A Novel puf-A Gene Predicted from Evolutionary Analysis Is Involved in the Development of Eyes and Primordial Germ-Cells

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Abstract

Although the human genome project has been completed for some time, the issue of the number of transcribed genes with identifiable biological functions remains unresolved. We used zebrafish as a model organism to study the functions of Ka/Ks-predicted novel human exons, which were identified from a comparative evolutionary genomics analysis. In this study, a novel gene, designated as puf-A, was cloned and functionally characterized, and its homologs in zebrafish, mouse, and human were identified as one of the three homolog clusters which were consisted of 14 related proteins with Puf repeats. Computer modeling of human Puf-A structure and a pull-down assay for interactions with RNA targets predicted that it was a RNA-binding protein. Specifically, Puf-A contained a special six Puf-repeat domain, which constituted a unique superhelix half doughnut-shaped Puf domain with a topology similar to, but different from the conventional eight-repeat Pumilio domain. Puf-A transcripts were uniformly distributed in early embryos, but became restricted primarily to eyes and ovaries at a later stage of development. In mice, puf-A expression was detected primarily in retinal ganglion and pigmented cells. Knockdown of puf-A in zebrafish embryos resulted in microphthalmia, a small head, and abnormal primordial germ-cell (PGC) migration. The latter was confirmed by microinjecting into embryos puf-A siRNA containing nanos 3’ UTR that expressed in PGC only. The importance of Puf-A in the maturation of germline stem cells was also implicated by its unique expression in the most primitive follicles (stage I) in adult ovaries, followed by a sharp decline of expression in later stages of folliculogenesis. Taken together, our study shows that puf-A plays an important role not only in eye development, but also in PGC migration and the specification of germ cell lineage. These studies represent an exemplary implementation of a unique platform to uncover unknown function(s) of human genes and their roles in development regulation.

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Introduction

Comparing human and mouse/rat genomic sequences, Nekrutenko et al. predicted new human protein-coding exons [1]. This approach takes advantage of the fact that in coding regions, synonymous substitutions occur much more frequently than non-synonymous ones. They predicted 13,711 novel exons that were present in both the rodent and human genomes, but the predicted transcripts remained to be validated and their biological functions remained to be demonstrated [2,3]. When we started this study, 4,768 of the originally predicted new exons were already recognized as genes or pseudogenes, so we used the remaining 8,943 potential novel human exons to search for zebrafish orthologs in a zebrafish database (http://www.sanger.ac.uk/Projects/D_rerio/). From this in silico analysis, we found 308 potential genes that had yet no defined biological function (unpublished data). In this study, we chose a novel puf-A gene from the 308 potential genes to characterize its function in zebrafish, mouse, and human.

Zebrafish (Danio rerio) has become a favorite vertebrate model for genetic and developmental studies due to its attributes such as a small body size, rapid development, frequent reproductive cycles (1~2 weeks), short maturation period (3 months), large-scale genetic screening, and easy maintenance [4]. In addition, zebrafish mutations are usually faithful phenocopies of many human disorders [5].

The Puf family is an evolutionarily conserved protein family named after Pumilio (Drosophila) and FBF (Fem-3 mRNA-binding Factor, Caenorhabditis elegans). Puf proteins have been found in various organisms, including yeast, C. elegans, Drosophila, zebrafish, Xenopus, mouse, and human, but their function is largely unclear. The first Puf protein, Pumilio, identified from Drosophila, was known to repress translation of hunchback mRNA in the posterior half of the Drosophila embryo, thereby permitting abdominal development [6]. In addition to its role in posterior patterning of embryos, Drosophila Pumilio functions in the development of germline stem cells [7]. Puf family members are usually identified by the presence of eight tandem Puf repeats of ~35–39 amino
acids [8] and the repeat binds to specific sequences in the 3' untranslated region (UTR) of a target mRNA.

In this study, we conducted various experiments to show that a novel puf-A gene is involved in eye and primordial germ-cell (PGC) development. Using the SMART server, we identified 14 puf-A-related proteins in human, mouse and zebrafish. We studied their phylogenetic relationships of these 14 proteins. Moreover, a computer modeling of human Puf-A predicted that it is a unique RNA-binding protein composed of six Puf repeats.

**Results**

Expression and cDNA cloning of the novel puf-A gene in zebrafish

In zebrafish, the puf-A gene was found to express at a high level by RT-PCR in the eyes and ovaries, and to a lesser degree in the brain, head kidney (pronephros), and testes (Fig. 1A). Moreover, this gene was found to express at all stages of zebrafish embryo development (Fig. 1B). In situ hybridization confirmed that puf-A was ubiquitously expressed in zebrafish embryos from fertilization to early somitogenesis, but at a later stage of embryo development, its expression was restricted primarily to the eyes and optic tectum (Fig. 1C).

In addition, in adult zebrafish ovaries, puf-A was found to be prominently expressed in early immature follicles that were small in size and nested with other developing oocytes (Fig. 1D). In general, the process of folliculogenesis could be divided into five stages, beginning with the early germine stage I (primary growth) cells that appeared in clusters, through the pre-vitellogenic stage II and the vitellogenic stage III, and ending with the mature or ovulated stages IV and V [9,10]. As shown in Fig. 1D, strong puf-A mRNA expression was noted in primitive stage I ovarian follicles that appeared spherical in shape with diameter less than 100 μm, nesting with other developing. But this expression declined sharply and became negligible in subsequent stages of oocyte development (e.g. stages II and III). The results of in situ hybridization of ovary cross-sections confirmed that puf-A mRNA expressed prominently in the cytoplasm of stage I follicles which appeared in clusters. In contrast, stage II and III ovarian follicles showed no discernible expression of the puf-A transcript (Fig. 1D). It seems that the expression of puf-A occurs when the first wave of follicles begin their process of folliculogenesis.

