syndecan 2 and Ets1b through their 3’ UTRs. These data suggest that the 3’ LCU-mediated fine-tuning system determines the appropriate level of Mex3b expression, which in turn contributes to neural patterning through regulating FGF signaling.

KEY WORDS: FGF, Mex3b, RNA-binding protein, Xenopus, Neural plate, Post-transcriptional regulation

INTRODUCTION
In developmental processes, embryonic induction and patterning are strictly regulated in a spatiotemporal manner by the expression of numerous genes. Gene expression can be regulated at multiple steps, including transcription, mRNA stability and translation. For gene expression to be established at an appropriate level, some of these regulatory processes must be under rapid and precise control. Because transcriptional regulation is not sufficient to determine the level of gene expression and its duration, post-transcriptional regulation is necessary for fine-tuning gene expression. Fine-tuning mechanisms, such as the regulation of mRNA stability and translational efficacy, are generally operated by cis-elements present mostly in 3’ untranslated regions (UTRs) and by trans-acting factors, such as RNA-binding proteins and miRNA (Kuersten and Goodwin, 2003; Sevignani et al., 2006). These regulators can adjust the amounts of gene products and the duration of gene expression more rapidly and precisely than can transcriptional regulation. With respect to signal transduction, maintaining certain levels of signal transduction components such as ligands, receptors, co-receptors, cytoplasmic transducers and transcription factors is thought to be crucial for transducing appropriate levels of signaling. Thus, although post-transcriptional regulation needs to be incorporated into the control mechanisms for determining the levels of signal transduction components, their mechanisms remain poorly understood.

RNA-binding proteins have been shown to play key roles in post-transcriptional control. RNA-binding proteins are classified into several families according to the types of RNA-binding domains they contain: Zn finger proteins; RNA recognition motif (RRM) proteins, such as ELAV/Hu, Musashi-1/Nrp1 and SRp38; and KH (hrNRP homology) domain proteins, such as Mex3. In vertebrate development, for example, ELAV family members are involved in neuronal differentiation by stabilizing target mRNAs (Perrone-Bizzozero and Bolognani, 2002). RNA-binding proteins are also involved in subcellular RNA localization in oocytes and asymmetrically dividing cells (Minakhina and Steward, 2005). Despite these studies, RNA-binding proteins have not been reported to be involved in functions of early vertebrate development such as anteroposterior (AP) patterning and signal transduction.

With respect to cis-elements in 3’ UTRs and trans-acting factors for the regulation of mRNA stability and translation, AU-rich elements (AREs), typically the AUUUA sequence, and their binding proteins have been well investigated. To date, many ARE-containing mRNAs, such as those for Fos and Myc, have been reported to be subject to rapid degradation by RNA-binding proteins, such as AUF1 and Tristetraprolin (TTP) (Barreau et al., 2005; Chen and Shyu, 1995). These mRNAs are all short-lived and are rapidly degraded when transcription is stopped. However, other types of destabilizing elements have not been fully investigated, especially in developmental processes.

In the course of seeking novel genes from a Xenopus anterior neuroectoderm cDNA library using expression pattern screening (Takahashi et al., 2005), we identified a gene encoding an RNA-binding protein, Mex3b, which possesses two KH domains and a RING finger domain. A notable feature of this gene is the possession of a long evolutionarily conserved region in the 3’ UTR, named 3’ long conserved untranslated region (3’ LCU), which is 800 nucleotides long and is highly conserved among vertebrates. Mex3 has been identified in Caenorhabditis elegans, ascidians and sea urchins, and a family of four paralogous proteins, MEX3A, MEX3B, MEX3C and MEX3D, has been identified in humans (Buchet-Poyau et al., 2007). Mex-3 mRNA is localized in the anterior blastomeres of C. elegans embryos, and its product, MEX-
analyses suggest that Mex3b is required for AP patterning and autoregulatory destabilization of the mRNA. Functional expression through the regulation of both translational enhancement/H11032

Plasmid construction and in vitro mRNA synthesis

The full-length clone of mex3b was obtained from Open Biosystems. The following plasmids were PCR-amplified and cloned into the RI and EcoRI sites of pCS2+ to construct pCS2-Mex3b. N-terminally Myc-tagged Mex3b constructs, pCS2-Myc-Mex3b and pCS2-Myc–ΔKH1-2, were made by inserting PCR products of the mex3b and ΔKH1-2 (aa 58-124 and 157-220) were deleted) CDSs into the EcoRI and XbaI sites of pCS2+MTmcs (made by H.T. and XbaI site of pCS2+AdN-GFP.

Embryo manipulations, β-gal staining and whole-mount in situ hybridization

Artificial fertilization, rearing of embryos and animal cap assays were performed as described previously (Shinga et al., 2001; Taira et al., 1992). FGF2/FGF or activin were used. Embryonic stages were determined according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). β-Gal staining and whole-mount in situ hybridization (ISH) was carried out as described previously (Suga et al., 2006; Takada et al., 2005).

