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Identification of *zRAP55*，a gene preponderantly expressed in Stages Ⅰ and Ⅱ oocytes of zebrafish

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Abstract: In an *in silico* search for gonand specific expressed genes, we have identified zRAP55 which is enriched in the ovary of zebrafish . zRAP55 encodes a protein of 382 amino acids with a highly conserved Lsm domain. zRAP55 protein shares more than 56% identities with that of other vertebrate species. RT-PCR results show that it is predominantly expressed in the ovary. *In situ* hybridization and immunohistochemistry studies reveal that *zRAP55* is ubiquitously dispersed throughout the cytoplasm of stages Ⅰ and Ⅱ oocytes, whereas no expression is observed in stages Ⅲ and Ⅳ oocytes. As an RNA associated protein, *zRAP55* might function in the control of protein translation at the early stages of oogenesis in zebrafish.

Key words: Lsm domain; *zRAP55*; Oogenesis; Immunohistochemistry; Zebrafish

在斑马鱼Ⅰ和Ⅱ期卵母细胞中优势表达基因 *zRAP55* 的鉴定

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摘要：通过性腺特异性表达基因的筛选，作者发现了一个在斑马鱼卵巢中富集的基因*zRAP55*。zRAP55蛋白由382个氨基酸组成并含有一个高度保守的Lsm区。zRAP55蛋白与其他脊椎动物一致性在56%以上。RT-PCR结果表明，zRAP55优势表达于卵巢中。原位杂交和免疫组织化学结果表明：在Ⅰ期和Ⅱ期卵母细胞中，zRAP55的阳性信号强烈，均匀地分布于整个细胞质中，但是在Ⅲ期和Ⅳ期卵母细胞中均检测不到信号。作为一个RNA相关蛋白，zRAP55可能在早期卵母细胞中具有调节蛋白质翻译的重要作用。

关键词：Lsm区；*zRAP55*；卵母细胞；免疫组织化学；斑马鱼
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Specialized cells called germ cells are the only ones that can give rise to an entirely new organism (Cinalli et al, 2008). Both types of the egg cells and the sperm cells are created from germ cells undergoing a particular program of meiosis and differentiation (Matova & Cooley, 2001) (Matova & Ooley, 2001). We already know that oocyte-specific factors play essential roles during oogenesis, folliculogenesis, fertilization and early embryonic development (Au et al, 2008). In addition, the abnormal expression of these genes may lead to deviant oogenesis or early embryonic development, it therefore seems important to screen and investigate oocyte-speci-

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fied genes.

Several genes that have been cloned and characterized are specifically or preponderantly expressed in gonads and they may play crucial roles in oogenesis and spermatogenesis (Suzuki, 2000; Gu et al, 2005; Mo et al, 2005; Zhou et al, 2006; Draper et al, 2007; Li et al , 2007; Wang et al, 2007). We show here that zebrafish *RAP55* (*zRAP55*, RNA-Associated Protein with a Molecular Mass of 55 k) is specifically expressed at early stages of Ⅰ and Ⅱ oocytes in the female adult zebrafish.

The zRAP55 protein contains an Lsm domain, which is conserved in the Sm-like proteins, Lsm domain associates with small nuclear RNA to form the core domain of the ribonucleoprotein particles responsible for RNA processing including pre-mRNA splicing, telomere replication and mRNA degradation (Bachellerie & Cavaille, 1997; Belfort & Weiner, 1997; Albrecht & Lengauer, 2004). In *Xenopus laevis*, domain analyses reveal that the Lsm domain of RAP55 protein is essential for the localization to P-bodies and translational repression (Tanaka et al, 2006). In *Drosophila*, the N-terminal Lsm domain of EDC3 protein is important for mRNA decapping (Tritschler et al, 2007). Our results suggested that *zRAP55* is predominantly expressed in the ovary, where it is specifically expressed in stages Ⅰ and Ⅱ oocytes. *zRAP55* might contribute to the regulation of protein translation at the early stages (Ⅰ and Ⅱ) of oogenesis.

