Characterization of ΔN-Zfp36l2 Mutant Associated with Arrest of Early Embryonic Development and Female Infertility

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Background: Zfp36l2 is critical for female fertility.
Results: ΔN-Zfp36l2 protein shuttles between the cytoplasm and nucleus, binds to RNAs containing AREs, and promotes deadenylation. ΔN-Zfp36l2 is more resistant to stimulus-induced down-regulation.
Conclusion: ΔN-Zfp36l2 protein is more stable, suggesting the molecular basis of the phenotype.
Significance: Mutations of Zfp36l2 protein results in developmental arrest suggesting that it is critical for regulation of embryonic development in mammals.

The zinc finger protein 36-like 2, Zfp36l2, has been implicated in female mouse infertility, because an amino-terminal truncation mutation (ΔN-Zfp36l2) leads to two-cell stage arrest of embryos derived from the homozygous mutant female gamete. Zfp36l2 is a member of the tristetraprolin (TTP) family of CCCH tandem zinc finger proteins that can bind to transcripts containing AU-rich elements (ARE), resulting in deadenylation and destabilization of these transcripts. I show here that the mouse Zfp36l2 is composed of two exons and a single intron, encoding a polypeptide of 484 amino acids. I observed that ΔN-Zfp36l2 protein is similar to both wild-type Zfp36l2 and TTP (Zfp36) in that it shuttles between the cytoplasm and nucleus, binds to RNAs containing AREs, and promotes deadenylation of a model ARE transcript in a cell-based co-transfection assay. Surprisingly, in contrast to TTP, Zfp36l2 mRNA and protein were rapidly down-regulated upon LPS exposure in bone marrow-derived macrophages. The AN-Zfp36l2 protein was substantially more resistant to stimulus-induced down-regulation than the WT. I postulate that the embryonic arrest linked to the ΔN-Zfp36l2 truncation might be related to its resistance to stimulus-induced down-regulation.

ΔN-Zfp36l2 (zinc finger protein 36 like 2), also known as TIS11D (1), ERF2 (2), and BRF2 (3), is critical for female fertility, because maternal expression of an amino-terminal truncated form leads to arrest of early embryonic development at the two-cell stage (4).

Zfp36l2 belongs to the tristetraprolin (TTP)2 (also known as ZFP36, TIS11, and GOS24) family of CCCH zinc finger proteins, which contain tandem zinc-binding motifs characterized by three cysteines followed by one histidine (CCCH) (5). TTP itself has an anti-inflammatory function, as TTP knock-out mice display a severe systemic inflammatory syndrome that is due, in large part, to increased production of tumor necrosis factor α (TNFα) (6, 7).

Another member of this protein family is Zfp36l1, also known as TIS11B (1), cMG1 (8), Berg36 (9), ERF1 (10), and BRF1 (11). Disruption of this gene in mice resulted in a lethal phenotype at approximately embryonic day 11, mainly due to failure of chorioallantoic fusion (12). Recently, a fourth member of this family, Zfp36l3, has been identified; its function is currently unknown, but its expression seems to be restricted to the rodent yolk sac and placenta (13, 14). These CCCH proteins can bind to the 3′-untranslated regions (3′-UTR) of certain mRNAs containing class II AU-rich elements (ARE, UUAU-UA(U/A)(U/A)) through their zinc fingers. Binding is then followed by degradation of the target mRNAs (15), ultimately resulting in decreased levels of translated protein (5).

Insight into one biological role for the third family member, Zfp36l2, came from a mouse model in which homozygous mutant females expressing a truncated form of Zfp36l2 (ΔN-Zfp36l2) lacking the amino-terminal 29 amino acids (29NTD) exhibited complete infertility (4). These females cycled and ovulated normally, and their ova could be fertilized; however, when they were crossed with WT males, their embryos were able to undergo the initial cleavage step but remained arrested at the two-cell stage. Because Xenopus Zfp36l2 (XC3H-3) can bind to and destabilize certain mRNAs in cell-free assays (16), and embryonic progression beyond the two-cell stage requires degradation of maternal transcripts coupled to zygotic gene activation (17–19), I have suggested that Zfp36l2 could mediate...
the destabilization of specific maternal transcripts in early embryonic development. Although the precise role played by the amino terminus of Zfp36l2 remains largely unknown, this sequence may contain a functional domain that is of critical importance for the control of early embryonic cell proliferation.

The correct structure of the gene in the mouse has also been uncertain. Previous findings from the ΔN-Zfp36l2 mutant model (4) suggested that mouse Zfp36l2 gene contains two exons, as seen in the human gene (20). However, the only published report on the mouse Zfp36l2 gene described a cDNA of 1,200 nucleotides originating from a single exon (1). This would be similar to the situation seen with Zfp36l3 (13). In this report, I confirm that the mouse gene, like the human, is composed of two exons separated by a single intron. I also show that the ΔN-Zfp36l2 protein appears to be more resistant to lipopolysaccharide (LPS)-induced down-regulation than the WT protein.

MATERIALS AND METHODS

Plasmids—A mouse genomic Zfp36l2 clone (MG-TIS11D) was obtained as described previously (21). The proposed 3′-UTR of the transcript, corresponding to bp 2202–3537 of GenBankTM RefSeq NM_001001862, was generated using 3′-rapid amplification of cDNA ends. The full-length cDNA was constructed by removing exon 1, the single intron, and the beginning of exon 2 from this genomic clone and replacing them by an EST clone (GenBankTM accession number AI327503) that contained a spliced sequence in which exon 1 was fused with the beginning of exon 2, as described (21).

Plasmid CMV.mZfp36l2-HA.tag, encoding the hemagglutinin (HA) tag fused to the carboxyl-terminal end of mouse Zfp36l2, was constructed by removing a fragment of 120 bp (SstI-Stsl fragment) at the 3′ end of the cDNA and replacing it with a ~120-bp PCR product containing an HA sequence in-frame with the last amino acid of Zfp36l2 followed by a stop codon and an XbaI site. After sequencing, the Zfp36l2-HA was excised from the pSK− vector and introduced into the KpnI-XbaI sites of the CMV.BGH3′/pBS+–modified vector (15). Similarly, a Zfp36l2 sequence fused with GFP was constructed as described above, except that the 120-bp fragment was replaced by a PCR product in which the stop codon was removed and a PspOMI site was introduced. After the 3′ end was sequenced to confirm the desired mutation, the EcoRI-PspOMI fragment was excised and introduced in-frame with GFP in the pEGFP-N1 vector from Clontech. The ΔN-Zfp36l2-GFP vector was constructed by PCR primer-overlapping mutagenesis, in which a KpnI site was created immediately before the second methionine; after sequencing, the ΔN-Zfp36l2 insert was introduced as described above into the pEGFP-N1 vector.