Plasmid construction and in vitro mRNA synthesis

The full-length clone of Xenopus mex3b (DBJ accession no. AB499902) was obtained from Open Biosystems. The following plasmids were PCR-amplified and cloned into the Clal and EcoRI sites of pCS2+ to construct pCS2-Mex3b. N-terminally Myc-tagged Mex3b constructs, pCS2-Myc-Mex3b and pCS2-Myc–ΔKH1-2, were made by inserting PCR products of the mex3b and ΔKH1-2 (aa 58-124 and 157-220) were deleted) CDSs into the EcoRI and XbaI sites of pCS2+MTmcs (made by H.T. and M.T.). C-terminally HA-tagged Mex3b constructs, pCS2+Mtx-HA and pCS2+-ΔKH1-2-HA, were made by inserting PCR products of the coding region of Mex3b and ΔKH1-2 CDSs into the Clal site of pCS2+mtcs4HAmcs (made by S. Matsuda and M.T.). To construct pCS2+myc-mex3b-3'LCU and pCS2+mex3b-HA-D for DNA expression, PCR products of the 3'LCU and subregion D were inserted into the XbaI site of pCS2+Myc-Mex3b and the EcoRI site of pCS2+Mtx-HA, respectively. The following constructs were used for GFP mRNA reporter experiments. The 3'LCU (nucleotide nos 1-798), BCD (nos 244-798), CD (nos 405-798), D (nos 577-798), ABC (nos 1-576), AB (nos 1-404), and A (nos 1-243) were PCR-amplified with the N25A3 clone (DBJ accession no. AB499903) and inserted into the XbaI site downstream of the GFP CDS of pCS2+AdN-GFP (made by M. Inamori and M. T.). To construct a destabilized GFP reporter plasmid, pCS105-GFP-PEST, the region encoding the PEST sequence of ornithine decarboxylase was PCR-amplified from a stage 17/18 Xenopus cDNA library and inserted into the EcoRI and XbaI sites downstream of the GFP CDS in pCS105-GFP (made by M. Osada and M. T.). To construct pCS105-GFP-PEST-3'LCU, +3'LCUrev, +3'LCU, and the corresponding regions were PCR-amplified and inserted into the XbaI site of pCS2+GFP-PEST. To construct pCS2+GFP-fg20-3'UTR, +sd2c-3'UTR, +ets1b-3'UTR, and +syntenin-3'UTR, those 3' UTR sequences were PCR-amplified from a stage 17/18 Xenopus cDNA library, and inserted into the XbaI site of pCS2+AdN-GFP.

Microarray analysis

The microarray tips that we used have 40,000 spots, each of which contained a 60mer oligonucleotide designed based on unique sequences near the 3' ends of known or unknown cDNA or expressed sequence tag (EST) clones deposited in GenBank/DDJB as Unigene. Total RNA was extracted from dissected neural plate tissue and reverse-transcribed with the oligo(dT) primer containing the T7 promoter. Cy3- or Cy5-labeled cRNA was synthesized from a cDNA pool and purified with RNasey (Qiagen). Cyber labeled cRNA was then fragmented and hybridized to microarray glasses (Agilent Technology) at 65°C overnight.

Western blot analysis

Injected embryos were homogenized with cell lysis buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 8 mM DTT, 40 μg/ml leupeptin, 20 μg/ml aprotinin, 2 mM PMSF, 0.1% NP-40). Protein bands on a polyvinylidene fluoride (PVDF) membrane were visualized using anti-Myc (9E10; BIOMOL) and anti-HA (Y-11; Santa Cruz) antibody as primary antibody and Alexa Fluor 680 anti-mouse IgG antibody (Molecular Probes) as secondary antibody, or using anti-GFP antibody conjugated with Irdy800 (ROCKLAND), and quantified with the Odyssey System (Aloka).

RNA immunoprecipitation

RNA immunoprecipitation experiments were performed as described previously (Liu and Harland, 2005). Embryos were lysed at stage 8.5 with homogenize buffer (15 mM HEPES, pH 7.6, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM EDTA, 44 mM sucrose, 8 mM DTT, 40 μg/ml leupeptin, 20 μg/ml aprotinin, 2 mM PMSF, 0.1μ/ml RNasin). Supernatants were incubated with anti-HA antibody (prepared from ascites of 9E10 cell-inoculated mice by Y. Goya, Y. Mii, M. Park and M.T.) for 1 hour, and then incubated with protein G agarose beads for another hour. The beads were washed three times with homogenize buffer, and treated with proteinase K in lysis buffer (0.5% SDS, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5, 50 mM NaCl) for 45 minutes. The resultant solution with the beads was treated with phenol/chloroform, and nucleic acids were isolated for RT-PCR with a GFP primer set (forwards: 5'-AACGGCCACAAGTTCAAGGGTGTCGCC-3'; reverse: 5'-CAGGACCATGTAGTCGCTTCTCG-3').

Antisense morpholino oligo experiments

Two antisense morpholino oligos designed for 25-nucleotide sequences upstream of the start codon of mex3b (Mex3bMO1, 5'-GCTCACCTCACTGTCCTAGCAGAC-3'; and Mex3bMO2, 5'-TTCCTCCAGTTACCCCTGAGTATTG-3'), and a five-base mismatched control oligo for Mex3bMO1 (5mmMO, 5'-GFTgACCTgAGTcCTACtACAC-3') were purchased from Gene Tools.

