Supplementary information

Translation Repression by Maternal RNA Binding Protein Zar1 is Essential for Early Oogenesis in Zebrafish

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Materials and Methods

Mutant generation and genotyping

The zebrafish *zar1* mutants were generated using the Tübingen strain with TALEN system or CRISPR/Cas9 system. The following primers were used to identify *zar1* mutants generated with TALEN system: for mutant screening a pair of primers (forward primer: 5'-CTTTCCCAAACCTCGAAAATCGT-3'; reverse primer: 5'-GGGGTGAGATTTGGGTTGATCTG-3') were used; for *zar1*^{gd5} mutant line specific genotyping two forward primers (5'-GCGAAAAATGGCTACATAT-3' to detect the WT allele and 5'-GCGAAAAATGGCTACATGG-3' to detect the mutant allele) and a reverse primer (5'-TCCGGGATTCTACTGGGGAGTAA-3') were used

Antibodies

Zar1 antibody was generated with full-length recombinant Zar1 protein purified from *E.coli* by immunizing rabbits and affinity purified with SulfoLink Immobilization Trial Kit (Thermo, 20325). Zar1 antibody was diluted 1:1000. Other antibodies and beads were purchased: 4E-T antibody (Abcam, ab6034, 1:500); tubulin antibody (Sigma, T6074, 1:4000); FLAG antibody (Sigma, F3165, 1:2000); anti-FLAG M2 affinity gel (Sigma, A2220); and protein A beads (Millipore, 16-156).

Quantitative proteomic analysis by iTRAQ

The ovary lysates were boiled for 10 min and centrifuged (12000 rpm, 10 min). The supernatant (100 μg protein / sample) were reduced (10 mM DTT, 37^{o}C, 1 h), alkylated (55 mM iodoacetamide, room temperature, 1 h, in the dark), and transferred to the Microcon YM-30 centrifugal filter units (EMD Millipore Corporation) where the lysis buffer was replaced with iTRAQ dissolution buffer. Samples were digested with trypsin (sequencing grade, 1:50, 37 ^{o}C, overnight). The resulting peptides were labeled, according to the manufacturer's manual with slight modifications, with iTRAQ Reagents (AB Sciex Inc.). Briefly, the peptides were incubated with the ethanol-dissolved iTRAQ reagents (2 h, room temperature), and then terminated by adding H_2O to 30% of the total reaction volume. The labeled samples were mixed together with equal ratios in amount and fractioned by reverse phase HPLC. The mixed peptides were resuspended in 0.5 ml of RP-HPLC solvent A (2% ACN, 5 mM ammonium formate, pH 10), and separated into 10 fractions using the Gemini-NX 5u c18 110A (P/No: 00G-4454-Y0 3*250 mm length, 5 m particle size, Phenomenex) on a Waters e2695 separations module system with a flow rate of 0.4 ml/min. A 97 min basic RP LC method was utilized for offline fractionation. The gradient consisted of an initial increase to 8% solvent B (1.1%B/min) (90% ACN/5 mM ammonium formate, pH 10) followed by a 38 min linear gradient (0.5% B/min) from 8% solvent B to 27% B and successive ramps to 31% B (1%B/min), 39% B (0.5%B/min), and 60% B (3%B/min). The separated peptides were further desalted with C18 StageTips, concentrated with a SpeedVac, resuspended with 0.1% formic acid.