Northern Blot Analysis—Dissected mouse tissues were rapidly frozen and pulverized in liquid nitrogen and processed using the RNeasy kit from Qiagen to extract total cellular RNA, according to the directions of the manufacturer. RNA samples (10 μg) were separated by electrophoresis in 1.2% agarose/formaldehyde gels and used for Northern blotting (22). The nylon membranes were hybridized with 32P-labeled Zfp36l2 probes comprising exon 1, the single intron, or exon 2 as described (4). Northern blots were analyzed using a Phosphorimager Typhoon 8600 and ImageQuant software (GE Healthcare).

Reverse Transcriptase (RT)-PCR and Real Time RT-PCR—A total of 1 μg of RNA was used to synthesize cDNA using the High Capacity cDNA archive kit (Applied Biosystems) according to manufacturer’s instructions. To quantify TTP and Zfp36l2, 9 ng of cDNA was combined with predesigned primer/probe sets and TaqMan Universal PCR Master Mix (Applied Biosystems). For the housekeeping gene, 4 ng of cDNA was used to detect GAPDH. All reactions were performed in triplicate in 96-well plates. Real time assays were run on an ABI 7000 sequence detector system (Applied Biosystems).

In Vitro Translated Zfp36l2 Labeled with 35S—To allow the linearization of the plasmid at the 3′ end using a preferred restriction enzyme, a pBluescript II SK− plasmid was constructed containing a T7 promoter at the 5′ end preceding the Zfp36l2 cDNA, followed by a unique restriction enzyme site (HindIII) downstream of the 3′ end of the stop codon. The RNA was transcribed from 1 μg of linearized plasmids (Zfp36l2 cDNA digested with HindIII) and used as a template to prepare in vitro translated 35S-cysteine-labeled Zfp36l2 in a rabbit reticulocyte lysate system or in wheat germ extract (both from Promega Biotech). The translated protein labeled with 35S-cysteine was loaded onto a 10% SDS-polyacrylamide gel and subjected to electrophoresis; the gel was then fixed and incubated with Autofluor (National Diagnostics), dried, and exposed to film.

Cell Culture, Transfections, and Protein Extracts—Primary embryonic fibroblasts were prepared from embryonic day 14–15 mouse embryos, and bone marrow–derived macrophages (BMM) were prepared from adult mice as described (7). Marrow cells were flushed from femurs of individual animals (2–5-month-old WT and ΔN-Zfp36l2 mice) and cultured in standard medium containing colony-stimulating factor for 3–4 weeks. Animal care procedures were in accordance with institutional guidelines for animal use at NIEHS (National Institutes of Health) and University of North Carolina.

Mouse RAW 264.7 cells (American Type Culture Collection) were cultured in Eagle’s minimum essential medium with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) and treated with lipopolysaccharide (LPS) (1 μg/ml, Sigma) for various times in four independent experiments. Finally, RAW cells were pretreated with 50 μM cycloheximide (Sigma) for 15 min to inhibit protein synthesis before LPS stimulation.

HEK 293 cells (American Type Culture Collection) were maintained in minimum essential medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) and treated with lipopolysaccharide (LPS) (1 μg/ml, Sigma) for various times in four independent experiments. Finally, RAW cells were pretreated with 50 μM cycloheximide (Sigma) for 15 min to inhibit protein synthesis before LPS stimulation.

HEK 293 cells (American Type Culture Collection) were maintained in minimum essential medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Transient transfection of 1 × 10⁶ cells with different Zfp36l2 constructs in calcium-phosphate precipitates was performed as described previously (15). The transfection mixture was allowed to stay on the cells for 20 h and then removed; after a further 24-h incubation period, cells were lysed for protein extraction.

Cytosolic protein extracts from cell lines, primary cells, and HEK 293 cells transfected with different constructs were pre-
pared in a buffer solution comprised of 50 mM β-glycerol phosphate (pH 8.2), 0.25 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 50 mM sodium fluoride, and 10 mM benzamidine-HCl, plus the following protease inhibitors: 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 μM pepstatin, 2 μM leupeptin, and 2 μg/ml aprotinin. The amount of protein was measured using a Bio-Rad assay.

**Cell Labeling and Immunoprecipitation**—Cell labeling and immunoprecipitation protocols have been described in detail elsewhere (6). Briefly, confluent 100- or 60-mm plates of cells were serum-deprived overnight in DMEM (Invitrogen) supplemented with 1% bovine serum albumin (BSA), then preincubated for 1 h in phosphorus-free DMEM without BSA, and stimulated with 10% (v/v) dialyzed FCS in the presence of [32P]orthophosphoric acid (0.2 mCi/ml) for 2 h. Next, the cells were briefly sonicated in a lysis buffer containing 50 mM Tris (pH 8.3), 0.10 M NaCl, 0.05 M NaF, 5 mM EDTA, 1% (w/v) Nonidet P-40, and protease inhibitors. Protein extracts were subjected to immunoprecipitation using protein A-Sepharose beads and a polyclonal peptide antiserum, N2-Zfp36l2-AS. Precipitated proteins were eluted from the beads, boiled in 4× loading buffer, and separated on 10% SDS-polyacrylamide gels, which were dried and used for autoradiography.

**Zfp36l2 Antiseras and Western Blot Analysis**—Four to 25 μg of protein extracts from HEK 293 cells overexpressing Zfp36l2, or 400–500 μg of protein extracts containing endogenous Zfp36l2, were mixed with a 4× SDS loading buffer, boiled, and then loaded onto 10 or 8% SDS-polyacrylamide gels. Western blots were performed using standard techniques. The nitrocellulose membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.5% Tween 20 and then probed with different rabbit antisera generated against synthetic peptides. Two amino- and two carboxyl-terminal peptides, for a total of four, were synthesized based on the Zfp36l2 protein sequence (Fig. 1B). Each of the four peptides were individually conjugated with keyhole limpet hemocyanin as a protein carrier and then injected into two different rabbits to produce a total of eight different rabbit antisera. Among the four peptides, three were unique to Zfp36l2 amino acid sequences and one was shared with another family member, Zfp36l1. The extreme amino-terminal peptide, corresponding to amino acids 5–16 translated from exon 1 (LLSPFYDIDFLC), was named N1-Zfp36l2-AS. The other amino-terminal sequence was located after the second methionine in the beginning of exon 2, corresponding to amino acids 58–80 (ASNLHALAHVPVPSGCSKPFGPQ, N2-Zfp36l2-AS). The Zfp36l2 carboxyl-terminal specific antiserum was raised using a peptide corresponding to amino acids 423–437 (PAPPSPPPFGQLPR, C1-Zfp36l2-AS); the fourth, an extreme carboxyl-terminal sequence shared between Zfp36l2 and Zfp36l1 and corresponding to amino acids 469–484 (RRLPFSRLSISDD) of mouse Zfp36l2, was used to raise a common carboxyl-terminal antiserum (C2-Zfp36l2-AS). Each primary antibody was used diluted to 10,000 or 1,250 for overexpressed or endogenous protein samples, respectively. Incubation of the membranes with the secondary goat anti-rabbit IgG HRP-conjugated (Bio-Rad) was diluted 1:25,000 or 1:50,000 and developed with a SuperSignal West Pico Chemiluminescent Substrate (Pierce) according to the manufacturer’s instructions.