Luciferase reporter assay

Embryos were co-injected with cdx4/Xcad3-luc, which contains the −546 promoter sequence and the entire intron 1 region of cdx4, or TOPFLASH DNA reporter (Haremaki et al., 2003; van de Wetering et al., 1997) together with mRNA or DNA expression plasmid into the two ventral animal blastomers at the four-cell stage. Five pools of three injected embryos or animal caps were assayed for luciferase activity at stage 12.5/13 using the luciferase assay system (Promega) according to the manufacturer’s protocol, as described (Yamamoto et al., 2003).

Real-time PCR

Real-time PCR analysis for quantitative RT-PCR (qRT-PCR) was performed using ABI PRISM 7000 or 7300 detection systems with Power SYBR Green PCR Master Mix and PCR primers as follows: reporter mRNA (designed for the SV40 polyadenylation region in the 3' UTR): forward, 5'-TCGTATTACGGATGATCAGACA-3'; reverse, 5'-TTGGTACAATAAAAGCAATACGATC-3'; ef1-α forward, 5'-CCCTGCTGGAG CCTCTTGAC-3'; reverse, 5'-GAGGCAGACGGAGAGGCTTAT-3'; SV40: TAAAGCAATAGCATC-3'. Error bars of normalized values were calculated as described (Yamamoto et al., 2003).
RESULTS
Characterization and expression of Xenopus mex3b

Our previous expression-pattern screening of an anterior neuroectoderm cDNA library (Takahashi et al., 2005) identified a clone, N25A3, which has a 0.8 kb conserved noncoding sequence. EST analysis of N25A3 and sequencing of its full-length cDNA clone revealed that the N25A3 sequence is a part of the 3’ UTR of an mRNA with a CDS that encodes 507 amino acids containing two KH domains and a RING finger domain. This gene was subsequently identified as the Xenopus ortholog of human MEX3B (also known as KKHD3) (Fig. 1A). The 3’ UTR of Xenopus mex3b is 2.2 kb long and, notably, the last 0.8 kb sequence is highly conserved among vertebrates and amphioxus (see Fig. S1 in the supplementary material). This sequence will be referred to as ‘3’ long conserved UTR’ (3’ LCU) (Fig. 1B,C). Database analysis suggests that such a long evolutionarily conserved 3’ UTR is very unusual, implying that the 3’ LCU plays an important and unique role in the regulation of mex3b mRNA. Because KH domains are believed to be an RNA-binding domain, we hypothesized that Mex3b protein regulates its target RNAs through KH domains and that the 3’ LCU of mex3b mRNA is involved in post-transcriptional regulation by RNA-binding proteins.

In Xenopus embryos, mex3b mRNA was detected in the animal region at the four-cell stage (Fig. 1D,E). At the early gastrula stages, mex3b was expressed in the entire ectoderm and the equatorial region (Fig. 1F,G). During gastrulation, the expression in the ectoderm region gradually became restricted to the neural plate during gastrulation (Fig. 1H,J). At later stages, expression was detected in the brain, branchial arches and tailbud (Fig. 1J). This expression pattern implies multiple functional roles of mex3b in embryogenesis.

The 3’ LCU promotes translation and destabilization of mRNA

The existence of 3’ LCU in the mex3b 3’ UTR prompted us to examine the function of this region in the regulation of mex3b mRNA. Because the 3’ LCU contains several AREs, which are common RNA-destabilizing elements, we first tested whether the 3’ LCU promotes mRNA degradation using GFP mRNA reporter constructs (Fig. 2A). Reporter mRNA was injected into the prospective neuroectoderm of Xenopus embryos at the four-cell stage and examined at the early neurula stage (stage 13) using WISH. Notably, compared to control mRNAs (GFP, GFP+3’ LCUrev and GFP+nc3’ UTR), GFP+3’ LCU mRNA was markedly degraded (Fig. 2B-E). qRT-PCR confirmed that GFP+3’ LCU but not GFP mRNA was significantly decreased between stages 12 (late gastrula) and 13 (Fig. 2H). These data suggest that the 3’ LCU has a destabilizing element that responds to some endogenous factors.

To narrow down the region required for the degradative activity, the 3’ LCU was divided into four subregions A, B, C and D, based on sequence conservation among vertebrates (see Fig. S1A in the supplementary material). The data showed that subregion A is necessary and sufficient for the degradative activity (Fig. 2F,G), and that this degradation occurs between stage 12 and 13 (see Fig. S2 in the supplementary material). Therefore, we named this element ‘late gastrula destabilizing element’ (LGDE).