Figures

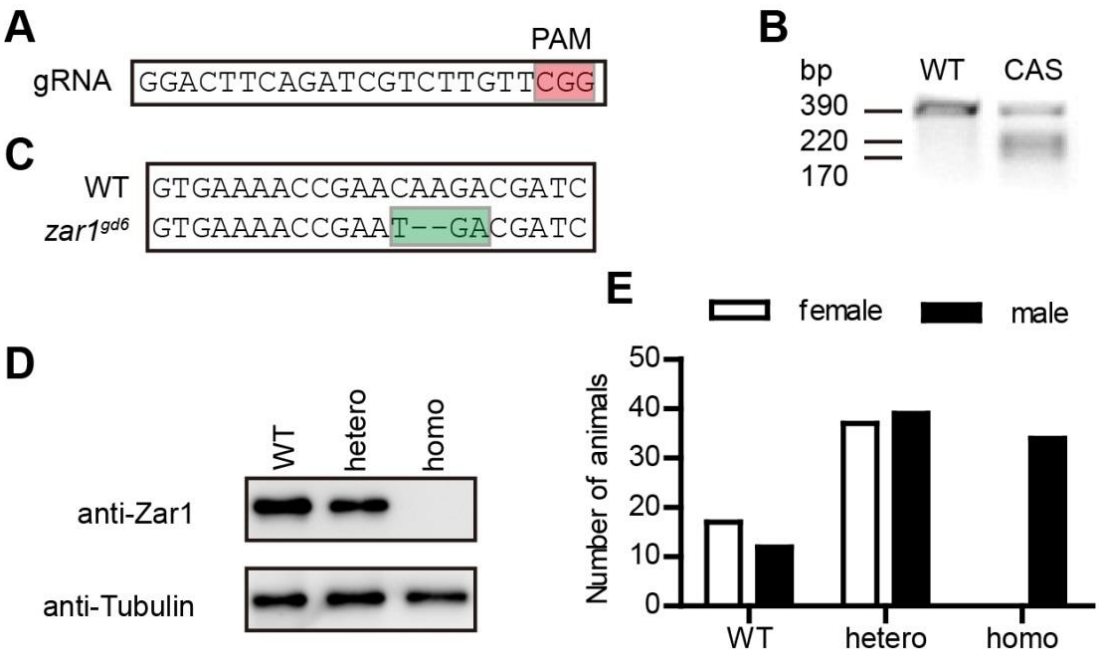

Fig. S1. *zar1* mutant generation with CRISPR/Cas9 system

(A) Selected gRNA sequence. DNA sequences highlighted in red are the protospacer-adjacent motif (PAM). (B) PCR fragments (amplified with the following primers: primer-F: 5'-AGGTCACAGAGACGGTTGACAGC-3' and primer-R: 5'-ACTTTCCCACCGTAGGTTGCAGT-3') were digested with T7 endonuclease I to test the gRNA. (C) DNA sequences of the *zar1*^{gd6} mutant line. Green highlighted DNA sequences are the premature stop codon. Two *zar1*^{gd6} mutant line specific forward primers (5'-CCAGTGAAAACCGAACAA-3' to detect the WT allele and 5'-CCAGTGAAAACCGAATGA-3' to detect the mutant allele) were used together with the reverse primer (primer-R) mentioned above to do genotyping. (D) Western blot of Zar1 in gonads at 25 dpf from wild-type (WT), *zar1*$^{gd6/+}$ heterozygotes (hetero) and *zar1*$^{gd6/gd6}$ homozygotes (homo). Gonads from 8 fish were mixed together and lysed for each genotype. (E) Analysis of the gender of *zar1*$^{gd6/gd6}$ homozygotes (homo), *zar1*$^{gd6/+}$ heterozygotes (hetero) and WT siblings.

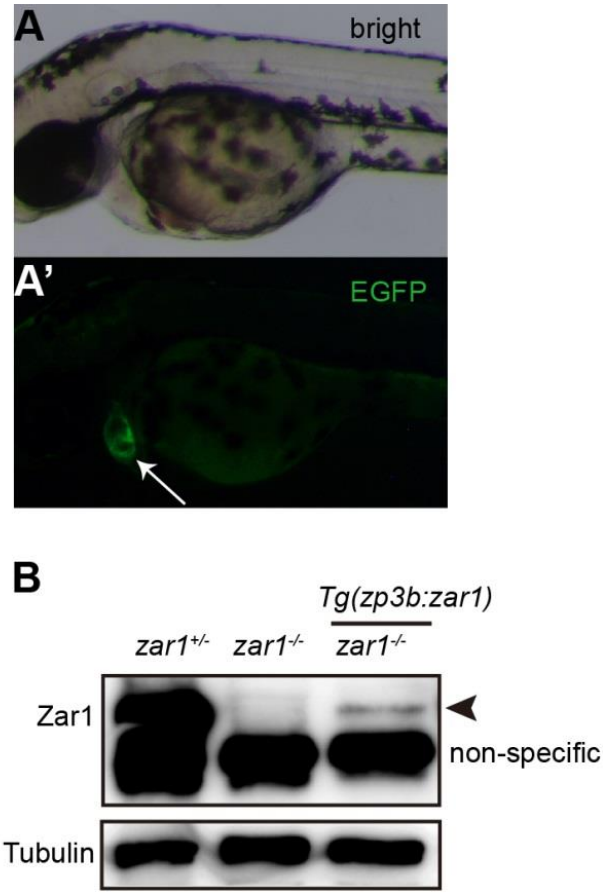

Fig. S2. Zar1 is expressed in *zar1* homozygous females with the *Tg(zp3b:zar1,cmlc2:EGFP)* transgene.