**RNA Electrophoretic Mobility Shift Assay**—Protein extracts prepared from HEK 293 cells transfected with either vector alone (BS+), Zfp36l2, or Zfp36l2-HA constructs driven by the CMV promoter were incubated with 2 × 10⁶ cpm of a mTFN-ARE RNA probe (based on bp 1309–1332 of GenBank™ accession number X02611), at room temperature for 20 min in 20 μl of lysate buffer (without a protease inhibitor). Heparin and yeast tRNA were added to final concentrations of 0.5 μg/μl and 50 ng/liter, respectively, for an additional 10 min. The RNA not associated with protein was digested with 100 units of RNase T₁ (Invitrogen) for 20 min at room temperature. The resultant reaction mixture was then loaded onto a 6% nondenaturing acrylamide gel and subjected to electrophoresis at 250 V for 90 min in 0.4× Tris borate/EDTA buffer (15). For the gel supershift assay, an HA antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or the N2-Zfp36l2 antiserum was added to the first incubation step together with the protein extract and the labeled probe for 20 min at room temperature. For the assays of Zfp36l2 in vitro translated using wheat germ extracts, a human TNF-ARE RNA probe (1331–1389 bp of NM_000594.2) was used.

**Cell-free Deadenylation of Polyadenylated ARE-containing Probes**—Assay and RNA probes have been described previously (23). Briefly, all three probes, ARE-A50, ARE, and V, contained the same 5’ sequences. Probe ARE-A50 consisted of 58 bases transcribed from the multiple cloning sites of the SK– vector, plus 24 bases of the core ARE from the mouse TNF mRNA, followed by 50 adenosines. Probe ARE was identical to ARE-A50 except that it did not contain a poly(A) tail. Probe V (vector) consisted only of 58 bases transcribed from the multiple cloning sites of SK–, but no ARE or poly(A) tail. All plasmids were linearized and used as templates in the Promega riboprobe in vitro transcription system. The capped RNA probes were transcribed in the presence of [α-32P]UTP (800 Ci/mmol) and Ribo m7G cap analog (Promega), and the resulting products were separated from free nucleotides on G-50 columns. The reaction mixtures were assembled on ice, and the reaction was started by adding 50 μl of probe (4 × 10⁶ cpm) to 5 μg of protein extracts in 50 μl in an assay buffer (1 mM HEPES (pH 7.6), 40 mM KCl, and 5% glycerol). The mixtures were incubated on ice or at 37 °C for 60 min. EDTA at a final concentration of 20 mM was added to terminate the reaction. The final reaction mixture was then extracted once with phenol/chloroform and an aliquot of 60 μl of the aqueous phase was mixed with 60 μl of formamide stop solution (95% formamide, 0.1% xylene cyanol) and then heated at 70 °C for 5 min. Aliquots of reaction products were analyzed on an 8% acrylamide gel containing 7 μM urea.

**Fluorescence Microscopy**—To observe the subcellular localization of Zfp36l2-GFP and ΔN-Zfp36l2-GFP fusion proteins, HEK 293 cells (4 × 10⁴/ml) were plated in two-well (4 cm²) LabTek glass chamber slides (Nagle Nunc International) and transfected using calcium phosphate precipitation as described previously (16), except for the amount of DNA, 1–10 ng per well of different GFP fusion constructs. The transfection mixture was allowed to stay on the cells for 16 h, and 1–8 h after the
removal of the transfection mixture, the cells were rinsed twice at room temperature in PBS, fixed for 10 min in 4% (v/v) paraformaldehyde, and then rinsed again with PBS before mounting with ProLong Anti-fade (Molecular Probes, Inc., Eugene, OR). Slides were dried and examined, and images were obtained using a Zeiss LSM510 confocal microscope (Zeiss, Thornwood, NY).

RESULTS

Mouse Zfp36l2 Gene and Its Transcript—Analysis of the mouse genome shows that mouse Zfp36l2 contains two exons, as does the human gene (20), and encodes a 484-amino acid protein (Fig. 1A, panel I). The initial description of mouse Zfp36l2 was from a 1,200-bp cDNA originating from a single exon, resulting in a predicted protein of 367 amino acids; and this protein has been used in other studies (24, 25). This cDNA lacked the coding sequence for 29 and 88 amino acids at the amino- and carboxyl-terminal ends, respectively.

I used Northern blot experiments with probes corresponding to exon 1, exon 2, and the 3′-UTR to characterize the WT and mutant transcripts. In the ΔN-Zfp36l2 mutant animals the small exon 1 was knocked out (Fig. 2A) resulting in a truncated protein of 455 amino acids (Fig. 1A, panel II). Cytosolic RNA from WT mouse bone marrow-derived macrophages failed to hybridize with a probe to the intron sequence (Fig. 2B), because this sequence is spliced out in the mature Zfp36l2 transcript. However, the transcript present in the ΔN-Zfp36l2 mice did hybridize to the intron probe (Fig. 2B) demonstrating that a portion of the original intron sequence is part of the 5′-UTR of the mutant transcript. Using 3′-rapid amplification of cDNA ends, I obtained the entire 3′-UTR, which contains only one typical polyadenylation signal (AATAAA) located 1810 bp downstream of the stop codon, in both the WT and mutant transcript (Fig. 1A).