We next examined whether the 3’ LCU affects the level of GFP protein. Surprisingly, embryos injected with GFP+3’ LCU or GFP+3’ LCUrev mRNA showed stronger GFP fluorescence at the neurula stage than those injected with GFP or GFP+nc3’ UTR mRNA (Fig. 2I-L). Because both GFP+3’ LCU and GFP+3’ LCUrev showed translational enhancement, secondary structures of the 3’ LCU RNA may be important. Using the 3’ LCU deletion constructs, however, we could not assign this translational enhancement activity to a single entity, because all deletion constructs examined had some activity (see Fig. S3 in the supplementary material). Western blot analysis revealed that the increased levels of GFP induced by the 3’ LCU were already detectable at the four-cell stage (1.5 hours after mRNA injection) and continued to be observed at later stages (Fig. 2Q). These data suggest that the 3’ LCU has elements, named ‘translational enhancer elements’ (TLEEs), that are distributed in subregions A to D. To examine whether the translational enhancement continued to the neurula stage, we created a destabilized GFP, in which the degradation sequence PEST of ornithine decarboxylase was fused to EGFP. GFP-PEST+3’ LCU mRNA-injected embryos exhibited decreased levels of GFP fluorescence at stage 14 (Fig. 2N), consistent with the expectation that GFP-PEST+3’ LCU mRNA is degraded at this stage and the GFP-PEST protein is unstable. By contrast, 3’ LCUrev still upregulated GFP-PEST protein levels compared with the non-conserved 3’ UTR (nc3’ UTR) (Fig. 2O,P), suggesting that the TLEEs (actually sequences complementary to TLEEs) also function at the early neurula stage. Taken together, these results suggest that the 3’ LCU has at least two types of regulatory elements: LGDE and TLEE.

Mex3b promotes mRNA degradation through the 3’ LCU and forms an autoregulatory loop

Because Mex3b has KH domains, we hypothesized that Mex3b could regulate its own mRNA through the 3’ LCU. To test this possibility, we examined whether forced expression of Mex3b
affects the level of reporter mRNA. Although GFP+3’LCU mRNA was degraded to some extent by endogenous factors through the LGDE as shown above, we found that co-injection of mex3b mRNA further promoted the degradation of GFP+3’LCU mRNA but not GFP mRNA (Fig. 3A,B). We also showed that this degradation is detectable at stage 9.5 as well as stage 12.5 (see Fig. S4A in the supplementary material), which differs from the LGDE-dependent degradation by endogenous factors after stage 12 (see Fig. S2 in the supplementary material). This indicates that Mex3b destabilizes reporter mRNA in a 3’LCU-dependent manner. By contrast, Mex3b was not able to either promote or reduce the translation of reporter mRNA at stage 7 (see Fig. S4B in the supplementary material) before Mex3b-dependent mRNA degradation occurred, suggesting that Mex3b does not affect the translational enhancement mediated by the TLEEs.

We next assigned a region of the 3’LCU that responds to Mex3b. As shown in Fig. 3C,D, Mex3b destabilized mRNAs, GFP+BCD, +CD and +D, which possess subregion D (panel C; data for +BCD and +CD are not shown), but did not destabilize mRNAs, GFP+ABC, +AB, and +A (panel D; data for +AB and +A are not shown). These data suggest that subregion D is necessary and sufficient for mRNA degradation by Mex3b, and therefore this element was named ‘Mex3b-responsive destabilizing element’ (MRDE). Thus, the 3’LCU has at least two distinct destabilizing elements, LGDE and MRDE, residing in subregions A and D, respectively.

To specify the sequence of MRDE, subregion D was divided into five regions, D1-D5, each of which is about 60 nucleotides long (see Fig. S5A in the supplementary material). Among them, only the D2 construct was destabilized, although weakly, by Mex3b (see Fig. S5B in the supplementary material). Notably, this weak destabilizing activity of D2 was enhanced by adding D1, D3 and D4 but not D5 (see Fig. S5B in the supplementary material). Furthermore, D2 contains a purine-rich stretch followed by a destabilizing sequence AUUUUAUUUAUUUA (see Fig. S5C in the supplementary material), which is similar to the binding site of human MEX3D in the BCL2 3’ UTR (Donnini et al., 2004). These data suggest that Mex3b binds to the core sequence of the MRDE in subregion D2 and that the other regions, D1, D3 and D4, are required for full destabilizing activity of the MRDE by binding putative endogenous co-factors of Mex3b.

To examine the physical interaction between Mex3b and the 3’LCU, we next performed RNA immunoprecipitation experiments. Embryos that had been injected with GFP+3’LCU or GFP mRNA as a target and mRNA for HA-tagged protein, were lysed and immunoprecipitated with the anti-HA antibody. Immunoprecipitated protein-RNA complexes were then subjected to RT-PCR for the mRNA. As shown in Fig. 3E, GFP+3’LCU mRNA was immunoprecipitated by Mex3b-HA (lane 1). By contrast, smaller amounts of GFP+3’LCU mRNA were brought down by the negative control proteins, ΔKH1+2-4HA and 4HA-globin (lanes 3 and 5), suggesting that Mex3b physically interacts with the 3’LCU through the KH domains. In addition, GFP mRNA was not brought down by Mex3b-4HA (lane 4), indicating that the KH domains of Mex3b specifically bind to the 3’LCU. We conclude from these results that Mex3b has autorepressive activity through the formation of a complex with the 3’LCU, resulting in a negative autoregulatory loop.

