Requirement of the 3′-UTR-dependent suppression of DAZL in oocytes for pre-implantation mouse development

Kurumi Fukuda1,2, Aki Masuda1, Takuma Naka3, Atsushi Suzuki3, Yuzuru Kato1,2,*; Yumiko Saga1,2,4*

1 Division of Mammalian Development, Genetic Strains Research Center, National Institute of Genetics, Mishima, Shizuoka, Japan, 2 Department of Genetics, SOKENDAI, Mishima, Shizuoka, Japan, 3 Division of Materials Science and Chemical Engineering, Graduate School of Engineering, Faculty of Engineering, Yokohama National University, Yokohama Kanagawa, Japan, 4 Department of Biological Science, Graduate School of Science, The University of Tokyo, Bunkyo-ku, Tokyo, Japan

* yukato@nig.ac.jp (YK); ysaga@nig.ac.jp (YS)

Abstract

Functional oocytes are produced through complex molecular and cellular processes. In particular, the contribution of post-transcriptional gene regulation mediated by RNA-binding proteins (RBPs) is crucial for controlling proper gene expression during this process. DAZL (deleted in azoospermia-like) is one of the RBPs required for the sexual differentiation of primordial germ cells and for the progression of meiosis in ovulated oocytes. However, the involvement of DAZL in the development of follicular oocytes is still unknown. Here, we show that Dazl is translationally suppressed in a 3′-UTR-dependent manner in follicular oocytes, and this suppression is required for normal pre-implantation development. We found that suppression of DAZL occurred in postnatal oocytes concomitant with the formation of primordial follicles, whereas Dazl mRNA was continuously expressed throughout oocyte development, raising the possibility that DAZL is dispensable for the survival and growth of follicular oocytes. Indeed, follicular oocyte-specific knockout of Dazl resulted in the production of normal number of pups. On the other hand, genetically modified female mice that overexpress DAZL produced fewer numbers of pups than the control due to defective pre-implantation development. Our data suggest that post-transcriptional suppression of DAZL in oocytes is an important mechanism controlling gene expression in the development of functional oocytes.

Author summary

Evolutionarily conserved DAZ family genes are indispensably involved in germline development. Dazl (deleted in azoospermia-like) is a member of the mammalian DAZ family of genes, and plays crucial roles in the sexual differentiation of primordial germ cells and spermatogenesis, and is implicated in the progression of meiosis II in ovulated eggs. Despite its importance for multiple processes during germline development, its participation in follicular oocyte development is enigmatic. This study addressed this issue and found that DAZL is translationally suppressed in postnatal oocytes in a 3′-UTR-dependent manner. This suppression is required for normal pre-implantation development. Our data suggest that post-transcriptional suppression of DAZL in oocytes is an important mechanism controlling gene expression in the development of functional oocytes.
Introduction

The production of functional oocytes is an essential process in the female ovary, by which genetic information is continuously passed to the next generations. For successful oocyte development, gene expression needs to be precisely regulated according to the developmental stages and environmental cues such as gonadotropin hormones. Oocytes that fail to regulate proper gene expression are degenerated during their development or are unable to proceed with embryonic cleavage even if they are fully developed [1]. Therefore, unveiling the mechanisms controlling the quality of oocytes is a crucial issue to understand the molecular basis of female reproduction.

Post-transcriptional gene regulation mediated by RNA-binding proteins is an important molecular mechanism involved in this process. An evolutionarily conserved and well-documented post-transcriptional event is the translational suppression and storage of maternal mRNAs with shorter poly (A) tails [2]. Although the genome is actively transcribed and proteins are produced during oocyte growth, transcription becomes inactive in full-grown oocytes and maternal mRNAs are used for protein synthesis in early zygote development [3]. These processes are orchestrated by a battery of relevant RNA-binding proteins, including cytoplasmic polyadenylation element binding proteins (CPEB), maskin, and other germ cell-specific RNA-binding proteins [4,5,6,7]. In addition to the maturation process, germ cell-specific RNA-binding proteins are also responsible for multiple processes in oogenesis. For instance, CPEB1 and Pumilio1 are involved in the progression of meiotic prophase I in the embryonic ovary [8,9], and MSY2 is involved in follicle development after birth in mice [10,11], suggesting the significant contribution of post-transcriptional gene regulation throughout oogenesis.

