Molecular Biology of the Cell
Vol. 21, 3137–3148, September 15, 2010

KHDC1B Is a Novel CPEB Binding Partner Specifically Expressed in Mouse Oocytes and Early Embryos

Congli Cai,* Keiko Tamai,† and Kathleen Molyneaux*

*Department of Genetics, School of Medicine, Case Western Reserve University, Cleveland, OH 44106; and †Daiich Sankyo Co. Ltd., Tokyo 103-8426, Japan

Submitted March 30, 2010; Revised July 9, 2010; Accepted July 16, 2010
Monitoring Editor: Kunxin Luo

mRNAs required for meiotic maturation and early embryonic development are stored in growing oocytes. These transcripts are translationally repressed until hormonal cues trigger ovulation. Errors in translation underlie some cases of human infertility and are associated with ovarian germ cell tumors. However, it remains unclear how maternal transcripts are kept quiescent in mammals. This study describes a potential translational regulator, KHDC1B. KHDC1B is a member of a small family of KH-domain containing proteins specific to eutherian mammals. Two family members, KHDC1A and 1B, are highly expressed in oocytes. KHDC1A and 1B bind polyU agarose and form oligomers like other KH-domain proteins. The functions of these proteins were tested by expression in *Xenopus* embryos. KHDC1A caused cell death, whereas KHDC1B caused cleavage arrest. This arrest phenotype was rescued by coexpression of the mouse translational regulator cytoplasmic polyadenylation binding protein 1 (mCPEB1). Coimmunoprecipitation and coimmunostaining experiments confirmed the functional interaction between KHDC1B and mCPEB1. Finally, KHDC1B levels and binding partners were shown to fluctuate with the cell cycle. KHDC1B, via its interaction with mCEPB1, may regulate translation of mRNA targets required for oocyte maturation.

INTRODUCTION

Oocyte maturation and early embryonic development take place in the absence of transcription and rely on maternally stored mRNAs and proteins. The temporal and in some cases the spatial expression of these stored mRNAs control cell cycle progression and embryonic patterning until zygotic gene expression is initiated. Consequently, the regulation of mRNA stability, localization, and translation serve as the driving forces behind oogenesis and early embryogenesis (Colegrove-Otero *et al*., 2005).

Multiple translational regulators including members of the DAZ (deleted in azoospermia), PUF (Pumilio and FBF), STAR/QKI (Signal transduction and activator of mRNA/Quaking), and Bicaudal C families have been shown to be vital for oocyte development with evidence accumulating from both invertebrate and vertebrate systems (Colegrove-Otero *et al*., 2005; Kimble and Crittenden, 2007). Perhaps the best studied of these factors, at least at the biochemical level, is cytoplasmic polyadenylation element binding protein (CPEB). CPEB is an RNA recognition motif (RRM) protein originally identified in *Xenopus* oocytes (Hake and Richter, 1994). CPEB controls the translation of specific mRNA targets during meiotic maturation (Stebbins-Boaz *et al*., 1996) and early embryonic development (Groisman *et al*., 2000). In growing oocytes, CPEB binds mRNA targets containing the cytoplasmic polyadenylation element (CPE), a U-rich motif (consensus UUUUUAU) found in the 3′-untranslated region (UTR) typically within 100 nucleotides of the polyadenylation site. Initially, CPEB acts as a translational repressor by recruiting the eIF4E (eukaryotic initiation factor 4E) binding protein Maskin to the RNP complex (Stebbins-Boaz *et al*., 1999). Maskin binds the cap-associated eIF4E, blocking its interaction with eIF4G and preventing assembly of the ribosome on the transcript. The hormonal signals that control oocyte maturation lead to the activation of the Aurora A/Eg2 kinase that phosphorylates CPEB (Mendez *et al*., 2000). Phospho-CPEB leads to activation of the cytoplasmic polyadenylase, xGld2 (Barnard *et al*., 2004; Papin *et al*., 2008), elongation of the polyA tail, displacement of Maskin, and assembly of an active translational complex.

The core role for CPEB in translational control during oocyte maturation appears to be conserved in both invertebrate and vertebrate model systems. However, many of the details of this process remain vague in mammals. An oocyte specific loss of mouse CPEB1 (mCPEB1) results in abnormal follicle growth suggesting a role for mCPEB1 before its canonical role in meiotic maturation (Racki and Richter, 2006). Additionally, there are four CPEB paralogs in mammals, and three are expressed in mouse oocytes (Evsikov and Marin de Evsikova, 2009b), complicating analysis in this system. It is also puzzling that *mGld2/Papd4* knockout females are fertile, leaving the identity of the cytoplasmic polyadenylase unclear in mice (Nakanishi *et al*., 2007). Finally, sequence comparisons have failed to identify a mammalian Maskin homologue. Transforming acidic coiled-coiled domain proteins (TACCs) share some sequence homology with Maskin but lack the eIF4E binding region, and it is unclear whether they participate in translational repression (de Moor *et al*., 2005). The discovery of both

This article was published online ahead of print in *MBoC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E10–03–0255) on July 28, 2010.

Address correspondence to: Congli Cai (cxc242@case.edu).

Xenopus (Minshall *et al*., 2007) and mouse (Evsikov and Marin de Evsikova, 2009a) eIF4Eb, an oocyte specific isoform of eIF4E shown to inhibit translation, has led to the development of Maskin-independent models for translational repression (reviewed in Standart and Minshall, 2008). In this study, we describe a novel family of KH domain proteins that, based on their expression and binding partners, are likely to participate in translational control during oocyte growth. Analysis of this gene family may help clarify how translational repression is controlled during oocyte growth in mammals.

MATERIALS AND METHODS

Harvesting Oocytes and Embryos

All animal procedures have been approved by the Case Western Institutional Animal Care and Use Committee. CD-1 mice (Charles River Laboratories, Wilmington, MA) were used to obtain tissues, oocytes, and early embryos for RNA and protein assays. Early stage embryos (from embryonic day 0.5–5.5), fetal ovaries, and testes were obtained from naturally mated mice. To obtain germinal vesicle (GV) oocytes, the ovaries were removed from 8-wk-old mice and transferred into prewarmed M2 medium containing 100 μM 3-Isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich, St. Lous, MO), which prevents GV oocytes from undergoing germinal vesicle breakdown (GVBD). The ovaries were punctured using 27 gauge needles and GV oocytes were harvested under a stereomicroscope. To obtain GVBD and metaphase I (MI) oocytes, GV oocytes were released from IBMX and cultured for 3 and 6 h, respectively. Metaphase II (MII) arrested oocytes were recovered from mice induced to ovulate by intraperitoneal injection of pregnant mare serum gonadotropin (PMSG, Sigma-Aldrich) and human chorionic gonadotropin (hCG, Sigma-Aldrich) as described (Hogan, 1994). Ovulated oocytes were released from the ampullae of oviducts 20 h post-hCG. The cumulus cells were dispersed by brief exposure to 0.1 M hyaluronidase (Sigma-Aldrich) and careful washing.

Xenopus Embryo Manipulation

Xenopus embryo injections were performed as described (Iioka *et al*., 2009). RNA was in vitro transcribed using SP6 mMESSAGEmachine (Ambion, Austin, TX). Embryos were microinjected with 5 nl per blastomere of in vitro synthesized RNA. To obtain sections of *Xenopus* embryos, the embryos were fixed in 100% methanol then rehydrated into phosphate-buffered saline (PBS) through a methanol series (75%, 50%, 25% methanol). The embryos were cryoprotected in 15 and 30% sucrose and embedded in Tissue-Tek OCT (optimum cutting temperature) compound (Fisher Scientific, Pittsburgh, PA). Embryos were sectioned at 10–12 μm.

RNA Isolation, cDNA Synthesis, and RT-PCR

Heart, liver, lung, kidney, brain, intestine, muscle, testes, and ovaries were obtained from 8-wk-old mice. Ovaries and testes from fetal or young mice, oocytes, and embryos were obtained as described above. Total RNA was extracted using Trizol following the manufacturer's protocol. After treatment with DNase I (Promega, Madison, WI), total RNA was quantified using the Quant-iT RiboGreen kit (Invitrogen). Reverse transcription was performed using the Superscript III First-Strand cDNA Synthesis kit (Invitrogen, Carlsbad, CA) in 20 μl reaction. The reaction products were diluted to 200 μl, and PCR was performed with primers for specific genes. *Khdc1a* primers: (F: CAA GGT CTG GGA AGG CTA CA; R: TGT CCC CCT TTG TTA AGT GC). *Khdc1b* primers: (F: GAC CAT GAG CCT TCA GCA CT; R: ACT CTG CTC AGG ACA CTG CTC). *Gapdh* primers: (F: AGG CCG GTG CTG AGT ATG TC; R: TCA GAT CCA CGA CGG ACA CA).

Histology and RNA in Situ Hybridization

In situ hybridization on ovary sections was performed as described previously (Yu *et al*., 2008). Ovaries from eight-week-old mice were fixed in 4% paraformaldehyde (PFA) for 1 h at 4°C. Ovaries were washed with PBS, cryoprotected in 30% sucrose, and embedded in OCT compound (Tissue-Tek). Tissues were sectioned at 10 μm on a Leica Cryostat. Sense and antisense RNA probes were transcribed in vitro with the DIG labeling kit (Roche, Indianapolis, IN) from linearized T-Easy vectors (Promega). The primers for generating probes are: *Khdc1a*-F: CAA GGT CTG GGA AGG CTA CA; *Khdc1a*-R: CAG CAT TGC CTA TAC CAG AGA; *Khdc1b*-F: ACT TCT AGG TGT AAG ATT CTG; *Khdc1b*-R: AGC CCA CTG ATG TAC ACC TAC.

Plasmids Used in the Study

pCS2-Flag or pCS2-HA vectors were derived from the pCS2 vector (*http://sitemaker.umich.edu/dlturner.vectors/home*) by adding two tandem Flag or HA tags to the original plasmids. The coding sequence of mouse *Khdc1a*, *Khdc1b*, *mCpeb1*, and *eIf4e* genes were inserted into pCS2-Flag or pCS2-HA vectors for transfections or to be linearized for in vitro transcription. For production of glutathione-S-transferas (GST)-fusion proteins, the coding sequences of mouse *Khdc1a* and *Khdc1b* genes were inserted into pGEX-4T-1 with BamHI and XhoI. pWPI, pMD2G and pCMV-dR8.74 lentivirus system (*http://tronolab.epfl.ch/page58115.html*) was used for establishing the NIH-3T3-KHDC1 stable cell lines. The Flag-tagged *Khdc1a* or *Khdc1b* coding sequence was inserted in the pWPI plasmid using the PmeI site.