**Figure 1. Expression of puf-A in zebrafish using RT-PCR and in situ hybridization.** (A) Gene expression in adult tissues of zebrafish was analyzed by RT-PCR and electrophoresis with puf-A primers (upper panel) or actin primers (lower panel, as the internal control). Notation: B, brain; E, eye; G, gill; H, heart; I, intestine; K, head kidney; L, liver; O, ovary; T, testis; N, negative control. (B) The puf-A gene was expressed in various stages of zebrafish embryo. C, cleavage; B, blastula; 1, 1 day post-fertilization (dpf); 2, 2 dpf; 5, 5 dpf; N, negative control. (B) The puf-A gene was expressed in various stages of zebrafish embryo. C, cleavage; B, blastula; 1, 1 day post-fertilization (dpf); 2, 2 dpf; 5, 5 dpf; N, negative control. (C) Whole-mount in situ hybridization with puf-A antisense riboprobe on zebrafish embryo. The puf-A expression at 10 h post-fertilization (hpf) (tailbud stage) and 24 hpf (25-somite stage) with lateral overview. The black arrow points to the eye and the red arrow to the optic tectum. (D) Whole-mount and cryo-section in situ hybridization with puf-A antisense riboprobe in adult ovaries. In adult ovaries, a staging series of oocyte development was characterized by the diameter of various oocytes [9,10]. Stage I, primary growth follicles (<0.1 mm); stage II, previtellogenic (0.1–0.30 mm); and stage III, vitellogenic (>0.30 mm).

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A full-length puf-A cDNA was cloned from RNA of zebrafish ovaries (Fig. S1). It is 2,053 bp in length and contains an open reading frame of 629 amino acids (Fig. S1). Further blasting in the NCBI and Ensembl websites demonstrated that the BAC clone #CH211-241o7 contained the full-length puf-A gene [ENS-SDARG00000063356 [Ensembl 44]] with 10 exons on a region of ~13.8 kb in chromosome 10: 5,573, 939 to 5,391,449 (Fig. 2A). The cDNA/protein sequence of zebrafish Puf-A corresponds to protein LOC394185 (gi:66377) in the NCBI with gene ID of 394185 (accession # of protein sequences, XP_695580.2, Table 1) with an exception of five amino acid residues. At residues 558–562, the sequence is “Glu-Arg-Phe-Ser-Arg” in Puf-A from our study (Fig. S1), while “Gly-Lys-Tyr-Lys-Met” in LOC394185 (Table 1); and this discrepancy arises from a difference in lengths of exons 16 and 17.

Phylogenetic relationships of Puf-A-related proteins

A search for Puf-A-related sequence fragments in the databases suggested that the Puf-A in zebrafish is a member of the Puf family. Each Puf protein contains a Puf domain that consists of several tandem Puf repeats of 36 amino acids [11,12]; the Puf domain has also been known as the pumilio homolog domain [12]. In total, we identified 14 Puf-related proteins of zebrafish, mouse, and human using the SMART server (Table 1 and Fig. 2B). A phylogenetic tree constructed using the PHYLIP package suggests that these 14 Puf proteins can be grouped into three clusters: (1) the Puf-A homolog cluster, (2) the C14orf21 homolog cluster, and (3) the PUM1/PUM2 homolog cluster (Fig. 2B).

Sequence similarities among these Puf proteins in each cluster were analyzed and categorized (Fig. 2B). In this study, the human and mouse Puf-A homologs, i.e., KIAA0020 and D19Bwg1575c in Table 1, are designated, respectively, as the human and murine Puf-A, respectively. BLASTP analysis revealed that human Puf-A shared 89% identity in the aligned 647 amino acid residues with murine Puf-A and 66% identity with zebrafish Puf-A in the aligned 621 residues. However, compared with human related proteins in the other two categories, human Puf-A and human C14orf21 showed no significant similarity and human Puf-A and Pumilio (PUM1) shared only 21% identity in the aligned 241 amino acid residues (Fig. 2B and Table 1). Similarly, human Puf-A and human PUM2 shared only 20% identity in the aligned 240 residues. Thus, members of the Puf-A cluster are similar to each other, but distinct from the members of the other two clusters. Based on the results of the phylogenetic and sequence similarity analyses, Puf-A homologs could be grouped into a single cluster (Fig. 2B).

On the other hand, within the cluster of C14orf21 homologs (Fig. 2B), human C14orf21 showed 94% identity with murine 2610027H16Rik in the aligned 581 residues and 34% identity with zebrafish LOC564287 in the aligned 619 residues. However, human C14orf21 showed no significant similarity and human Pumilio (PUM1), and human C14orf21 and human PUM2 shared only 23% identity in the aligned 140 amino acid residues. Thus, C14orf21 homologs could be grouped into a single cluster (Fig. 2B).

As to the cluster of PUM1/PUM2 homologs (Fig. 2B), human Pumilio (PUM1) and PUM2 shared 75% identity in the aligned 1,076 residues and human and mouse Pumilio (PUM1) shared 98% identity in the aligned 1,189 residues. In zebrafish, there were four Puf proteins in this cluster (Fig. 2B and Table 1) and their similarity analyses were described in Data S1. The results described above support the clustering of the homologs of PUM1 and PUM2 into one group in our phylogenetic tree analysis (Fig. 2B). The multiple sequence alignments of these 14 Puf proteins were shown in Fig. S2.