1 Materials and Methods

1.1 Animals

Adult zebrafish (*Danio rerio*) were raised and maintained on a 14/10 h light/dark cycle at 28.5℃ and staged as previously described (Kimmel et al, 1995; Wang et al, 2005). Adult male New Zealand white rabbits were purchased from the Experimental Animal Center of Wuhan University.

1.2 *In silico* subtraction

A software was programmed by our lab to search some previously uncharacterized genes that were specifically or predominantly expressed in germ cells of zebrafish or mouse (Gu et al, 2005; Mo et al, 2005; Li et al, 2007; Wang et al, 2007). By the same way, a gene which is preponderantly expressed in ovary of zebrafish was identified in this paper.

1.3 RT-PCR

Total RNA was isolated from the various tissues of the adult zebrafish. Reverse transcription polymerase chain reaction (RT-PCR) was carried out to observe the expression of *zRAP55* mRNA in these organizations by using the following primers: zRAP55RTf (5′-tgc tat tgt cca gtc ttc tgt cgg-3′) and zRAP55RTr (5′-ttc tgg tct tcg atc tct ttc tcc-3′) (Song et al, 1999).

1.4 Bioinformatics analysis of zRAP55

The nucleotide sequence and deduced amino acid sequence of *zRAP55* were analyzed using the NCBI blast N server, ensembl blast X server, Tmpred program, Compute tools and EXPASY Proteomics respectively. Multiple alignments were carried out by using software Clustal W (Thompson et al, 1994).

1.5 Expression of zRAP55 fusion protein and preparation of its antiserum

Primers were designed based on the nucleotide sequence of zRAP55 (The GenBank accession number: NM_200171): zRAP55F (5′-agc gga act ccc tac atc-3′) and zRAP55R (5′-ctc gta ggc agc acc aaa ccc-3′), with EcoRI and XhoI sites on them, the PCR product was inserted into pGEX-6P-1 expression vector to generate fusion proteins. The bacterial culture was induced with IPTG (final concentration is 1 mmol/L), then the recombinant GST-fusion proteins were purified to raise antiserum by immunization of rabbit (Koyano et al, 1997; Li et al, 2007).

1.6 *In situ* hybridization and western blots

Whole mount *in situ* hybridization (WISH) and western blots are conducted as standard protocol (Mo et al, 2005; Yang et al, 2010). Fluorescent double *in situ* hybridization was performed essentially as described (Jowett, 2001).

1.7 Immunohistochemistry

The ovary tissue was isolated from adult zebrafish and fixed in 4% PFA/PBS (pH 7.2) for 24 h at 4℃. Sections of 18 μm were prepared and were rinsed three times for 5 min with PBST (PBS with 1‰ Triton X-100 and 5% serum of newborn calf) and then subsequently blocked with PBS with 10% serum of newborn calf for 2 hr. The sections were incubated in the blocking solution containing the primary antibody (1:100) overnight at 4℃. Controls were incubated with similarly diluted preimmune serum. The sections were then washed with PBST for 4 times at least 10 min each. Thereafter, they were incubated with the FITC-labeled goat anti–rabbit IgG antibody (diluted 1:500; Proteintech Group, Inc. China. Product # 00003-2) overnight at 4℃. Finally, sections were rinsed again with PBS for four times mounted using vectashield (Vector Laboratories, Inc. U.S.A .Product # H-1000) and observed under a Leica fluorescence microscope.

2 Results

2.1 Genomic structure analysis of zRAP55

The cDNA of *zRAP55* is 1699 nucleotides in length and contains an open reading frame of 1,149 nucleotides, with a 115 bp 5′untranslated region (5′UTR) and a 3′untranslated region (3′UTR) of 435 bp, and the 3′ untranslated region contains a polyadenylation signal (AATAAA) (Fig. 1).The *zRAP55* gene consists of nine exons and eight intervening introns, which span approximately 13.9 kb (997242-1011149 bp) on the zebrafish chromosome 23. The exon/ intron structure of the gene is determined by comparison of the cDNA to the genomic sequence at National Center for Biotechnology Information (NCBI), and the exon–intron boundaries strictly agree with the "GT-AG" rule (Tab. 1).The open reading frame initiates in exon 1 and stops in exon 8 (Fig. 1).

