Zebrafish Dnd protein binds to 3'UTR of *geminin* mRNA and regulates its expression

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**INTRODUCTION**

RNAs in cells are associated with RNA-binding proteins (RBPs) to form ribonucleoprotein (RNP) complexes (1). The RBPs influence the structure and interactions of the RNAs and play important roles in their splicing, polyadenylation, sequence editing, transport, mRNA stability, mRNA localization and translation (1, 2). Eukaryotic genes encode a large number of RBPs, each of which has unique RNA-binding activity and protein-protein interaction characteristics (1). The remarkable diversity of RBPs appears to have increased during evolution in parallel to the increase in the number of introns, and allows eukaryotic cells to utilize them in an enormous array of combinations giving rise to a unique RNP for each RNA (1, 3).

**RESULTS AND DISCUSSION**

Identification of the target mRNAs of ZDND protein by SNAAP technique

The *Dnd* gene is required for PGC survival and migration in zebrafish (6). As an RNA-binding protein, ZDND is predicted to regulate a series of genes that are essential for those processes. In order to explore the function of *ZDnd*, the SNAAP technique was used to identify the target mRNAs of *ZDnd*. Because the target mRNAs of many RBPs have not been identified and different RBPs may bind the same target...
mRNAs, GST protein was used as a control. In order to provide the natural circumstances of competitor proteins and increase the binding specificity of the protein-mRNA, the total embryo extract was chosen to enable the identification of true mRNA substrates (12). As a result, 13 target mRNAs were bound by GST-ZDnd protein (Table 1).

To avoid false positive result of the SNAAP technique, RT-PCR was used to further confirm the target mRNA of ZDnd protein. The result showed that all target genes could be amplified using the cDNA reverse transcribed from the embryos total RNA. 8 target genes, including ankrd16, atp5i, cdc42se1, cod, hypothetical protein, ell2, tk1 and gmnin, were amplified using the RNA bound by GST-ZDND protein, indicating that all the target genes expressed in the embryo and ZDnd could bind these 8 target mRNAs (Fig. 1).

**Geminin mRNA was bound by ZDND protein via 3'UTR sequence**

Although the approaches used above enable identification of mRNAs which were bound by ZDND, the binding sites of these mRNAs remained unknown. Of the 8 target mRNAs, gmnin was chosen for the further study. Geminin is a multifunctional protein, which takes part in the cell proliferation, embryogenesis and oncogenesis. Geminin inhibits DNA replication through association with Cdt1 to prevent the incorporation of MCM complex into prereplication complex (13). In addition, geminin interacts directly with Six3 and Hox homeodomain proteins during embryogenesis and inhibits their functions (14). Geminin regulated the function of Hox through a transient association with the Hox repressive Polycomb complex (14, 15). Meanwhile, depletion of geminin causes centrosome overduplication and mitotic defects (16). In contrast, overexpression of wild-type geminin in cancer cells in culture did not produce a cell cycle block (17).

In addition, as shown in (Fig. 1E, F), the HEK293 cells were transfected with PVAX-ZDnd and PVAX-LUC-3'UTR as experimental group, and the HEK293 cells transfected with only PVAX-LUC-3'UTR were served as the negative control. The cells were collected at 24 h, 48 h and 72 h. And then, RT-PCR was used to check the expression of mRNA, and the dual-luciferase assay was used to check the activity of luciferase, respectively. The results indicated that the expression of mRNA and the activity of luciferase in experimental group are higher than the negative control, showing that ZDnd can impact the expression of geminin.

In our study, we used 8-cell-stage embryo to prepare the total embryo extraction. Our result of the whole-mount in situ hybridization indicates that geminin is non-specifically expressed in the animal pole in the zebrafish embryo at the 4-cell, 8-cell, 1k-cell and the sphere stage (Fig. 2), and at these stages ZDnd is also expressed in the animal pole (6), suggesting that ZDnd may be involved in the regulation of geminin.

To address how ZDnd regulate the expression of geminin, the 5'UTR region, the coding sequences (CDS) and the 3'UTR region of the geminin have been transcribed in the presence of DIG-UTP. The labeled RNAs were subsequently incubated with GST-ZDND or the control GST. The copurified RNAs were isolated and resolved on a 1.5% agarose gel. As shown

