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RESEARCH ARTICLE

Translation repression by maternal RNA binding protein Zar1 is essential for early oogenesis in zebrafish

Liyun Miao[1,2,3,4], Yue Yuan[1,3,4], Feng Cheng[1,*], Junshun Fang[1], Fang Zhou[1], Weirui Ma[1,‡], Yan Jiang[5], Xiahe Huang[1], Yingchun Wang[1], Lingjuan Shan[1], Dahua Chen[6] and Jian Zhang[1,3,4,§]

ABSTRACT

A large amount of maternal RNA is deposited in oocytes and is reserved for later development. Control of maternal RNA translation during oocyte maturation has been extensively investigated and its regulatory mechanisms are well documented. However, translational regulation of maternal RNA in early oogenesis is largely unexplored. In this study, we generated zebrafish *zar1* mutants that result in early oocyte apoptosis and fully penetrant male development. Loss of p53 suppresses the apoptosis in *zar1* mutants and restores oocyte development. *zar1* immature ovaries show upregulation of proteins implicated in endoplasmic reticulum (ER) stress and the unfolded protein response (UPR). More importantly, loss of Zar1 causes marked upregulation of zona pellucida (ZP) family proteins, while overexpression of ZP proteins in oocytes causes upregulation of stress-related *activating transcription factor 3* (*atf3*), arguing that tightly controlled translation of ZP proteins is essential for ER homeostasis during early oogenesis. Furthermore, Zar1 binds to ZP gene mRNAs and represses their translation. Together, our results indicate that regulation of translational repression and de-repression are essential for precisely controlling protein expression during early oogenesis.

KEY WORDS: Zar1, Zona pellucida, Oogenesis, p53, Zebrafish, Translational control, ER stress, Unfolded protein response, Apoptosis, Sex differentiation

INTRODUCTION

In contrast to spermatogenesis, oogenesis is associated with increasing cell volume and accumulating maternal molecules such as RNAs and proteins, which are essential for oogenesis itself and early embryogenesis. Maternal gene expression is temporally and spatially controlled (Becalska and Gavis, 2009; Curtis et al., 1995; Evans and Hunter, 2005; Li et al., 2010; Richter and Lasko, 2011). In most species, maternal RNAs play central roles during early embryogenesis owing to the absence of zygotic transcription at initial embryonic stages. To accomplish their functions during early embryogenesis, some of the maternal RNAs are extensively regulated post-transcriptionally during oocyte maturation and early embryogenesis. For example, maternal mRNA translation can be regulated by cytoplasmic polyadenylation (Richter, 2007; Richter and Lasko, 2011). Maternal RNA stability can also be regulated by micro RNAs, 3′ untranslated region (UTR) length and even codon usage (Barckmann and Simonelig, 2013; Mishima and Tomari, 2016; Norbury, 2013), which can further temporally control protein expression.

In addition to cytoplasmic components, oocytes also maintain complex cell surface structures including the glycoprotein-rich zona pellucida (ZP), which surrounds oocyte plasma membrane and is vital for oocyte integrity. At fertilization in mouse, ZP proteins (such as ZP2 and ZP3) are required to bind to spermatozoa and to initiate acrosome reaction (Conner et al., 2005). FIGα activates transcription of the mouse genes *Zp1*, *Zp2* or *Zp3* through an E-box in their promoters (Liang et al., 1997; Soyal et al., 2000). In zebrafish, the vitelline envelope (equivalent to mammalian ZP) starts to form in stage IB follicles. ZP glycoproteins are encoded by multicopy ZP genes whose expression is tightly regulated at transcriptional and translational levels, yet their regulators remain unclear (Mold et al., 2009).

Zebrafish oocyte development is closely associated with sex determination (Liew and Orban, 2013). Adult zebrafish possess only testes or ovaries, but juveniles are initially bipotential. Undifferentiated gonads are ovary-like with early stage oocytes (Maack and Segner, 2003; Takahashi, 1977). These oocytes grow to full-size in females, but degenerate and are replaced by spermatogenesis in males (Uchida et al., 2002). Zebrafish without germ cells develop into male adults, indicating germ cells are essential for female development (Campbell et al., 2015; Draper et al., 2007; Houwing et al., 2007; Siegfried and Nüsslein-Volhard, 2008; Slanchev et al., 2005). When oocytes are depleted in juveniles, zebrafish also develop into males (Dranow et al., 2013; Hartung et al., 2014; Houwing et al., 2008; Rodríguez-Marí et al., 2010, 2011; Shive et al., 2010; White et al., 2011). Attenuation of apoptosis pathways by p53 deficiency restores ovarian development in otherwise all male *fancl* and *brca2* mutants (Rodríguez-Marí et al., 2010, 2011; Shive et al., 2010). In addition, germ cells and oocytes are also required to maintain female phenotype in adult zebrafish (Dranow et al., 2016, 2013). Separately, activation of the estrogen pathway suppresses apoptosis in mouse and fish ovaries (Janz and Van Der Kraak, 1997; Kim et al., 2009). Estrogen is also essential for zebrafish female development (Dranow et al., 2016; Guiguen et al., 2010). Furthermore, juvenile fish exposed to estrogen are likely to become females (Larsen et al., 2009; Örn et al., 2003).

Maternal genes have been studied in a number of model organisms. *Zar1* (*zygotic arrest 1*) was initially identified as a maternal gene in mouse. *Zar1* null female mice generate fully grown oocytes and the eggs can be fertilized, but the resulting embryos fail to develop beyond the 2-cell stage, suggesting that *Zar1* is required for very early embryogenesis (Wu et al., 2003a). The molecular

[1]State Key Laboratory of Molecular Developmental Biology, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China. [2]University of Chinese Academy of Sciences, Beijing 100101, China. [3]Center for Life Sciences; School of Life Sciences, Yunnan University, Kunming 650500, China. [4]State Key Laboratory for Conservation and Utilization of Bio-Resources, Kunming 650500, China. [5]Jilin University, Changchun 130012, China. [6]Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China.
*Present address: University of California, San Francisco, CA 94143, USA.
‡Present address: Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA.

§Author for correspondence (jianzhang84@genetics.ac.cn)

J.Z., 0000-0002-5867-9808

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regulatory mechanism of ZAR1 in mouse oocytes is largely unknown, but *in vitro* results suggest that its *Xenopus* homolog may function as a RNA binding protein to regulate RNA translation (Yamamoto et al., 2013). ZAR1 proteins are conserved in vertebrates and contain an atypical plant homeodomain (PHD) zinc finger (Znf) domain in the C-terminus (Wu et al., 2003b). Here, we show that Zar1 functions by repressing ZP gene translation, thereby preventing overload of ZP proteins in early oocytes. Our results indicate that Zar1 is essential for early oogenesis.

RESULTS

Zebrafish *zar1* null mutants exclusively develop into adult males

To study the function of Zar1, we first examined *zar1* mRNA expression. Zebrafish *zar1* is expressed in ovaries but absent in testes (Fig. 1A). *zar1* mRNA is highly expressed in primary growth (stage I) and cortical alveolus (stage II) oocytes (oocyte staging according to Selman et al., 1993) (Fig. 1A). Zar1 protein was also detected in Stage I and II oocytes but not in Stage III oocytes (Fig. 1B). We generated several *zar1* mutant lines using TALEN (Fig. 1C-E) and CRISPR/Cas9 (Fig. S1) technology (Chang et al., 2013; Huang et al., 2011; Li et al., 2011). We focused on the $zar1^{gd5}$ (Fig. 1E) and $zar1^{gd6}$ mutants (Fig. S1C). Immunoblotting results indicate that Zar1 protein is absent in both $zar1^{gd5/gd5}$ (Fig. 1F) and $zar1^{gd6/gd6}$ homozygotes (Fig. S1D).