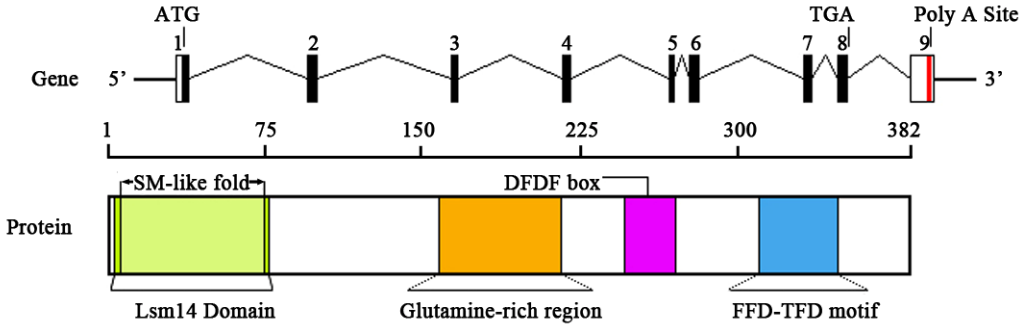

Fig. 1 Schematic representation of *zRAP55* gene and its encoded protein

Exons are indicated by boxes, solid boxes indicate *zRAP55* coding regions; open boxes represent the 5′ and 3′ untranslated region; "∧"shows introns; poly A (AATAAA) is depicted as red box. Green box denotes Lsm14 domain; glutamine-rich region is presented as orange box; purple box indicates DFDF box; blue box denotes FFD-TFD motif.

2.2 Predicted protein sequence analysis of zRAP55

The predicted zRAP55 protein contains 382 amino acids with a molecular weight of 41.55 k and the theoretical isoelectric point is 10.11. A transmembrane region is predited at amino acids 124 − 142.

Multiple sequence alignment reveals that the zRAP55 protein shares high sequence identities with several known protein, including *Xenopus tropicalis* (59.7%), *Mus musculus* (57.3%), *Homo sapiens* (57.2%) and *Rattus* (56.7%). The Lsm domain is highly conserved (Fig. 2). Comparative analysis on protein sequence of Lsm domain from different species, we found that the Lsm domain from zRAP55 shares 92.5% identities with the Lsm domain from *Mus musculus*, *Homo sapiens* and *Rattus*. Further, Lsm domain of zRAP55 shares the high homology (91% identity) with that of *Xenopus tropicalis*. In addition, the glutamine-rich region was only identified in the zRAP55 protein but not in the proteins from other species (*Mus musculus, Xenopus tropicalis, Homo sapiens* and *Rattus*).

2.3 The expression of zRAP55 mRNA in adult zebrafish tissues

By analyzing the tissue distribution of *zRAP55* mRNA using RT-PCR, we identified that the *zRAP55*

Tab. 1 The Exon-Intron analysis of *zRAP55*

Number	Length (bp)	cDNA position	Splice acceptor	Splice donor	Intron length (bp)
1	236	1 − 236		CTGGCGAAAGgtgagtctta	
2	164	237 − 400	tttgtgtcagTGAGGTCTTT	TATTGTCCAGgtgtgtgtta	2157
3	136	401 − 536	tgttcatcagTCTTCTGTCG	CTGGGTTTGGgtcagtgaat	2480
4	156	537 − 693	tctgtgtcagGTCTGCATGG	CAGAGAGGAGgtacaccgtt	1686
5	87	694 − 780	tggagtgcagATAGTTCATC	AGAAAACCAGgtaagagggt	1841
6	162	781 − 943	actctttcagGAACCCGCCG	AGCGTTCAAGgtattcagtt	370
7	157	944 − 1101	gttctgtcagAGCCCAGATC	TCCGCTCCAGgtacatcaac	2115
8	174	1102 − 1276	tcctccctagGCGAACCACG	CACACTATGAgtgagtctga	466
9	408	1277 − 1685	cctcctccagATGTCCTGCT		1113

Uppercase letters indicate the partial exon sequences while lowercase letters show the partial intron sequences. The donor and acceptor splice sites are indicated by red color letters.