<table>
<thead>
<tr>
<th>Name</th>
<th>SNAAP primer</th>
<th>Accession number</th>
<th>Tissue</th>
<th>Function</th>
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<tbody>
<tr>
<td>atp5i</td>
<td>H-T11C, H-AP7</td>
<td>BC124416.1</td>
<td>Widespread/reproductive system</td>
<td>Catalysis of the transfer of hydrogen ions from one side of a membrane to the other</td>
</tr>
<tr>
<td>cdc42se1</td>
<td>H-T11C, H-AP31</td>
<td>BC044443.1</td>
<td>Widespread</td>
<td>Any process that modulates the surface configuration of a cell</td>
</tr>
<tr>
<td>ankrd16</td>
<td>H-T11C, H-AP3</td>
<td>BC092927.1</td>
<td>Widespread</td>
<td>Elemental activities, such as catalysis or binding, describing the actions of a gene product at the molecular level. A given gene product may exhibit one or more molecular functions</td>
</tr>
<tr>
<td>cod</td>
<td>H-T11A, H-AP8</td>
<td>AY996924.1</td>
<td>Mitochondrial</td>
<td>Aerobic respiration electron transport chain oxidation reduction transport</td>
</tr>
<tr>
<td>hypothetical protein</td>
<td>H-T11A, H-AP3</td>
<td>AY391441.1</td>
<td>Widespread</td>
<td>ATP binding; kinase activity; thymidine kinase activity; transferase activity</td>
</tr>
<tr>
<td>tk1</td>
<td>H-T11G, H-AP31</td>
<td>BC044148.1</td>
<td>Widespread</td>
<td>Regulation of transcription, DNA-dependent</td>
</tr>
<tr>
<td>ell2</td>
<td>H-T11G, H-AP3</td>
<td>BC056534.1</td>
<td>Widespread</td>
<td>Embryonic development; translational elongation</td>
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<td>rplp2l</td>
<td>H-T11G, H-AP1</td>
<td>NM_001100436</td>
<td>Widespread/ reproductive system</td>
<td>Negative regulation of DNA replication</td>
</tr>
<tr>
<td>gmnin</td>
<td>H-T11G, H-AP2</td>
<td>NM_200086</td>
<td>Widespread/ reproductive system</td>
<td>Ergosterol biosynthetic process; lipid transport; transport</td>
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<td>sigmar1</td>
<td>H-T11G, H-AP28</td>
<td>NM_200977</td>
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<td>Chromatin silencing; protein amino acid deacetylation; regulation of transcription, DNA repair pathway</td>
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<tr>
<td>sir6</td>
<td>H-T11G, H-AP30</td>
<td>BC153391</td>
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<td>DNA replication; deoxynucleoside diphosphate metabolic process; oxidation reduction</td>
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<td>rm2</td>
<td>H-T11C, H-AP1</td>
<td>NM_131450</td>
<td>Widespread/ reproductive system</td>
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in Fig. 3A, the ZDND binding site was located at the 3'UTR region of the *geminin* mRNA. But the GST protein failed to bind the 3'UTR region. Moreover, when purified GST-ZDND protein was incubated with increasing amounts of Digoxigenin-labeled 3'UTR of *geminin* mRNA, more RNA-protein complex band was formed (Fig. 3B). These data demonstrated that the ZDND binding site was located at the 3'UTR region of *geminin*. The copurification experiment with the use of recombinant protein demonstrated that this binding was direct and did not require other protein.

In order to identify the specific nucleotide sequence bound by ZDND, the 3'UTR was further divided into 4 segments, including 3'UTR-1, 3'UTR-2, 3'UTR-3 and 3'UTR-4 (Fig. 3C). The results indicated that the binding site was residing in 3'UTR-2 and 3'UTR-4 (Fig. 3D). Analysis of the sequence of these four fragments indicated that the nucleotides from 864 to 1177 were perfect matches to the 3'UTR-2 and 3'UTR-4 sequences.
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Fig. 3. (A) Digoxigenin-labeled 3'UTR of geminin mRNA is incubated with purified proteins GST (lanes 2 and 3) and GST-DND (lanes 4 and 6). Lane 1 and 5 contain Free RNA only. (B) GST-ZDND proteins is incubated with increasing amounts of Digoxigenin-labeled 3'UTR of geminin mRNA: Lanes 1, 2, 3, 4 and 5 contain 0, 100, 500, 1,000, 3,000 fmol of Digoxigenin-labeled 3'UTR of geminin mRNA, respectively. (C) The 3'UTR of geminin mRNA is divided into four smaller fragments. The fragments are named 3'UTR-1, 3'UTR-2, 3'UTR-3 and 3'UTR-4, respectively. (D) Various fragments derive from full-length geminin cDNA are subcloned into pGEM-T-easy vector, and about 100 fmol DIG-labeled RNAs probes bound by DND at a final concentration of 300 nM are carried out by EMSA, respectively. (E) ZDND binds the 67 nucleotides of geminin mRNA from 864 to 931. GST-ZDND protein is incubated with digoxigenin-labeled 67nt (lane 2 and 3) and Δ3'UTR (lane 4 and 6) of geminin mRNA. Lane 1 contains GST-ZDND protein only. (F) ZDND increases the translation of geminin by binding its 3'UTR. HEK-293 cells were transfected with the indicated constructs. Relative luciferase activity is the ratio between firefly luciferase and renilla luciferase, adjusted to 100%.