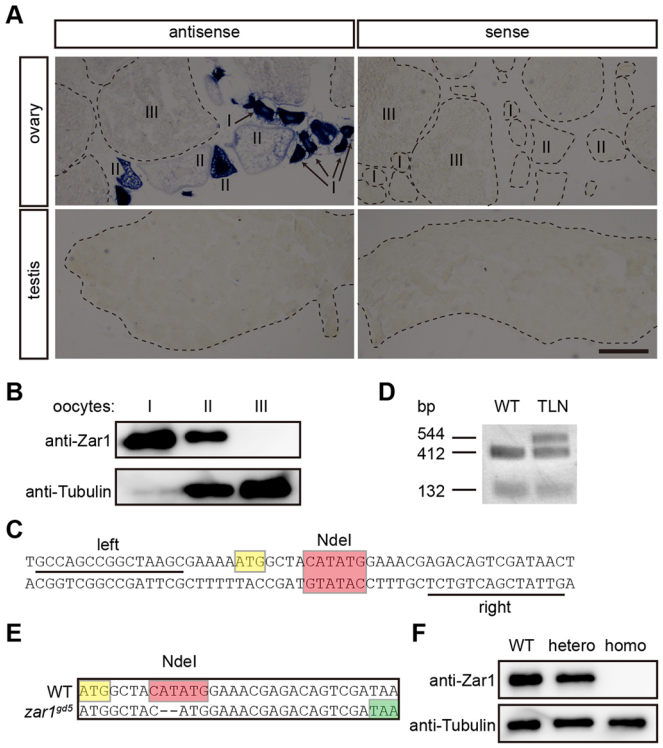

Fig. 1. Generation of a *zar1* mutant with TALENs in zebrafish. (A) *In situ* hybridization on cryosections of wild-type gonads with *zar1* antisense probe and sense probe. Scale bar: 200 µm. (B) Immunoblotting to detect Zar1 protein in stage I, stage II and stage III oocytes. Ten oocytes were lysed for each stage. (C) The TALEN sequences for *zar1* mutant generation. (D) Digestion of PCR products from wild-type (WT) and *zar1* TALEN mRNA injected embryos (TLN) with *Nde*I restriction enzyme. (E) DNA sequences of the $zar1^{gd5}$ mutant fish line. A premature stop codon was generated. (F) Western blot of Zar1 in gonads from WT, heterozygotes (hetero) and homozygotes (homo) at 25 dpf. DNA sequences highlighted in yellow are the start codon; red, *Nde*I recognition sites; green, premature stop codon.

We intercrossed $zar1^{gd5/+}$ heterozygotes to obtain $zar1^{gd5/gd5}$ homozygotes. No morphological difference was observed between $zar1^{gd5/gd5}$ homozygotes and siblings during embryonic and juvenile stages (data not shown). To our surprise, we could not detect adult female *zar1* homozygotes based on external morphology. In contrast, sex ratios in sibling wild-type and heterozygotes were normal (Fig. 2A). Furthermore, histological analysis of adult gonads indicated no ovary in *zar1* homozygotes (Fig. 2B-D). It should be noted that all $zar1^{gd5/gd5}$ homozygous males were fertile. There is no histological difference in testes between the homozygotes and siblings (Fig. 2C,D). Analysis of $zar1^{gd6/gd6}$ homozygotes further suggests that loss of Zar1 causes the all-male phenotype (Fig. S1E). Results in this study were generated using $zar1^{gd5}$, unless otherwise indicated.

To confirm that the all-male phenotype was due to Zar1 deficiency, we used an oocyte-specific *zp3b* (*zpc*) promoter (Onichtchouk et al., 2003) to drive *zar1* transgene expression in oocytes. *EGFP* driven by the heart-specific *cmlc2* promoter was inserted into the transgenic construct *Tg(zp3b:zar1,cmlc2:EGFP)* to facilitate screening of transgenic zebrafish (Fig. 2E). EGFP-positive embryos, indicating embryos carrying *Tg(zp3b:zar1,cmlc2:EGFP)*, were selected at 48-72 h post fertilization (Fig. S2A-A′). EGFP-positive *zar1* homozygous males were crossed with EGFP-positive *zar1* heterozygous females. As expected, all EGFP-negative $zar1^{-/-}$ homozygotes were male. In contrast, females were recovered from EGFP-positive $zar1^{-/-}$ homozygotes (Fig. 2F). Accordingly Zar1 expression, albeit at relatively low level, was detected in EGFP positive $zar1^{-/-}$ homozygotes (Fig. S2B). Ovaries of the rescued *zar1* homozygous females were morphologically similar to those of the heterozygous females (Fig. 2G,H). Together, these results confirm that the all-male phenotype results from Zar1 deficiency.

Loss of Zar1 results in female-to-male sex reversal

Two possibilities could account for the all-male phenotype of *zar1* mutants: *zar1* homozygous females might have died during development or *zar1* homozygous females could have reversed to males. To resolve this issue, we crossed *zar1* heterozygous females with *zar1* homozygous males. If the all-male phenotype is caused by female lethality, the number of homozygotes should be about 50% of the number of heterozygotes, assuming the allele segregates in a Mendelian manner. If it is caused by sex-reversal, the numbers of homozygotes and heterozygotes should be similar. The observed survival rate was 91.6% (174 out of 190), indicating that the all-male phenotype in *zar1* mutants is not caused by female lethality. The number of *zar1* homozygotes was similar to that of *zar1* heterozygotes (Fig. 3A). Among heterozygotes, about 50% were female. As expected, no females were observed among *zar1* homozygotes (Fig. 3A). These results indicate that the all-male phenotype in *zar1* mutants is caused by female-to-male sex reversal.

To better understand the sex reversal process in *zar1* homozygotes, we examined their gonads at different developmental stages. Undifferentiated gonads in *zar1* homozygotes resembled those in control siblings (heterozygotes and wild type) at 22 days post fertilization (dpf) (Fig. 3B,C). Only stage I oocytes were detected in these undifferentiated gonads. At 33 dpf, sex is determined, but gonads are immature (Rodríguez-Marí et al., 2010). At 33 dpf, similar to the control gonads, *zar1* mutant gonads were either immature ovaries (Fig. 3D,E) or immature testes (Fig. 3F,G). From 22 dpf to 33 dpf, oocyte volume increased similarly both in *zar1* mutants and in control siblings. Most oocytes in *zar1* homozygotes resembled the control oocytes. However aberrant vesicles started to appear in *zar1* mutant oocytes (Fig. 3E, arrows), suggesting that

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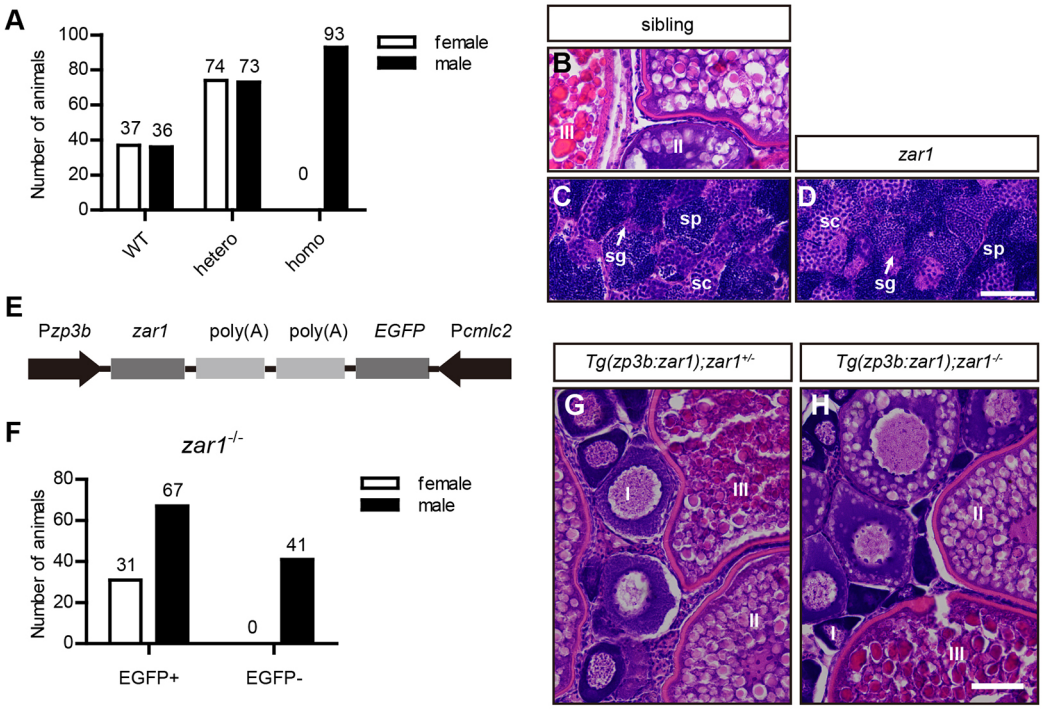