Computer modeling of human Puf-A

Based on the crystal 3D structure of the human Pumilio domain with 1.9 Å resolution [13], we conducted a computer modeling of the Puf-domain for human Puf-A (KIAA0020) using its 336 amino acid residues from Asp-151 to Ile-486 (Fig. 2C). The quality of this modeling evaluated by the VADAR server showed that 100% of the residues were in the allowed regions of the Ramachandran diagram [14].

The computer model of human Puf-A predicted its structure to be composed of six Puf repeats, each of which constitutes a unique superhelix, half doughnut-shaped Puf domain (Fig. 2C). The six Puf repeats are distributed in two separate regions from Leu-165 to Glu-273, and from Ala-350 to Glu-460 (R1 to 3 and R4 to 6, respectively; shown in blue in Fig. 2C). These six repeats are structurally aligned with corresponding repeats of the template used in this computer modeling. Moreover, each repeat has three helices and the second helix, which is located at the inner, concave face of the model, and interacts with RNA (yellow in Fig. 2C), exhibiting characteristic features of a conventional Puf repeat [13].

On the other hand, the sequence from Glu-274 to Glu-349, which represents the middle region of this model (RL1 and RL2; shown in magenta in Fig. 2C), contains no typical Puf repeats identifiable by the SMART server. Detailed analysis of this model showed that this middle region possesses a length of segment close to two tandem Puf repeats (76 residues) and each of these “repeat-like” structures exhibits features of three-helix similar to a typical Puf repeat. It is concluded that this middle region mimics two Puf repeats structurally. Thus, the overall structure of Puf-A features a six-Puf-repeat domain with an intermediate region of two repeat-like segments so that it displays a topology similar to the conventional eight-repeat Pumilio homolog domain [13]. Furthermore, this computer model of human Puf-A predicts that it is a new RNA-binding protein, distinctly different from the Pumilio domain.

In addition, the values of electrostatic potentials on the molecular surface of this model of human Puf-A were calculated. As shown in Fig. 2D, an asymmetric distribution of electrostatic potentials was noted for the Puf domain of Puf-A: its concave surface has predominately positive basic electrostatic potentials (shown in blue; left panel of Fig. 2D), presumably for RNA binding, while the convex surface in this model is acidic (red) with partly hydrophobic (white) areas (right panel). Similar properties of the electrostatic surface of this model were also observed in the crystal structure of Pumilio [13].

Eye defects in the MO knockdown of the puf-A gene

To examine the biological function of the puf-A gene, zebrafish embryos at the 1~4 cell stage were injected with one of the two puf-A-MO antisense oligonucleotides, MO1 and MO2 (see “Materials and Methods” and Fig. S1 for locations of target). As illustrated in Fig. 3A, MO1 morphants clearly showed small eyes, a cilia (Fig. 3B), and a brain edema at 1 and 2 dpf. Relative to the eye size of WT fish, there were significant reductions in eye size among the morphants in a dose-responsive manner (~40% reduction with 5 ng puf-A-MO1; Fig. 3B). The puf-A-MO2 gave results similar to those of the puf-A-MO1.

In order to further demonstrate the efficiency of MO1 and MO2, in vitro transcription/translation of puf-A was performed in the presence or absence of 0~200 nM MOs. It was shown that these MOs blocked puf-A translation in vitro, especially at high concentration (Fig. 3C). The specificity was further confirmed by the experiment in which the addition of capped puf-A RNA partially but significantly rescued the phenotype of eye size in MO-induced morphants in vivo (p value<0.00007; Fig. 3D).
Figure 2. Genetic map of the puf-A locus of zebrafish, the unrooted phylogenetic tree, computer modeling of human Puf-A and its electrostatic surface representation. (A) Genetic map of the zebrafish puf-A locus and the exon/intron structure of the puf-A transcript were constructed through blasting the puf-A cDNA sequence to the genome databases of the NCBI and Ensembl websites. The puf-A (zgc: 66377) is ENSDARG00000063356 in chromosome 10: 5,373,938 to 5,391,448 (Ensembl 44). (B) The unrooted phylogenetic tree of human (Hs), mouse (Mm) and zebrafish (Dr) Puf proteins. Phylogenetic analysis was performed using the PHYLIP 3.67 package as described in Method. These Puf proteins could be grouped into three clusters: (1) the Puf-A cluster, (2) the C14orf21 cluster, and (3) the PUM1/PUM2 homolog cluster. (C) Modeling the Puf domain of human Puf-A. This model, built by MODELLER 9v3 as described in Methods, represents the corresponding Puf domain for binding with RNA (yellow). This Puf domain of Puf-A contains six Puf repeats distributed in two regions (R1 to 3 and R4 to 6; blue), and each region contains three repeats. The magenta color refers to the middle region of the Puf domain. The N and C terminal ends of this Puf domain are indicated. (D) Electrostatic surface representation of the Puf domain. The electrostatic potentials were calculated by DELPHI as described. The left panel shows the areas on the concave surface with positive potentials (blue) which interacts with RNA (yellow). The right panel represents the convex surface, where the negative potentials are shown mainly as the acidic (red) and a few hydrophobic (white) areas.