Polynucleotide Binding Assay

KHDC1 proteins were expressed in bacteria and purified with the B-PER GST Fusion Protein Purification Kit (Pierce, Rockford, IL) according to the manufacture's protocol. The PolyU binding assay was performed as described (Chen *et al*., 2001) with the following modifications. Purified GST fusion protein (100 ng) and 50 μl poly U agarose beads were added to 0.5 ml RNA binding buffer (10 mM Tris-HCl at pH 7.5, 100 mM NaCl, 2.5 mM MgCl2, 0.5% Triton X-100, and 0.1 mg/ml BSA). The mixture was rotated at 4°C for 30 min. The beads were spun down and washed five times with ice-cold binding buffer. Proteins bound to the beads were released by boiling in 1× SDS loading buffer and resolved on SDS-PAGE. Proteins were detected by Western blotting using a polyclonal antibody directed against GST (Abcam, Cambridge, MA).

Cell Culture, Transfection, and Establishing the KHDC1 Stable Cell Lines

NIH-3T3, 293T, and HeLa cells were cultured in DMEM containing 10% fetal bovine serum (FBS) at 37°C and 5% $C0_2$. Transfection was conducted using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). The NIH-3T3-KHDC1 stable cell lines were established as described (Wiznerowicz and Trono, 2003). Briefly, 293T cells were cotransfected with pCMV-dR8.74, pMD2G, and either pWPI-KHDC1A or pWPI-KHDC1B. Twenty-four and 48 h after transfection, the medium containing lentivirus was filtered and added to NIH-3T3 cells. Infected cells were passaged and analyzed by fluorescence-activated cell sorting (FACS). GFP-positive cells were maintained as stable cells.

Cell Synchronization and FACS Analysis

Cells were synchronized at G2/M by thymidine-nocodazole treatment. Cells were synchronized at G1/S by double-thymidine treatment (Whitfield *et al*., 2002). To collect cells at different stages, the cells were released from block and collected at different time points. DNA content of synchronized cells was determined by staining with 50 μg/ml propidium iodide in the presence of 0.1 mg/ml RNaseA, followed by flow cytometry (Coulter Epics XL, Beckman). The data were analyzed with WinMDI software.

Coimmunoprecipitation and Western Blot Analysis

293T cells were cotransfected with different plasmid combinations as necessary. Cells were washed with ice-cold PBS and lysed in EBC buffer: 50 mM Tris-HCl, (pH 7.5), 120 mM NaCl, 0.5% Nonidet P-40 supplemented with protease inhibitor cocktail (Roche) and PhosSTOP Phosphatase Inhibitor Cocktail (Roche). Lysates were clarified by centrifugation at 16,100 × *g* for 15 min at 4°C, and the supernatant was incubated with 2 μg indicated antibody at 4°C for 2 h with rotation. Protein G agarose beads (20 μl; Roche) were added for 1 h at 4°C with rotation. Then the beads were washed by EBC buffer 3 times and boiled 10 min at 95°C in 1× SDS loading buffer.

For immunoblot analysis, proteins were resolved by SDS-PAGE and transferred to Immobilon-P membrane (Millipore, Billerica, MA). The filters were blocked in TBST (10 mM Tris [pH 8.0], 150 mM NaCl, 0.1% Tween 20) supplemented with 5% (wt/vol) powdered milk for 1 h at room temperature and then incubated with primary antibody at room temperature for 1.5 h or 4°C overnight. Anti–pan-KHDC1rabbit serum (Rajpal *et al*., 2003) and affinity purified KHDC1A polyclonal antibodies (GenScript, Piscataway, NJ) were used at 1:2000. Anti-GST (Abcam), anti-HA (Abcam), and anti–β-tubulin (Sigma) antibodies were used at 1:10,000. The monoclonal anti-Flag antibody was used at 1:5000. The filters were then washed three times with TBST at room temperature and incubated with a horseradish peroxidase-conjugated goat anti-mouse or rabbit secondary antibody (GE, Piscataway, NJ) diluted 1:5000 in blocking buffer for 1 h at room temperature. The filters were then washed three times with TBST, and bound secondary antibody was detected by ECL plus (GE) according to the manufacturer's protocol.

Immunofluorescence Staining

Frozen sections of mouse ovaries and *Xenopus* embryos were prepared as above. The sections were rehydrated and permeabilized with 0.1% Triton X-100. Oocytes, early embryos, and cultured cells were fixed with 4% paraformaldehyde (PFA) and permeabilized with 0.1% Triton X-100. Then all samples (sections, oocytes, embryos, or cells) were blocked in 3% BSA/PBS at room temperature for 1 h and incubated in diluted primary antibody at room temperature for 1.5 h or at 4°C overnight. The mAb against mCPEB1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) (sc-137146); KHDC1 antibodies are described above. Samples were then washed with PBS

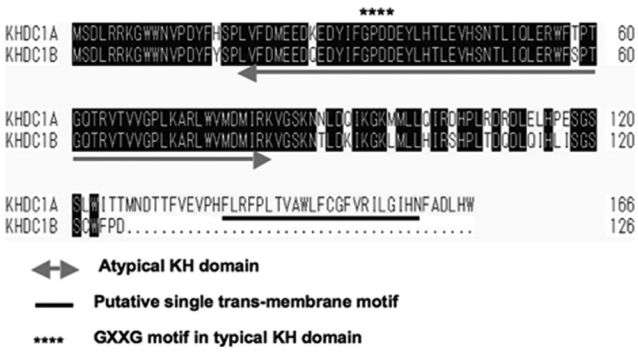

Figure 1. Alignment of KHDC1A and KHDC1B proteins. Overall the proteins are 65% identical (black-boxed amino acids). Identity is highest in the N-terminal region containing the KH domain (arrow).

three times and incubated in diluted secondary antibody (Cy5 conjugated anti-rabbit IgG and FITC conjugated anti-mouse IgG) (Jackson Immunoresearch Laboratories, West Grove, PA) at room temperature for 1 h. Samples were then washed as above and mounted in VECTASHIELD Mounting Medium (Vector labs, Burlingame, CA). Images were collected with the TCS SP2 confocal microscope (Leica, Bannockburn, IL) or DM6000 fluorescence microscope (Leica) and analyzed with Volocity software (Perkin Elmer-Cetus, Waltham, MA).

RESULTS

The Khdc1 *Gene Family Encodes for Putative RNA Binding Proteins*

Transcripts for *Khdc1a* (*KH domain containing 1A*) were detected in a transcriptional profiling screen for genes expressed in purified primordial germ cells (PGCs) (Molyneaux *et al*., 2004). Using q-RT-PCR, we confirmed that expression of *Khdc1a* is elevated in E10.5 and E12.5 PGCs relative to expression in the somatic cells (Supplemental Figure 1). Searching GenBank revealed that *Khdc1a* is a member of the small KH domain containing 1 family originally described by Pierre *et al.* (2007). There are three members in this family, *Khdc1a* (gene ID: 368204), *Khdc1b* (gene ID: 98582), and *Khdc1c* (gene ID: 433278). All three genes are adjacent to each other on mouse Chromosome 1A4. In silico analysis identified 40 expressed sequence tugs (ESTs) for *Khdc1a* in cDNA derived from spleen, ovary, testis, fertilized eggs, and early embryos. In contrast, ESTs for *Khdc1b* were restricted to the ovary, oocytes, and early stage embryos. *Khdc1c* is very similar in sequence to *Khdc1a* making expression difficult to detect unambiguously. In fact, UniGENE has listed mRNAs for *Khdc1c* under the *Khdc1a* reference. It is currently unclear whether *Khdc1c* is expressed or is a psuedogene, so we have confined our analysis to *Khdc1a* and *Khdc1b*.

Both *Khdc1a* and *Khdc1b* have three exons. The ORF (open reading frame) of mouse *Khdc1a* is 501 base pairs long, encoding a 166-aa protein. The ORF of mouse *Khdc1b* is 381 base pairs long, encoding a 126-aa protein. As shown in Figure 1, both KHDC1 proteins contain a N-terminal domain with similarity to the K homology (KH) nucleotide binding motif; however, an invariant GXXG loop shown to be required for the RNA binding activity of the KH domain is changed (Lewis *et al*., 2000). Compared with KHDC1B, KHDC1A has a unique hydrophobic C-terminal extension predicted by InterProScan analysis to be a transmembrane helix motif.

Khdc1 mRNAs Are Enriched in Oocytes and Early Stage Embryos

In silico analysis suggested that *Khdc1a* and *Khdc1b* are expressed in a limited subset of tissues. To confirm this, we used RT-PCR to examine *Khdc1a* and *1b* expression in a panel of adult mouse tissues (Figure 2A). *Khdc1a* was highly expressed in ovaries along with weak expression in several other tissues such as brain, lung, and testis. In contrast, *Khdc1b* was only observed in ovaries. There was no *Khdc1b* detected in the other tissues examined including testes.

Next, we examined the expression of *Khdc1* mRNAs in oocytes and early stage embryos. As shown in Figure 2B, *Khdc1a* and *Khdc1b* have similar expression patterns. The mRNAs of both genes are enriched in germinal vesicle (GV) and metaphase II (MII) stage oocytes along with one-cell and two-cell stage embryos. The mRNA levels of both genes were decreased in 4–8 cell stage embryos and could not be detected in blastocysts.

To see when *Khdc1* expression in ovaries initiates, we performed a developmental time series (Figure 2C). Weak *Khdc1a* expression could be detected in the fetal gonads. Expression of *Khdc1a* increased dramatically in postnatal day 2 ovaries. Additionally, *Khdc1b* expression initiated at postnatal day 2 and increased as the animals matured. This expression pattern indicates that *Khdc1a* and *Khdc1b* are likely to play a role in oocyte maturation or early embryonic development.

To further confirm expression of *Khdc1a* and *Khdc1b* in growing oocytes, we performed in situ hybridization on cryosections of mouse ovaries. Consistent with the RT-PCR results and previous observations (Pierre *et al*., 2007), the signal was enriched in oocytes (Figure 2D). The solid inset and spaced inset show a high magnification view of preantral and antral follicles, respectively, highlighting the strong expression of *Khdc1* genes in later stage follicles.