Fig. 2. Loss of Zar1 causes all-male phenotype in zebrafish. (A) Analysis of genders of *zar1* homozygotes (homo), heterozygotes (hetero) and wild-type siblings. (B-D) Histological analysis of gonads of *zar1* homozygotes and sibling controls by H&E staining. About half of the gonads (20/37) from wild-type and *zar1* heterozygotes were ovaries (B) while the other half (17/37) were testes (C). All the 13 gonads from *zar1* homozygotes were testes (D). (E) Schematic diagram of the *Tg(zp3b:zar1)* transgenic construct. *egfp* coding sequences were placed under control of the *cmlc2* promoter to help visually identify transgenic fish. (F) Gender analysis of $zar1^{-/-}$ homozygotes with or without EGFP signal, indicating *Tg(zp3b:zar1)* transgene. Females were recovered only from $zar1^{-/-}$ homozygotes with the *Tg(zp3b:zar1)* transgene. (G,H) H&E staining of ovaries of $zar1^{+/-}$ heterozygotes and $zar1^{-/-}$ homozygotes on the *Tg(zp3b:zar1)* transgenic backgrounds. Ovaries from $zar1^{-/-}$ homozygotes rescued with the *Tg(zp3b:zar1)* transgene are normal histologically. *Tg(zp3b:zar1)*, *Tg(zp3b:zar1, cmlc2:EGFP)*; sg, spermatogonia; sc, spermatocytes; sp, sperm; I,II,III, oocyte stage I, II or III. Scale bars: 40 µm.

ovarian development defects occurred as early as 33 dpf. At 39 dpf, stage II oocytes appeared in *zar1* heterozygous ovaries and wild-type ovaries (Fig. 3H). In contrast, oocyte growth appeared arrested in the mutants (Fig. 3I) with obvious aberrant vesicles (Fig. 3I, arrows). The vesicles resemble cortical granules (CGs). To identify the nature of the vesicles, we used *Maclura pomifera* agglutinin (MPA), which recognizes CG contents (Becker and Hart, 1999). The aberrant vesicles in the mutant oocytes were indeed MPA positive (Fig. 3L), indicating they are CGs. The CGs in the mutants are larger than those in sibling controls. In addition, the CGs are found in smaller oocytes in the mutants, suggesting that they accumulate prematurely. We also noticed that CG number in mutant oocytes is far less than that in sibling oocytes (Fig. 3M).

At 50 dpf, *zar1* mutant ovaries became ovotestis, containing both oocytes and spermatocytes (Fig. 3N,O). At 60 dpf, when oocytes reached stage III in control ovaries, spermatogenesis dominated in the *zar1* mutant ovotestis with a few residual stage I oocytes (Fig. 3P,Q). Testis development in *zar1* mutants resembled that in sibling controls (Fig. 3F,G,J,K, and Fig. S3). The percentage of immature ovaries at 33-41 dpf in *zar1* homozygotes (54.5%) is similar to that in *zar1* heterozygous controls (56.9%) (Fig. S4). These results confirm that the all-male phenotype in *zar1* mutants is due to female-to-male sex reversal.

Apoptosis in *zar1* mutants is mediated through the p53 pathway

We hypothesized that degeneration of *zar1* homozygous oocytes is mediated by apoptosis. To test this, we used the TUNEL assay to examine apoptosis in immature ovaries at 37-40 dpf. Few apoptotic cells could be seen in immature ovaries of *zar1* heterozygotes but apoptosis was readily detected in *zar1* homozygous ovaries (Fig. 4A-C). p53-mediated germ cell apoptosis causes gonad transformation in several zebrafish mutants (Rodríguez-Marí et al., 2010, 2011; Shive et al., 2010). To test whether apoptosis in *zar1* homozygotes is mediated by p53 (also known as Tp53), we crossed $zar1^{-/-}$ males with $p53^{M214K/M214K}$ ($p53^{-/-}$) females and then crossed $p53^{+/-};zar1^{+/-}$ F1 progenies. Of the $p53^{-/-};zar1^{-/-}$ double mutant offspring, 8 out of 14 were female while all the other 28 mutant siblings ($p53^{+/-};zar1^{-/-}$ or $p53^{+/+};zar1^{-/-}$) were male (Fig. 4D), suggesting oogenesis arrest in $zar1^{-/-}$ mutants is suppressed by p53 deficiency. The ovaries of the $p53^{-/-};zar1^{-/-}$ double mutant were indistinguishable from control ovaries histologically (Fig. 4E,F), but the chorions of $p53^{-/-};zar1^{-/-}$ double mutant eggs failed to lift upon activation (Fig. S5). As expected, apoptosis was also blocked in $p53^{-/-};zar1^{-/-}$ double mutant ovaries (Fig. 4G-J). Thus, p53-mediated apoptosis contributes to oocyte degeneration in the $zar1^{-/-}$ mutants.

Estrogen treatment restores oogenesis in *zar1* mutants

Estrogen is required for zebrafish female development (Dranow et al., 2016; Guiguen et al., 2010). Furthermore, juvenile fish exposed to 17α-ethinylestradiol (EE2), a synthetic estrogen agonist, usually develop into females (Örn et al., 2003). To explore whether estrogen can affect *zar1* mutant ovarian development, we used EE2 (10 ng/l, 20-60 dpf) to treat *zar1* homozygous juveniles. Females were recovered from EE2-treated *zar1* homozygous mutants, although the percentage of female in *zar1* homozygotes is not as high as that in *zar1* heterozygotes (Fig. 5A). No obvious

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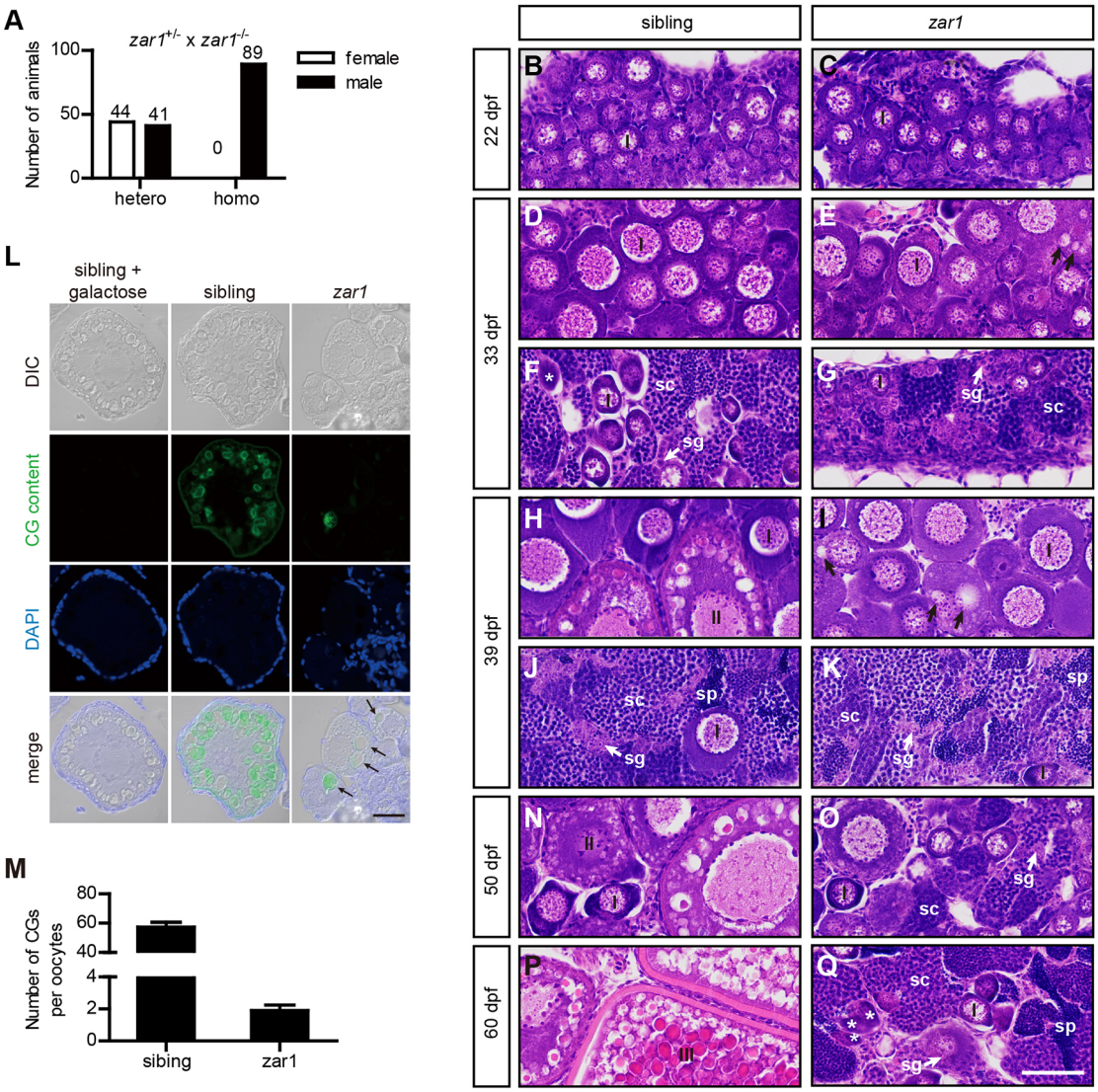