I next examined the expression of the transcript in tissues and found a single 3.6-kb Zfp36l2 transcript (Fig. 2C), consistent with the gene model in Fig. 1A. High levels of expression were observed in typical lymphoid tissues such as thymus, spleen, small and large intestines, and Peyer’s patches and with low levels of expression in brain, liver, testis, skeletal muscle, heart, and kidney. The gel was stained with acridine orange before transferring to the nylon membrane to check the loading amount of RNA per lane (Fig. 2C).

Zfp36l2 is highly expressed in oocytes (Fig. 2D) during embryo development (26) and in female reproductive organs (4, 26). These data demonstrated that only specific tissues expressed abundant levels of Zfp36l2 mRNA; among these, mouse oocytes showed a 7-fold increase when compared with bone marrow-derived macrophages (Fig. 2D).

Thus, mouse full-length protein is 484 amino acids long, whereas the mutant protein is 455 amino acids, i.e. 30 amino acids shorter and likely uses the only methionine in exon 2, located at amino acid 30 of the wild-type gene. The identity of these proteins was confirmed by the antibody experiments described below.

Immunochemical Characterization of Mouse Zfp36l2—HEK 293 cells, which do not express ZFP36 family members, were transfected with mTTP, mZfp36l1, and mZfp36l2 constructs.

These protein extracts were used to characterize a panel of Zfp36l2 antisera (Fig. 3, A–D). After an initial characterization of these antibodies, they were then used to define the nature of WT and mutant Zfp36l2 proteins. I used anti-peptide antibodies directed at amino- and carboxyl-terminal sequences, as well as two internal sequences of Zfp36l2 (Fig. 1B). A mixture of preimmune sera (Fig. 3D) was used as control at a 5-fold greater concentration than the Zfp36l2-specific peptide antisera. They
did not react with any of the transfected TTP family member proteins (Fig. 3D).

The amino-terminal antibody (N1-Zfp36l2-AS), directed against the sequence encoded by exon 1, reacted with a band of about 60 kDa in protein extracts from HEK 293 cells transfected with a construct for the full-length Zfp36l2 (Fig. 3A, lane 2). This antiserum is specific for Zfp36l2, as it failed to react with transfectants for the empty vector (Fig. 3A, lane 1) and other TTP family members (Fig. 3A, lanes 4 and 5) or with ΔN-Zfp36l2 (Fig. 3A, lane 3). Importantly, N1-Zfp36l2-AS reacted with the endogenous wild-type protein of the same approximate 58 kDa in macrophage cell extracts from WT (arrow) but not from ΔN-Zfp36l2 homozygous mice (Fig. 3E, left panel), confirming that the endogenous mouse Zfp36l2 protein encodes an amino-terminal sequence originated from exon 1.

The carboxyl-terminal peptide antiserum (C2-Zfp36l2-AS) reacted with transfected and expressed ΔN-Zfp36l2 and Zfp36l2 (Fig. 3B, lanes 2 and 3) as well as with mouse Zfp36l1 (Fig. 3B, lane 5), in which the last 14 carboxyl-terminal amino acids are identical to those of Zfp36l2. This antiserum did not react with mouse TTP (Fig. 3B, lane 4) or mock-transfected cells (Fig. 3B, lane 1). It detected both the endogenous wild-type Zfp36l2 protein and the amino-terminal truncated protein present in mouse macrophages from WT and mutant animals, respectively (Fig. 3E, right panel). This antibody also cross-reacted with Zfp36l3, a 100-kDa protein, expressed exclusively in murine placenta (13). The antiserum C1-Zfp36l2-AS, raised against a peptide with a sequence unique to Zfp36l2, upstream of its extreme carboxyl terminus (Fig. 1B) reacted with transfected and expressed Zfp36l2 and ΔN-Zfp36l2 (Fig. 3C, lanes 2 and 3) but not with other TTP family members (Fig. 3C, lanes 4 and 5). This antibody failed to recognize the endogenous protein in Western blots (data not shown).

The antiserum N2-Zfp36l2-AS raised against a peptide with a sequence encoded by the beginning of exon 2 (Fig. 1B) reacted
with overexpressed Zfp36l2 (Fig. 3F, lane 2, left panel) but failed to recognize the endogenous protein in Western blots (data not shown). Nevertheless, N2-Zfp36l2-AS was effective in immunoprecipitating and supershifting the Zfp36l2 protein. The N2-Zfp36l2-AS immunoprecipitated Zfp36l2 in a dose-dependent manner (Fig. 3G, lanes 2–4) and displayed specificity (Fig. 3H, lane 8), as it did not IP mouse TTP or Zfp36l1 (Fig. 3I, lanes 2 and 5). TTP is known to be a phosphoprotein (27, 28), so I used 32P-labeling to show that N2-Zfp36l2-AS could also immunoprecipitate endogenous Zfp36l2 from mouse embryonic fibroblasts and BMM (Fig. 3I, two left panels), and this interaction was inhibited by the competing immunogenic peptide (Fig. 3I, two right panels).

∆N-Zfp36l2 binds to and promotes deadenylation of an ARE probe—Next, I evaluated the interaction of the truncated protein, ∆N-Zfp36l2, with an ARE-containing RNA probe derived from the mouse TNFα sequence (15). It was shown earlier that the Xenopus ortholog of Zfp36l2, XC3H-3, can bind to an ARE-RNA probe in a mobility shift assay (16). Although overall the Xenopus protein differs from the mouse protein by 39% (Fig. 1B), the RNA binding domain (amino acids 132–202) is >90% identical, suggesting that they would bind similarly to mRNAs.

Three lines of evidence demonstrate that the Zfp36l2 protein binds AU-rich RNAs. First, increasing amounts of mouse Zfp36l2 protein resulted in a proportional increase of RNA-protein complex formation (Fig. 4A, lanes 3–5), suggesting that Zfp36l2 is part of the RNA binding complex, similar to the original findings with TTP (15). Extracts from HEK 293 cells transfected with the empty vector resulted in two complexes (Fig. 4A, lane 2), likely due to endogenous ARE-binding proteins present in this cell (C1 and C2). Note that the complexes C1 and C2 migrated faster than the Zfp36l2-ARE probe complex (Fig. 4A) and that the TTP family members are not endogenously expressed in the HEK 293 cells. Two results demonstrate that this mobility shift was due to Zfp36l2 binding to the ARE probe. First, anti-HA antibodies shifted the migration of the Zfp36l2-HA fusion protein-ARE complex (Fig. 4B, lanes 4 and 5) as did the N2 antibody to the Zfp36l2 protein (Fig. 4C, lane 5, upper arrow). Second, immunodepletion of Zfp36l2-HA with either HA antibody (Fig. 4D, lanes 6 and 7) or the N2 antibody to the Zfp36l2 (lanes 8 and 9) abolished the binding of Zfp36l2 to ARE-RNA (Fig. 4D, lanes 6–9, SS). Thus, as expected, full-length mouse Zfp36l2 can bind ARE-containing mRNAs probes.