The 3’LCU acts as a system for fine-tuning gene expression

We expected that translational enhancement initially predominates over destabilization of mRNA to increase the amount of Mex3b, whereas, when the level of Mex3b becomes high enough to destabilize mex3b mRNA, the rate of production of Mex3b is reduced. This combined regulation provides a buffering action, which leads to fine-tuning gene expression. To test this hypothesis, we designed the experiment that mimics the endogenous state, using pCS DNA expression constructs that have the CMV promoter and Mex3b-4HA (lane 4), indicating that the KH domains of Mex3b specifically bind to the 3’LCU. We conclude from these results that Mex3b has autorepressive activity through the formation of a complex with the 3’LCU, resulting in a negative autoregulatory loop.

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and protein (Fig. 4C,F), but both levels were much less with the 3'LCU or subregion D than the control. These data suggest that the negative autoregulatory loop operates through the MRDE in the 3'H11032LCU and subregion D. Timecourse experiments also confirmed that the accumulation of Mex3b protein is delayed by subregion D (see Fig. S6 in the supplementary material). We noticed that an injection of 100 pg of CMV-

myc-mex3b+3'H11032LCU slightly upregulated the Mex3b protein level compared with the control (compare the protein bands of the western blot in Fig. 4C), supporting the translational enhancement activity of the 3'H11032LCU. Taken together, these data suggest that the translational enhancement and the negative autoregulatory loop through the 3'LCU form a fine-tuning system to maintain an appropriate level of Mex3b protein by their buffering action.

Effects of overexpression and knockdown of Mex3b on its own mRNA and on early embryogenesis

We next asked whether this negative autoregulatory loop through the 3'LCU operates in vivo and what the role of Mex3b is in early Xenopus embryogenesis. To address these questions, we first overexpressed Mex3b in the dorsal mesoderm or dorsal ectoderm region by injecting mRNA into the dorsal equatorial region or the dorsal animal pole region, respectively, at the four-cell stage (Takada et al., 2005). Overexpression of Mex3b in the dorsal mesoderm resulted in the loss of Xbra expression at the early gastrula stage (Fig. 5A), but it did not affect the expression of gsc and Xlim1 (see Fig. S7A,B in the supplementary material). Because Xbra expression is known to be induced by both FGF and activin/Nodal signaling (Smith et al., 1991), whereas gsc and Xlim1 are induced by activin/Nodal but not by FGF signaling (Cho et al., 1991; Taira et al., 1992), the data suggest that Mex3b specifically inhibits FGF signaling. Overexpression of Mex3b in the neuroectoderm reduced endogenous mex3b expression (Fig. 5B), which is in good agreement with its autorepression activity. Mex3b also decreased, although not strongly, the expression of the anterior marker otx2 (Fig. 5C) and the posterior marker genes cdx4/Xcad3 and hoxd1 (Fig. 5D,E) at the neurula stage, but did not affect a pan-neural marker, sox2 (Fig. 5F), a neural crest marker, msx1, and an anterior mesoderm marker, crescent (data not shown). Notably, forced expression of Myc-ΔKH1+2, which lacks KH domains, increased the level of the endogenous mex3b mRNA (Fig. 5G), suggesting that Myc-ΔKH1+2 functions as a dominant-negative mutant. At tailbud stages, overexpression of Mex3b in the dorsal mesoderm and neuroectoderm both exhibited a short axis phenotype (Fig. 5H-J). These data suggest that Mex3b is involved in AP patterning of the neural plate and in mesoderm formation.

To analyze the requirement for Mex3b during early embryogenesis, we next performed loss-of-function experiments using antisense morpholino oligos (MOs). We designed two MOs, mex3b MO1 and mex3b MO2, which targeted different sites of the mex3b 5'H11032UTR, and a five-base mismatched MO (5mmMO) as a control. We first examined the specificity of mex3b MO1 and 2417

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showed that it specifically inhibited the protein synthesis from 5′ UTR-mex3b mRNA (see Fig. S7D in the supplementary material). Because both mex3bMO1 and mex3bMO2 gave similar phenotypes, we used a mixture of them (referred to as mex3bMOs) for subsequent experiments.

Injection of mex3bMOs in the dorsal equatorial region markedly decreased the expression of Xbra (Fig. 5K) but not of chordin, gsc and Xlim1 (see Fig. S7J-L in the supplementary material) and caused severe open-yolk-plug phenotypes at the tailbud stages (Fig. 5P). This result is somewhat unexpected, because overexpression of Mex3b also inhibits Xbra expression (Fig. 5A; and see Discussion). However, the effect of mex3bMOs on Xbra expression is specific, because rescue experiments showed that the inhibition of Xbra expression by the MOs was recovered by co-injection with mex3b mRNA (see Fig. S7E-I in the supplementary material).