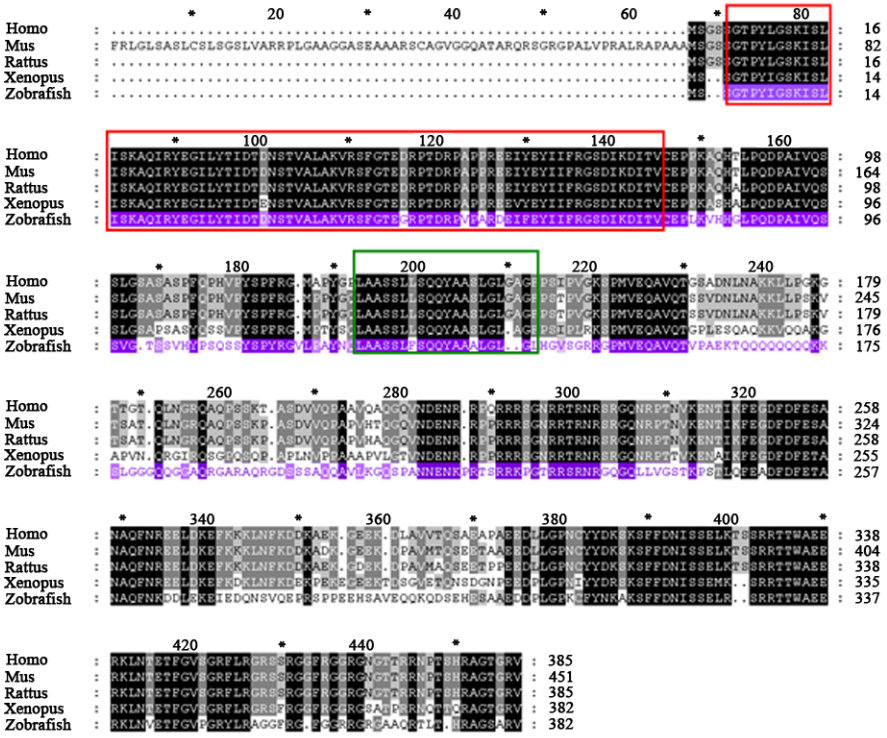

Fig. 2 Multiple protein sequence alignment in different species

The homologs of complete protein sequences from the *homo* (Accession No: NP_653304), *Mus* (Accession No: AAH40823), *Rattus* (Accession No: EDL88837), *Xenopus* (Accession No: NP_001025676) and the *zebrafish* (Accession No: NM_200171) are aligned and the identical residues are shaded. Red box shows the Lsm domain which is highly conserved. The antigen region of zRAP55 is indicated by purple font. The green box represents transmembrane region.

mRNA is abundantly present in ovary and very weak in kidney and liver, but not in brain, testis and heart (Fig. 3).

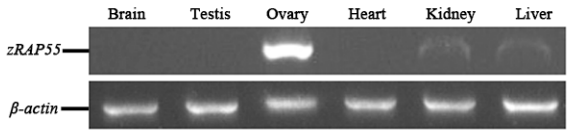

Fig. 3 The RT-PCR analysis of *zRAP55* expression in multiple tissues

The expression pattern shows that the *zRAP55* mRNA is strongly detected in ovary and is very weak in kidney and liver, but not in brain, testis and heart.

To analysis the *zRAP55* expression level in the ovary, whole mount double fluorescence *in situ* hybridization (FISH) was carried out , the signal was obviously appeared in early stages Ⅰ and Ⅱ oocytes. But there existed no detectable signals throughout the stages of Ⅲ and Ⅳ oocytes (Fig. 4A-C). Whole mount *in situ* hybridization (WISH) with DIG-labeled RNA probes also denoted the significant expression of *zRAP55* in stages of Ⅰ and Ⅱ and fade in older stages (Fig. 4D).