KHDC1 Proteins Are Expressed in Oocytes and Early Embryos

Two antibodies were used to examine the distribution of KHDC1 proteins (Figure 3). First, a rabbit polyclonal antibody (pan-KHDC1) raised against full-length KHDC1A (Rajpal *et al*., 2003) was tested and found to recognize both KHDC1A and 1B expressed in 293T cells (Figure 3A). Second, a rabbit polyclonal antibody specific for KHDC1A (αKHDC1A) was generated using a peptide (RDHPLRDRDLELHP) corresponding to 103 aa to 116 aa of KHDC1A, a region not exhibiting high sequence similarity to KHDC1B (Figure 1). Using the isoform specific antibody, we demonstrated that KHDC1A is highly expressed in the ovary but is also moderately expressed in other tissues including the liver and testis (Figure 3B). There appear to be two isoforms of KHDC1A expressed in the testis, the expected size 19-kDa form and a lower-molecular-weight isoform that might be the product of tissue specific processing. This lower-molecular-weight form was also detected in mature oocytes and early embryos (Figure 3C). Protein levels decline in E3.5 (blastocyst) and E4.5 embryos. Immunofluorescence using pan-KHDC1 of mouse ovary sections revealed that the KHDC1 proteins are enriched in oocytes (Figure 3D). KHDC1 proteins can be detected in oocytes within growing and antral follicles (Figure 3D). In most cases, the KHDC1 proteins were cytoplasmic, but in 14% of follicles the proteins were located within the nucleus of oocytes (Figure 3D). Additionally, KHDC1B accu mulated in nuclei when overexpressed in HeLa cells (Figure 7A).

Whole mount immunostaining of oocytes and early embryos revealed that the KHDC1 proteins are expressed in

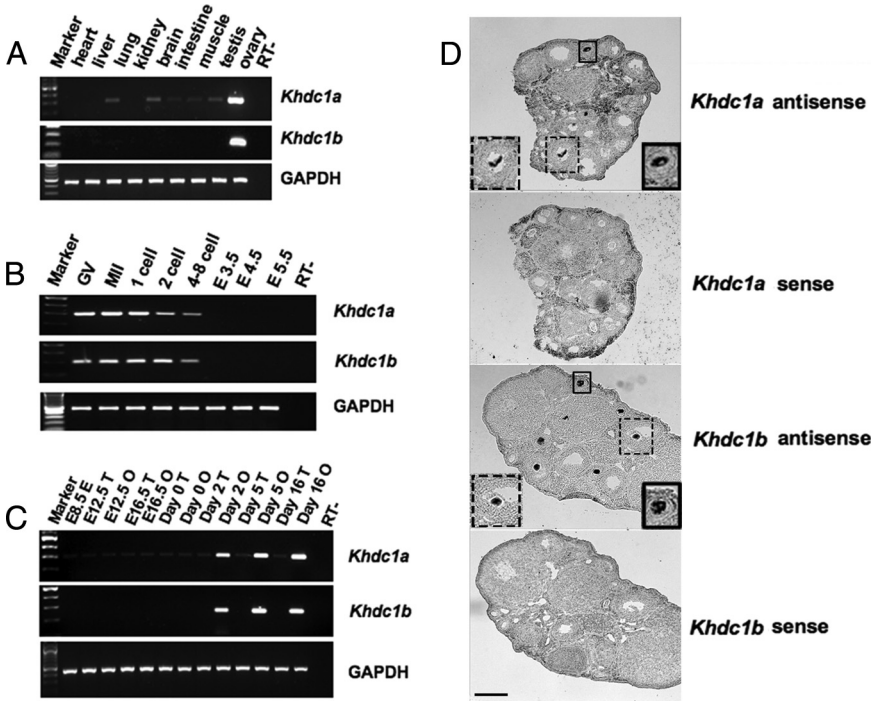

Figure 2. *Khdc1* family members are expressed in oocytes and early embryos. (A) RT-PCR for *Khdc1a* and *Khdc1b* expression in the indicated tissues. *Gapdh* expression is shown as a loading control. RT- indicates the negative control sample run without reverse transcriptase. (B) RT-PCR for *Khdc1a* and *Khdc1b* expression in isolated germinal vesicle (GV) and meiotic M-phase II (MII) oocytes and embryos. The stage of embryo is indicated by the cell number or by embryonic day. (C) RT-PCR for *Khdc1a* and *Khdc1b* in fetal (E12.5–16.5) and postnatal (Day 0–16) testes (T) and ovaries (O). E8.5 indicates whole embryonic day 8.5 embryos. (D) In situ hybridization for *Khdc1a* and *Khdc1b* in adult ovary sections. Sense probes were used as controls. Solid insets are higher magnification preantral follicles. Preantral follicles contain growing oocytes surrounded by one or a few layers of granulosa cells. Dashed insets are higher-magnification antral follicles. Antral follicles represent the final stage in follicle growth, contain transcriptionally and translationally quiescent oocytes surrounded by multiple layers of granulosa cells, and contain a fluid-filled space termed the antrum. Scale bar = 240 μm.

GV, GVBD, and MII oocytes as well as embryos up to E3.5 (Figure 3E). Intriguingly, the KHDC1 proteins localized to structures resembling germinal granules in the cytoplasm. Germinal granules (a.k.a. polar granules) are large protein and RNA complexes that are maternally deposited and direct germ cell specification in *Drosophila*, *C. elegans*, and *Xenopus* (Chuma *et al*., 2009). Evidence for maternal specification of germ cells is lacking in mice, but some studies have identified structures resembling germinal granules in mouse oocytes (Swetloff *et al*., 2009). In addition to appearing in granules, the KHDC1 proteins were also enriched in the cortex and in the perinuclear area of GVBD oocytes, MII oocytes, and 1-cell stage embryos (see arrows in Figure 3E, subpanels b, c, d, and e).

KHDC1 Proteins Have Polynucleotide Binding and Oligomerization Activity

The KH domain is a nucleotide binding domain, and most KH domain proteins associate with single-stranded RNA or DNA (Ryder *et al*., 2004; Galarneau and Richard, 2005). For example, Quaking I protein (QKI) regulates splicing and translation of mRNAs by binding to its target sequence NACUAAY-N_{1-20}-UAAY. The presence of an atypical KH domain in the KHDC1 proteins suggests that they might be RNA-binding proteins. To test this possibility, we analyzed whether these proteins can bind to homopolymer RNA-agarose. We purified GST fused KHDC1A and KHDC1B and tested their binding activity using poly-U as a target. As shown in Figure 4A, GST-KHDC1A and GST-KHDC1B have poly-U binding activity, however, as a control, GST itself could not bind to poly-U. This demonstrates that KHDC1A and KHDC1B possess nucleotide binding activity and are likely to bind endogenous mRNA targets.

KH domains are typically found in multiple copies, two in fragile X mental retardation protein (FMRP) (Siomi *et al*., 1993), three in hnRNP K and other members of the Poly rC binding protein family (Choi *et al*., 2009), and up to 14 in members of the vigilin family. These domains cooperate with each other and regulate the affinity and specificity for target RNAs (Chmiel *et al*., 2006). Other KH domain proteins such as members of the STAR family contain only one KH domain, and it is proposed that multimerization facilitates their RNA-binding activity (Chen *et al*., 1997). To test whether KHDC1A can form multimers, HA-tagged KHDC1A was cotransfected with or without Flag-tagged KHDC1A. Immunoprecipitations using antibodies directed against the Flag tag resulted in the precipitation of Flag-KHDC1A and coprecipitation of HA-KHDC1A, indicating that KHDC1A can form multimers (Figure 4B, lanes 1 and 2). Similar experiments proved that KHDC1B can also form multimers (Figure 4B, lanes 5 and 6). Furthermore, KHDC1A and KHDC1B can interact with each other (Figure 4B, lanes 3 and 4).

KHDC1 Proteins Interact with the Cytoplasmic Polyadenylation Machinery

The later stages of oocyte maturation and early embryonic development occur in the absence of transcription and consequently rely on the activities of a large number of RNA-binding proteins involved in translational regulation. The activity of many of these factors was initially characterized in *Xenopus*, making this a valuable system for testing the role of putative translational regulators (Brown, 2004). To test the activity of KHDC1A and KHDC1B, we ectopically expressed these proteins in *Xenopus* embryos. RNAs encoding for mouse *Khdc1a* or *Khdc1b* were injected into one cell of a two-cell embryo. By stage 6.5, embryos injected with *Khdc1a* exhibited small and strongly pigmented cells near the injection site (Figure 5A). By stage 8.5, these effected cells had died and the embryos failed to gastrulate (Figure 5A and data not shown). This phenotype resembles the induction of apoptosis achieved by overexpressing Bix3 (Trindade *et al*., 2003) and would be consistent with the fact that overexpression of KHDC1A has been shown to induce apoptosis in tissue culture cells (Rajpal *et al*., 2003). In contrast to KHDC1A, ectopic expression of KHDC1B resulted in large blastomeres at the injection site (Figure 5A). This large-cell