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hybridization showed that most brain regions were normal at 1dpf, during embryo development. As shown in Fig. S5A, lon/midbrain marker) etc were used to characterize the eye defects and hindbrain marker), mab21l2 hindbrain marker), rx3 retina marker), six3b retina/diencephalon/midbrain marker etc were used to characterize the eye defects during embryo development. As shown in Fig. S5A, in situ hybridization showed that most brain regions were normal at 1dpf, but some regions (like optic tectum and eyes) developed abnormal defects that occurred at 2dpf. For example, six3b expressed only in ganglion cell layer of eye tissues in wild type 2dpf; but this gene expression was found in retina, optic tectum and hindbrain. In contrast, in morphants, no mab21l1 expression was found in the entire eye tissue or optic tectum (Fig. S5A). Furthermore, expression of another marker, mab21l1, was found in retina, optic tectum and hindbrain. In contrast, in morphants, no mab21l1 expression was found in the entire eye tissue or optic tectum (Fig. S5A). Furthermore, as shown in Fig. S5B, puf-A knockdown promoted apoptosis in eye tissues at 1dpf as compared to control. It seemed that cell death occurred prior to retinal differentiation which occurred approximately 28–30 hpf [15]. Moreover, the other retinal differentiation markers (ath5/atoh7) were not expressed in morphants as late as 36 hpf in in situ experiments [pictures not shown], suggesting that the puf-A knockdown led to specific differentiation defects in eyes, not simply delayed development.

Subsequently, at the development stages of 3 and 5 dpf, eye sections of WT fish and morphants were further examined (Fig. 3E). In WT zebrafish, the retina comprises several layers of differentiated cells including retinal ganglion cells, the inner plexiform layer, amacrine cells, bipolar, outer plexiform layer, rods and cones, and pigmented cells. In contrast, morphants with puf-A gene knockdown exhibited features of an undifferentiated retina with loss of detailed architecture and a significant reduction in eye size. Structures such as the rod and cone layers were not concentrically organized and retinal ganglion cells and plexiform layers were not readily discernible (Fig. 3E).

**Defects of primordial germ-cell development in the MO and siRNA knockdown**

During embryo development, primordial germ cells (PGC) follow a unique developmental path that is characterized by specification and migration of these cells to colonize the gonads where they differentiate into gametes. To investigate whether puf-A is involved in PGC development, the puf-A MO was used to knockdown its expression in

**Table 1. Information on the putative Puf proteins in humans, mice, and zebrafish.**

| Species | Puf proteins | Gene ID | Gene location | Accession #s of protein sequences | Length (amino acids) | Number of Puf repeats |
|---------|--------------|---------|---------------|----------------------------------|----------------------|-----------------------|
| Human   | Puf-A (KIAA0020) | 9933    | 9p24.2 | Q15397                      | 648                  | 6                     |
|         | C14orf21 | 161424  | 14q12 | Q86U38                       | 636                  | 7                     |
|         | Pumilio (PUM1) | 9698    | 1p35.2 | Q14671                      | 1,186                | 8                     |
|         | PUM2      | 23369   | 2p22-p21 | Q8TB72                      | 1,066                | 8                     |
| Mouse   | Puf-A (D19Bwg1357e) | 52874  | 19 | Q88K59                       | 647                  | 6                     |
|         | 2610027L16Rik | 67842   | 14 | Q88MC4                       | 636                  | 5                     |
|         | Pumilio (PUM1) | 80912   | 4 | Q80U7B                       | 1,189                | 8                     |
|         | PUM2      | 80913   | 12 | Q80U5B                       | 1,066                | 8                     |
| Zebrafish | Puf-A | 394185   | 10 | XP_695880.2                  | 629                  | 6                     |
|         | LOC564287 | 564287  | 3 | XP_692728.2                  | 604                  | 5                     |
|         | LOC568777 | 568777  | 16 | XP_697221.2                  | 457                  | 4                     |
|         | LOC567494 | 567494  | 13 | NP_001096040.1               | 1,106                | 6                     |
|         | LOC569578 | 569578  | 20 | XP_698067.2                  | 164                  | 3                     |
|         | LOC798171 | 798171  | 18 | XP_001338629.1               | 182                  | 3                     |

*Annotations described in the Entrez Gene database at NCBI.

**Accession number used in SwissProt.

2Accession number used in the RefSeq database.

3The Puf repeats were identified by the SMART server.

4According to the annotations in Entrez Gene database, the old gene symbol for puf-A is “zgc:66377” and the name for protein is “hypothetical protein LOC394185”. doi:10.1371/journal.pone.0004980.t001

Furthermore, in order to circumvent the potential issue of “off-target effects” of MOs, not only a wide range of MOs (1 to 10 ng/embryos) was used for gene knockdown experiments, but also a 3 bp mismatch puf-A (5mmMO1) was employed as a negative control for MO1.**
Figure 3. The phenotypes of *puf-A* morphants in the zebrafish. (A) Zebrafish embryos at the 1–4-cell stage were treated with 5 ng *puf-A* morpholino (MO1) by microinjection. The phenotypes of the wild-type and morphants are shown in lateral view at 1, 2, 3, and 5 days post-fertilization (dpf) after treatment. Black arrows point to the eyes. (B) Various amounts of MO1 were microinjected into zebrafish embryos, and the eye size was measured at 1 or 2 dpf and compared to the eye size of control fish. The “relative eye size” was defined by the value of eye size in MOs relative to the average size of eyes in normal embryos of WT fish. The average value of eye size in normal embryos at 1dpf was considered as 1. Error bars represent the standard error of the mean. ** refer to p<0.01 Student’s t-test. (C) 0, 2, 200 nM *puf-A-MO1* or *puf-A-MO2* were added to the *puf-A* generated through *in vitro* transcription/translation reactions. One microliter of the reaction mixture was separated on 10% SDS/PAGE, blotted, incubated with streptavidin-AP, and developed with NBT-BCIP reagents. (D) The 5 ng control- or *puf-A-MO1* was used for microinjection. In addition, 200 pg of capped *puf-A* RNA was co-injected with *puf-A-MO1* to check the specificity of MO knockdown. The “relative eye size” was defined as above. The eye size was measured at 2 dpf. Control-MO, n = 20 embryos; *puf-A-MO1*, n = 59; *puf-A-MO1*+ capped RNA, n = 65. The p value in Student’s t-test for the difference between *puf-A-MO* and *puf-A-MO1*+mRNA was <0.00007. (E) Transverse histological sections of zebrafish wild-type and morphant (*puf-A-MO1*) eyes stained with hematoxylin and eosin at 3 and 5 dpf.