(A, A') Heart specific EGPF signal (arrow) seen in transgenic embryos. (B) Western blot to detect Zar1 protein in adult ovaries. Zar1 was expressed in the transgene rescued adult *zar1* homozygous ovaries (arrowhead). Compared with Zar1 expression level in *zar1* heterozygous mutant ovaries (hetero), Zar1 expression level in transgene rescued *zar1* homozygous (homo) mutant ovaries was low. The smaller bands are non-specific.

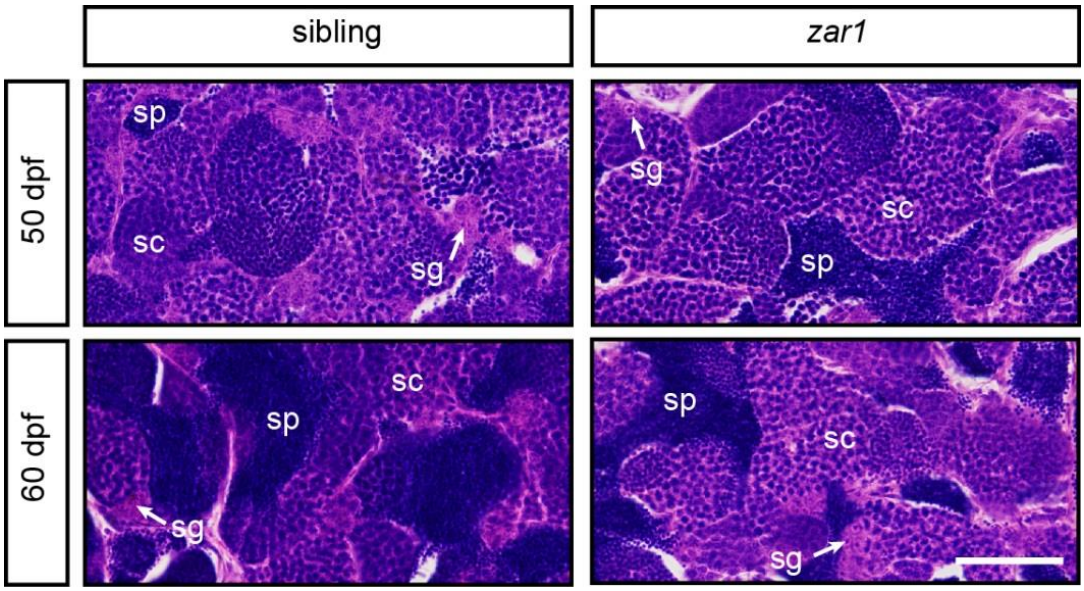

Fig. S3. Spermatogenesis is not affected in *zar1* mutant males.

Histology analysis of testes of *zar1* homozygous males and control siblings at 50 dpf and 60 dpf. Spermatogenesis in *zar1* homozygous males resembles that in control siblings. sg: spermatogonia; sc: spermatocytes; sp: sperm; Scale bar: 0.04 mm.