In addition, I did RNA binding assays using in vitro translated Zfp36l2 protein from the heterologous wheat germ system. This resulted in a shift of the human TNF-ARE probe in a dose-dependent manner (Fig. 4E, lanes 2–4), which was clearly distinct from the endogenous proteins in the wheat germ that endogenous and overexpressed Zfp36l2 protein. Primary cell lines of mouse embryonic fibroblasts (MEF) and BMM or the HEK 293 cells transfected with Zfp36l2 construct were labeled in vivo with 32P as described under “Materials and Methods” and subjected to immunoprecipitation with preimmune serum (PI) or N2-Zfp36l2-AS antiserum (AS). N2-Zfp36l2-AS (arrow), but not preimmune serum, was able to immunoprecipitate endogenous (three left panels) and overexpressed Zfp36l2 (right panel). The amount of immunoprecipitated material was reduced by a third of the original amount in the presence of the competing peptide (+), two right panels.

**FIGURE 3.** Immunoreactivity and specificity of each antiserum for Zfp36l2. Western blot analysis using protein extracts from HEK 293 cells transfected with empty vector (lane 1) or each member of mTTP family as follows: full-length Zfp36l2 (lane 2), ∆N-Zfp36l2 starting at the second methionine (lane 3), TTP (lane 4), and Zfp36l1 (lane 5) are shown in A–D. Protein extracts were prepared as described under “Materials and Methods.” A, N1-Zfp36l2-AS recognizes only full-length Zfp36l2 (lane 2) but not ∆N-, TTP, or Zfp36l1, lanes 3–5, respectively. B, C2-Zfp36l2-AS detects both versions of Zfp36l2 protein (lanes 2 and 3) and Zfp36l1 (lane 5). C, C1-Zfp36l2-AS recognized different versions of Zfp36l2 (lanes 2 and 3) but not TTP or Zfp36l1 (lanes 4 and 5, respectively). D, preimmune serum does not immunoreact with any CCCH family member. E, Western blot analysis using protein extracts from BMM of WT and mutant (Mut) mice were prepared as described under “Materials and Methods.” N1-Zfp36l2-AS recognized the WT endogenous protein but not the mutant present in the ∆N-Zfp36l2 mouse (arrow, left panel). C2-Zfp36l2-AS immunoreacted with WT and ∆N-Zfp36l2 proteins (right panel), and nonspecific bands are indicated by small arrows, F, N2-Zfp36l2-AS detected an ~60-kDa band in protein extracts from overexpressed Zfp36l2 (lane 2) but not with the empty vector (lane 1). This band recognition was blocked by the competing peptide (pep) (+) (right panel), and nonspecific bands are indicated by small arrows. G, N2-Zfp36l2-AS immunoprecipitated in vitro translated Zfp36l2. Zfp36l2 translated in vitro, using a rabbit reticulocyte system, and labeled with 32P was immunoprecipitated in a dose-dependent manner (arrow). Lanes 1–4 show products containing the same amount of in vitro translated Zfp36l2 protein incubated either with preimmune serum (lane 1) or with decreasing amounts of the antisem: 1:50, 1:100, or 1:500, lanes 2–4, respectively. As a negative control (lane 5), in vitro translated protein that did not contain exogenous Zfp36l2 mRNA was subjected to IP with the antiserum in a dilution of 1:100. As a positive control, a small amount of crude protein extract (not IP) containing mRNA of Zfp36l2 was loaded in lane 6. H, N2-Zfp36l2-AS immunoprecipitated Zfp36l2 but not the other two family members, TTP or Zfp36l1. A small amount of total in vitro translated protein (not IP) from the rabbit reticulocyte system containing TTP, Zfp36l1, and Zfp36l2 was loaded in lanes 3, 6, and 9, respectively. A fixed dilution (1:50) of the antiserum (lanes 2, 5, and 8) or preimmune serum (lanes 1, 4, and 7) was used to immunoprecipitate TTP, Zfp36l1, or Zfp36l2, respectively. A negative control, similar to the one in lane 5 of the upper panel, was loaded into lane 10. The numbers on the left side of the figures are numeric values for molecular mass controls expressed in kDa. I, N2-Zfp36l2-AS immunoprecipitated
Mouse Zfp36l2 and ΔN-Zfp36l2 Truncation

FIGURE 4. Interaction of Zfp36l2 protein with RNA. RNA electrophoretic mobility shift assays were done incubating protein extracts from HEK 293 cells transfected with mouse Zfp36l2 or empty vector (V) with 2 × 10⁶ cpm of 32P-labeled mTNFα (1309–1332 bp) ARE probe. RNA-protein complexes were resolved in a 6% acrylamide nondenaturing gel. A, increasing amounts of protein extracts containing overexpressed Zfp36l2 resulted in increased shift of the probe electrophoretic migration (lanes 3–5, arrow) in regard to the original probe migration (lane 1, P). Protein extracts from HEK 293 cells transfected with the empty vector (V) result in the formation of two complexes (C1 and C2, double arrows). B, HEK 293 cells were transfected with Zfp36l2-HA construct. Protein extracts were incubated as described previously, except for the presence of increasing amounts of HA-antibody (HA-Ab). The HA-Ab shifted the migration of the Zfp36l2-TNFα ARE complex (supershift, SS, lanes 4 and 5). C, N2-Zfp36l2-AS was able to shift the Zfp36l2-HA-TNFα ARE complex, in a similar fashion as the HA-Ab (SS, in lane 5). D, Immunodepletion of Zfp36l2 from protein extracts of HEK 293 cells overexpressing Zfp36l2-HA abolished the shift of TNFα probe. The Zfp36l2 protein present in the extracts of HEK 293 cells transfected with Zfp36l2-HA was incubated with the HA-Ab (lanes 6 and 7) or with N2-Zfp36l2-AS (lanes 8 and 9), then immunoprecipitated using protein A-Sepharose beads. The immunodepleted protein extracts (lanes 6–9) were used in the RNA electrophoretic mobility shift assays. In lanes 6 and 8, the Zfp36l2-immunodepleted extracts were incubated with RNA probe plus HA-Ab, and in lanes 7 and 9 the complexes were incubated with the N2-Zfp36l2-AS and resolved in a 6% acrylamide nondenaturing gel. The ability of these antibodies to shift the protein-TNFα-probe complex was virtually gone in the Zfp36l2-immunodepleted extract (lanes 6–9, SS). Unspecific recognition was observed in the AS (arrowhead). The same protein extracts containing Zfp36l2 before the immunodepletion resulted in a shift (lanes 3) or in a supershift in the presence of HA or N2-Zfp36l2-AS (lanes 4 and 5, SS). E, recombinant Zfp36l2 protein shifted a human TNFα ARE probe. Increasing amounts of recombinant Zfp36l2 (5, 10, and 15 μl, lanes 2–4, respectively) from a WGS containing Zfp36l2 mRNA shifted a 32P-labeled human TNFα (1331–1389 bp) ARE probe (upper arrow). As a negative control, 15 μl of wheat germ system that did not contain exogenous Zfp36l2 mRNA was incubated with TNF ARE probe, and this resulted in no shift (lane 1). Unspecific complexes, C1 and C2, were present in lanes 1–4 (lower arrows). Note that the intensity of C1 and C2 complexes varies according to the volume of WGS used in reaction. F, expression of recombinant Zfp36l2 protein from WGS. Recombinant proteins using WGS with or without exogenous Zfp36l2 mRNA were labeled with 35S, loaded in a 10% SDS-PAGE, and excised to film. As a negative control, in vitro translated protein that did not contain exogenous Zfp36l2 mRNA was loaded in lane 1. Increasing amounts of recombinant Zfp36l2 protein from WGS were loaded in lanes 2–4, respectively.