Importantly, injection of mex3bMOs into the prospective neuroectoderm region increased the endogenous expression of mex3b, strongly suggesting that Mex3b destabilizes its own mRNA in vivo. We also found that mex3bMOs decreased the expression of anterior markers, otx2 and rax (Fig. 5M,N) possibly by increasing posteriorizing signals. However, mex3bMOs did not expand cdx4 expression in an anterior direction (Fig. 5O), leading to the possibility that the anterior boundary of cdx4 is determined by negative regulation, which could not be overcome by increasing posteriorizing signals. In fact, we have observed that FGF20, which has the ability to inhibit otx2 expression (see Fig. S7M in the supplementary material), was not able to expand cdx4 expression in the dorsal region (see Fig. S7N in the supplementary material; indicated by black arrow), but expanded it in the lateral region (see Fig. S7N in the supplementary material; indicated by magenta arrow), implying that cdx4 is positively regulated by FGF signaling but negatively regulated for the anterior boundary by other signaling pathways. At the tailbud stage, small eye phenotypes were observed (Fig. 5Q,Q′), consistent with otx2 and rax reduction at the neurula stage. Together with the results of the gain-of-function experiments, these data suggest that Mex3b is required for AP patterning in the neural plate and mesodermal differentiation in the marginal zone, and that the negative autoregulatory loop of Mex3b is operating in vivo.

We hypothesized that the negative autoregulation of mex3b mRNA occurs through the 3′LCU in vivo. If this is the case, excess amounts of 3′LCU should deplete possible 3′LCU-binding proteins (3′LCU-BPs), including Mex3b, from the cell, and excess amounts of the complementary strand of the 3′LCU (3′LCUrev) should hybridize to the 3′LCU of mex3b mRNA to prevent 3′LCU-BPs from binding. To test this possibility, we injected a large amount of GFP+3′LCU or GFP+3′LCUrev mRNA into embryos and showed that both injected mRNAs increased the amount of endogenous mex3b mRNA (Fig. 5R–T). These data indicate that the level of mex3b mRNA in vivo is negatively regulated through the 3′LCU.

**Mexc3b affects the FGF responsiveness of the cell**

The phenotypes caused by overexpression and knockdown of Mex3b suggest the possible relationship between Mex3b and FGF signaling, because cdx4 and Xbra are direct targets of FGF signaling (Haremaki et al., 2003; Northrop and Kimelman, 1994; Pownall et al., 1996; Smith et al., 1991). To examine whether Mex3b inhibits FGF signaling, we first performed animal cap assays. As shown in Fig. 6A, Mex3b inhibited Xbra expression induced by FGF2, similar to a dominant-negative FGFR1, XFD. In control experiments, Mex3b did not inhibit gsc expression induced by activin (Fig. 6B), suggesting that Mex3b specifically inhibits FGF signaling. To further address this, we performed reporter analysis using cdx4-luc,
in which the luciferase gene is driven by the cdx4 promoter and intron 1 containing FGF response elements; this reporter is upregulated in the dorsal ectoderm in an FGF-dependent manner or activated by FGF signaling in dissociated animal cap cells (Haremaki et al., 2003). As shown in Fig. 6C, expression of fgf8 or fgf4 upregulated luciferase activity of cdx4-luc in the ventral ectodermal region, and this activity was inhibited by co-injection of mex3b mRNA (Fig. 6C). Conversely, mex3bMOs further upregulated luciferase activity induced by FGF8 (Fig. 6D). Because expression of cdx4-luc is reportedly affected by Tcf3 or β-catenin (Haremaki et al., 2003), we asked whether Mex3b modulates canonical Wnt signaling. TOPFLASH reporter analysis showed that Mex3b did not inhibit activation of the reporter gene by Wnt8 (data not shown), suggesting that Mex3b mRNA destabilizes Reporter mRNA remaining in the embryos were examined at stage 12.5/13. Note that the 3’ UTR of fgf20 (A), syntenin (B), sdc2 (C) or ets1 (D) destabilized reporter mRNA to some extent in response to endogenous factors (left panels), but only the 3’ UTR of sdc2 (C) or ets1 (D) responded to Mex3b for mRNA destabilization (right panels). (F) FGF reporter assay. Reporter DNA cdx4/Xcad3-luc (100 pg) was co-injected with fgf4 DNA (10 pg), sdc2 mRNA (100 pg) and ets1 (100 pg) as indicated, and injected embryos were assayed for luciferase activity at stage 12.5/13.

assays but it did not do so (see Fig. S8B in the supplementary material), suggesting that Mex3b mainly affects a FGF signal transducer(s) downstream of, or parallel with, ERK, or both.

**Microarray screening of Mex3b target mRNAs**

The data so far suggest that Mex3b regulates FGF signaling by destabilizing some mRNAs in a similar manner to autorepression. To identify such putative target mRNAs of Mex3b, we performed differential screening using *Xenopus* oligo DNA microarray. Target mRNAs, if any, would be expected to be regulated in a
fashion similar to that of mex3b mRNA and to be decreased in Mex3b-overexpressed embryos and increased in mex3b MO-injected embryos. Based on this expectation, we carried out two sets of differential screening with (1) wild-type Mex3b versus MexcΔKH1+2 and (2) 5mmMO versus mex3b MOs. mRNA or MO was injected into the prospective neuroectoderm region of embryos with GFP mRNA as a tracer, and the embryos in which GFP fluorescence was detected in the neural plate were collected at the early neurula stage. To maximize the difference in the levels of affected mRNAs, the neural plate was isolated from these embryos. Total RNA was extracted from these neuroectoderm pieces and verified by semi-quantitative RT-PCR for mex3b mRNA.