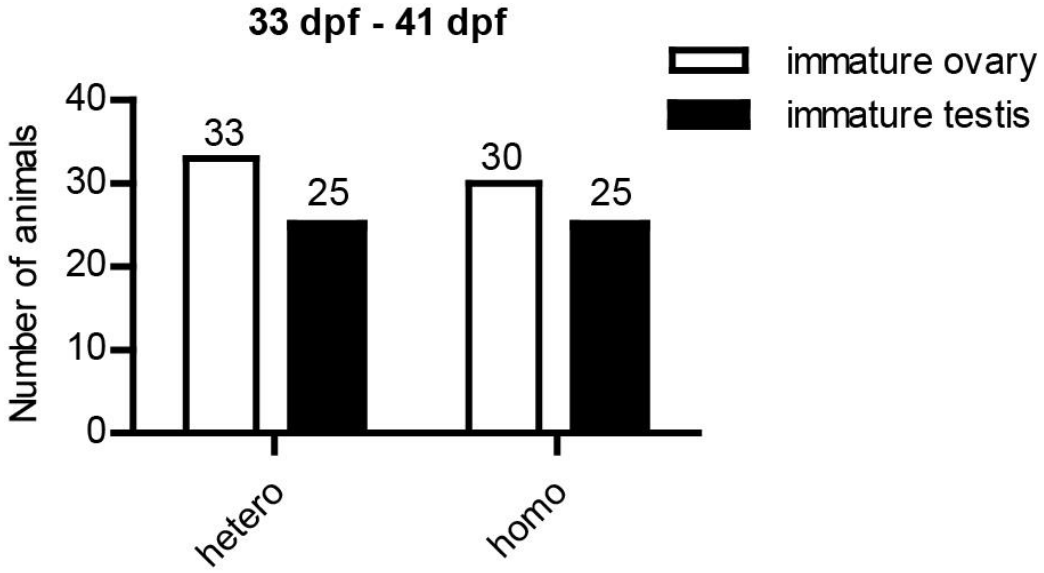

Fig. S4. Sex ratio in juveniles at 33-41 dpf is not affected in *zar1* mutants.

Sex ratios in *zar1* homozygotes (homo) and *zar1* heterozygotes (hetero) at 33 dpf-41 dpf. Sex of *zar1* homozygotes and *zar1* heterozygotes was determined at 33-41 dpf according to H&E staining results.

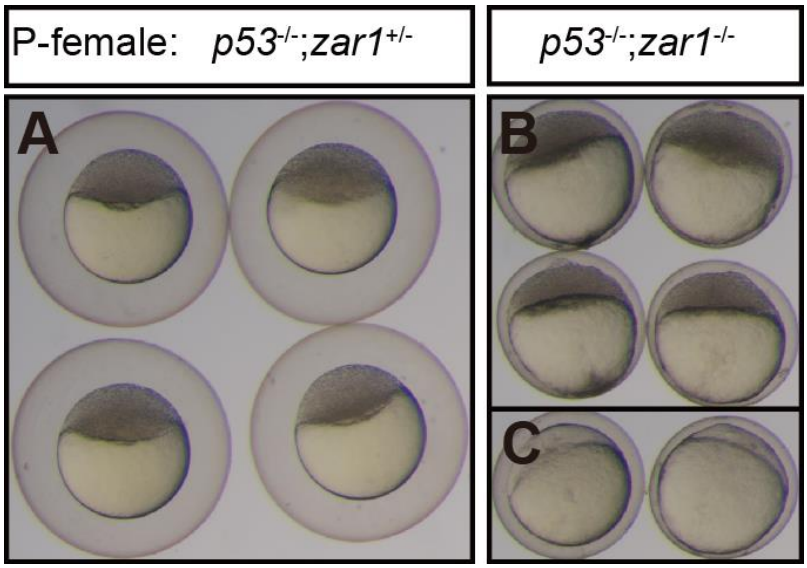

Fig. S5. The chorions of eggs from *p53*$^{-/-}$;*zar1*$^{-/-}$ failed to elevate normally.

Five *p53*$^{-/-}$;*zar1*$^{+/-}$ females and five *p53*$^{-/-}$;*zar1*$^{-/-}$ females were crossed with wild-type males. The chorions of eggs from *p53*$^{-/-}$;*zar1*$^{+/-}$ lifted normally (A). The chorions of eggs from *p53*$^{-/-}$;*zar1*$^{-/-}$ failed to lift. Cleaved embryos (B) and uncleaved embryos (C); P-female: genotypes of the mothers