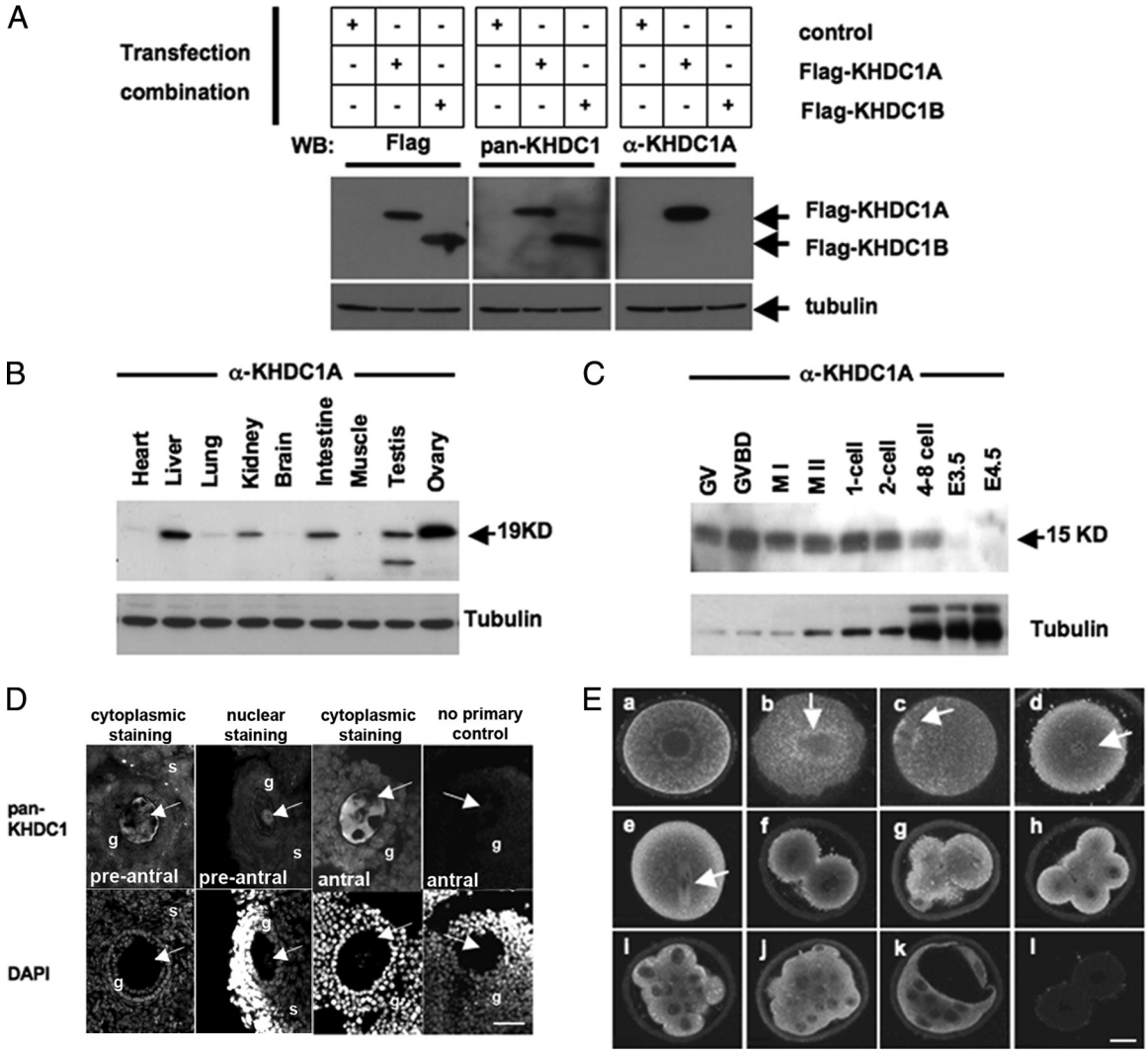

Figure 3. Expression profile for KHDC1 proteins. (A) Antibody specificity was tested by Western blotting. 293T cells were transfected with the indicated plasmids. The pan-KHDC1 antibody recognizes both 1A and 1B. The peptide antibody (α-KHDC1A) is specific for 1A. (B) KHDC1A is expressed in multiple tissues as detected using the α-KHDC1A antibody. The predicated size for KHDC1A is 19.6 kDa. In addition to the full-length form, a 15 kDa isoform of 1A is detected in testis. Tubulin is shown as a loading control. (C) The α-KHDC1A antibody detects a 15-kDa isoform in germinal vesicle (GV), germinal vesicle breakdown (GVBD), and meiotic M-phase I (MI) and meiotic M-phase II (MII) stage oocytes. Expression was also detected in early cleavage stage embryos. (D) Immunostaining for pan-KHDC1 in frozen sections taken from adult ovaries. Preantral follicles containing oocytes surrounded by two layers of granulosa cells or antral follicles surrounded by multiple layers of granulosa cells are shown. Staining is either cytoplasmic or nuclear, and arrows indicate the position of the oocyte. The antibody also stains granulosa cells (g) and ovarian stroma (s). An antral follicle processed without primary antibody is shown as a negative control. Scale bar = 82 μm. (E) Immunostaining for pan-KHDC1 during meiotic maturation and early embryonic development. Staining is shown in germinal vesicle (a), germinal vesicle breakdown (b), and meiotic M-phase II (c) stage oocytes and 1-cell (d and e), 2-cell (f), 4-cell (g), 8-cell (h), 16-cell (i), morula (j), and blastocyst (k) stage embryos. A two-cell embryo processed without primary antibody (l) is shown as a negative control. Arrows indicate perinuclear staining. Scale bar = 20 μm.

phenotype is characteristic of perturbations affecting cell cycle progression, which in *Xenopus* embryos is controlled by translational regulators (Mendez and Richter, 2001). Embryos injected with *Khdc1b* die shortly after midblastula transition (Figure 5D).

Cell division requires coordination of both cytoplasmic and nuclear processes. To see whether KHDC1B expression affects the cytoplasmic or nuclear aspects of division, we examined the distribution of microtubules (Figure 5B) and DNA (DAPI) (Figure 5C) in *Khdc1b*-injected embryos. The MT cytoskeleton in the cleavage-arrested cells was completely disrupted (Figure 5B). Mitotic asters could be readily identified in control cells, whereas KHDC1B expressing cells typically contained many small foci of β- tubulin staining. Additionally, regions expressing KHDC1B had fewer nuclei indicating arrest of both nuclear division and cytokinesis (Figure 5C). The above phenotypes bear a striking resemblance to phenotypes resulting from inhibition of the translational regulator, CPEB (Groisman *et al.*, 2000). In *Xenopus*, CPEB controls cell cycle progression before the midblastula transition. It has been shown to regulate polyA tail length and translation of Cyclin B1 and other target mRNAs involved in cell cycle control (Richter, 2007). To test whether KHDC1B overexpression perturbs the cell cycle by interfering with CPEB activity, we performed rescue experiments. The majority of *Khdc1b*-injected embryos have a cleavage

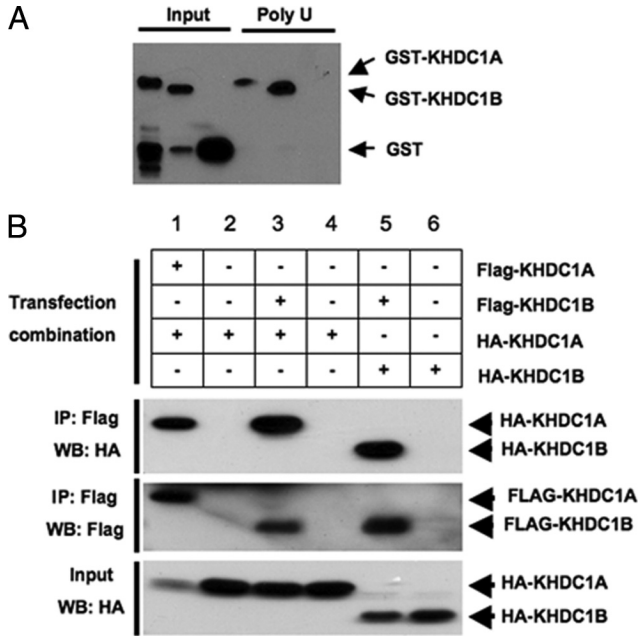

Figure 4. KHDC1A and 1B bind polyU and can multimerize like typical KH domain containing proteins. (A) GST-KHDC1A and GST-KHDC1B bind PolyU agarose. GST alone is shown as a control. (B) HA-KHDC1A coimmunoprecipitates with Flag-KHDC1A (lane 1) or Flag-KHDC1B (lane 3) expressed in 293T cells. HA-KHDC1B also coimmunoprecipitates with Flag-KHDC1B (lane 5). This demonstrates that KHDC1 proteins can form homo or hetero multimers.

arrest phenotype, and only 5.4% of these embryos survive past MBT. However, coinjection of mCPEB1 RNA reversed this phenotype resulting in a nearly complete rescue of survival (91.6%) (Figure 5, D and E). Curiously, coexpression of mCPEB1 was also able to partially rescue the cell death phenotype caused by KHDC1A expression. Only 1.3% of embryos expressing KHDC1A survived to MBT. However, 55.3% of embryos coinjected with mCPEB1 survived. Note that this rescue was not as efficient as that achieved for KHDC1B. Also, note that expression of the weakly related KH-domain protein ESG1/DPPA5 (Tanaka *et al*., 2006; Pierre *et al*., 2007) did not perturb *Xenopus* development indicating that the KHDC1 phenotypes are specific and not just the result of expressing high levels of an RNA-binding protein.

KHDC1 Proteins Are Part of the CPEB Protein Complex

Analysis of the KHDC1 proteins in *Xenopus* indicate that these proteins may regulate CPEB activity. To further test this hypothesis, we examined whether mCPEB1 and KHDC1 proteins can physically interact. Both Flag-KHDC1A and Flag-KHDC1B were able to coimmunoprecipitate with HA-mCPEB1 when expressed in *Xenopus* embryos or in 293T cells (Figure 6A). The interaction between Flag-KHDC1A and mCPEB1 in *Xenopus* appeared to be slightly weaker than the interaction between KHDC1B and mCPEB1. This is consistent with the fact that the KHDC1A phenotype was only partially rescued by mCPEB1 expression (Figure 5E). The interaction between KHDC1B and mCPEB1 was not dependent on RNA (Figure 6B). However, it is the C-terminal RNA-binding domain of mCPEB1 that is required for association (Figure 6, C and D).

KHDC1 Proteins Colocalize with mCPEB1 in Xenopus Embryos, Mammalian Cell Lines, and Mouse Oocytes

The interaction between KHDC1 and CPEB was further analyzed by coimmunostaining. Flag-tagged KHDC1 proteins and HA-tagged-mCPEB1 were expressed in *Xenopus* embryos (Figure 7, A and B) or HeLa cells (Figure 7, C or D), and the subcellular distribution of the proteins were examined by indirect immunofluorescence using anti Flag antibodies (FITC, green) and anti HA antibodies (Cy5, magenta). The distribution of endogenous KHDC1 proteins and mCPEB1 were also examined in mouse oocytes (Figure 7, E and F) by indirect immunofluorescence using the pan-KHDC1 antibody (Cy5, magenta) and a mouse mAb against mCPEB1 (FITC, green). In *Xenopus*, CPEB was reported to be a microtubule binding protein and localizes to the mitotic spindle (Groisman *et al*., 2000). Coimmunostaining for KHDC1 proteins and mCPEB1 revealed colocalization of these proteins in structures resembling the spindle in *Xenopus* blastomeres (Figure 7, A and B). KHDC1B expression disrupts cell division, and the spindle shown in Figure 7B is tripolar. In HeLa cells, overexpressed KHDC1A formed large aggregates in the cytoplasm that only partially overlapped with mCPEB1 expression. KHDC1B colocalized with mCPEB1 in small cytoplasmic granules. However, a portion of KHDC1B was found to accumulate in the nucleus where it did not colocalize with mCPEB1. In germinal vesicle stage oocytes, KHDC1 proteins were found in cytoplasmic granules as previously shown (Figure 3E), and these structures overlapped with the distribution of mCPEB1. The staining intensities for cytoplasmic KHDC1 proteins and mCPEB1 both drop in MI stage oocytes, and the proteins do not colocalize as well as in GV oocytes (Figure 7F). Also, in MI stage oocytes, mCPEB1 staining was enriched on the meiotic spindle similar to its distribution in *Xenopus* (Figure 7A), but endogenous KHDC1 staining was absent from the spindle.