2.4 The expression of zRAP55 protein

For the investigation of zRAP55 protein expression in ovary of zebrafish, an antibody panel with epitopes spread over the zRAP55 protein (Fig. 2) was selected and the rabbit polyclonal antibody zRAP55 was raised against amino acids 3 − 244 of the zebrafish zRAP55.

Analysis of the distribution characteristic of zRAP55 protein in ovary by fluorescence immunohistochemistry revealed that *zRAP55* is highly expressed in early stages Ⅰ and Ⅱ oocytes and the signal is evenly distributed throughout the cytoplasm (Fig. 5B, C and D). with the progression of oocyte growth, no signal could be detected in stages Ⅲ and Ⅳ oocytes, same result was got by whole mount immunohistochemistry using the intact ovary (Fig. 5E). In western blots, the band of 41.55 k was specifically recognized in ovary but absent from other tissues (Fig. 5F).

3 Discussion

Oocyte development in the ovary can be divided into four stages in zebrafish (Selman et al, 1993; Zeng &

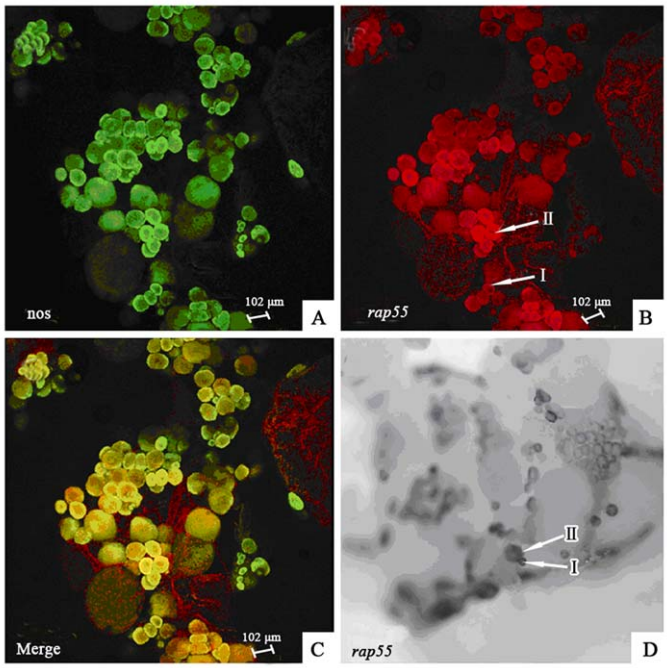

Fig. 4 The expression of *zrap55* and zebrafish *nanos* mRNA in ovary of adult zebrafish

A−C: Double fluorescence *in situ* hybridization results for *zrap55* (red) and *nanos* (green). The arrowheads showing the strong expression in oocytes of stages Ⅰ and Ⅱ, 40×. D: WISH using DIG-labeled probes showing *zRAP55* expression in stages Ⅰ and Ⅱ oocytes (arrowheads), 40×.