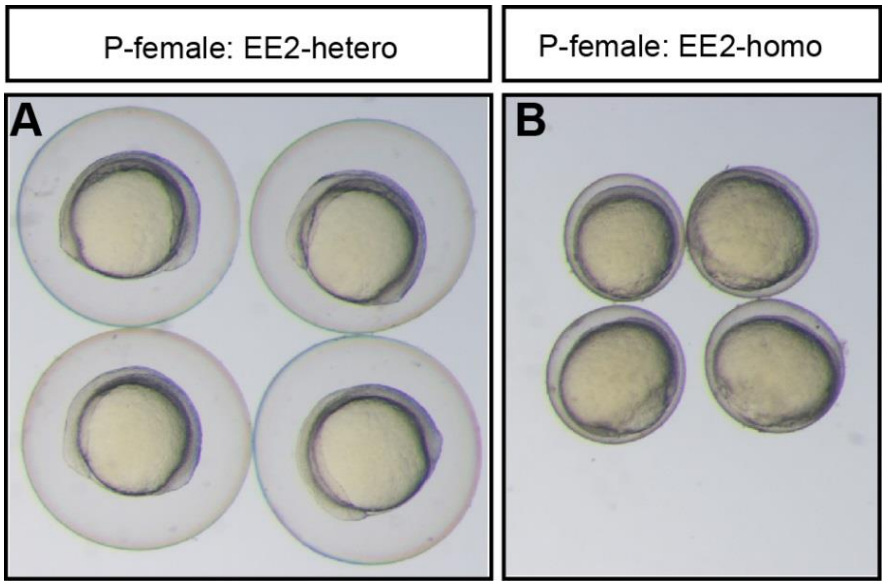

Fig. S6. The chorions of *zar1* mutant eggs failed to elevate normally.

EE2 treated females were crossed with wild-type males. Embryos from 6 heterozygous females (EE2-hetero) and 6 homozygous females (EE2-homo) were analyzed. The chorions of *zar1* homozygotes failed to lift properly compared with those of *zar1* heterozygotes. P-female: genotypes of the mothers.

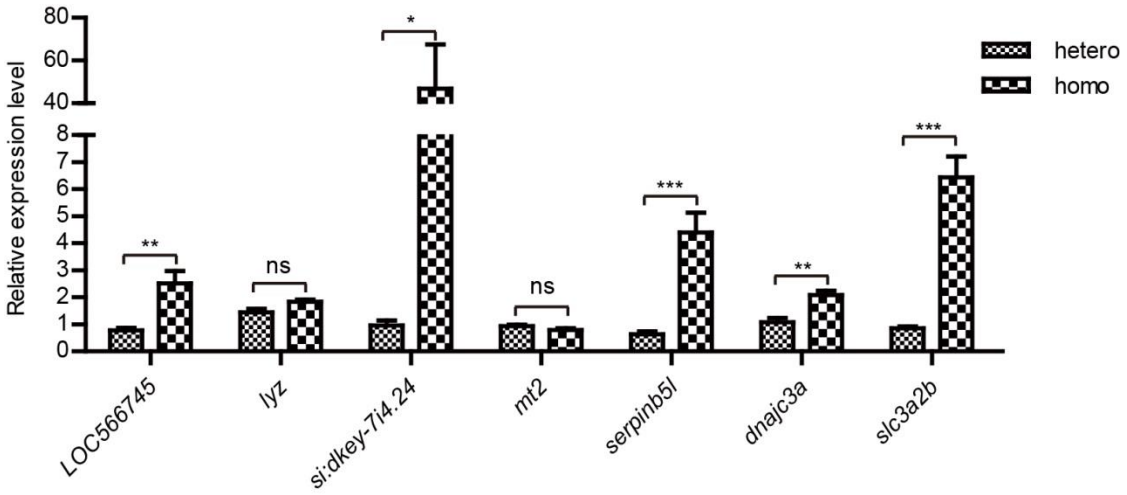

Fig. S7. qPCR to confirm the iTRAQ result.

Statistical analysis of mRNA levels of upregulated genes in *zar1* homozygotes (homo), according to iTRAQ result. Ovaries were isolated from *zar1* homozygotes (homo) and *zar1* heterozygotes (hetero) at 33 dpf. Seven out of the first 25 upregulated genes were tested. Internal control: *ef1a*; ns: not significant; *: P < 0.05; **: P < 0.01; ***: P < 0.001.