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In the early stage of zebrafish embryos. In situ hybridization with zebrafish PGC-specific vasa RNA was employed as a marker to monitor primordial germ-cell (PGC) development in zebrafish. The vasa expression in wild-type (WT) and morphants (MO1 at 5 ng/embryo) at 16 and 20 hpf embryos in dorsal view. Anterior is to the left for 16 hpf and left bottom for 20 hpf. The morphants exhibited prominent abnormalities with either a reduction in PGC numbers (50.4%, n = 125 embryos) or abnormal patterns of migration (34.4%, n = 125) indicating the failure of PGC navigation towards their destined sites. (B) Upper: the construction of puf-A siRNA with nanos 3’ UTR, vasa expression and normal eye size in embryos 2 dpf after injection. The embryos displayed a marked reduction in PGC numbers (80.9%, n = 115 embryos) and abnormal patterns of migration (11.3%, n = 115 embryos). Middle: construction of puf-A siRNA without nanos 3’ UTR, vasa expression and small eye size in 2dpf embryos. Bottom: construction of control siRNA with nanos 3’ UTR, vasa expression and normal eye size in embryos after injection.

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Expression of the puf-A gene in eye tissues of adult mice

The puf-A gene was identified in the mouse genome as the mouse D19Bwg1357e (Table 1). As puf-A MO knockdown led to abnormal differentiation in zebrafish eye, puf-A expression in mouse eyes was investigated. The in situ hybridization analysis showed that puf-A was expressed in retina ganglion cells of mice, and to a lesser degree, in the pigmented cells of mice as well (see arrows in Fig. 5), suggesting that the Puf-A protein may play an important role in the function of vertebrate eyes.

Identification of the mRNA targets for puf-A in zebrafish

The biotinylated puf-A was prepared from in vitro transcription/translation and then purified through immobilization on streptavidin magnetic beads. Afterwards, the purified biotinylated puf-A were mixed with 10 ug mRNA mixtures from embryos and ovaries. After fix and PBS wash, the residual RNA pulled down by biotinylated Puf-A was amplified, subcloned and sequenced. Using this pull-down assay, many potential RNA targets for puf-A bindings were found and listed in Table 2 with their gene IDs and symbols. We further showed that there was a reciprocal relationship for the expression of puf-A and one of its potential RNA targets, prdm1a. (Jui-Chin Chang and John Yu, unpublished observations). Therefore, these results and computer modeling predicted that puf-A is a RNA binding protein.

Discussion

The zebrafish has become one of the top vertebrate models for genetic and developmental studies because it is highly prolific and amenable to micromanipulation and gene knockdown. In this study, the zebrafish was used as a model for analyzing the structure and functions of a novel gene, puf-A. This has proved to be an efficient strategy for a detailed analysis of the function of a gene. This approach provides an outstanding platform for understanding the functions of novel genes and their roles in controlling development of an organ or organism.

It was found that the puf-A gene was primarily expressed in the eyes and ovaries and to a lesser degree in the brain and kidneys of adult zebrafish. In the eyes of adult zebrafish and mice, the Puf-A protein was mainly expressed in retina ganglion cells. During embryogenesis, the formation of retinal neurons follows a phylogenetically conserved order, and all six retinal neuron types are generated from common multipotent progenitors, with retinal ganglion cells being the first neurons to occur [20,21]. In this study, zebrafish morphants of 3- and 5-dpf embryos showed incomplete differentiation patterns in the retina, suggesting that the Puf-A protein may have important roles in the development of retinal progenitors.

Additionally, during embryonic development, knockdown of the puf-A gene led to a reduction in the number of PGCs and their abnormal migration, suggesting that Puf-A is involved in the maintenance and migration of these primitive germ cells. The adult zebrafish ovary is a useful vertebrate model to study oocyte development and its regulation [22]. In this study, the expression of puf-A was predominantly in stage I follicles in adult ovaries and became undetectable in stage II and III follicles during subsequent oocyte development. It was noted that the most primitive germ line stem cells, oogonia, were not readily distinguishable from stage I follicles. Thus, the transition of oogonia into stage I follicles was

| Gene IDs | Gene symbols | Names or descriptions | Locations (chromosome) |
|----------|--------------|----------------------|-----------------------|
| 792333   | zgc:193933   | ovary-expressed homeobox protein | 24 |
| 323473   | prdm1a       | PR domain containing 1, with ZNF domain | 16 |
| 568830   | spata2       | spermatogenesis associated 2 | 23 |
| 327196   | txt10        | testis expressed 10 | 16 |
| 321726   | rbb4         | retinoblastoma binding protein 4 | 19 |
| 566947   | ddx3         | DEAD (Asp-Glu-Ala-Asp) box polypeptide 3 | 9 |
| 114438   | zp2.2        | zona pellucida glycoprotein 2.2 | 20 |

*detailed description of this pull-down assay was described in Text and Methods and Materials.