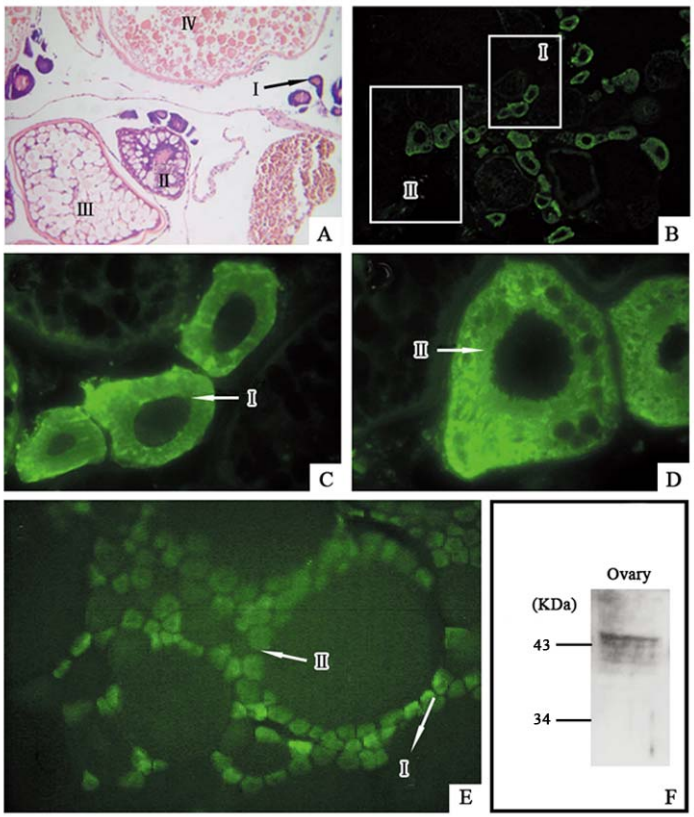

Fig. 5 The expression of ZRAP55 protein in ovary of adult zebrafish

A: H&E staining for ovary, 100×. B,C,D: *zRAP55* is expressed in oocytes of stages Ⅰ and Ⅱ by fluorescence immunohistochemistry (B, × 40; C, D, × 400). E: Wholemount fluorescence immunohistochemistry for *zRAP55* in ovary, the white line shows the expression of *zRAP55* in oocytes of stages Ⅰ and Ⅱ, × 40. F: The western blots demonstrates the band of 41.55 k in ovary.

Gong, 2002). Stage Ⅰ is a stage of primary growth when the large nucleus (germinal vesicle) is surrounded by cytoplasm. Stage Ⅱ (cortical alveoli stage) is characterized by the appearance of cortical alveoli and the enrichment of vitelline envelope. In stage Ⅲ (vitellogenic stage), yolk proteins and yolk bodies with crystalline yolk appear in oocytes. In stage Ⅳ (maturation stage), the yolk becomes non-crystalline as they undergo final meiotic and the eggs are able to fertilize. At present, some oocyte-specific genes which expressed in early oocyte development have been cloned and they play essential roles during early oogenesis and embryonic development in the zebrafish (Koyano et al, 1997; Ramachandra et al, 2007). For example, Nanos1 is expressed in early stage oocytes (stage Ⅰ) and is required for the continued production of oocytes in zebrafish (Draper et al, 2007). XSox3 protein is specially expressed in Ⅰ and Ⅱ oocytes and may be concerned with early oogenesis as a transcription factor (Koyano et al, 1997). Our results indicated that the *zRAP55* is expressed at the early stages of oogenesis (stages I and Ⅱ) (Fig. 5) .Thus, *zRAP55* is likely to play a role at early stages of oogenesis in zebrafish.

Sm and Sm-like proteins of the Lsm (like Sm) domain family are suggested to function in RNA-processing tasks. Five novel groups of Lsm domain proteins (Lsm12 − 16) with long C-terminal tails were discovered and all of them were commonly involved in RNA metabolism. Lsm14 is part of mRNP particles and exclusively found in oocytes (Lieb et al, 1998). RAP55 mediates translational repression through its Lsm domain which recruits RAP55-bound mRNAs to specific sites for translational repression in the oocytes (Albrecht & Lengauer, 2004). The RNA-binding Sm and Sm-like proteins are characterized by mRNA decapping and pre-mRNA splicing activities (Bravo et al, 2005). Recently it is testified that RAP55 is oocyte-specific as a component of ribonucleoprotein particles. *Xenopus RAP55* is observed in the oocytes and early embryos and its N-terminal region including the Lsm domain plays an important role for the localization to P-bodies and translational repression (Tanaka et al, 2006). It is possible that *zRAP55* is involved in regulation of protein translation in stages Ⅰ and Ⅱ oocytes. *zRAP55* gene can also be used as an ideal molecular marker at the early stages of oogenesis.

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