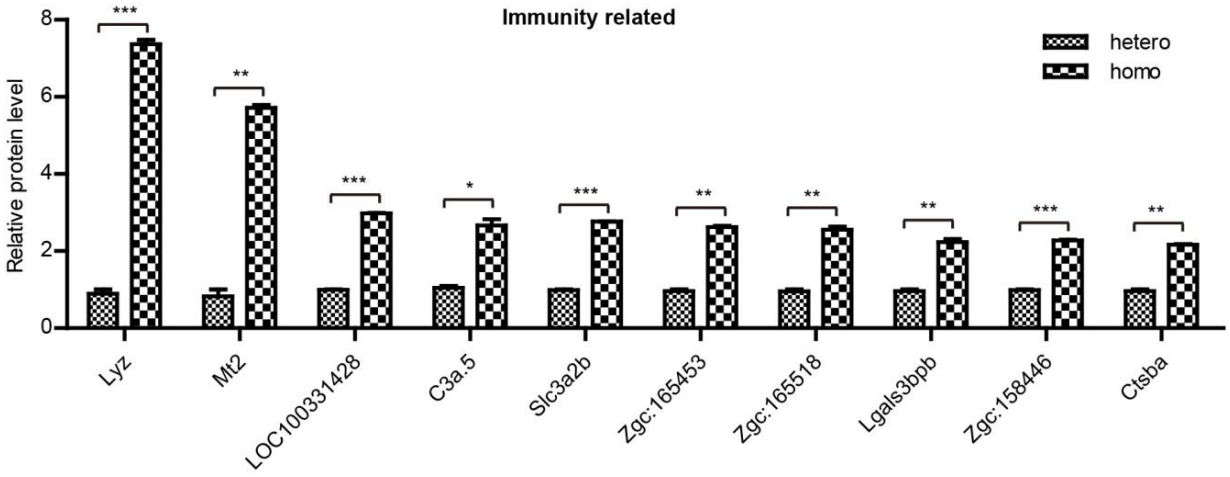

Fig. S8. Immunity related proteins are upregulated in *zar1* mutants.

Statistical analysis of immune related proteins upregulated in *zar1* homozygotes (homo) compared to heterozygotes (hetero) determined by iTRAQ. *: P < 0.05; **: P < 0.01; ***: P < 0.001.

Fig. S9. Mutation of the Zar1 Znf domain.

The 8 cysteines in Zar1 Znf domain are mutated to alanines.