bound the AU-rich probe (Fig. 4E, lane 1). Note that the intensity of C1 and C2 complexes varies according to the volume of WGS used in reaction. Expression of recombinant Zfp36l2 protein from WGS was confirmed by autoradiography (Fig. 4F). This result, obtained using a heterologous system, excludes the possibility that intermediate proteins in the extract are required for the RNA binding. The recombinant purified protein would have been the preferable way to address “direct binding” of Zfp36l2 to RNA. However, it has not been possible to express this protein or other CCCH family members as recombinant proteins able to actively bind to RNA.

I next compared the effects of the full-length Zfp36l2 and ΔN-Zfp36l2 truncation in three assays: binding to ARE-containing RNA probes in a gel-shift assay, stimulated deadenylation of ARE-containing polyadenylated RNA probes in a cell-free assay, and deadenylation of a modified ARE transcript in cell extracts. The ΔN-Zfp36l2-GFP protein bound to the ARE-RNA probe in a manner similar to WT-Zfp36l2-GFP, as assessed by gel-shift assays (Fig. 5A). Both proteins shifted the ARE probe to similar region of the gel and with similar intensities (Fig. 5A, lanes 2 and 3). ΔN-Zfp36l2-GFP also stimulated the deadenylation of an ARE-containing polyadenylated RNA probe to approximately the same extent as either TTP-GFP or WT-Zfp36l2 in this cell-free assay (Fig. 5B, lanes 5, 8, and 9). Likewise, ΔN-Zfp36l2 was as effective as WT-Zfp36l2 in promoting deadenylation of a transfected TNFα reporter mRNA (15) in intact cells (Fig. 5C). The protein extracts used in Fig. 5, A and B, displayed comparable levels of expression of the Zfp36l2 WT and truncated proteins in Western blot (Fig. 5D).

Thus, the ΔN-Zfp36l2 protein is functional with respect to RNA binding and promotion of RNA deadenylation, like WT and the other TTP family member proteins.

Amino-terminal Truncation in ΔN-Zfp36l2 Does Not Alter Subcellular Localization—Alteration in the subcellular distribution of ΔN-Zfp36l2 could explain the arrest of the ΔN-Zfp36l2 embryos at the two-cell stage. To test whether there was a difference in the subcellular localization of the truncated protein, HEK 293 cells were transfected with WT-Zfp36l2-GFP and ΔN-Zfp36l2-GFP plasmids; extracts from the transfected cells were used for Western blots (Fig. 6A) and intact cells for confocal microscopy (Fig. 6, B–F). GFP fusion proteins were chosen because two of the antibodies N2-Zfp36l2-AS and C1-Zfp36l2-AS failed to work in immunohistochemistry staining. One of the best antibodies, N1-Zfp36l2-AS, could not be used for this purpose because it recognizes the WT but does not recognize the ΔN-Zfp36l2 protein. The most sensitive antiserum, the common carboxyl-terminal antiserum (C2-Zfp36l2-AS), recognizes both Zfp36l2 protein. The most sensitive antiserum, the common carboxyl-terminal antiserum (C2-Zfp36l2-AS), recognizes both Zfp36l2 protein. The most sensitive antiserum, the common carboxyl-terminal antiserum (C2-Zfp36l2-AS), recognizes both Zfp36l2 protein. The most sensitive antiserum, the common carboxyl-terminal antiserum (C2-Zfp36l2-AS), recognizes both Zfp36l2 protein.

Thus, the ΔN-Zfp36l2 protein is functional with respect to RNA binding and promotion of RNA deadenylation, like WT and the other TTP family member proteins.

N-Zfp36l2-GFP

not contain exogenous Zfp36l2 mRNA was loaded in lane 1. Increasing amounts of recombinant Zfp36l2 protein from WGS were loaded in lanes 2–4, respectively.
Mouse Zfp36l2 and ∆N-Zfp36l2 Truncation

