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

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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 azoospermmia), PUF (Pumilio and FBF), STAR/QKI (Signal transduction and activation 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 elf4E (eukaryotic initiation factor 4E) binding protein Maskin to the RNP complex (Stebbins-Boaz et al., 1999). Maskin binds the cap-associated elf4E, blocking its interaction with elf4G and preventing assembly of the ribosome on the transcript. The hormonal signals that control oocyte maturation lead to the activation of the Aurora A/Ep1 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 (Evskiv et al., 2007). If CPEB paralogs are conserved in the bivalvia, then it is also puzzling that mGld2/Pagd4 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 elf4E binding region, and it is unclear whether they participate in translational repression (de Moor et al., 2005). The discovery of both KH domain proteins. The functions of these proteins were tested by expression in 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 mCPEB1, may regulate translation of mRNA targets required for oocyte maturation.
Xenopus (Minshall et al., 2007) and mouse (Evsikov and Marin de Esvikova, 2009a) eIF4E, 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

Harrowing 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-week-old mice and transferred into prewarmed M2 medium containing 100 μM 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich, St. Louis, 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, GV oocytes were released from IBMX and cultured for 3 and 6 h, respectively. Metaphase II (MI) 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). Oulated oocytes were released from the ampulla 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 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 30% sucrose, and embedded in OCT compound (Tissue-Tek). Embryos were microinjected with 5 nl of the purified KHDC1A polyclonal antibodies (GenScript, Piscataway, NJ) were added to 0.1 mg/ml RNaseA, followed by flow cytometry (Coulter Epics XL, Beckman). The data were analyzed with WinMDI software.

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 in 5% CO2. Transfection was performed using Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen). The NIH-3T3-KHDC1 stable cell lines were established as described (Witzenowiecz 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 cell lines.

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.

Immunoprecipitation and Western Blot Analysis

For immunoprecipitation 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-KHDC1 rabbit serum (Rajpal et al., 2003) and affinity purified KHDC1A polyclonal antibodies (GenScript, Piscataway, NJ) were used at 1:2000. Anti-α-tubulin (Sigma) antibodies were used at 1:100,000. 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 Tris-buffered saline with 1% BSA. 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. Sections were dehydrated 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. All samples (sections, oocytes, embryos, or cells) were blocked in 5% BSA/PBS at room temperature for 1 h and incubated in diluted primary antibody at room temperature for 1.5 h or 4 °C overnight. The samples were then washed with PBS at room temperature for 1 h and incubated in diluted primary antibody at room temperature for 1.5 h or 4 °C overnight. The samples were then washed with PBS at room temperature for 1 h and incubated in diluted primary antibody at room temperature for 1.5 h or 4 °C overnight. The samples were then washed with PBS at room temperature for 1 h and incubated in diluted primary antibody at room temperature for 1.5 h or 4 °C overnight. The samples were then washed with PBS at room temperature for 1 h and incubated in diluted primary antibody at room temperature for 1.5 h or 4 °C overnight. The samples were then washed with PBS.
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 Velocity 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 pseudogene, 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 GXGX 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.

KLHDC1 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 (aKHDC1A) 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...
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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) tests (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 inlets are higher magnification preantral follicles. Preantral follicles contain growing oocytes surrounded by one or a few layers of granulosa cells. Dashed inlets 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.

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; Galanro and Richard, 2005). For example, Quaking I protein (QKI) regulates splicing and translation of mRNAs by binding to its target sequence NACUAAAY-N_{1,25}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) (Siohi 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 expressed in tissue culture cells (Rajpal 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
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 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 predicted 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.
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).

KHDC1B Levels and Binding Partners Fluctuate with the Cell Cycle

The exact protein components of the cytoplasmic 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 activity. The exact protein components of the cytoplasmic 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 activity.
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 9.5–9.5). (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.
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 predominantly 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 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.
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 of KH domain proteins.

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.
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...
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 azoosperma 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.

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