Fig. 3. Zar1 deficiency causing all-male phenotype is due to female-to-male sex reversal. (A) Analysis of genders of *zar1* homozygotes (homo) and heterozygous siblings (hetero). *zar1*$^{+/-}$ heterozygous females were crossed with *zar1*$^{-/-}$ homozygous males and genders of their progenies were analyzed. (B-K,N-Q) Gonad development of *zar1* homozygotes and control siblings analyzed by H&E staining. At 22 dpf, zebrafish gonads are undifferentiated. Gonads of *zar1* homozygotes and control siblings are indistinguishable histologically (B,C). At 33 dpf, WT gonads differentiate into immature ovaries and immature testes. Minor developmental abnormalities are observed in *zar1* homozygotes. Oocytes in *zar1* homozygotes are similar to those in WT in size and morphology, but aberrant vesicles (arrow indicated) are observed in ooplasm of a few *zar1* homozygous oocytes (D,E). Testis development in *zar1* homozygotes is normal compared with that in control siblings (F,G). At 39 dpf, ovarian developmental abnormality in *zar1* homozygotes becomes more pronounced (H,I), while testis development in *zar1* homozygotes is similar to the controls (J,K). Immature ovaries in control siblings contain stage I and stage II oocytes (H) while oocytes in *zar1* homozygotes resemble stage I oocytes, indicating oogenesis arrest in *zar1* homozygotes. In addition, aberrant vesicles (arrow) in *zar1* homozygotes increase significantly in size and number. (L) MPA (*Maclura pomifera* agglutinin) staining of ovary sections. Juveniles at 37-40 dpf fixed with 4% PFA were embedded in paraffin and sections stained with MPA; 0.5 M D-galactose inhibited MPA staining. MPA specifically stains CGs in control siblings. The aberrant vesicles in *zar1* mutant ovaries are MPA positive (arrows). (M) Comparison of CG numbers (mean±s.e.m.) between *zar1* mutants (n=11) and siblings (n=11). At 50 dpf, stage II oocytes are observed in heterozygous and wild-type ovaries (N), but large numbers of oocytes underwent atresia in *zar1* homozygotes, and spermatogonia and spermatocytes appear among oocytes, indicating transitional ovaries (ovotestis) (O). At 60 dpf, stage III oocytes were seen in the control ovaries (P); in contrast, very few oocytes remain in ovotestis of *zar1* mutants and spermatogenesis has progessed further (Q). At 22 dpf, six juveniles were analyzed for each genotype. At 33 dpf, 39 dpf, 50 dpf and 60 dpf, 20 juveniles per stage were analyzed for each genotype. Stars indicate degenerating oocytes. sg, spermatogonia; sc, spermatocytes; sp, sperm. Scale bar: 40 µm.

histological difference could be detected between EE2-treated *zar1* mutant ovaries and control ovaries (Fig. 5B,C), yet the chorions of eggs from EE2-treated *zar1* mutants failed to lift properly upon activation (Fig. S6).

Estrogen treatment has been shown to suppress apoptosis in mouse and fish ovaries (Janz and Van Der Kraak, 1997; Kim et al., 2009). Similarly, in human breast cancer cells, estrogen downregulates the P53 target genes, including *ATF3*, *BGT2* and *TRAF4*, which are involved in P53-mediated apoptosis (Bailey et al., 2012). We found that at 33-34 dpf, *btg2* mRNA was downregulated while *traf4a* mRNA had similar levels of expression in *zar1* mutant ovaries and control sibling ovaries (Fig. 5D). Only *atf3* was upregulated in *zar1* mutant ovaries (Fig. 5E). Moreover, EE2 treatment was able to repress the *atf3* upregulation in *zar1* mutant ovaries (Fig. 5E). These results suggest that estrogen functions through suppression of stress-related genes to restore oogenesis.

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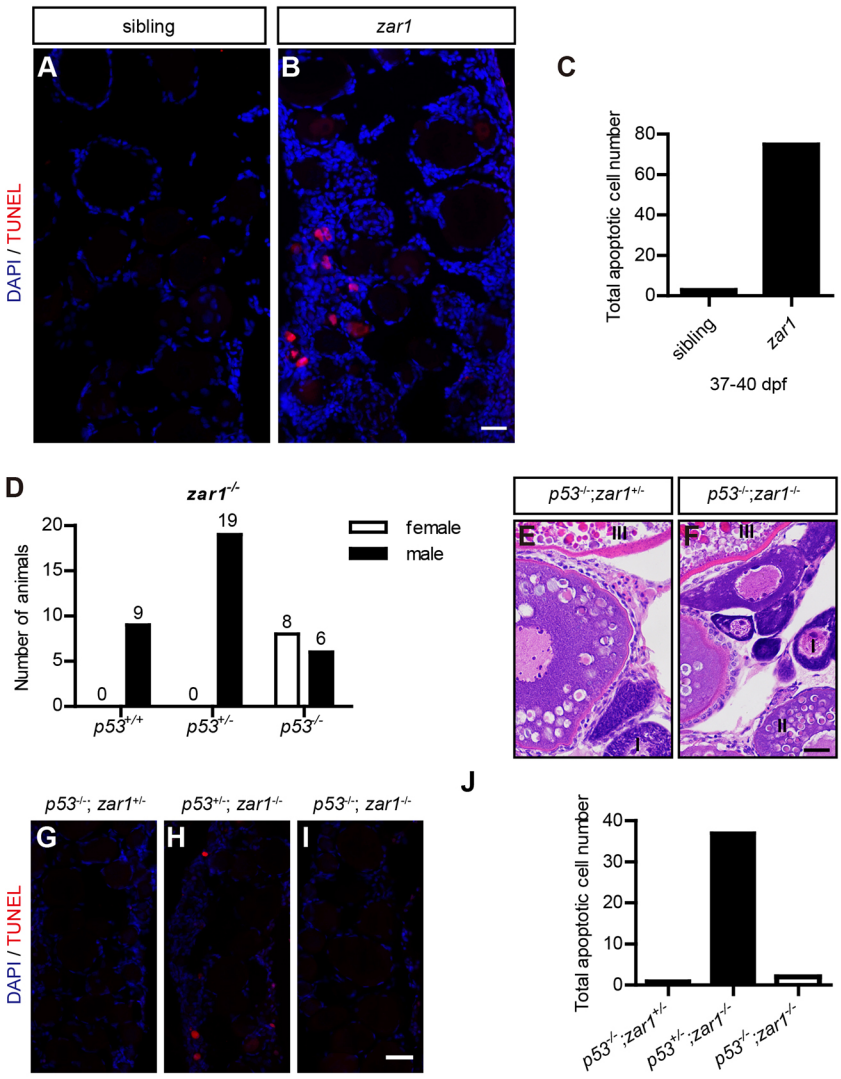

Fig. 4. Zar1 deficiency causing all-male phenotype is due to p53-mediated apoptosis. (A-C) TUNEL staining of ovary sections of *zar1* homozygotes and control siblings at 37-40 dpf. Obvious apoptotic cells are observed in immature ovaries in *zar1* homozygotes (A), but not in sibling controls (B). Quantification of apoptotic cells is shown in C; 18 sections from 6 juveniles were counted for each genotype. (D) Gender analysis of $zar1^{-/-}$ homozygotes on different *p53* genotype backgrounds ($p53^{+/+}$, $p53^{+/-}$ and $p53^{-/-}$). Females are only observed in $p53^{-/-};zar1^{-/-}$ double homozygous mutants. (E,F) H&E staining of sections of $p53^{-/-};zar1^{+/-}$ and $p53^{-/-};zar1^{-/-}$ adult ovaries. $p53^{-/-};zar1^{-/-}$ ovaries are morphologically normal. (G-J) TUNEL staining of ovary sections of $p53^{-/-};zar1^{-/-}$ ovaries at 37-40 dpf with $p53^{-/-};zar1^{+/-}$ and $p53^{+/-};zar1^{-/-}$ ovaries as controls. (J) Quantification of apoptotic cells. Nine sections from three juveniles were counted for each genotype. Scale bar: 20 µm.