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not investigated in this study. Taken together, these findings indicated that in zebrafish Puf-A not only regulates PGC development but may also play a role in germline stem cells up to stage I follicles.

In total, 14 puf-related proteins of zebrafish, mice, and humans were identified by the SMART server. The Puf-A in this study with its newly identified roles in eyes and PGCs corresponds to the zebrafish LOC594185, mouse D19Bwg1357e, and human KIAA0020. There are three groups of Puf-related proteins: the Puf-A homolog cluster; the C14orf21 homolog cluster, and the PUM1/PUM2 homolog cluster. The Puf-A and C14orf21 homologs could be separated into two homolog clusters and the proteins of their members were similar to each other within the same cluster, but easily distinguishable from members of the other clusters. For example, the Puf-As in humans, mice, or zebrafish were similar to each other, but showed only ~21% identity with the human Pumilio (PUM1) protein. In the PUM1/PUM2 homolog cluster, there are more than one member proteins in each animal species, especially in zebrafish (six homologous genes in the PUM1/PUM2 cluster have been annotated, but await further characterizations of their proteins).

The Puf family proteins are characterized by their tandem Puf repeats with ~35–39 amino acids in each repeat. Each repeat consists of three α-helices, which bind to its RNA recognition residues [8]. A typical RNA recognition motif such as the Pumilio homolog domain usually contains eight tandem Puf repeats. Our computer modeling indicated that the Puf domain of the Puf-A homologs in humans, mice, zebrafish and yeast (data not shown) consists of six Puf repeats and the topographic characteristics predicted it to be a new RNA binding protein. Furthermore, using a pull-down assay, we had found potential RNA targets for puf-A bindings, conceivably leading to the suppression of target gene expression. In fact, Puf8p, the homolog protein of puf-A in yeast, was shown to be involved in the repression of ASH1 mRNA [23]. This model of human Puf-A also suggested that its Puf domain exhibited a structural feature with six Puf-repeats and a middle region of the Puf domain that mimics exactly two additional Puf repeats. In addition, the asymmetric distribution of the electrostatic potentials of the amino acid side chains on the surfaces of the Puf domain of Puf-A suggests that the concave surface of the protein in Drosophila and C. elegans have been reported to be penguin and puf-12, respectively. The function of the penguin protein in Drosophila is still unknown, while knockdown of puf-12 via RNAi in the C. elegans caused early larval arrest and egg laying abnormalities (Egl) (http://www.wormbase.org/).

Materials and Methods

Animals

Breeding and maintenance of AB strain zebrafish, as well as collecting and staging of embryos, were done according to standard procedures [27]. Some embryos were reared in egg water treated with 0.003% 1-phenyl-2-thiourea (PTU) to inhibit pigmentation [27]. Developmental times refer to hours (hpf) or days (dpf) post-fertilization.

RT-PCR and cDNA cloning of puf-A from zebrafish

Total RNA was extracted from zebrafish embryos and adult tissues using Tri-reagent (Sigma, St. Louis, MO, USA). Reverse transcription was performed using the Superscript pre-amplification system (Gibco BRL, Grand Island, NY, USA) as described in the manufacturer’s instructions. The cDNA product was amplified by PCR with specific primer sets for puf-A or β-actin. The puf-A forward primer was 5’-GGTCCACAGAAAGCGCGACG-3’ and the reversed primer, 5’-CGAACATCTACCTCCCTTACG-3’. The β-actin forward primer was 5’-TCACACCTCTTCAACAGGACTCGCG-3’ and the reversed primer, 5’-GAAGCTGCTAGCCTCTCCGTCAG-3’. To obtain the puf-A complete cDNA, rapid amplification of 5’- and 3’-cDNA ends (5’-RACE and 3’-RACE) was performed with total RNA of the ovaries using the SMART cDNA amplification kit (Clontech Laboratories, Palo Alto, CA, USA). The RACE products were cloned into pGEM-T easy vectors (Promega, Madison, WI, USA) and sequenced. The cDNAs of puf-A full-length and puf-A without 5’-UTR region were reconstructed into pBluescript SK minus vector using the 5’- and 3’-RACE products.

Retrieval of putative Puf-protein sequences

Zebrafish, murine and human Puf protein sequences were retrieved from the SMART server (http://smart.embl-heidelberg.de) by the analysis of Pumilio-conserved domains in both the normal and genomic modes. Four individual sequences were found to contain the Puf domain in humans and mice. Additionally, there were six putative Puf-related proteins identified in zebrafish using the SMART server. More detailed information about these Puf proteins and their accession numbers is given in Data S1 and Table 1.

Phylogenetic analysis

A multiple-sequence alignment for these Puf-related protein sequences was generated by CLUSTAL X2.0, using the BLOSUM series matrix [28]. The option for a negative matrix was turned on, while the other parameters remained at the default setting. The BLASTP algorithm with the BLOSUM62 matrix, which was implemented in BLAST at NCBI, was used for the sequence similarity analysis. A phylogenetic tree of putative Puf proteins was constructed using algorithms with PHYLIP vers. 3.67 [29] (see Data S1). The final unrooted tree diagram was prepared using DENDROSCOPE vers. 1.2.4 [30].

Modeling the Puf domain of human Puf-A (KIAA0020)

In order to model the three-dimensional (3D) structure of human Puf-A, the mGenTHREADER method of the PSIPRED server [31,32] was used for predicting secondary structures and making sequence alignments. Initially, the structural information of human Pumilio homolog domain (Protein Data Bank code: 1IB2 and 1MBY) [8,13] was used as modeling templates. Even though the sequences of human Pumilio (PUM1) is only 21% identity with human Puf-A, the crystal structure of Pumilio homolog domain is similar to Puf-A detected by mGenTHREADER with p value<0.0001.