931 were the common portion of the binding sequences.

**ZDnd regulates the translation by binding to 3'UTR of geminin**

As shown in Fig. 3A, ZDND can bind to the 3'UTR of geminin mRNA directly, suggesting that ZDND may regulate the expression of geminin through regulating its translation.

The Dual-luciferase assay was used to investigate whether DND could impact the expression of geminin. As shown in Fig. 3F, the translation of the luciferase increased 3.8-fold relative to the PVAX, LUC-3'UTR control cotransfected with PVAXo-ZDnd and PVAX-LUC-3'UTR, indicating that the translation of mRNAs containing the 3'UTR of geminin was specifically regulated by ZDnd.

Compared with cotransfection of PVAX-LUC3'ΔDnd (the coding region without RNA-binding domain of Dnd, amino acids 138-298) and PVAX-LUC-3'UTR, cotransfection of PVAX-LUC3'ΔDnd and PVAX-LUC-3'UTR enhanced the luciferase activity by 3.6-fold, suggesting that the increase of luciferase expression was not caused by a general effect on transcription or translation efficiency but rather specific effect that ZDnd enhanced the translation. In addition, Fig. 1E, F showed that the expression of mRNA and the activity of luciferase in experimental group are higher than the negative control, also suggesting that ZDnd can increase the stability of the target mRNA (Fig. 1 supplemental data). More than that, transfection of PVAX-LUC-3'UTR resulted in the reduction of luciferase activity compared with the transfection of PVAX-LUC-A3'UTR (without the 67 nucleotides from 864 to 931). Bioinformatics analysis of the 3'UTR of geminin showed that an ARE was found in the URR. The promoter and 5'UTR sequence are uniform in the reporter vector, so we exclude that the expression difference is in the transcription level.

**The 67-nucleotide region from 864 to 931 of the 3'UTR of geminin mRNA was the ZDND binding site**

As shown in Fig. 3C, the nucleotides from 864 to 931 were the common portion of the binding sequence by analyzing the sequences of the four fragments. To investigate whether this 67nt fragment can be bound by ZDND, GST-ZDND was incubated with digoxigenin-labeled 67nt mRNA (Fig. 3E). Furthermore, using Dual-luciferase assay proved that translation of the luci-
ferase was increased 3.9-fold relative to PVAX2-LUC-3'UTR when it was fused to the 67nt mRNA of geminin 3'UTR, indicating that the 67-nucleotide region from 864 to 931 of the geminin 3'UTR mRNA was the ZDND binding site (Fig. 3E).

Bioinformatics analysis of 3'UTR sequence of geminin showed that the 864-931nt region in the geminin mRNAs is a URR, in which the content of uridine is high to 60.29%. Our result was consistent with the previous result that URR mediated Dnd binding and function in human cells and in PGCs of zebrafish (11).

Our present data indicates that ZDnd regulates translation of geminin through binding to the URRs in the 3'UTR of geminin mRNA. Furthermore, the identification of ZDnd substrate mRNAs will enable a detailed mechanistic assessment of the consequence of ZDND binding to each target mRNA, and these investigations will provide valuable insights into roles for Dnd in embryogenesis or in cancer cells.

MATERIALS AND METHODS

Zebrafish strain and fish maintenance
Zebrafish (Danio rerio) of the AB genetic background was provided by National Zebrafish Resources of China. Embryos were obtained by natural mating and cultured in embryo medium (18). The Zebrafish were maintained, raised, and staged as previously described (19).

Preparation of total embryo extraction
Preparation of total embryo extraction was carried out according to the method described by Jiao et al. (20). The zebrafish embryos were collected at 8-cell-stage. And then washed twice in phosphate-buffered saline (PBS) and placed into lysis buffer (100 mM NaCl, 10 mM MgCl2, 30 mM Tris-HCl, 1 mM dithiothreitol (DTT), protease inhibitor cocktail, 40 U/ml RNase ZDnd) and 0.5% Triton X-100) (21). After centrifuged for 5 min the supernatant was collected, supplemented with 5% glycerol, and centrifuged with 500 × g for 5 min; the beads were subsequently washed 5 times in PBS; and then, the bound proteins were eluted by washing solution (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0) at 4°C for 20 min with gentle agitation; after the centrifugation at 500 × g for 5 min, the target proteins were collected.