Loss of Zar1 triggers ER stress and the unfolded protein response (UPR)

The ATF4-ATF3-CHOP cascade has been implicated in ER stress and the UPR (Jiang et al., 2004; Schröder and Kaufman, 2005). ER is involved in protein folding, post-translational modification and secretory activities. ER homeostasis is essential for normal cell functions (Wang and Kaufman, 2012). ER homeostasis can be disrupted by misfolded proteins and abnormally elevated secretory protein synthesis. Under ER stress, cells activate the UPR to alleviate ER burden by reducing protein translation, increasing protein degradation and generating additional chaperones to assist protein folding. When the UPR fails to restore ER homeostasis, cells may undergo apoptosis (Breckenridge et al., 2003; Shore et al., 2011; Szegezdi et al., 2006). The UPR functions through three major pathways, initiated by three ER-localized transmembrane proteins, to restore ER homeostasis. One of them is initiated by protein kinase RNA-like ER kinase (PERK). Activation of PERK contributes to the accumulation of activating transcription factor 4 (ATF4) (Bettigole and Glimcher, 2015; Sano and Reed, 2013), which upregulates ATF3 and CHOP expression. We checked the expression level of the CHOP-encoding gene *ddit3* and found that, like *atf3*, *ddit3* was upregulated in *zar1* mutants (Fig. 6A). These results suggest that loss of Zar1 causes ER stress in ovaries.

The lack of specific antibodies against zebrafish antigens prevents us from using immunological methods to analyze ER stress and the UPR targets at the protein level. To further study cellular stress in *zar1* mutant ovaries, we quantitatively compared proteomes of *zar1* homozygous ovaries with that of *zar1* heterozygous ones using isobaric tags for relative and absolute quantitation (iTRAQ) technology. We chose ovaries at 33 dpf based on the following considerations: (1) prior to 33 dpf, ovaries and testes are nearly indistinguishable morphologically; (2) at 33 dpf, oocyte sizes are similar in both groups yet aberrant cortical granules start to appear in the mutants, indicating initial oocyte defects. More than 5300 proteins were identified in ovaries from the two genotypes (Table S3), 325 proteins show differential expression ($P<0.05$) (Table S4). A total of 42 proteins were increased or decreased by more than twofold, with five proteins, including Zar1, downregulated and 37 proteins upregulated in homozygous $zar1^{-/-}$ ovaries. We examined seven upregulated proteins and analyzed their mRNA expression (Fig. S7). Five of them were also transcriptionally upregulated. RNA levels of the other two were similar between the two groups, suggesting that protein upregulation occurs at the post-transcriptional level.

Among the 37 upregulated proteins are ER stress- and UPR-related proteins, such as Dnajc3a, Vapb and Pdia4 (Fig. 6B and

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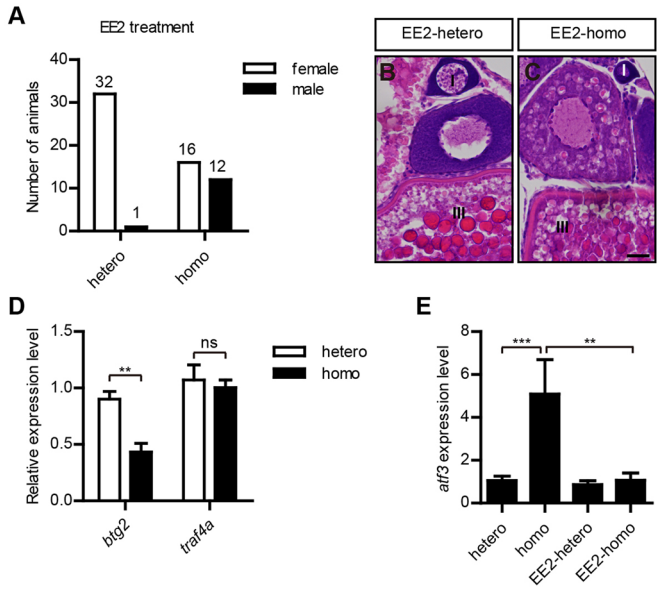

Fig. 5. EE2 treatment restores ovarian development in *zar1* homozygous mutant. (A) Statistics for sex ratio of *zar1* homozygous and heterozygous mutants after EE2 treatment. (B,C) H&E staining of ovarian tissue in EE2-treated *zar1* homozygotes and heterozygotes. Similar to *zar1* heterozygous ovaries, the treated homozygous mutant ovaries possess oocytes at all stages. (D) *btg2* and *traf4a* expression analyzed by qPCR in *zar1* homozygotes (n=6) and heterozygotes (n=6). (E) *atf3* expression analyzed by qPCR in *zar1* homozygotes (n=7) and heterozygotes (n=8) with or without EE2 treatment. Gene expression was normalized to expression of *elongation factor 1 alpha (ef1a)*. hetero, *zar1* heterozygotes; homo, *zar1* homozygotes; EE2-hetero, EE2-treated *zar1* heterozygotes (n=6); EE2-homo, EE2 treated *zar1* homozygotes (n=6). Data are mean±s.e.m. **P<0.01, ***P<0.001; ns, not significant. Scale bar: 20 µm.

Table S1), indicating that ER stress and the UPR occurred. If the PERK-ATF4-ATF3-CHOP UPR cascade is a major contributor for oogenesis arrest, we would expect to restore female development by inhibiting PERK activity. To this end, we used PERK inhibitors (GSK2606414 and ISRIB) to treat *zar1*$^{-/-}$ homozygous juveniles. In both treatments, females were recovered in *zar1* mutants (Fig. 6C,D), suggesting that ER stress and the UPR play an important role in *zar1* oogenesis arrest.

Zar1 regulates translation of ZP proteins

In addition to the three upregulated ER stress-related proteins (Fig. 6A and Table S1), there are another 34 upregulated proteins, among which 23 proteins have been annotated in various databases. Of these, 17 can be classified into two categories (Table S1). Seven proteins are ZP glycoproteins (Fig. 7A), and ten proteins have been implicated in immune regulation (Fig. S8). We speculated that upregulation of ER stress, the UPR and immunity-related proteins are secondary events. We hypothesize that overexpression of ZP glycoproteins in the *zar1* mutants may overwhelm translational and post-translational machineries in early oocytes and cause ER stress and the UPR. To test this hypothesis, we overexpressed Zp3b protein in zebrafish oocytes by injection of *zp3b-flag* mRNA (Fig. 7B). Indeed, *zp3b-flag* injected oocytes showed higher *atf3* expression compared with oocytes injected with control *rfp-flag* mRNA (Fig. 7C), suggesting that ZP overexpression causes the UPR, possibly by overwhelming ER capacity.

We noticed that mRNA levels of the seven ZP genes were not upregulated in *zar1* homozygous mutants, with the *zp2l1* transcript level actually downregulated (Fig. 7D), indicating that upregulation of ZP protein occurs post-transcriptionally. One possible role for Zar1 to fit into this model is to function as a translational repressor. As a first step to test the hypothesis, we examined whether Zar1 protein is associated with ZP mRNAs. We first confirmed that affinity-purified Zar1 antibodies can specifically immunoprecipitate endogenous Zar1 in juvenile ovaries (Fig. 8A). We compared mRNAs precipitated by the antibody between *zar1* mutants and heterozygous siblings. The results indicate that all seven ZP mRNAs are significantly enriched in the Zar1 protein precipitate, whereas control mRNAs (*ef1a* and *gapdh*) recovered similarly between the two lysates (Fig. 8B). Furthermore, in a yeast three-hybrid assay (Bernstein et al., 2002; Chen et al., 2014), Zar1 was shown to activate *lacZ* reporter expression in the presence of either *zp2l1* or *zp3b* RNAs (Fig. 8C,D), suggesting direct protein-RNA interaction. To check whether the Zar1 Znf motif is required for the binding, we mutated all of the eight cysteines in the Znf domain to alanines (Fig. S9). The Zar1 Znf domain mutant (Zar1-mu) failed to activate the reporter, suggesting the Znf domain is required for the binding (Fig. 8C,D). Next, we tested whether Zar1 could repress ZP mRNA translation in zebrafish oocytes. Using a

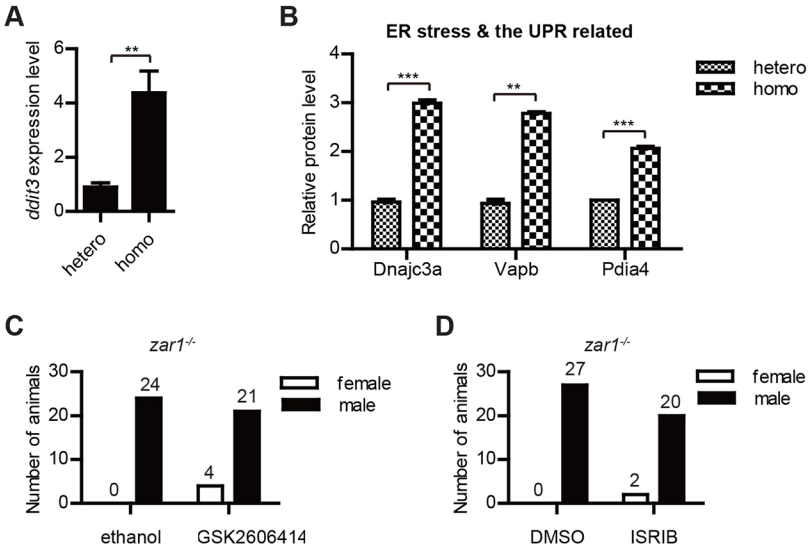

Fig. 6. ER stress and the UPR may be responsible for deprivation of female development of zar1 homozygous mutants. (A) Transcriptional upregulation of *ddit3*, examined with qPCR, in mutant ovaries at 33-34 dpf (hetero, n=6; homo, n=6). Internal control: *ef1a*. (B) ER stress- and UPR-related proteins are upregulated in *zar1* homozygous (homo) ovaries compared with heterozygous (hetero) ovaries at 33-34 dpf as determined by iTRAQ. (C,D) PERK inhibitor treatment restores female development in zar1 homozygous mutants. Juveniles were treated with two PERK inhibitors from 22 dpf to 60 dpf. Final concentration: GSK2606414, 6 nM; ISRIB, 50 nM. **P<0.01, ***P<0.001.