Then 3D structure of Puf domain in human Puf-A was constructed by MODELLER 9v3 [33] (see Data S1). A segment of the RNA ligand from 1MBY [13] was assembled into the resulting model to represent the potential RNA binding site. Furthermore, the electrostatic potentials were calculated using DELPHI [34] with default parameters setting in CHIMERA [33]. The color spectrum mapped onto the domain surface ranged from ~7 kT/e (dark red) to +7 kT/e (dark blue). Finally, 3D structural diagrams in this study were prepared using CHIMERA [35]. The convex and concave surfaces represent the presentation of the model that had been rotated 180° about the vertical axis.
In situ hybridization of eyes and ovaries

The collection and staging of embryos were performed as described. Embryos were fixed overnight at 4°C in 4% paraformaldehyde buffered with 1× phosphate-buffered saline (PFA/PBS). In addition, the ovaries and eyes were removed from zebrafish or mouse after anesthetization and decapitation, and placed in 4% PFA/PBS. After being treated with 30% sucrose, specimens were embedded in OCT. Frozen sections (7 and 10 μm thick for mouse and zebrafish, separately) were collected onto coated slides. In situ hybridization was performed using an InsituPro automated system (Intavis, Koeln, Germany). Whole-mount and section in situ hybridization were carried out using a digoxigenin (DIG)-labeled RNA probe and anti-DIG antibody conjugated with alkaline phosphatase as described previously [36,37]. After hybridization, slides were incubated with anti-DIG antibody conjugated with AP, and developed with NBT-BCIP reagents. The in situ hybridization analysis of the cyrossections of adult zebrafish eyes was carried out with a zebrafish puf-A riboprobe after fluorescein (Flu) labeling. After hybridization, slides were incubated with anti-Flu-AP, and developed with FastRed reagents.

The following DIG-labeled RNA probes were prepared from linearized plasmids using the DIG RNA labeling kit (Roche, Basel, Switzerland): (1) an antisense probe of the puf-A gene prepared from KpnI-digested pBluescript SK- puf-A (full-length, 2,053 bp) using T3 RNA polymerase, (2) a puf-A sense probe prepared from BamHI-digested pBluescript SK- puf-A using T7 RNA polymerase, and (3) a vasa antisense probe prepared from XbaI-digested pBluescript SK- vasa (a gift from Dr. Bon-chu Chung, Academia Sinica) with T7 RNA polymerase. Follicles at different stages of development were identified according to the different-sized diameters of the follicles [9,10].

Morpholino (MO) knockdown

Zebrafish embryos were obtained by natural mating and MO microinjection was performed at the stage of 1–4 cells. The puf-A-MO1 antisense oligonucleotide 5'-AATGAGCCATGTGTGACTGACAAACA-3' was designed to direct against the 5’ UTR of the puf-A gene, and the puf-A-MO2 antisense oligonucleotide was 5’-TTTACCCTCCTATAATGGACACATGGT-3’ that directed against the 5’ UTR and part of coding region including ATG. The 5 bp mismatch MO1 as a negative control for MO (i.e. puf-A 5mmMO1) was 5’-AATGAGCCATGTGTGACTGACAAACA-3’. Embryos positioned in an agarose injection chamber were injected with 5–10 ng of MO in 4.6 nl using a Narishige micromanipulator and needle holder (Narishige, Tokyo, Japan). For the experiment, eye size was determined by photographing lateral views of anesthetized larvae and was normalized to the average eye size of age-matched WT fish.

In vitro transcription/translation

An in vitro transcription/translation assay was carried out with the TNT Quick coupled reticulocyte lysate system together with the Transcend™ biotinylated lysine-tRNA (Promega, Madison, WI, USA) and then purified through immobilization on streptavidin magnetic beads (Promega) with 5 times PBS wash. Afterwards, the purified biotinylated puf-A were mixed with 10μg mRNA mixtures from embryos and ovaries. After formaldehyde fix (final 1% concentration), glycine treatment (final 125mM concentration) and 5 times PBS wash, the residual RNA pulled down by biotinylated puf-A was amplified by using Full Spectrum Complete Transcriprome RNA Amplification kit (System Biosciences, Mountain View, CA, USA) as described in the manufacturer’s instructions. The PCR products were cloned into pGEM-T easy vectors (Promega) and sequenced.

Rescue experiment of morphants

Rescue experiments were performed by injecting the synthesized capped puf-A RNA with puf-A-MO1. The capped puf-A RNA that did not contain a 5’ UTR region was prepared from a Bluescript SK-plasmid after BamHI digestion using the mMessage mMachine kit (Ambion, Austin, TX, USA). For the rescue experiments, 200 pg of capped puf-A RNA was microinjected with puf-A-MO1 into zebrafish embryos, and the eye size was measured at 2 dpf.

Paraffin embedding and sectioning of mouse

Eyes from mouse were collected and placed in 4% paraformaldehyde. Tissue sections (3 μm thick) from paraffin-embedded tissue blocks were placed on charged slides, deparaffinized in xylene, rehydrated through graded alcohol solutions and stained with hematoxylin and eosin (H&E).