KHDC1A and 1B, despite sharing 65% identity (Figure 1), have distinct subcellular localizations (Figure 7, C and D) and activities (Figure 5B) when ectopically expressed. To test the structural basis for this, we performed domain swap experiments (Supplemental Figure 2). We tested the activity and subcellular localization of a truncated form of KHDC1A lacking the C-terminal extension. Additionally, we fused the KHDC1A extension to the C terminus of KHDC1B. The truncated form of 1A behaved like 1B (Supplemental Figure 2). It caused cell cycle arrest in *Xenopus* and was able to accumulate in the nucleus when expressed in HeLa cells. Likewise the KHDC1B-fu protein behaved like 1A. This demonstrates that the unique C-terminal extension of 1A represents an important structural motif.

KHDC1B Levels and Binding Partners Fluctuate with the Cell Cycle

The exact protein components of the cytoplamic polyadenylation complex depend on the stage of the cell-cycle. For instance, the overall protein levels of the CPEB-inhibitor Maskin were shown to oscillate during the cell-cycle (Groisman *et al*., 2000). Additionally, phosphorylation of CPEB by the M-phase kinase aurora A is proposed to regulate its ability to bind to CPSF and mediate polyadenylation (Groisman *et al*., 2000). To test whether the KHDC1 proteins are cell cycle-regulated, we generated a KHDC1 stable cell lines and examined KHDC1 protein levels at different cell cycle stages. The cells were synchronized with double thymidine at G1/S stage and were released by addition of fresh media. KHDC1A protein levels remained steady throughout the cell cycle (Figure 8A) whereas KHDC1B levels oscillated (Figure 8B) with low levels in S phase and peak levels at the M/G1 transition.

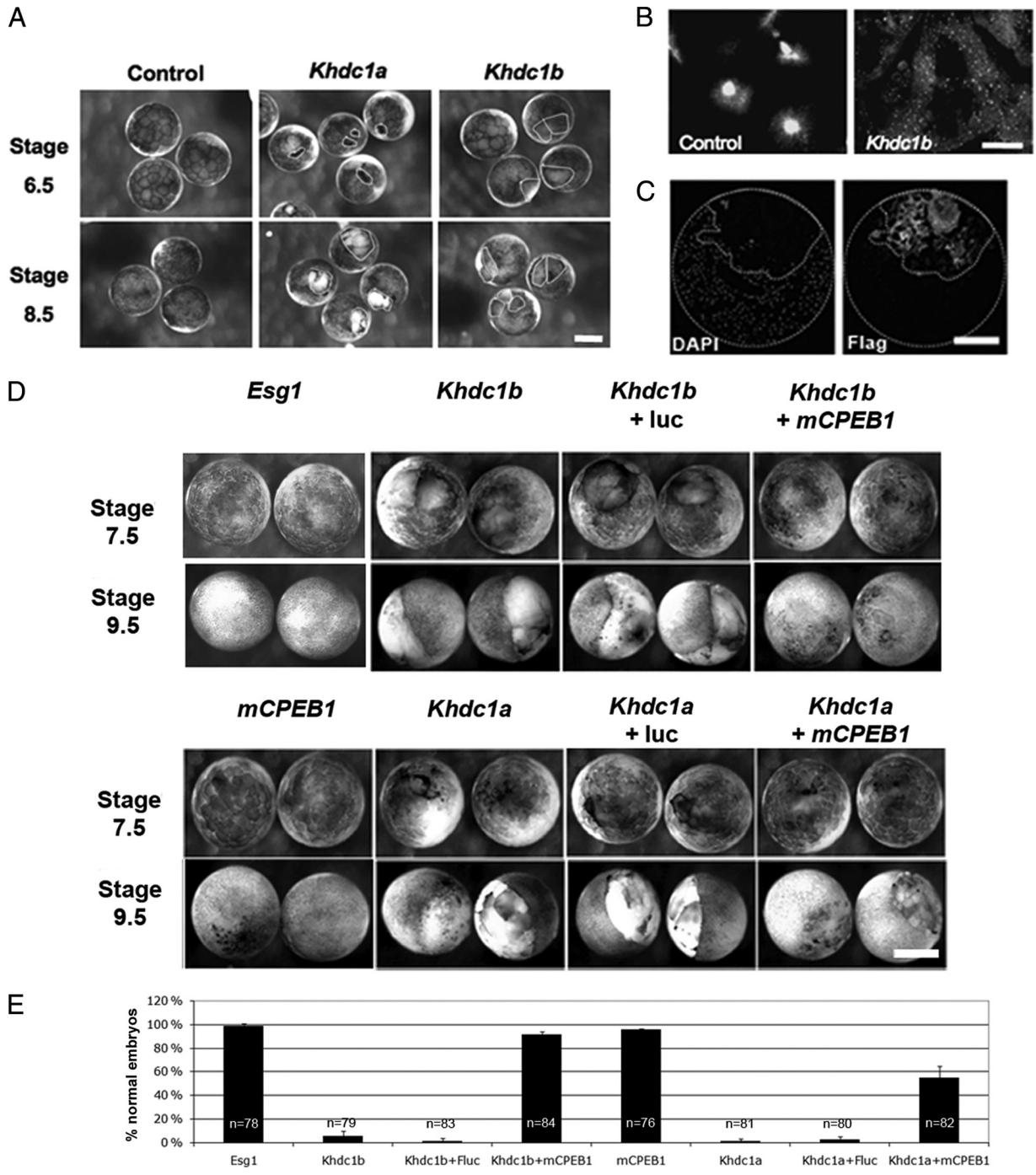

Figure 5. Ectopic expression of KHDC1 proteins perturb *Xenopus* development. (A) 1 ng of Flag-*Khdc1a* or Flag-*Khdc1b* RNA was injected into one cell of two-cell stage *Xenopus* embryos. Phenotypes were scored at stage 6.5 or at stage 8.5. KHDC1A expression caused a small cell phenotype by stage 6.5 (outlines) and cell death by 8.5. KHDC1B expression caused cleavage arrest (outlines) and eventually cell death after stage 8.5 (see D). Scale bar = 500 μm. (B) Ectopic expression of KHDC1B altered microtubule distribution. Frozen sections from control or *Khdc1b*-injected embryos were stained for β-tubulin. Control cells have normal mitotic spindles. *Khdc1b*-injected cells have many small foci of β-tubulin. Scale bar = 100 μm. (C) Ectopic expression of KHDC1B altered the distribution of nuclei. The KHDC1B expressing region marked by Flag staining contains very few nuclei. Dotted lines indicate the edge of the tissue section and the Flag expressing region. Scale bar = 250 μm. (D-E) mCPEB1 rescues the KHDC1 phenotypes. 1 ng of the indicated mRNAs was injected into one cell of two-cell stage embryos and embryos were cultured to midblastula transition (stage 9–9.5). (D) Expression of the control KH domain protein ESG1 did not perturb *Xenopus* development. Expression of KHDC1B by itself caused cell cycle arrest and death by MBT. Coinjection of a control mRNA encoding for luciferase did not rescue the KHDC1B phenotype. However, coinjection of *mCpeb1* rescued both the cell cycle arrest phenotype and embryo survival. Expression of mCPEB1 by itself did not perturb *Xenopus* development. Expression of KHDC1A caused a small cell phenotype and embryonic death. Expression of mCPEB1 partially rescued the KHDC1A phenotype. Scale bar = 500 μm. (E) Percent of control and injected embryos exhibiting normal development to midblastula transition (MBT). n = the total number of embryos counted. Data were collected from three independent experiments, and error bars show SD.