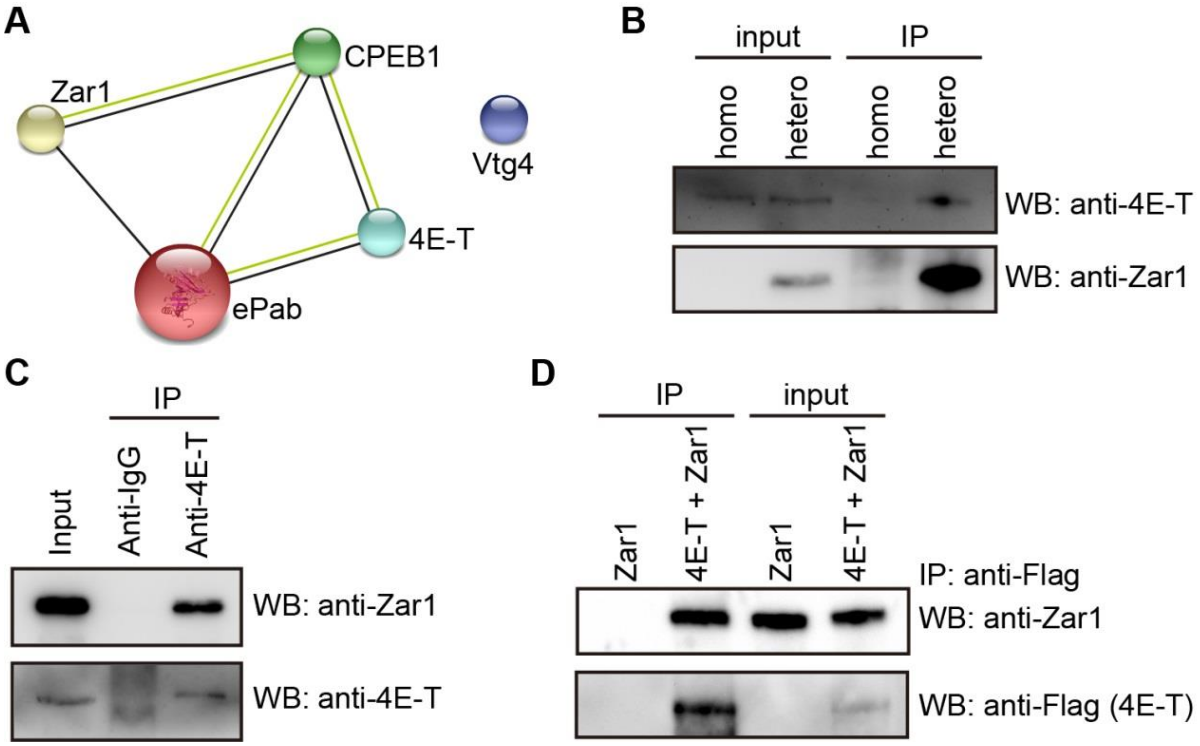

Fig. S10. Interaction between Zar1 and translation initiation factor 4E transporter.

(A) Association analysis of Zar1 with the immunoprecipitated proteins by searching STING database (evidence view). (B) 4E-T was immunoprecipitated by the Zar1 antibody from *zar1* heterozygous ovaries (hetero, $p53^{-/-};zar1^{+/-}$) but not from *zar1* homozygous ovaries (homo, $p53^{-/-};zar1^{-/-}$). (C) Zar1 was immunoprecipitated from ovaries by a 4E-T antibody but not by a control antibody. (D) Zar1 was immunoprecipitated by the Flag antibody from HEK293 cells co-transfected with *zar1* and *eif4enif1-flag* plasmids but not from cells transfected with *zar1* plasmid alone.

Tables

Table S1. The three categories of upregulated proteins (> 2 fold over controls) in *zar1* mutants

Category	Accession	Protein Name	Gene symbol
ER stress & the UPR related	Q6P0U6	DnaJ (Hsp40) homolog, subfamily C, member 3a	*dnajc3a*
	Q6P2B0	Vesicle-associated membrane protein-associated protein B/C	*vapb*
	Q6P3I1	Protein disulfide-isomerase A4	*pdia4*
Zona pellucida proteins	Q5TYP2	Zona pellucida glycoprotein 3a, tandem duplicate 2	*zp3a.2*
	A7MBW8	Zona pellucida glycoprotein 2, like 1	*zp2l1*
	A8WG31	pellucida sperm-binding protein 3	*LOC100148225*
	F1R4N4	Egg envelope glycoprotein-like	*si:dkey-19b23.11*
	A7MBS3	Zona pellucida glycoprotein 3b	*zp3b*
	B0R0H4	ZPC domain containing protein 1	*si:ch211-14a17.7*
	Q5TYX2	ZPA domain containing protein	*si:dkeyp-50f7.2*
Immunity related	Q24JW2	Lysozyme	*lyz*
	Q1LV07	Metallothionein	*mt2*
	F1QYN0	Complement C3-like	*LOC100331428*
	F1QLN6	Complement component c3a, duplicate 5	*c3a.5*
	Q803G1	Solute carrier family 3, member 2b	*slc3a2b*
	E7FCS3	Alpha-2-macroglobulin-liked, duplicate 1	*a2m1*
	F1QF63	Alpha-2-macroglobulin-liked, duplicate 2	*a2m2*
	F1Q6K5	Lectin, galactoside-binding, soluble, 3 binding protein b	*lgals3bpb*
	A2VD28	Complement factor B	*cfb*
	Q6PH75	Cathepsin B	*ctsba*

Table S2. Sequences of primers used in qPCR and RT-PCR.