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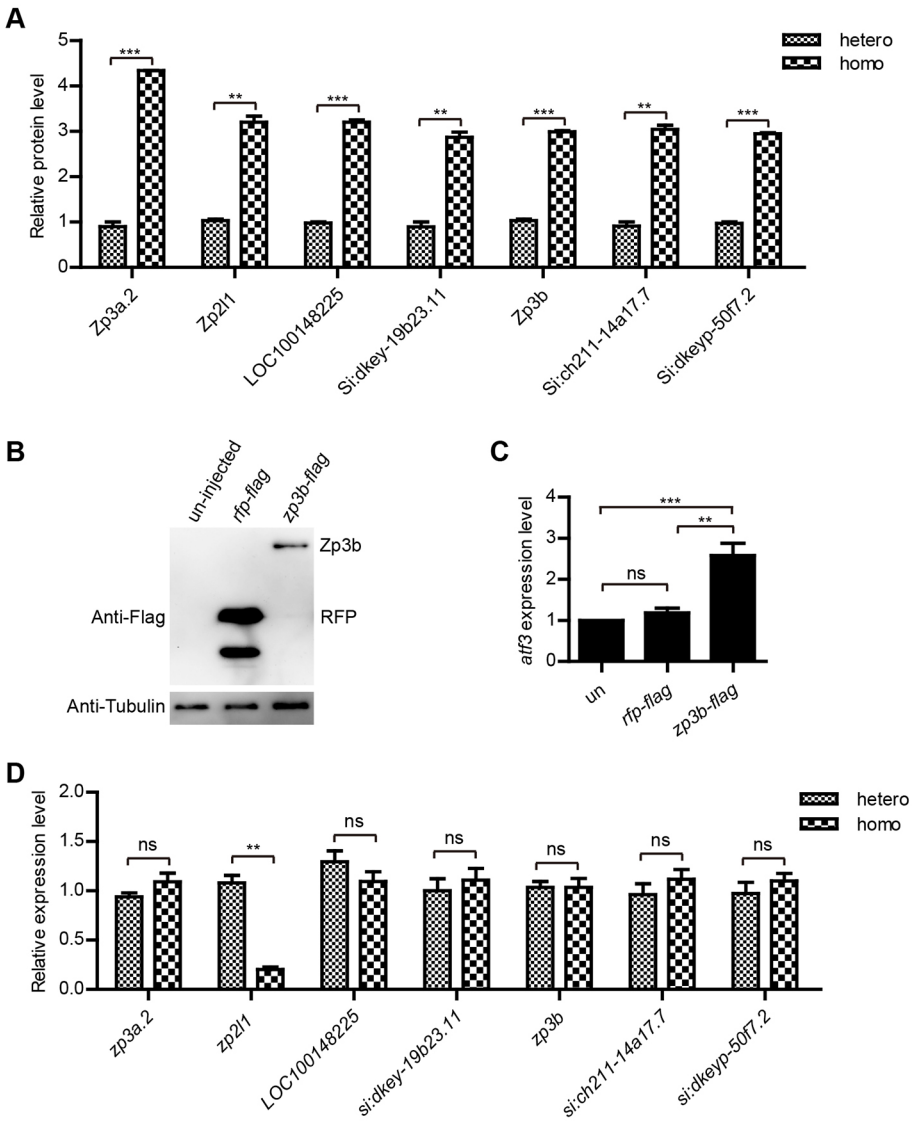

Fig. 7. Translational upregulation of zona pellucida proteins may cause ER stress. (A) ZP proteins are upregulated in *zar1* homozygous (homo) ovaries compared with heterozygous (hetero) ovaries at 33-34 dpf determined by iTRAQ. (B,C) Overexpression of *zp3b* in oocytes causes upregulation of *atf3*. Stage IV oocytes were injected with 600 pg *rfp* or *zp3b* mRNAs. Injected oocytes were cultured in OCM for 4 h and collected for RNA and protein extraction with TRIzol reagent. (B) Western blot to confirm the translation of injected mRNAs. (C) qPCR to detect *atf3* expression in injected oocytes. (D) qPCR to detect ZP gene transcription (hetero, *n*=6; homo, *n*=6). Internal control: *ef1a*. ***P*<0.01, ****P*<0.001; ns, not significant. Data are mean±s.e.m.

ZP-luciferase reporter, we showed that wild-type Zar1, but not Zar1-mu, downregulated ZP gene translation (Fig. 8E-G).

Next, using immunoprecipitation and mass spectrometry (MS), we identified four Zar1-interacting candidates: vitellogenin 4 (Vtg4), poly(A) binding protein cytoplasmic 1-like (Pabpc1l), eukaryotic translation initiation factor 4E transporter (Eif4enif1/4E-T) and cytoplasmic polyadenylation element-binding protein 1 (Cpeb1) (Fig. S10 and Table S5). Results from a STRING database search (Franceschini et al., 2013) indicate that Zar1, Pabpc1l (ePAB), 4E-T and Cpeb1 (CPEB) share an association network (Fig. S10A). ePAB, 4E-T and CPEB are all involved in translational regulation and ovarian development (Gray et al., 2000; Guzeloglu-Kayisli et al., 2012; Kamenska et al., 2014; Kasippillai et al., 2013; Voeltz et al., 2001). The interaction of Zar1 with CPEB and ePAB has been reported in *Xenopus* (Cook and Charlesworth, 2015). We confirmed the interaction between Zar1 and 4E-T both *in vivo* and *in vitro* (Fig. S10B-D). These results suggest that Zar1is likely to interact with translational regulatory factors to function in a common complex that regulates oogenesis.

Taken together, these results suggest that in early oocytes (stage I and II), Zar1 is required to negatively regulate ZP protein expression through direct interactions between ZP mRNA and Zar1. The lack of Zar1 in early oocytes causes excess ZP mRNA translation, and the resulting ZP proteins overwhelm ER capacity and cause the UPR and ultimately, apoptosis (Fig. 8H).

DISCUSSION

Maternal effect genes are relatively less studied compared with somatic genes in vertebrates. Previously, maternal ZAR1 was shown to be essential for early embryogenesis and was proposed to regulate protein translation (Wu et al., 2003a; Yamamoto et al., 2013). In this study, we reveal that zebrafish Zar1 is essential for very early oogenesis. Loss of Zar1 causes early oogenesis arrest and female-to-male sex reversal. Both genetic ablation of *p53* and estrogen treatment restore oogenesis and female fertility. Mechanistically, Zar1 binds to ZP mRNAs and represses their translation, whereas ZP protein overexpression in oocytes may cause ER stress and the UPR. Our results reveal previously unappreciated functions of Zar1 during early oogenesis.