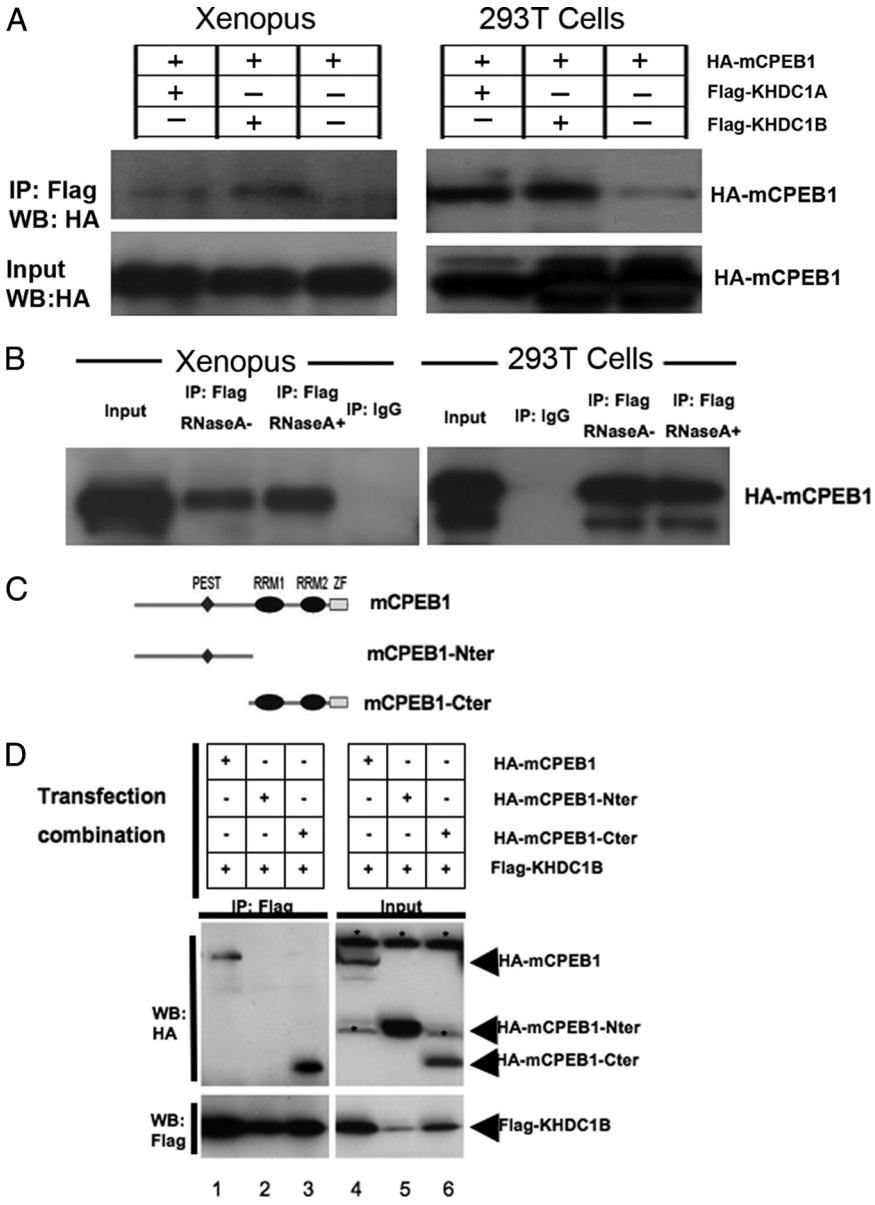

Figure 6. KHDC1 proteins associate with mCPEB1. (A) Flag-KHDC1A and Flag-KHDC1B coimmunoprecipitate with HA-mCPEB1 expressed in *Xenopus* embryos or in 293T cells. (B) The interaction between KHDC1B and mCPEB1 is independent of RNA. (C) Full-length mCPEB1 and the deletion constructs used to test for interaction with KHDC1B. The PEST domain is a motif that regulates protein stability. The RRM (RNA recognition motif) and ZF (zinc finger) domains control RNA binding. (D) HA-mCPEB1 and HA-mCPEB1-Cter coimmunoprecipitate with Flag-KHDC1B when coexpressed in 293T cells. HA-CPEB-Nter did not interact with KHDC1B in this assay. * in the input indicates nonspecific signal. For input, 5% of the total lysate was loaded.

In addition to exhibiting cell cycle–dependent changes in protein levels, KHDC1B binding partners may also be cell cycle–dependent. eIF4E is a known component of the CPEB complex and is a target of the translational repressor Maskin (Stebbins-Boaz *et al*., 1999). KHDC1B was found to weakly associate with the translational regulator eIF4E; however, when cells were synchronized at metaphase by nocodazole, the interaction was strengthened (Figure 8C). These data demonstrate that, like Maskin, KHDC1B may associate with eIF4E under specific conditions.

DISCUSSION

In this study, we describe the expression pattern and activity of two related KH-domain proteins. The founding member of this family, *Ndg1/Khdc1a* was originally identified in T-cells in a screen for target genes of the orphan nuclear receptor NUR77 (Rajpal *et al*., 2003). Pierre *et al.* (2007) identified two additional family members in mice and proposed that the KHDC1 family was part of a larger superfamily of RNA binding proteins expressed in oocytes of eutherian mammals. We have confirmed that both *Khdc1a* and *Khdc1b* are expressed in mouse oocytes but find that *Khdc1a* has a wider expression profile with mRNA detected in lung, brain, testes, and ovaries. KHDC1A protein was detected in liver, kidney, intestine, testes, and ovaries. In addition to the expected sized protein, we detected a smaller-molecular-weight isoform in testes and late stage oocytes. We speculate that this smaller-weight form might represent a splice variant or cleavage product specific to germ cells. We do not know whether KHDC1B protein is expressed because we currently lack an antibody specific for this family member.

The KHDC1 proteins probably have both nuclear and cytoplasmic functions. In growing follicles the KHDC1 proteins are predominately cytoplasmic, but we did observe intense KHDC1 staining within the nucleus of some oocytes. This probably represents KHDC1B protein considering that 1B was the only form to exhibit nuclear localization when transiently

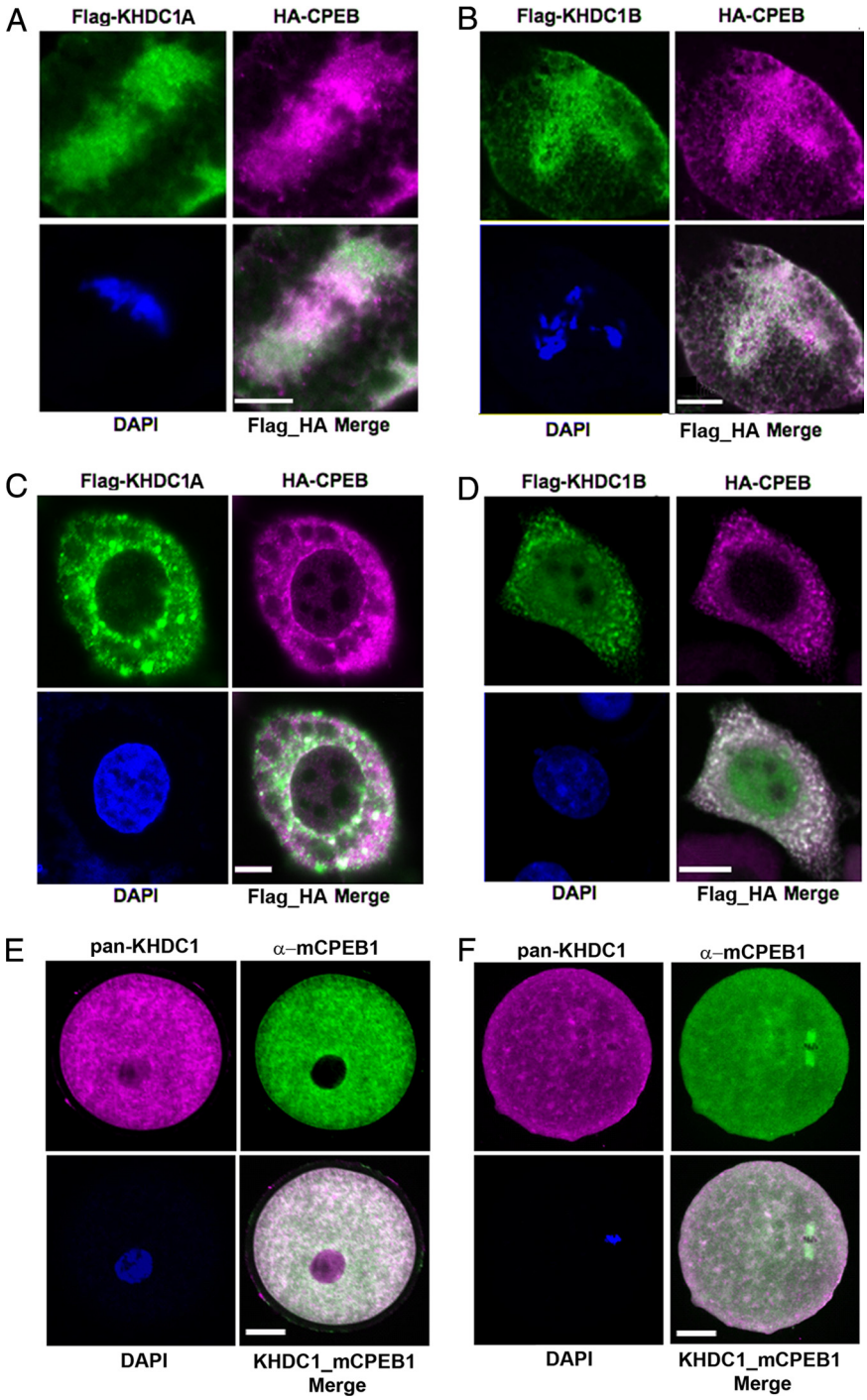

Figure 7. KHDC1 proteins colocalize with mCPEB1 in *Xenopus* embryos, mammalian cells, and mouse oocytes. 1 ng of HA-*mCpeb1* RNA was injected together with (A) 1 ng of Flag-*Khdc1a* or (B) 1 ng of Flag-*Khdc1b* mRNA into one cell of two-cell stage *Xenopus* embryos. Embryos were allowed to develop to stage 6, and protein localization was examined by immunostaining for the Flag (FITC, green) and HA (Cy5, magenta) tags. Images are single optical sections taken via confocal and are representative of the expression pattern seen in the majority of cells. A single blastomere from an injected region is shown. mCPEB1 and KHDC1 proteins accumulated around the spindle in dividing cells. Note the tripolar spindle structure resulting from KHDC1B expression. Scale bar in A = 10 μm. Scale bar in B = 14 μm. (C) Flag-KHDC1A and HA-mCPEB1 were coexpressed in HeLa cells and protein localization was detected as above. Scale bar = 6 μm. (D) Flag-KHDC1B and HA-mCPEB1 were coexpressed in HeLa cells, and their distribution was detected by immunostaining. Scale bar = 10 μm. (E) Immunostaining using the pan-KHDC1 antibody (Cy5, magenta) and a mouse mAb against mCPEB1 (FITC, green) reveal that the endogenous proteins colocalize in GV oocytes. Scale bar = 40 μm. (F) KHDC1 and mCPEB1 also partially colocalize in MI stage oocytes. Note that mCPEB1 is enriched on the spindle, but endogenous KHDC1 proteins are not. Scale bar = 40 μm.

expressed in HeLa cells (Figure 7). It appears that the C-terminal extension of KHDC1A blocks nuclear localization (Supplemental Figure 2) either through tethering the protein in the cytoplasm or by facilitating nuclear export. We favor a tethering mechanism as the C-terminal extension does not contain a known export sequence. Instead, there is a potential transmembrane motif that may anchor the protein to intracellular membrane compartments such as the ER or golgi.

In the cytoplasm of oocytes, KHDC1 proteins were enriched in cytoplasmic granules, in the cortex of oocytes, and within the perinuclear region. The pattern of KHDC1 protein localization underwent subtle shifts during oocyte maturation with more KHDC1 localized to the perinuclear region during GVBD, MII stages, and the first embryonic cell division. This may hint at a role of KHDC1 proteins in regulating progression through the meiotic cell cycle.