Deleted in azoospermia-like (DAZL) is a member of the evolutionarily conserved DAZ family of RNA-binding proteins that acts as a translational activator in mice [12]. Biochemical and structural analyses showed that DAZL binds to the U-rich region of its target’s 3’-UTR [13,14,15], and genetic analyses revealed that DAZL is indispensable for gametogenesis in both males and females [16,17,18]. As DAZL is reportedly required for sexual differentiation of primordial germ cells, progression of meiotic prophase I in embryonic female germ cells [19], and for the progression of meiosis in maturing oocytes [20], it is believed that DAZL is involved in female germ cell development throughout oogenesis. However, the role of DAZL in follicular oocytes remains unknown because Dazl-deficient oocytes die due to the failure of meiotic progression in the embryonic ovary [16,21]. Moreover, although the previous immunohistochemical analysis demonstrated that DAZL was expressed in both embryonic and follicular oocytes in postnatal ovaries [16], it was also noted that DAZL signals were not detectable by western blotting in ovaries 1 to 2 weeks after birth [22]. Therefore, further analysis is required to clarify this contradiction.

In this study, we investigated DAZL expression in embryonic and postnatal ovaries, and found that DAZL was translationally suppressed in a 3’-UTR-dependent manner in follicular oocytes. Genetic analysis by knocking out the Dazl gene in a follicular oocyte-specific manner indicated that Dazl is dispensable for follicular growth, maturation, and fertilization. On the
other hand, the 3’-UTR-dependent suppression of DAZL in follicular oocytes is required for the progression of normal pre-implantation development. Our data clarify the previously ambiguous expression pattern of DAZL in the postnatal ovary, and simultaneously demonstrate the significance of the post-transcriptional suppression of DAZL in follicular oocytes.

**Results**

**DAZL is post-transcriptionally suppressed in postnatal oocytes**

To examine Dazl/DAZL expression in detail, we performed quantitative reverse transcription-polymerase chain reaction (RT-qPCR) and western blotting analyses using ovaries from embryos until juvenile stages (Fig 1A and 1B). RT-qPCR data showed that Dazl mRNA was constantly expressed and expression differences were less than 2-fold among all stages investigated (Fig 1A). On the other hand, DAZL expression was markedly changed during oocyte development (Fig 1B). Although it was abundant in embryonic ovaries, with the strongest expression at embryonic day (E) 15.5, the expression declined in newborn ovaries. Afterwards, DAZL expression further declined and was hardly detectable in 1- and 2-week ovaries, which is consistent with previous descriptions [22].

As DAZL expression was significantly decreased in the newborn ovary and onward, we next asked whether this reduction in DAZL was correlated with the formation of primordial follicles. In the embryonic ovary, oocytes are connected with each other by intercellular bridges (ICBs). Within a few days after birth, ICBs are broken and each oocyte is enclosed by pre-granulosa cells, resulting in the formation of primordial follicles [23]. Thus, we examined the expression changes of DAZL in perinatal ovaries by immunostaining DAZL together with a granulosa cell marker, forkhead box protein L2 (FOXL2), from E18.5 to 1W ovaries. Strong DAZL signals were observed in most oocytes until the day of birth (P0), when a large number of oocytes were still connected with each other. However, at one day after birth (P1), its expression began to decrease in some oocytes (Fig 1C, open arrowheads). The expression of DAZL was further decreased at two days after birth (P2), at which point, many oocytes formed primordial follicles (Fig 1C, yellow arrowheads) and exhibited weaker expression than cystic-oocytes (Fig 1C, white arrowheads). Thereafter, the weakened DAZL expression was observed in 1-week ovaries. These data suggest that DAZL is decreases in oocytes coinciding with the development of primordial follicles.

**DAZL is not required for oogenesis in the postnatal ovary**

Both immunostaining and western blotting analyses revealed that DAZL was decreased in oocytes shortly after birth, which raised the possibility that DAZL is dispensable for follicular development. To test this possibility, we used conditional Dazl knockout (cKO) mice (Fig 2A). Dazlflox mice were crossed with a postnatal oocyte-specific Cre mouse line, Gdf9-iCre, which expresses improved Cre recombinase from P2 oocytes [25]. The Dazl gene was successfully disrupted by Gdf9-iCre, as evidenced by RT-qPCR and western blotting, in which both Dazl mRNA and DAZL protein were hardly detectable in Dazl cKO ovaries (Fig 2B). We also confirmed that our Dazl KO (Dazllox/lox) mouse line recapitulated the phenotype of previous Dazl knockout females (S1 Fig)[16]. On histological analysis, Dazl cKO ovaries as well as control ovaries contained both primordial and growing follicles (Fig 2C). Notably, cKO ovaries did not have any significant differences in the number of primordial or growing follicles (Fig 2D). These data suggest that DAZL is dispensable for the survival and growth of follicular oocytes.