FIGURE 5. RNA functional assays of Zfp36l2 and ∆N-Zfp36l2. A, in a gel shift assay, protein extracts from HEK 293 cells transfected with GFP (vector) or Zfp36l2-GFP or ∆N-Zfp36l2-GFP (respectively, lanes 2–3) were incubated with a 32P-mTNFα-ARE probe for 20 min at room temperature. The products were separated on an 8% nondenaturing polyacrylamide gel followed by autoradiography. Both protein extracts containing Zfp36l2-GFP or ∆N-Zfp36l2-GFP formed complexes with the labeled RNA probe, shifting the migration position of the free probe (lane 4, P) to an upper region (arrow). B, cell-free deadenylation of polyadenylated ARE-containing probes. Protein extracts of HEK 293 cells transfected with different constructs, as indicated in the figure, were incubated with 32P-labeled ARE-A50 RNA probe at 37°C in the presence (lane 4, P) to an upper region (arrow), B, cell-free deadenylation of polyadenylated ARE-containing probes. Protein extracts of HEK 293 cells transfected with different constructs, as indicated in the figure, were incubated with 32P-labeled ARE-A50 RNA probe at 37°C in the presence (+) or absence (−) of EDTA to inhibit cellular exonucleases for 60 min. The probes were then purified and subjected to electrophoresis on urea-polyacrylamide gels, followed by autoradiography. The arrows indicate the migration position of the polyadenylated probe (ARE-A50), deadenylated product of the probe (ARE), and empty vector (V). C, dose-response effect of WT and ∆N-Zfp36l2. A fixed amount of 1 µg of CMV-mTNFα was co-transfected into HEK 293 cells with increasing amounts of the expression vector Zfp36l2-GFP (left panel) or ∆N-Zfp36l2-GFP (right panel). Each condition was adjusted to a final concentration of 3 µg of transfected DNA by addition of vector alone. Twenty four hours after the removal of the transfection mixture, total cellular RNA was harvested. Each lane contains 10 µg of total RNA subjected to Northern blot assay and hybridized sequentially with Zfp36l2, TNFα and cyclophilin 32P-labeled probes. The upper arrow indicates Zfp36l2 fusion constructs; the double arrows indicate the two species of TNFα mRNA, and the lower arrow indicates the cyclophilin transcript used to normalize the samples. D, Western blot analysis and protein quantification; 25 µg of protein extracts from HEK 293 cells co-transfected with 50 ng of BS+ (vector), GFP, Zfp36l2-GFP, or ∆N-Zfp36l2-GFP constructs in the presence of 1 µg of CMV-mTNFα were probed with a GFP-antibody. The bands were quantified and plotted in a bar graph.

FIGURE 6. Expression of Zfp36l2-GFP and ∆N-Zfp36l2-GFP. HEK 293 cells were transfected with GFP or GFP fusion plasmids, and samples were simultaneously processed for Western blotting and confocal microscopic analysis. A, Western blots containing 25 µg per lane of cytotoxic protein extracts from transfected HEK 293 cells were probed with an anti-GFP antibody. Each lane corresponds, respectively, to cells transfected with empty vector (lane 1), GFP (lane 2), Zfp36l2-GFP (lane 3), and ∆N-Zfp36l2-GFP (lane 4). Note the similar levels of expression of the WT and ∆N-Zfp36l2 fusion proteins. B, shows a confocal image of HEK 293 cells expressing GFP alone, and the other panels show WT-Zfp36l2-GFP (C and E) and ∆N-Zfp36l2-GFP (D and F). Bars represent 10 µm.

was expressed at levels similar to those of WT-Zfp36l2-GFP in HEK cells (Fig. 6A, lanes 4 and 3, respectively). The subcellular localization of expressed ∆N-Zfp36l2-GFP (Fig. 6, D and F) and WT-Zfp36l2-GFP (Fig. 6, C and E) was indistinguishable; both proteins displayed a cytoplasmic subcellular pattern (Fig. 6, C–F). In a previous publication (21), we showed that all three CCCH zinc finger proteins, TTP, Zfp36l1, and Zfp36l2, shuttle between the nucleus and the cytoplasm despite being GFP-tagged, suggesting that the GFP tag does not interfere with their cytoplasmic subcellular localization or with their shuttling ability.

Zfp36l2 is a shuttling protein sensitive to leptomycin B, which selectively inhibits the nuclear export receptor CRM1 (21). Removal of the 29 amino acids at the amino-terminal sequence of Zfp36l2 did not affect its shuttling properties (data not shown). Therefore, it is unlikely that the embryo arrest seen
Stimulus-induced Reciprocal Regulation of Zfp36l2 and TTP mRNAs—Lipopolysaccharide (LPS) is a prototypical inducer of TTP mRNA, promoting a large increase in its levels within 1 h in macrophages and monocytes (29–31). I thus investigated the pattern of Zfp36l2 mRNA expression after LPS stimulation in bone marrow-derived macrophages from WT and ΔN-Zfp36l2 mice using Northern blot and real time PCR (RT-PCR). Surprisingly, unlike TTP, the Zfp36l2 mRNA was rapidly down-regulated upon exposure of bone marrow-derived macrophages to LPS. After 15 min of LPS exposure, there was a slight induction (~1.5-fold) of Zfp36l2 expression; however, 60 min after addition of LPS, the levels of Zfp36l2 mRNA were decreased ~4-fold from the basal values (Fig. 7A).

Although ΔN-Zfp36l2 mRNA is expressed at lower basal levels than the WT transcript, its response to LPS was similar to that seen with the WT transcript. The ΔN-Zfp36l2 transcript was also down-regulated by LPS (Fig. 7A). Similar results were obtained using RT-PCR (Fig. 7B).

The TTP mRNA expression profile after LPS stimulation of bone marrow-derived macrophages from WT and ΔN-Zfp36l2 mice was indistinguishable (Fig. 7B). However, the major difference between the effects of LPS on the regulation of TTP and Zfp36l2 mRNAs is illustrated in Fig. 7C. LPS slightly increased Zfp36l2 mRNA levels, followed by a rapid and marked down-regulation, whereas TTP mRNA levels are massively induced followed by a return to base line by 2 h. The LPS-induced down-regulation of Zfp36l2 mRNA was preceded by a significant induction of TTP mRNA at 30 min. This time course raises the interesting possibility that TTP might be involved in the down-regulation of Zfp36l2 mRNA levels. However, I found that down-regulation of Zfp36l2 mRNA was independent of TTP, as it also occurred in TTP-KO bone marrow-derived macrophages after LPS exposure.  

Down-regulation of Zfp36l2, but Not ΔN-Zfp36l2, by LPS—To evaluate the effect of LPS on Zfp36l2 protein levels, I initially examined Zfp36l2 protein levels in a mouse macrophage cell line, RAW 264.7. Unlike TTP (28), Zfp36l2 protein levels decreased over time after LPS exposure (Fig. 8A), with a half-maximal decrease seen at about 3 h (Fig. 8, A and C, black squares).

The effects of LPS on the levels of the WT-Zfp36l2 and ΔN-Zfp36l2 proteins were evaluated using bone marrow-derived macrophages from wild-type and the ΔN-Zfp36l2 mutant mice, respectively. ΔN-Zfp36l2 protein was resistant to LPS-induced down-regulation, although the wild-type protein was down-regulated at a similar rate as in RAW cells (Fig. 8, B and C). This result suggests that one difference between the wild-type and mutant protein is that the mutant protein is more stable than the wild-type protein when cells are stimulated with LPS. Note that there is shift in mobility of both proteins as a result of LPS treatment.