KH domain proteins can exhibit both RNA and ssDNA binding and participate in many aspects of nucleic acid biology including transcription, DNA repair, splicing, translation, and RNA stability (Valverde *et al.*, 2008). We have demonstrated that both KHDC1A and KHDC1B have nucleic acid binding activity despite having a variant KH domain lacking the GXXG motif. This is consistent with previous studies on the nucleic acid binding activity

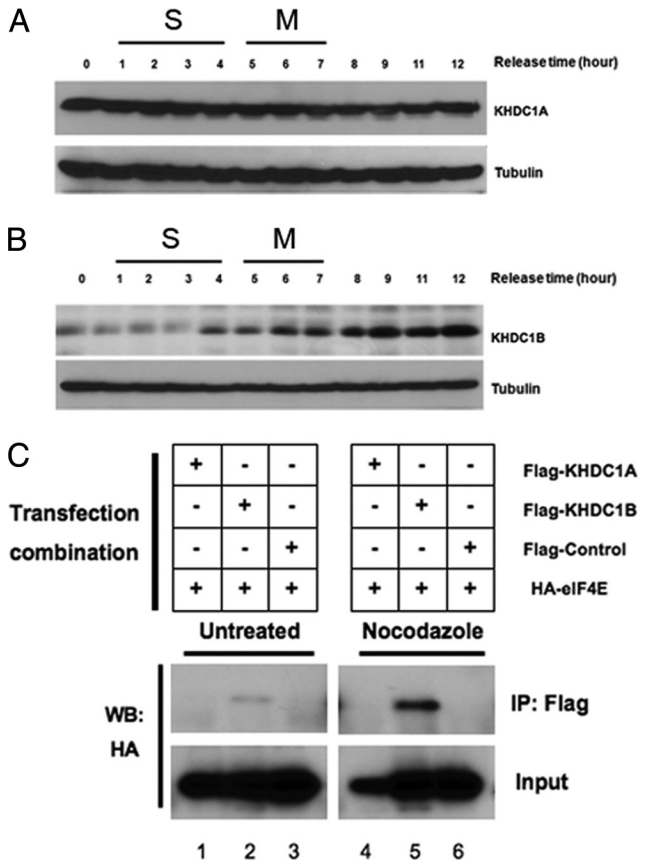

Figure 8. KHDC1B protein levels and binding partners are cell cycle regulated. (A) NIH-3T3 cells stably expressing Flag-KHDC1A were synchronized using double thymidine arrest. The Western blot shows Flag-KHDC1A expression at the indicated times after release from arrest. The cell cycle was determined by flow cytometry, and the approximate positions of S and M phases are shown. (B) NIH-3T3 cells stably expressing Flag-KHDC1B were synchronized, and protein levels were detected as in A. KHDC1B protein levels are elevated in late M and G1 phase. (C) KHDC1B, but not KHDC1A interacted with eIF4E in M-phase cells. In untreated cells, HA-eIF4E associated weakly with Flag-KHDC1B. The precipitation efficiency was enhanced if the cells were treated with nocodazole to arrest the cells in M-phase.

of SCP160. SCP160 is a KH domain in *Saccharomyces cerevisiae*, containing seven conserved KH domains with the GXXG loop and seven diverged KH domains, in which the GXXG loop is interrupted. Mutational analysis revealed that both types of KH domains are essential for SCP160 function; furthermore, the diverged KH domains could functionally replace conserved KH domains (Brykailo *et al*., 2007).

This nucleic acid binding activity of KHDC1 proteins may be modulated by their ability to form oligomers (Figure 4) as proposed for other KH domain–containing proteins (Zorn and Krieg, 1997; Ramos *et al*., 2002). In fact, recent evidence suggests that the KH domain itself can serve as a protein–protein interaction domain in addition to binding nucleic acids (Git and Standart, 2002). Oocytes are the only cell type where both KHDC1A and KHDC1B are expressed and hence the only tissue type where hetero-oligomers are possible. It will be interesting to test whether this interaction is important for regulating oocyte specific targets and whether KHDC1A exhibits different localization or activity in tissues lacking KHDC1B.

Consistent with previous reports (Rajpal *et al*., 2003), KHDC1A induced what appeared to be an apoptotic response when expressed in *Xenopus* embryos. Additional KH-domain proteins have been proposed to regulate apoptosis. In particular, a specific splice isoform of the QKI protein (QKI-7) can induce apoptosis in cultured cells (Pilotte *et al*., 2001). Curiously, this effect is ameliorated by heterodimerization of QKI-7 with other QKI isoforms, and this regulation appears to be controlled via nuclear localization of QKI protein complexes. Perhaps similar regulatory interactions exist between the different isoforms of KHDC1A or between KHDC1A and 1B proteins.

Unlike KHDC1A, KHDC1B did not induce apoptosis. Instead, it caused a cell cycle arrest phenotype consistent with a role in translational regulation. Furthermore, we have shown that KHDC1B interacts with mCPEB1, a core component of the cytoplasmic polyadenylation complex known to regulate oocyte maturation and embryonic cell cycle progression. In *Xenopus*, the members of the CPEB complex are well characterized and include the poly(A) polymerase GLD2, the deadenylating enzyme PARN, the scaffold protein symplekin, and the eIF4E-binding protein and translational inhibitor Maskin. Known CPEB mRNA targets in *Xenopus* include the cell cycle regulators *c-mos* and *Cyclin B1.* In mice, mCPEB1 appears to regulate follicle growth by controlling the polyadenylation and translation of multiple target genes including *Gdf9* (Racki and Richter, 2006), but the molecular details of this regulation are unclear. Based on translational paradigms established in other invertebrate

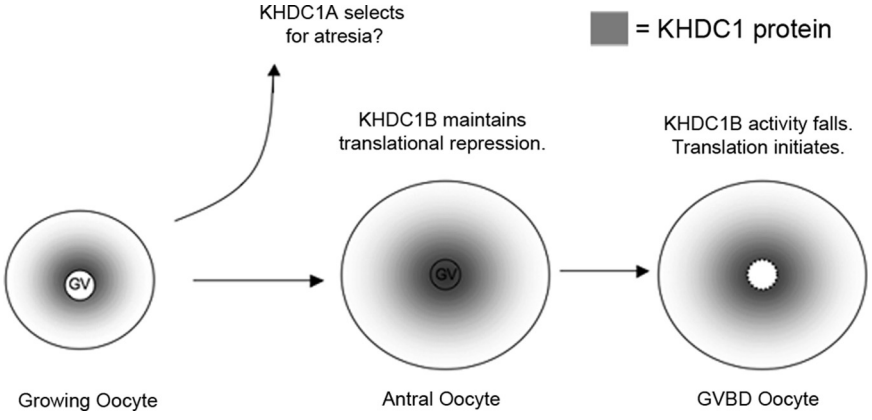

Figure 9. A model for the function of KHDC1 proteins during oocyte maturation. KHDC1 proteins accumulate in the cytoplasm of growing oocytes. If 1A activity dominates, oocytes may undergo cell death. If 1B activity dominates, 1B protein can enter the nucleus where it may assemble into CPEB containing RNP complexes selecting them for repression (Lin *et al*., 2010). Cell survival is rescued and the oocyte matures into a translationally quiescent form. When hormonal cues trigger ovulation, KHDC1B activity falls in response to cell-cycle changes. This triggers activation of cytoplasmic polyadenylation and translation of mRNAs required for progression through meiosis.

and vertebrate systems, we suggest two possibilities for KHDC1B in mouse. The first possibility is that KHDC1B serves as a translational repressor in oocytes and may fulfill a role similar to Maskin. KHDC1B exhibits eIF4E binding activity, and like Maskin KHDC1B protein levels are regulated in a cell-cycle–dependent manner (Figure 8). Also of note, we have observed an apparent shift in KHDC1 protein interaction with CPEB during oocyte maturation (Figure 7). In GV-stage oocytes, the two proteins are highly colocalized in the cytoplasm. However, in MI stage oocytes CPEB accumulates on the spindle, but KHDC1 staining is excluded. The second possibility is that KHDC1 proteins function like GLD1. GLD1 was originally identified in *C. elegans* and is a single KH domain protein and member of the QKI/STAR family. It also acts as a translational repressor (Biedermann *et al*., 2009), but it may have an activating role as well by helping to recruit the GLD2 polymerase to specific targets during entry into meiosis (Kimble and Crittenden, 2007).

In conclusion, we have characterized two related KH domain proteins that appear to have distinct functions (Figure 9). KHDC1A may play a role in apoptosis. In the adult ovary, the majority of oocytes that initiate growth are never ovulated. Instead, they undergo atresia and are lost (Markstrom *et al*., 2002). Perhaps KHDC1A acts to coordinate cell death during this process. KHDC1B appears to play a role in translational control during oocyte maturation. Based on overexpression studies in *Xenopus*, it appears to act as an inhibitor of CPEB, and like CPEB (Lin *et al*., 2010) we propose that it has both nuclear and cytoplasmic functions. These conclusions are based largely on overexpression systems due to the challenges associated with performing biochemistry on mouse oocytes. However, knockout models will eventually help elucidate the specific role of KHDC1 family members during oogenesis and might reveal novel roles for these proteins in other tissue compartments. Additionally, analysis of this gene family may eventually lead to a deeper insight into factors influencing human fertility. The translational regulators DAZ and the KH domain protein FMR (fragile X mental retardation protein) are associated with azoospermia and premature ovarian failure respectively (Fox and Reijo Pera, 2001; Wittenberger *et al*., 2007). By analogy, changes in KHDC1 expression might underlie some cases of idiopathic infertility in humans.

ACKNOWLEDGMENTS

We acknowledge Astar Winoto for the gift of the pan-KHDC1 antibody and George Stark for the pWPI lentivirus system. We thank Weiying Yu for help with the section in situs and Jinhua Shen for help with the cell cycle assays. We thank Radhika Atit and Hua Lou for their comments on the manuscript. We thank Patti Conrad for microscopy assistance and acknowledge the use of the Leica widefield and confocal microscopes in the Genetics Department Imaging Facility at Case Western Reserve University, funded by the National Center for Research Resources (NIH-NCRR) Shared Instrumentation Grant program (1S10RR021228 and 1S10RR017980). Financial support for this project was provided by start-up funds from Case Western Reserve University.

REFERENCES

Barnard, D. C., Ryan, K., Manley, J. L., and Richter, J. D. (2004). Symplekin and xGLD-2 are required for CPEB-mediated cytoplasmic polyadenylation. Cell *119*, 641–651.

Biedermann, B., Wright, J., Senften, M., Kalchhauser, I., Sarathy, G., Lee, M. H., and Ciosk, R. (2009). Translational repression of cyclin E prevents precocious mitosis and embryonic gene activation during *C. elegans* meiosis. Dev. Cell *17*, 355–364.

Brown, D. D. (2004). A tribute to the *Xenopus laevis* oocyte and egg. J. Biol. Chem. *279*, 45291–45299.

Brykailo, M. A., Corbett, A. H., and Fridovich-Keil, J. L. (2007). Functional overlap between conserved and diverged KH domains in *Saccharomyces cerevisiae* SCP160. Nucleic Acids Res. *35*, 1108–1118.

Chen, T., Cote, J., Carvajal, H. V., and Richard, S. (2001). Identification of Sam68 arginine glycine-rich sequences capable of conferring nonspecific RNA binding to the GSG domain. J. Biol. Chem. *276*, 30803–30811.