To select possible Mex3b target mRNAs, we used criteria of a 1.5-fold difference in set 1 (wt-Mex3b/Myc versus ΔKH1+2) and a 1.2-fold difference in set 2 (5mmMO versus mex3b MOs) so that selected genes include mex3b, which is considered as an internal positive control in this microarray experiment. We assigned Xenopus and/or mouse RefSeq ID(s) for 6826 genes from the 28,310 tags in total. These genes were divided into three groups according to the following conditions: group 1, candidates of Mex3b target genes (ΔKH1+2/Mex3b ratio of more than 1.5 or a mex3b MOs/M5mmMO ratio of more than 1.2; 221 genes; see the footnote of Table S1 in the supplementary material); group 2, negative controls, the genes with expression that would not be regulated by Mex3b (ΔKH1+2/Mex3b ratio of less than 1.1 and a mex3b MOs/M5mmMO ratio of less than 1.1; 155 genes); and group 3, the remaining 6447 genes. We performed GOstat analysis (Beissbarth and Speed, 2004), which categorizes genes based on gene ontology (GO) terms. However, we could not find any specific GO terms concentrated in the group 1 genes, and thereby could not expect any common functions of Mex3b targets from microarray analysis (see the footnote of Table S2 in the supplementary material). These data imply that Mex3b directly or indirectly regulates various mRNAs, which are involved in diverged cellular functions.

**sdc2 and ets1 as Mex3b target mRNAs**

Because functional analyses suggest the involvement of Mex3b in FGF signaling, we used the microarray data to seek possible FGF-signaling components. We picked fgf20, ets1 and syntenin (syndecan-binding protein) from the 221 possible targets (in both set 1 and set 2), and sdc2 from set 1 (752 tags). We first focused on fgf20. However, although fgf20 mRNA was downregulated by forced expression of Mex3b in the whole embryo (see Fig. S7C in the supplementary material), mRNA reporter analysis showed that the 3' UTR of fgf20 did not respond to Mex3b for destabilization (Fig. 7A). Therefore, we directly analyzed 3' UTRs of sdc2, ets1b and syntenin. We found that Mex3b specifically destabilizes the 3' UTRs of sdc2 and ets1b but not of syntenin mRNAs (Fig. 7B-D).

**DISCUSSION**

In this study, we have analyzed the function of the RNA-binding protein Mex3b and its 3' UTR regulatory region, the 3'LCU, in early Xenopus embryogenesis. Based on our data, we propose the following model of post-transcriptional regulation of mex3b mRNA (Fig. 8). The 3'LCU has at least three distinct elements. The first type is the translational enhancer element (TLEE), distributed in four subregions A to D; the second type is the late gastrula destabilizing element (LGDE) in subregion A; and the third type is the Mex3b-responsive destabilizing element (MRDE) in subregion D. Of these elements, the negative autoregulatory loop through the MRDE is a key component in the maintenance of the level of Mex3b protein. The TLEEs also contribute to the maintenance of the protein level by rapid upward regulation of mex3b mRNA translation when the protein level is decreased. The LGDE affects the mRNA level through some as yet unidentified trans-acting factors, which act at the late gastrula stage and perhaps later. Thus, these three distinct elements constitute a fine-tuning system for mex3b mRNA that determines and maintains an appropriate level of Mex3b by providing a buffering action against fluctuations. Mex3b at a certain level in turn controls the half-life of target mRNAs including sdc2 and ets1b, which are probably involved in FGF responsiveness. This model provides a new mechanism for fine-tuning the control of mRNAs as well as a novel role of post-translational regulation in determining the level of cellular responsiveness to signaling molecules.

Several types of post-transcriptional autoregulation by RNA-binding proteins have been reported. For example, Drosophila ELAV binds to its own 3' UTR, as does the mouse homolog Mel-N1, and this binding increases the stability of mRNA by competing with other factors that destabilize the mRNA (Abe et al., 1996; Borgeson
and Samson, 2005; Samson, 1998). TTP destabilizes its own mRNA as well as other mRNAs through binding to AUUUA sequences (Brooks et al., 2004). The poly(A) binding protein PABP downregulates its own translation or decreases the amount of its own mRNA in a cell-type-specific manner (Hornstein et al., 1999). Among them, the Mex3b autoregulation mechanism is similar to that of TTP. However, Mex3b has a characteristic 3'UTR, 3'LCU, that possesses translational enhancer elements in addition to autoregulatory elements.

Derepression mechanisms for translational enhancement are well known. The best example of a derepression mechanism is that of the cytoplasmic polyadenylation element (CPE) during oocyte maturation. Before maturation, the CPE-binding protein (CPEB) inhibits translation by binding to the CPE, whereas, when maturation begins, translation is initiated by derepression of CPEB activity upon its phosphorylation by Aurora kinase (Kuersten and Goodwin, 2003). By contrast, the 3'LCU of mex3b enhances translation, possibly by the binding of trans-acting factors to the secondary structures of the 3'LCU. Recently, it has been reported that the 3' UTR of nanos2 enhances its translation, as distinct from derepression (Tsuda et al., 2006), but the mechanism of enhancement is unknown. More recently, it has been revealed that microRNAs upregulate the translation of TNFα mRNA through recruiting Argonaute and FXR1 (Vasudevan et al., 2007). In all cases, further studies are necessary to clarify the mechanisms of translational enhancement through the 3' UTR. Thus, our data provide a novel 3'-regulatory module that is composed of elements for both positive regulation and negative autoregulation. Furthermore, we have shown that this regulatory module is actually operating in developing embryos.