LPS-induced down-regulation of Zfp36l2 was independent of protein synthesis, as pretreatment of RAW 264.7 cells with cycloheximide did not alter the LPS effect on Zfp36l2 levels (Fig. 8, D and E). Thus, in addition to down-regulation of the Zfp36l2 mRNA, the protein is rapidly degraded when cells are stimulated with LPS, although the mutant protein is stable under these conditions.

DISCUSSION

Here I have demonstrated that the structure of the mouse Zfp36l2 gene conforms to its human ortholog (20) and encodes a protein with 89% identity at the amino acid level, which is highly conserved among different vertebrates (Fig. 1B). Mouse Zfp36l2 can bind to typical mRNA-containing ARE probes, although the specific mRNA targets for Zfp36l2 remain unknown. Zfp36l2-RNA complexes already assembled under in vitro conditions were not disrupted by an antibody binding to the amino or carboxyl terminus (Fig. 4C), suggesting that the ends of the termini do not interfere with ARE-RNA binding under these conditions. This result is consistent with previous findings that removing 96 or 153 amino acids of human TTP from the amino- and carboxyl-terminal ends, respectively, did not disrupt its RNA binding (16), as measured by similar assays used here.

Removal of 29 amino acids from the amino terminus of Zfp36l2 results in female infertility and in expression of a shorter protein, ΔN-Zfp36l2, in the homozygous animals (4).
To better understand its mechanism, I compared a number of biochemical properties of the mutant and wild-type proteins. The subcellular localization of the two proteins and their ability to bind RNA and promote deadenylation was alike.

Zfp36l2 has a similar overall structure as TTP, with a central RNA binding domain; however, its amino- and carboxyl-terminal portions are larger. The amino-terminal region of TTP is required for deadenylation (32). Removal of the amino-terminal region of TTP (100 amino acids), the prototype of the family, results in reduced mRNA decay function, and there is evidence suggesting that this protein domain functions as a binding platform for mRNA decay enzymes (33). I found that ∆N-Zfp36l2 binds and promotes deadenylation of ARE-mRNA similarly to WT-Zfp36l2. Thus, using these assays, the absence of the 29NTD portion of Zfp36l2 does not affect its RNA binding properties and its ability to promote deadenylation of ARE-mRNA in vitro.

The 29NTD is part of the pfam Tis11B_N (Tis11B-like protein, amino terminus), which is also present in Zfp36l1 and many other proteins. The 29NTD covers only one-fifth of the Tis11B_N domain of Zfp36l2, and the deletion results in stabilization of the protein in LPS-stimulated macrophages. The ∆N-Zfp36l2 mutant does not result in a nonfunctional or “dead” protein in terms of the classical function of the TTP-like CCCH zinc finger proteins, because the ∆N-Zfp36l2 protein can bind and deadenylate ARE-mRNA.

Interestingly, I observed that Zfp36l2 mRNA and protein were rapidly down-regulated in macrophages upon LPS exposure (Figs. 7C and 8) in contrast to TTP (28, 30, 34). Zfp36l2 protein levels decline with a 3-h half-life after LPS treatment. Strikingly, ∆N-Zfp36l2 protein levels are not decreased after LPS treatment, although the mRNA is down-regulated like the wild type. As a result, the mutant accumulates to much higher levels than the wild-type protein in LPS-treated macrophages (Fig. 8B). Thus, the first 29 amino acids are essential for the degradation of Zfp36l2 under these conditions.

Because ∆N-Zfp36l2 was able to regulate an RNA reporter containing an AU-rich element (Fig. 5), the finding that the mutant protein has altered stability in response to signals is consistent with the expression of this mutant protein resulting in altered regulation of its mRNA targets in vivo leading to infertility. Although the physiological mRNA targets for this protein remain unknown, my hypothesis is that the Zfp36l2 protein acts to affect the stability of an unknown group of mRNAs expressed during the oocyte to embryo transition. Identifying target mRNAs in this system is problematic given the small amounts of material to which one has access. Altered regulation of these RNA targets may be the basis for the arrest of the embryos derived from the mutant females at the two-cell stage. Note that this is the point at which zygotic gene activation and maternal RNA degradation occur. Recently, in Caenorhabditis elegans five CCCH zinc finger proteins were shown to be degraded during early embryogenesis (35). Specifically, degradation of OMA-1, OMA-2, and MEX-5 is required for normal embryogenesis in C. elegans (36, 37). The decay of these proteins may be related to the degradation of maternal mRNAs and/or to zygotic gene activation (38). OMA-1 degradation involves phosphorylation by the DYRK kinase MBK-2 (36). It is possible that ∆N-Zfp36l2 lacks the signal that is required to target Zfp36l2 to the proteasome, modulating its degradation.

The biochemical characteristics of Zfp36l2, in conjunction with its maternal effect during early development, suggest that it may have a similar role in embryogenesis as the CCCH zinc finger proteins of C. elegans, which are involved in the fate of the early blastomere (39–43). Thus, early development is critically dependent on proper metabolism of maternal mRNAs, which is mediated by maternal RNA-binding proteins. I speculate that the two-cell stage blockage observed in embryos

**Mouse Zfp36l2 and ∆N-Zfp36l2 Truncation**

![Graphs and data showing the effects of Zfp36l2 and ∆N-Zfp36l2 in macrophages](image)
derived from the ΔN-Zfp36l2 mutant females might be associated with sustained levels of the protein at this early developmental stage. The 29NTD also contains an unconventional leucine-rich repeat. In general, leucine-rich repeats are short motifs composed of 20–29 amino acids that provide a structural framework for the formation of protein-protein interactions (44). Therefore, it is possible that the 29NTD may sense and/or transduce a yet unknown developmental signal(s), which is critical for the progression of the embryo beyond the two-cell stage. Animals lacking the whole Zfp36l2 protein are pan-cytopenic; their hematopoietic stem cells seem to be unable to proliferate or differentiate in vivo, suggesting an arrest of these primordial cells (26). These animals die at an early age, and hence it is not possible to determine whether they also would have fertility defects. The wild-type protein likely plays important roles in many different tissues, and the mutant protein must be capable of functioning successfully in these tissues. Many maternal effect genes display an oocyte-restricted expression (45–47); Zfp36l2 mRNA is expressed in numerous but not all mouse tissues, in addition to oocytes. Further understanding of the genetic and/or biochemical mechanisms underlying the ΔN-Zfp36l2 mutation is of great interest, because it leads to complete female infertility in mice, due to a blockage of the early embryonic development at the two-cell stage. The targets of this protein that result in developmental arrest are likely to be critical for regulation of embryonic development in mammals.

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