Chen, T., Damaj, B. B., Herrera, C., Lasko, P., and Richard, S. (1997). Self-association of the single-KH-domain family members Sam68, GRP33, GLD-1, and Qk *1*, role of the KH domain. Mol. Cell. Biol. *17*, 5707–5718.

Chmiel, N. H., Rio, D. C., and Doudna, J. A. (2006). Distinct contributions of KH domains to substrate binding affinity of Drosophila P-element somatic inhibitor protein. RNA *12*, 283–291.

Choi, H. S., Hwang, C. K., Song, K. Y., Law, P. Y., Wei, L. N., and Loh, H. H. (2009). Poly(C)-binding proteins as transcriptional regulators of gene expression. Biochem Biophys Res Commun *380*, 431–436.

Chuma, S., Hosokawa, M., Tanaka, T., and Nakatsuji, N. (2009). Ultrastructural characterization of spermatogenesis and its evolutionary conservation in the germline: germinal granules in mammals. Mol. Cell. Endocrinol. *306*, 17–23.

Colegrove-Otero, L. J., Minshall, N., and Standart, N. (2005). RNA-binding proteins in early development. Crit. Rev. Biochem. Mol. Biol. *40*, 21–73.

de Moor, C. H., Meijer, H., and Lissenden, S. (2005). Mechanisms of translational control by the 3′ UTR in development and differentiation. Semin. Cell Dev. Biol. *16*, 49–58.

Evsikov, A. V., and Marin de Evsikova, C. (2009a). Evolutionary origin and phylogenetic analysis of the novel oocyte-specific eukaryotic translation initiation factor 4E in Tetrapoda. Dev. Genes Evol. *219*, 111–118.

Evsikov, A. V., and Marin de Evsikova, C. (2009b). Gene expression during the oocyte-to-embryo transition in mammals. Reprod. Dev. *76*, 805–818.

Fox, M. S., and Reijo Pera, R. A. (2001). Male infertility, genetic analysis of the DAZ genes on the human Y chromosome and genetic analysis of DNA repair. Mol. Cell. Endocrinol. *184*, 41–49.

Galarneau, A., and Richard, S. (2005). Target RNA motif and target mRNAs of the Quaking STAR protein. Nat. Struct. Mol. Biol. *12*, 691–698.

Git, A., and Standart, N. (2002). The KH domains of *Xenopus* Vg1RBP mediate RNA binding and self-association. RNA *8*, 1319–1333.

Groisman, I., Huang, Y. S., Mendez, R., Cao, Q., Theurkauf, W., and Richter, J. D. (2000). CPEB, maskin, and cyclin B1 mRNA at the mitotic apparatus: implications for local translational control of cell division. Cell *103*, 435–447.

Hake, L. E., and Richter, J. D. (1994). CPEB is a specificity factor that mediates cytoplasmic polyadenylation during *Xenopus* oocyte maturation. Cell *79*, 617–627.

Hogan, B. (1994). Manipulating the mouse embryo: a laboratory manual. Cold Spring Harbor Laboratory Press: Plainview, N.Y.

Iioka, H., Doerner, S. K., and Tamai, K. (2009). Kaiso is a bimodal modulator for Wnt/beta-catenin signaling. FEBS Lett. *583*, 627–632.

Kimble, J., and Crittenden, S. L. (2007). Controls of germline stem cells, entry into meiosis, and the sperm/oocyte decision in *Caenorhabditis elegans*. Annu Rev. Cell Dev. Biol. *23*, 405–433.

Lewis, H. A., Musunuru, K., Jensen, K. B., Edo, C., Chen, H., Darnell, R. B., and Burley, S. K. (2000). Sequence-specific RNA binding by a Nova KH domain: implications for paraneoplastic disease and the fragile X syndrome. Cell *100*, 323–332.

Lin, C. L., Evans, V., Shen, S., Xing, Y., and Richter, J. D. (2010). The nuclear experience of CPEB: implications for RNA processing and translational control. RNA *16*, 338–348.

Markstrom, E., Svensson, E., Shao, R., Svanberg, B., and Billig, H. (2002). Survival factors regulating ovarian apoptosis – dependence on follicle differentiation. Reproduction *123*, 23–30.

Mendez, R., Murthy, K. G., Ryan, K., Manley, J. L., and Richter, J. D. (2000). Phosphorylation of CPEB by Eg2 mediates the recruitment of CPSF into an active cytoplasmic polyadenylation complex. Mol. Cell *6*, 1253–1259.

Mendez, R., and Richter, J. D. (2001). Translational control by CPEB: a means to the end. Nat. Rev. Mol. Cell Biol. *2*, 521–529.

Minshall, N., Reiter, M. H., Weil, D., and Standart, N. (2007). CPEB interacts with an ovary-specific eIF4E and 4E-T in early *Xenopus* oocytes. J. Biol. Chem. *282*, 37389–37401.

Molyneaux, K. A., Wang, Y., Schaible, K., and Wylie, C. (2004). Transcriptional profiling identifies genes differentially expressed during and after migration in murine primordial germ cells. Gene Expr. Patterns *4*, 167–181.

Nakanishi, T., Kumagai, S., Kimura, M., Watanabe, H., Sakurai, T., Kashiwabara, S., and Baba, T. (2007). Disruption of mouse poly(A) polymerase mGLD-2 does not alter polyadenylation status in oocytes and somatic cells. Biochem. Biophys. Res. Commun. *364*, 14–19.

Papin, C., Rouget, C., and Mandart, E. (2008). *Xenopus* Rbm9 is a novel interactor of XGld2 in the cytoplasmic polyadenylation complex. FEBS J. *275*, 490–503.

Pierre, A., Gautier, M., Callebaut, I., Bontoux, M., Jeanpierre, E., Pontarotti, P., and Monget, P. (2007). Atypical structure and phylogenomic evolution of the new eutherian oocyte- and embryo-expressed KHDC1/DPPA5/ECAT1/OOEP gene family. Genomics *90*, 583–594.

Pilotte, J., Larocque, D., and Richard, S. (2001). Nuclear translocation controlled by alternatively spliced isoforms inactivates the QUAKING apoptotic inducer. Genes Dev. *15*, 845–858.

Racki, W. J., and Richter, J. D. (2006). CPEB controls oocyte growth and follicle development in the mouse. Development *133*, 4527–4537.

Rajpal, A., Cho, Y. A., Yelent, B., Koza-Taylor, P. H., Li, D., Chen, E., Whang, M., Kang, C., Turi, T. G., and Winoto, A. (2003). Transcriptional activation of known and novel apoptotic pathways by Nur77 orphan steroid receptor. EMBO J. *22*, 6526–6536.

Ramos, A., Hollingworth, D., Major, S. A., Adinolfi, S., Kelly, G., Muskett, F. W., and Pastore, A. (2002). Role of dimerization in KH/RNA complexes: the example of Nova KH3. Biochemistry *41*, 4193–4201.

Richter, J. D. (2007). CPEB: a life in translation. Trends Biochem. Sci. *32*, 279–285.

Ryder, S. P., Frater, L. A., Abramovitz, D. L., Goodwin, E. B., and Williamson, J. R. (2004). RNA target specificity of the STAR/GSG domain post-transcriptional regulatory protein GLD-1. Nat. Struct. Mol. Biol. *11*, 20–28.

Siomi, H., Siomi, M. C., Nussbaum, R. L., and Dreyfuss, G. (1993). The protein product of the fragile X gene, FMR1, has characteristics of an RNA-binding protein. Cell *74*, 291–298.

Standart, N., and Minshall, N. (2008). Translational control in early development: CPEB, P-bodies and germinal granules. Biochem. Soc. Trans. *36*, 671–676.

Stebbins-Boaz, B., Cao, Q., de Moor, C. H., Mendez, R., and Richter, J. D. (1999). Maskin is a CPEB-associated factor that transiently interacts with elF-4E. Mol. Cell *4*, 1017–1027.

Stebbins-Boaz, B., Hake, L. E., and Richter, J. D. (1996). CPEB controls the cytoplasmic polyadenylation of cyclin, Cdk2 and c-mos mRNAs and is necessary for oocyte maturation in *Xenopus*. EMBO J. *15*, 2582–2592.

Swetloff, A., Conne, B., Huarte, J., Pitetti, J. L., Nef, S., and Vassalli, J. D. (2009). Dcp1-bodies in mouse oocytes. Mol. Biol. Cell *20*, 4951–4961.

Trindade, M., Messenger, N., Papin, C., Grimmer, D., Fairclough, L., Tada, M., and Smith, J. C. (2003). Regulation of apoptosis in the *Xenopus* embryo by Bix3. Development *130*, 4611–4622.

Tanaka, T. S., Lopez de Silanes, I., Sharova, L. V., Akutsu, H., Yoshikawa, T., Amano, H., Yamanaka, S., Gorospe, M., and Ko, M. S. (2006). Esg1, expressed exclusively in preimplantation embryos, germline, and embryonic stem cells, is a putative RNA-binding protein with broad RNA targets. Dev. Growth Differ *48*, 381–390.

Valverde, R., Edwards, L., and Regan, L. (2008). Structure and function of KH domains. FEBS J. *275*, 2712–2726.

Whitfield, M. L., Sherlock, G., Saldanha, A. J., Murray, J. I., Ball, C. A., Alexander, K. E., Matese, J. C., Perou, C. M., Hurt, M. M., Brown, P. O., and Botstein, D. (2002). Identification of genes periodically expressed in the human cell cycle and their expression in tumors. Mol. Biol. Cell *13*, 1977–2000.

Wittenberger, M. D., Hagerman, R. J., Sherman, S. L., McConkie-Rosell, A., Welt, C. K., Rebar, R. W., Corrigan, E. C., Simpson, J. L., and Nelson, L. M. (2007). The FMR1 premutation and reproduction. Fertil Steril *87*, 456–465.

Wiznerowicz, M., and Trono, D. (2003). Conditional suppression of cellular genes: lentivirus vector-mediated drug-inducible RNA interference. J. Virol. *77*, 8957–8961.

Yu, W., McDonnell, K., Taketo, M. M., and Bai, C. B. (2008). Wnt signaling determines ventral spinal cord cell fates in a time-dependent manner. Development *135*, 3687–3696.

Zorn, A. M., and Krieg, P. A. (1997). The KH domain protein encoded by quaking functions as a dimer and is essential for notochord development in *Xenopus* embryos. Genes Dev. *11*, 2176–2190.