We have shown that Mex3b can destabilize mRNAs for sdc2 and ets1 and inhibits FGFind. To date, various negative and positive regulators of FGFind have been reported, including the negative regulators Sprouty, Sef and Mkp3 (Furthauer et al., 2002; Hacohen et al., 1998; Kawakami et al., 2003) and the positive regulators of FGFind have been reported, including ets1.

What is the role of Mex3b in FGFind signal transduction? In AP patterning of the neuroectoderm, FGFind signals act as morphogens to regionalize the neuroectoderm along the AP axis in a concentration-dependent manner (Dubrulle and Pourquié, 2004; Kengaku and Okamoto, 1995). In this context, Mex3b is required to control the levels of key FGFind components at the mRNA level to fine-tune the level of FGFind signaling. The effects of such fine-tuning mechanisms at post-transcriptional levels may be relatively subtle compared with transcriptional regulation, but they are absolutely necessary for normal development, as we have shown in this study using overexpression and knockdown of Mex3b. In the mesoderm, overexpression of Mex3b decreases Xbra expression, which is consistent with the requirement of FGFind signaling for Xbra expression. However, knockdown of Mex3b also decreases Xbra expression. We do not have sufficient data to explain this result, but one possibility is that Mex3b morpholinos downregulates Xbra expression through upregulation of some unidentified Mex3b targets, which directly or indirectly inhibits Xbra expression.

Our data indicate that subregion D of the 3'LCU is necessary and sufficient for the negative autoregulation of Mex3b. Interestingly, only subregion D is conserved among human MEX3B, MEX3C, MEX3D and Xenopus mex3c, suggesting that the autoregulation is a common mechanism of Mex3 family members. Supporting this idea, we found that Mex3b can destabilize GFP reporter mRNA containing subregion D of Xenopus mex3c (see Fig. S5 in the supplementary material). We also found that the amphioxus mex3 gene has the 3'LCU-like sequence (see Fig. S1 in the supplementary material). Therefore, the entire 3'LCU was probably acquired in the ancestral mex3 gene before two rounds of whole genome duplication occurred during vertebrate evolution, and subregions A-C have been changed or lost in mex3 family members other than mex3b. In invertebrates, C. elegans mex-3 does not have the 3'LCU. Mex-3 is anteriorly localized in C. elegans embryos and inhibits the posterior fate. Thus, even if the protein level of Mex-3 is in excess in the anterior region, it does not affect the posterior formation, implying that C. elegans Mex-3 does not require the fine-tuning mechanisms through the 3'LCU. By contrast, Xenopus Mex3b is expressed in the entire neuroectoderm and is likely to be involved in maintaining an appropriate level of FGFind signaling. In this situation, for AP patterning to be precisely determined, an appropriate level of Mex3b protein is necessary. It should be noted that in C. elegans Mex-3 downregulates mRNA of caudal, possibly by directly binding to its 3' UTR, whereas in Xenopus Mex3b indirectly downregulates Cdx4/Xcad3, probably through inhibiting FGFind signaling. Thus, our data suggest the interesting possibility that the existence of the 3'LCU enables Mex3b to regulate the AP patterning of neural tissue through the fine-tuning of FGFind responsiveness.

To date, it has been shown that human MEX3D (TINO) destabilizes the mRNA for BCL2, which inhibits apoptosis, and that MEX3C is putatively involved in ‘essential hypertension’. Because subregion D is conserved in MEX3C and MEX3D mRNAs, our data concerning mex3b autoregulation may be applicable to the understanding of molecular mechanisms of other developmental processes and human diseases in which mex3 genes are involved.

Acknowledgements
We thank D. Turner, R. Rupp and J Lee for pcPS2+, pCS2+MT and pCS2+nβ-gal, E. De Robertis for gsc; Y. Sasai for sox2 and chd; H. Okamoto for the Xcad3 luciferase reporter construct; H. Ide for fgf8; M. Pannese for pGEM3-Xotx2, K. Yokoyama for pXFGF-20 and pSP56-XFGF-20; J. Smith for Xbra, M. Jamrich for Ro, H. Sive for hox1, and M. Hagiwara and T. Nojima for discussion. This work was supported in part by a Grant-in-Aid for Science Research from the Ministry of Education, Science, Sports, and Culture of Japan (M.T.); by research fellowship of the Japan Society for the Promotion of Science (M.T.); by research fellowship of the Japan Society for the Promotion of Science (H.T.); and by the Global COE Program (Integrative Life Science Based on the Study of Biosignaling Mechanisms), MEXT, Japan (H.T. and H.M.).

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/14/2413/DC1

References