Zar1 proteins are conserved in vertebrates, but their *in vivo* targets and molecular functions were largely unknown prior to this study. There are clear functional differences between zebrafish Zar1 and its mouse homolog. In mouse, *Zar1* mutants develop normally, yet loss of maternal ZAR1 results in two-cell arrest

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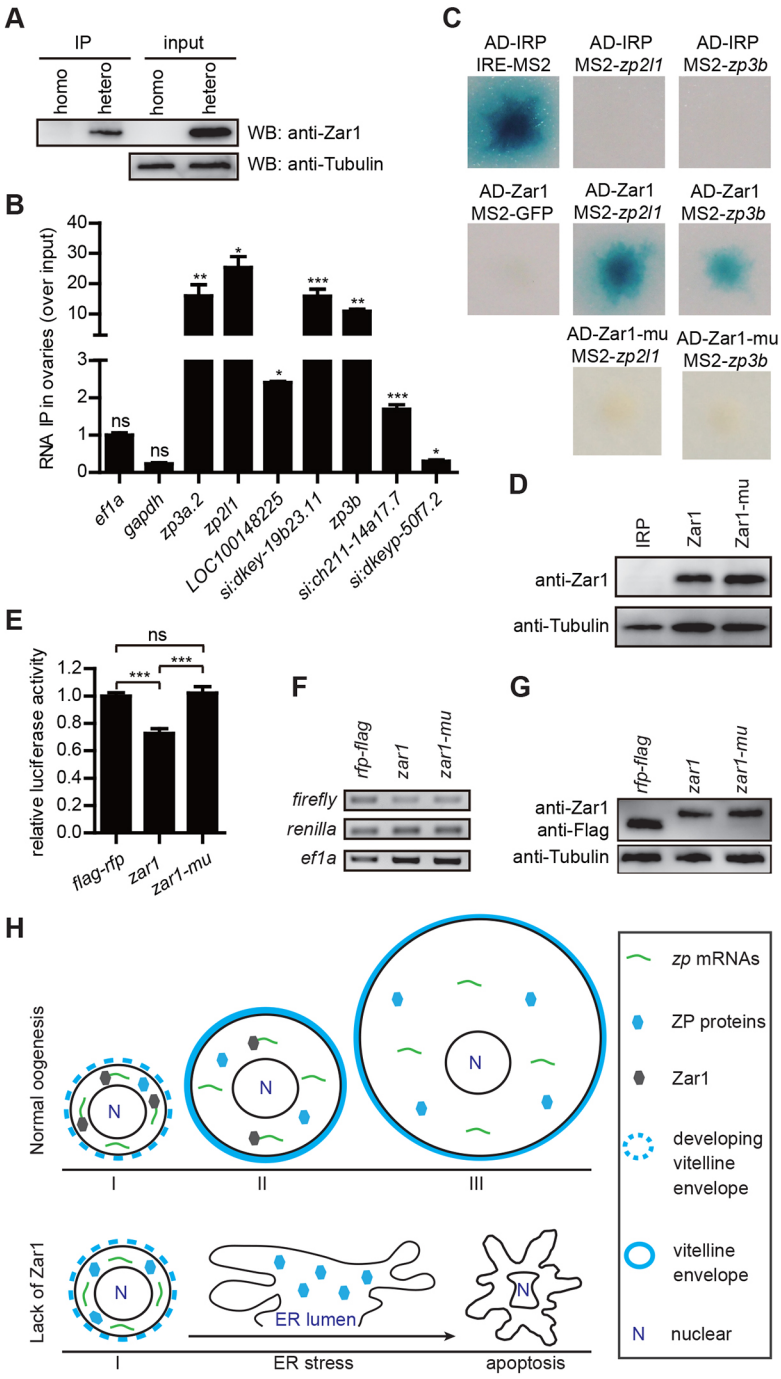

Fig. 8. Zar1 represses translation of ZP proteins in zebrafish oocytes. (A-D) Zar1 binds to ZP gene mRNAs. (A,B) RNA immunoprecipitation with anti-Zar1 antibody from *zar1* homozygous ovaries (homo) and heterozygous ovaries (hetero) at 33-34 dpf. (A) Zar1 protein was precipitated by anti-Zar1 antibody in heterozygous ovaries. (B) qPCR analysis of ZP gene mRNAs immunoprecipitated from heterozygous ovaries with anti-Zar1 antibodies. Relative level of immunoprecipitated ef1a mRNA compared with input *ef1a* mRNA was assigned as 1. (C) Analyzing interaction of zebrafish Zar1 and ZP mRNAs with a yeast three-hybrid system. (D) Western blot analysis indicates expression of Zar1 and Zar1-mu in yeast. (E-G) Zar1 represses translation of Zp proteins in oocytes. (E) Statistics of relative luciferase activity. *zar1* mRNA injection represses Zp3b translation. Mutation of Zar1 Znf domain abolishes its translational repression activity. (F) RT-PCR of injected RNA reporters. (G) Western blot analysis shows expression of RFP-Flag, Zar1 and Zar1-mu. (H) A model of translational regulation of ZP mRNAs. In early oocytes (stage I and II), Zar1 binds to ZP gene mRNAs and negatively regulates their translation. Later, Zar1 proteins are gradually degraded and the translational repression of ZP mRNAs is removed, allowing enough ZP proteins to be used in the vitelline membrane development. The lack of Zar1 in early oocytes causes excess ZP gene translation and the resulting ZP proteins overwhelm the ER, which causes the UPR and apoptosis. **P*<0.05, ***P*<0.01, ****P*<0.001; ns, not significant;

(Wu et al., 2003a). In contrast, zebrafish *zar1* mutants show oogenesis arrest and female-to-male sex reversal. We also noticed that even a very low level of *zar1* transgene expression in the homozygous mutants was sufficient to restore ovarian development. Like the *fancl* mutants, the *zar1* mutants showed p53-mediated apoptosis and female-to-male sex reversal. In both *zar1* and *fancl* mutants, p53 deficiency can restore ovarian development. The $zar1^{-/-};p53^{-/-}$ double mutants also showed a chorion elevation defect. In contrast, it is unknown whether a similar chorion phenotype occurs in $fancl^{-/-};p53^{-/-}$ double homozygous females. Oocyte clearing-out time is also different between *fancl* mutants (32 dpf) and *zar1* mutants (50 dpf). The difference could be due to a requirement for Fancl in overall genome stability (Rodríguez-Marí et al., 2011), while Zar1 is involved in translational control of a limited numbers of RNAs.

Undifferentiated gonads in *zar1* homozygotes are indistinguishable from wild-type gonads, indicating that the lack of Zar1 does not affect early gonad development prior to sex differentiation. In *zar1* homozygotes, oocyte size at 33 dpf is much larger than that at 22 dpf, indicating that loss of Zar1 does not impair initial gonad differentiation into ovaries. Furthermore, immature ovary to immature testis ratio (at 33-41 dpf) in *zar1* homozygotes is approximately 1:1, which is similar to that in *zar1* heterozygotes, suggesting that Zar1 is not required for initial sex differentiation. The appearance of aberrant cortical granules in *zar1* oocytes at 33 dpf is the initial sign of defective oogenesis, but the mutant

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oocytes could last more than 2 weeks before they were cleared out, suggesting that detrimental factors accumulate gradually in the oocytes. Several studies support the hypothesis that oocytes are essential for ovarian development and may suppress testis development (Dranow et al., 2016, 2013; Hartung et al., 2014; Houwing et al., 2008; Rodríguez-Marí et al., 2010, 2011; Shive et al., 2010; White et al., 2011). We hypothesize that gradual loss of oocytes leads to testis development in *zar1* homozygous females and eventually causes female-to-male sex reversal.

p53-mediated apoptosis is attributed to oocyte clearance in zebrafish *fancl* and *brca2* mutants (Rodríguez-Marí et al., 2010, 2011; Rodríguez-Marí and Postlethwait, 2011; Shive et al., 2010). Apoptosis in *zar1* mutants is also mediated by p53 (Fig. 4). In *zar1* mutant ovaries, upregulation of ER stress- and the UPR-related genes suggests that loss of Zar1 causes ER stress and the UPR. This hypothesis was partially supported by *atf3* upregulation upon overexpression of ZP proteins in oocytes (Fig. 7C). Both EE2 treatment and p53 deficiency restore ovarian development and female development in *zar1* homozygous females. Previous studies suggest that in some organisms, estrogen can suppress oocyte apoptosis (Janz and Van Der Kraak, 1997; Kim et al., 2009). Hydroxysteroid (17-β) dehydrogenase 1, an enzyme known to catalyze estrogen synthesis (Mindnich et al., 2004), is less abundant in *zar1* mutant ovaries, suggesting that loss of Zar1 may affect estrogen synthesis. EE2 represses the upregulation of *atf3* in *zar1* mutants, suggesting some crosstalk between the UPR and estrogen pathways, although additional functions of EE2 cannot be ruled out.