gene	primers
atf3	F: 5'-TCAATGGCTACTGAATTCCAACTG-3'
	R: 5'-TTCTTGTGGCATGTTATGTGGAC-3'
ddit3	F: 5'-AGTTGGAGGCGTGGTATGAAGAC-3'
	R: 5'-GTCAACCAGGTGAGCGAACAG-3'
btg2	F: 5'-CATTAGAAACCAGACAAATCCTCGT-3'
	R: 5'-GGAGCGGTGCTGTGGTTAAG-3'
traf4a	F: 5'-GAACTCTGGACTCGACATGCTCA-3'
	R: 5'-ATTGCACAAGGCTCATCTTCCTC-3'
LOC566745	F: 5'- GAGTTGCAACTTGAGAAGCTCTTC-3'
	R: 5'-TCCTCCAACCCAAACCCAGATC-3'
lyz	F: 5'-AGAATTTGTGCAAAGTGGCCTGT-3'
	R: 5'-AAGAATCCCAGGTTTCCCATGAT-3'
si:dkey-7i4.24	F: 5'-CCGCTCTGAAACAAAAGCAGTGG-3'
	R: 5'-CGCTTAGATGGATGGGGTCAGGT-3'
mt2	F: 5'-GACTGGAACTTGCAACTGTGGTG-3'
	R: 5'-GGGCAGCAAGAACAACAACTCTT-3'
serpinb5	F: 5'-TGATACAGGCAATGGTGGAGTTG-3'
	R: 5'-CTGTCGAGGAATCTGCTCCTAGC-3'
zp2l1	F: 5'-TTTCTGACTTTGGGTCGTTGTTG-3'
	R: 5'-GGGAACAAAGTGGTCAGGTAACG-3'
LOC100148225	F: 5'-GCCTTCATGTTCCAGGATACACC-3'
	R: 5'-CCCAGTAAGTTGGCCTTCAACAC-3'
dnajc3a	F: 5'-AGACAACTTCCAGGATGCAGAGG-3'
	R: 5'-GTCCAGAACCAAACGGATTGAAC-3'
zp3b	F: 5'-TTGTGTTCATCGACTGGTGTGTG-3'

	R: 5'-ACTCCTGCTATCTGCAAGACATC-3'
Slc3a2b	F: 5'-GGAGTGATGTCGTAGCCCTCATT-3'
	R: 5'-GAGGAGCAAGTCCACACCAGTTT-3'
zp3a.2	F: 5'-ACCCAGGACTGCATTCACTGC-3'
	R: 5'-ACAAGTTCAGGAGAACTCTAATC-3'
si:dkey-19b23.11	F: 5'-TATTCCTCTGACGCAAAGCCAGT-3'
	R: 5'-GTCCACTGAAAGGGTTTCAAGGT-3'
si:ch211-14a17.7	F: 5'-GCAGTGTTTCATGCTGAATTTGG-3'
	R: 5'-GAGACCACTCTTTTGGCCTTTCA-3'
si:dkeyp-50f7.2	F: 5'-CAGGCCTAAATTCAGCAATGACC-3'
	R: 5'-GCGGTTGTGAGGTAAACGAAATC-3'
firefly	F: 5'-GTTGACCGCCTGAAGTCTCTGAT-3'
	R: 5'-CCACGATCTCTTTTTCCGTCATC-3'
renilla	F: 5'-ATTGAATCGGACCCAGGATTCTT-3'
	R: 5'-TTTCATCAGGTGCATCTTCTTGC-3'

Table S3. Proteomic comparison between *zar1* homozygotes and *zar1* heterozygotes.

Proteomic analysis with iTRAQ to compare *zar1* homozygous ovaries (homo) with *zar1* heterozygous ovaries (hetero). 10 ovaries for each genotype were isolated at 33 dpf and analyzed with iTRAQ. Two replicates were performed for each genotype. The UniProt proteome sequences for Danio rerio were used for the database searching.

Click here to Download Table S3

Table S4. Summary of differentially expressed proteins based on the iTRAQ result.

Analysis of differentially expressed proteins between *zar1* homozygous ovaries (homo) and *zar1* heterozygous ovaries (hetero) determined by iTRAQ. 325 proteins show differential expression ($P < 0.05$). 42 proteins changed by more than 2 folds, among which 37 proteins were upregulated and 5 proteins were down-regulated in homozygous ovaries compared with the control ovaries.

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Table S5. Mass Spectrometry (MS) analysis of proteins precipitated by the Zar1 antibody.

MS analysis of immunoprecipitated proteins from *zar1* heterozygotes and *zar1* homozygotes. Immunoprecipitation with the Zar1 antibody was performed using *zar1* heterozygous mutant ovaries ($p53^{-/-}$;$zar1^{+/-}$) and *zar1* homozygous mutant ovaries ($p53^{-/-}$;$zar1^{-/-}$) followed by MS analysis. 59 proteins from *zar1* heterozygous ovaries and 44 proteins from *zar1* homozygous ovaries were precipitated.

Click here to Download Table S5