Puf-A silencing in the zebrafish PGCs

To silence the puf-A expression in zebrafish with small interfering RNA (siRNA), the pcDNA6.2-GW/EmGFPMiR (Block-iT Pol II miR RNAi Expression Vector Kits, Invitrogen) was used to construct the puf-A siRNA plasmid according to the user manual. The region of nt1076 to 1090 for zebrafish puf-A was chosen for the engineered puf-A siRNA plasmid. The commercial pC6 6.2-GW/EmGFPMiR-neg control plasmid served as the “control siRNA”. In addition, the 3’ UTR fragment of nanos prepared from the PCR product of pGEM-T Easy-nanos plasmid (a gift from Dr. Bon-chu Chung) was subcloned into Xhol site of the puf-A siRNA and control siRNA plasmids, separately, to generate the plasmid with either puf-A siRNA or control siRNA containing nanos 3’ UTR. Therefore, there are four siRNA plasmids: puf-A siRNA with or without nanos 3’ UTR and their two respective control plasmids without puf-A.

Then, the PCR products generated from these four siRNA plasmids using forward primer (ACAAGTTTTGACACAAAAAGCAGGCT) and reverse primer (ACACCTTTGTACACGAAACGCTTGG) were subcloned into pGEM-T Easy vector using a TA cloning kit (Promega). Afterwards, using the mMessage MMachine kit (Ambion), the RNAs with puf-A containing either or no nanos 3’ UTR and their controls without puf-A were prepared separately. Finally, 100–200 pg of these puf-A siRNAs and control siRNAs (with or without nanos 3’ UTR) were microinjected into one-cell stage of zebrafish embryos, and the phenotypes and vasa expression were observed under microscope.

Identification of the mRNAs that are targets of puf-A in zebrafish

Briefly, biotinylated puf-A was prepared from in vitro transcription/translation kit using the TNT Quick coupled reticulocyte lysate system together with the Transcend™ biotinylated lysine-tRNA (Promega, Madison, WI, USA) and then purified through immobilization on streptavidin magnetic beads (Promega) with 5 times PBS wash. Afterwards, the purified biotinylated puf-A were mixed with 10μg mRNA mixtures from embryos and ovaries. After formaldehyde fix (final 1% concentration), glycine treatment (final 125mM concentration) and 5 times PBS wash, the residual RNA pulled down by biotinylated puf-A was amplified by using Full Spectrum Complete Transcriprome RNA Amplification kit (System Biosciences, Mountain View, CA, USA) as described in the manufacturer’s instructions. The PCR products were cloned into pGEM-T easy vectors (Promega) and sequenced.

Supporting Information

Data S1

Found at: doi:10.1371/journal.pone.0004980.s001 (0.5 MB DOC)
**Figure S1** cDNA nucleotide sequence of the zebrafish puf-A gene. The full-length sequence of zebrafish puf-A cDNA was identified using 5′- and 3′-RACE. The 5′-untranslated region (UTR) and 3′-UTR are shown in lowercase letters and the coding region (nucleotides 45–1924) in uppercase letters. The stop codon is marked with an *. The deduced amino acid sequence (629 amino acids) is shown below the nucleotide sequence. At residues 558–562, the sequence is “Glu-Arg-Phc-Seq-Arg” in bold letters. Blue arrow indicates the location of MO1 target site; black arrow refers to the MO2 target site.

Found at: doi:10.1371/journal.pone.0004980.s002 (8.20 MB TIF)

**Figure S2** Multiple sequence alignments of Puf proteins. Sequences of 14 Puf proteins of human (Hs), mouse (Mm), and zebrafish (Dr) were aligned by CLUSTAL X as described in Methods. Protein names are shown at the left of the alignment data, and the residue numbers are shown at the right side. The quality scores of alignment are represented as column graph under the ruler to indicate the level of similarity among these proteins. The color scheme for the consensus residues was applied the default settings.

Found at: doi:10.1371/journal.pone.0004980.s003 (9.43 MB TIF)

**Figure S3** The in vitro and in vivo analyses for the specificity of puf-A MO1. (A) Various amounts of puf-A-5mmMO1 (5 bp mismatch control: 0, 2, and 200 nM) were added to the in vitro transcription/translation reactions for puf-A. One microliter of the reaction mixture was separated on 10% SDS/PAGE, blotted and probed with puf-A siRNA and in the last line, ppuf-A siRNA negative control. In Western blot, the siRNA was the control siRNA (pdsRNA 6.2-GW/EmGFP-miR -neg control plasmid) as negative control. In the middle line, ppuf-A siRNA and in the last line, ppuf-A siRNA containing nanos 3′-UTR were used to suppress the puf-A expression. Upper panel showed the Western blot after reaction with anti-Flag antibodies, while lower panel showed Western blot for β-actin as internal control.

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**Figure S4** The puf-A siRNA suppressed specifically the zebrafish puf-A expression in 3T3 cell line. The 3T3 cell line was co-transfected with pFlag-puf-A and different siRNAs. In the first line of Western blot, the siRNA was the control siRNA (pdsRNA 6.2-GW/EmGFP-miR -neg control plasmid) as negative control. In the middle line, ppuf-A siRNA and in the last line, ppuf-A siRNA containing nanos 3′-UTR were used to suppress the puf-A expression. Upper panel showed the Western blot after reaction with anti-Flag antibodies, while lower panel showed Western blot for β-actin as internal control.

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**Author Contributions**

Conceived and designed the experiments: MWK WHL JY. Performed the experiments: MWK SHW JCC LJJ HHIL. Analyzed the data: MWK SHW JCC ALTY WHL JY. Contributed reagents/materials/analysis tools: MWK SHW JCC LJJ HHIL. Wrote the paper: MWK SHW JCC ALTY WHL JY.

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