In *Xenopus laevis*, Zar1 binds to the translational control sequence (TCS) of *Wee1* and *Mos* mRNAs and represses their translation in immature oocytes (Yamamoto et al., 2013). However, we did not recover Wee1 and Mos in the iTRAQ analysis. This may be due to the detection limitation of iTRAQ or Wee1 and Mos may not be expressed during early oogenesis in zebrafish. ZP mRNAs are highly enriched in Zar1 immunoprecipitates in early ovaries (Fig. 8B). Furthermore, Zar1 probably binds to ZP mRNAs directly, as shown in the yeast three-hybrid assay (Fig. 8C). The finding that Zar1 with a mutated Znf domain failed to bind to ZP mRNAs to repress its translation in oocytes suggests that the Znf domain mediates the binding of Zar1 to ZP mRNAs. The exact molecular mechanism by which Zar1 represses ZP mRNA translation remains to be investigated. There are a number of ways that RNA binding proteins can exert their translational inhibitory functions towards their RNA targets. In general, depolyadenylation is considered to be a mechanism to repress mRNA translation, while polyadenylation is used to initiate and enhance translation (Richter, 2007). We noticed that all seven ZP mRNAs have relatively short 5′UTRs and 3′UTRs (most of them are under 100 bp), suggesting that Zar1 may not use ZP mRNA UTRs to regulate translation. However, there might be common binding motifs/secondary structures in ZP mRNAs for Zar1 binding. Bioinformatics and experimental analyses of ZP mRNA structures are warranted. More recently, Amon and colleagues showed that the RNA binding protein Rim4 can form amyloid-like aggregates that translationally repress its target RNAs in gametogenesis (Berchowitz et al., 2015). This mode of mRNA-specific repression of translation may be used in zebrafish oogenesis. Zar1 contains a putative low complexity/disordered region, which is usually found in amyloid-like proteins or proteins capable of forming hydrogel (Courchaine et al., 2016; Kato et al., 2012). Whether Zar1 can form amyloid-like structures or hydrogel will be examined experimentally.

Zar1 was reported to be associated with known translation factors, such as CPEB and ePAB (Cook and Charlesworth, 2015). Similarly, we found that zebrafish Zar1 coimmunoprecipited with Cpeb1, ePAB, and 4E-T. ePAB and CPEB have been shown to control mRNA translation and oogenesis (Gray et al., 2000; Guzeloglu-Kayisli et al., 2012; Voeltz et al., 2001). Human 4E-T controls mRNA decay and represses translation of bound mRNAs (Kamenska et al., 2014). Moreover, 4E-T mutations are implicated in human primary ovarian insufficiency (Kasippillai et al., 2013). Previous studies in *Xenopus laevis* show that CPEB forms an RNP complex and interacts with Pabpc1l and 4E-T (Kim and Richter, 2007; Minshall et al., 2007; Standart and Minshall, 2008). We propose that Zar1, as a component of a maternal translational complex, may recruit other translational regulators and repress ZP mRNA translation in early oocytes. Future studies will examine how the translational complex is regulated in zebrafish oocytes.

MATERIALS AND METHODS

Zebrafish and maintenance

All animal studies in this report were approved by the Institutional Review Board of the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences. Zebrafish husbandry and manipulations were performed as described (Westerfield, 2000). Zebrafish *zar1* mutants were generated using the Tübingen strain with TALEN system or CRISPR/Cas9 system using primers as detailed in the supplementary Materials and methods. Genotyping for the *p53* mutant was performed as reported (Berghmans et al., 2005).

Generation of *Tg(zp3:zar1,cmlc2:EGFP)* transgenic zebrafish

The 412 bp promoter of zebrafish *zp3b* (*zpc*) (Onichtchouk et al., 2003) was amplified by PCR with reported primers and cloned in the upstream of *zar1* coding sequence. The *egfp* sequence under the control of *cmlc2* promoter was cloned into *zar1* construct in different directions. The plasmid containing *cmlc2* promoter was reported previously (Chen et al., 2010). The transgene was introduced into zebrafish genome with the *Tol2* transposon system (Kawakami et al., 2004).

qRT-PCR and statistical analysis

Total RNA was extracted from the isolated gonads using TRIzol reagent (Thermo Fisher Scientific). cDNA was synthesized from 2 µg total RNA with M-MLV reverse transcriptase. qPCR was performed using SsoFast EvaGreen Supermix (Bio-Rad) in 10 µl reactions. *ef1a* and *gapdh* were amplified with reported primers (McCurley and Callard, 2008). Additional primers are listed in Table S2. Ovaries from at least six fish were analyzed for each genotype and treatment. The two-tailed unpaired *t*-test was used to determine whether the difference between the two groups was significant.

RNA *in situ* hybridization on cryosections

Animals were sacrificed with standard operations (Westerfield, 2000). Ovaries and testes were isolated and fixed in 4% paraformaldehyde (PFA) overnight at 4°C. Cryosection *in situ* hybridization was performed as described (Simmons et al., 2007). The full-length *zar1* coding sequences were used to synthesize DIG-labeled probe (Roche).

Histology, immunocytochemistry and TUNEL assay

For juveniles at 22-60 dpf, heads and tails were removed and the middle body parts containing gonads were fixed in Bouin's solution (Sigma) overnight at 4°C. For adults, ovaries and testes were isolated and fixed in Bouin's solution overnight at 4°C. Fixed tissues were embedded in paraffin and sectioned at 10 µm. Hematoxylin and Eosin (H&E) staining was then performed on the sections. Samples for TUNEL assay (Roche) were fixed in 4% PFA. A list of antibodies and sources is provided in the supplementary Materials and methods.

RNA immunoprecipitation (RIP)

Ovaries were homogenized and lysed with RIP buffer (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 0.5% NP-40, 2 mM EDTA, RNase inhibitor and protease inhibitors were added fresh) on ice for 30 min.

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Antibodies were added to the supernatant and incubated for 4 h with gentle rotation at 4°C. Protein A beads were then added, incubated for 1.5 h and washed four times (10 min each). The suspension was divided into two parts for protein and RNA preparation.

Juvenile treatment with EE2 and inhibitors

EE2 (Sigma, E4876), GSK2606414 (Selleck, S7307) and ISRIB (Selleck, S7400) were diluted to working concentration with system water. Thirty juveniles were put in a 10 liter tank containing the working solution. Fish water was renewed by dripping 20 liters of working solution per day. The juveniles were treated from 20 dpf or 22 dpf to 60 dpf and then transferred to zebrafish housing system and their gender was determined 1 month later.

Oocyte isolation and microinjection

Ovaries were isolated from adult females and transferred into fish oocyte culture medium (OCM; 20 mM HEPES, 0.2 mg/ml of BSA, 75% L-15 medium, pH 7.5) (Mold et al., 2009). Oocytes were dissociated by gentle pipetting. Oocytes at desired stage were collected, microinjected and cultured in OCM. Stage II oocytes were used to test the function of Zar1. As oocytes earlier than stage IV are easily stressed during *in vitro* manipulation, only healthy stage IV oocytes were used to test the relationship between ZP gene overexpression and ER stress.

Luciferase assay

zp3b was cloned downstream of Firefly luciferase encoding sequences (*firefly*) in pCS2 vector. *firefly-zp3b*, *rfp-flag*, *zar1* and *zar1-mu* (Znf domain mutated) mRNAs were synthesized *in vitro* using the SP6 Transcription Kit (Thermo, AM1340). *Renilla* mRNAs were synthesized *in vitro* using T7 Transcription Kit (Thermo, AM1344). *firefly-zp3b* mRNAs (200 ng/µl) and *Renilla* mRNAs (70 ng/µl) were mixed with *rfp-flag* mRNAs (210 ng/µl), *zar1* mRNAs (260 ng/µl) or *zar1-mu* mRNAs (260 ng/µl). The mixed mRNAs were injected into stage II oocytes (0.2 nl per oocyte). The injected oocytes were cultured in OCM for 4 h. Luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega, E1910).

Quantitative proteomic analysis by iTRAQ

At 33 dpf, 10 ovaries for each genotype ($zar1^{-/-}$ and $zar1^{+/-}$) were pooled and homogenized in denaturing buffer (1%SDS, 1 mM sodium orthovanadate, 1 mM sodium fluoride, protease inhibitors) on ice. The iTRAQ analysis was performed as described (Bi et al., 2014; Udeshi et al., 2013) (see supplementary Materials and methods for details). The UniProt proteome sequences for *Danio rerio* were used for the database searching.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

L.M. and J.Z. designed the experiments and wrote the paper; L.M., Y.Y., F.C., J.F., F.Z. W.M., Y.J., X.H. and L.S. performed the experiments; L.M., X.H., Y.W., D.C. and J.Z. analyzed the data.

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Data availability

Raw data of the iTRAQ proteome analyses are available in Figshare under accession number 4253888 (available at: https://figshare.com/articles/Translation_Repression_by_Maternal_RNA_Binding_Protein_Zar1_is_Essential_for_Early_Oogenesis_in_Zebrafish_rar/4253888).

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.144642.supplemental

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