SNAAP screening
The SNAAP screening technique has been also described by Jiao et al (20). After GST and GST-ZDND binding to glutathione Sepharose beads and removing unbound proteins, the washed beads were incubated with 300 μg of embryos total extraction precleared with 20 μl of beads. After binding at 4°C for 1 h, the beads were washed with RBB (RNA Binding Buffer, 10 mM Tris-HCl, pH 7.5, 1.5 mM MgCl2, 150 mM KCl/0.25% Triton X-100 followed by a 10 min wash in RBB/0.25% Triton X-100 containing 1 mg/ml heparin. The beads were subsequently washed 5 times in RBB/0.25% Triton X-100 and the bound RNA was then extracted by standard procedures.

Riboprobe generation
Zebrafish geminin full-length cDNA was purchased in the form that it was cloned in the pME18S-FL3 vector. The 5'UTR, CDS and 3'UTR of geminin were amplified from the pME18S-FL3-geminin vector and cloned into the pGEM-T-easy vector and sequenced. According to the insert direction and the restriction site, the optimal restriction enzyme was chosen to linearize the sequence.

RT-PCR
The RNAs associated with GST-ZDND, GST proteins and the embryos total RNA were subjected to RT-PCR with specific primers, respectively (Supplemental Data. Table 1).

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Electrophoretic mobility shift assay
The electromobility shift protocol was described by King et al (22). RNA synthesis and labeling were performed with an SP6/T7 Transcription and DIG RNA Labeling Kit (Promega). The binding buffer for ZDND-geminin interaction studies was buffer A (50 mM Tris-HCl pH 7.0, 150 mM NaCl, 67 μg/ml yeast tRNA, 0.25 mg/ml bovine serum albumin, 1.5 μg/ml heparin) + 2% 2-mercaptoethanol. All RNAs used in the EMSA were heat treated at 90°C for 3 min, followed by rapid cooling on ice. Potential protein-RNA complexes were allowed to form at 37°C for 10 min. After incubation, 2.5 μl of 6 x loading buffer (Ambion) was added, the samples were immediately loaded on to a 1.5% agarose gel in 1 x TBE buffer. The gel was subjected to electrophoresis on ice until separation was achieved. The protein-RNA complexes and the free RNAs were then electroblotted to a positively charged nylon membrane and subsequently cross linked to the membrane by short-wave ultraviolet radiation. Detection of the RNA on the membrane was performed by washing the blot with a detergent solution, followed by a block solution. The membrane was then probed with anti-digoxigenin-AP conjugate to bind digoxigenin-labeled RNA. The blot was firstly washed twice in de-tingent solution, followed by a block solution. The membrane was performed by washing the blot with a de-tingent solution, followed by a block solution. The membrane was then probed with anti-digoxigenin-AP conjugate to bind digoxigenin-labeled RNA. The blot was firstly washed twice in the MAB buffer, and then washed in the detection buffer for 5 min. After the substrate (CDP-Star) was added to initiate the chemiluminescence reaction, the membrane was wrapped with a plastic wrap and exposed to X-ray film.

Plasmid construction
The full-length coding regions of ZDnd and Z\(\Delta\)Dnd were generated from pGEX-ZDnd using a 5' primer with a EcoRI and a 3' primer with a Xhol restriction site and subcloned into the same sites within the vector PVAX (Invitrogen), generating PVAX-ZDnd and PVAX-\(\Delta\)Dnd.

To construct the PVAX-LUC-geminin (3'UTR) plasmid, the full-length 3'UTR of geminin was PCR amplified from pGEM-geminin, using a 5' primer with a BamHI and a 3' primer with the EcoRI restriction sites and subcloned into the same sites of PVAX-LUC.

Dual-luciferase assay
To perform in vitro assay experiment, HEK293 cells were used as a model system to detect the luciferase activity. HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL) and 1% penicillin-streptomycin (Gibco BRL) at 37°C in 5% CO\(_2\). All transfections were performed by using Lipofectamine 2000 (Invitrogen). For luciferase analysis with 100 ng of renilla control plasmid, 100 ng plasmid DNA was used per transfection. Three independent experiments were set up in every term.

Dual luciferase-activity assays were performed 48 hr after transfection according to the manufacturer’s directions (Promega). The results were represented as means and standard deviation (SD) from three independent experiments.

The results were assessed by the student t test and One-Way ANOVA using SPSS11.04 software.

Acknowledgements
This work was supported by National Program of High-tech Research and Development (863 Program) (grant no. 2008 AA02Z102), National Natural Science Foundation of China (grant no. 90919006 and 30770812) and Trans-Century Training Program Foundation for the Talents by the Ministry of Education of China (grant no. NCET-07-0580).

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