In order to evaluate the reproductive capability of Dazl cKO oocytes, we next crossed Dazl cKO females with wild-type (WT) males. We found that Dazl cKO females were fertile and...
produced a normal number of pups (Fig 2E). The average litter size delivered from Dazl cKO females (11.3±0.62) was almost identical with that from WT (12.0±2.83). We also confirmed that all progeny delivered by Dazl cKO females were heterozygotes for the Dazl<sup>1lox</sup> allele (n = 258). These results were surprising because a previous report stated that Dazl knockdown in MII oocytes results in the defective progression of the oocyte to zygote transition [20]. However, MII oocytes derived from Dazl cKO females did not have abnormal spindle morphology (S2 Fig). These data indicate that DAZL is not required for the maturation of oocytes or subsequent fertilization.
Fig 2. Postnatal oocyte-specific Dazl knock-out mice do not exhibit any defects. (A) A schematic diagram of the generation of conditional Dazl knockout mice. Dazl$^{flox/flox}$ females were crossed with Dazl$^{flox/flox}$;Gdf9iCre male mice to obtain Dazl$^{flox/flox}$;Gdf9iCre (conditional Dazl knockout) females. Blue arrows indicate the position of primers used for RT-qPCR in (B). (B)
Western blotting analysis for WT and Dazl cKO MII oocytes (upper panels), and RT-qPCR analysis for Dazl in 1 and 5W ovaries using a primer set that amplifies only the WT allele (lower graph, n = 3). Error bars represents S.D. Significance level of changes are indicated (two-tailed student’s t-test; \(^* P < 0.005\)). (C) Periodic acid-Schiff (PAS) staining of control (left) and cKO (right) ovaries at 5W after birth. PrF, primordial follicle; PF, primary follicle; SF, secondary follicle; and AF, antral follicle. Scale bars, 100μm. (D) Follicle counting analysis of control (white bar, n = 9) and Dazl cKO (black bar, n = 6) ovaries at 5 weeks after birth. Error bars represents S.D. ns, no significant difference between control and Dazl cKO ovaries (two-tailed student's t-test). (E) Average litter size of control (n = 3) and Dazl cKO females (n = 5). Error bars represent S.D. ns, same as in (D).

https://doi.org/10.1371/journal.pgen.1007436.g002

**DAZL is suppressed in a 3' -UTR dependent manner**

In embryonic male germ cells, Dazl is post-transcriptionally suppressed in a 3'-UTR-dependent manner by a male-specific RNA-binding protein, NANOS2 [26]. As DAZL decreases in postnatal ovaries, it is possible that Dazl is also post-transcriptionally suppressed in a 3'-UTR-dependent manner by unidentified mechanisms in follicular oocytes. In order to test this possibility, we used our bacterial artificial chromosome (BAC)-carrying transgenic mouse line, in which the FLAG tag was inserted at the C-terminus of Dazl and the Dazl 3' -UTR was flanked with FRT sequences (Dazl 3F, Fig 3A upper) [26]. The significance of the Dazl 3' -UTR for its expression was assessed by crossing the BAC transgenic female with a Rosa-Flp male (Dazl 3F; Flp, Fig 3A lower). RT-qPCR showed that the amount of Flag-Dazl mRNA was increased in Dazl 3F; Flp ovaries after birth (Fig 3B). However, the effect of removing the 3' -UTR was not clear because the difference in Flag-Dazl mRNA expression levels between Dazl 3F and Dazl 3F; Flp was less than 2-fold, and the total Dazl expression level (Flag-Dazl + endogenous Dazl) was not changed between Dazl 3F and Dazl 3F; Flp except in the P0 ovary (Fig 3B and 3C). In contrast to the small increase in the mRNA level, FLAG-DAZL expression was greatly increased after birth (Fig 3D). Although FLAG-DAZL (filled arrowheads) decreased in Dazl 3F ovaries from P0 onward, which was consistent with the reduction in endogenous DAZL (open arrowhead), its expression was continuously observed in P0, 1W, and 2W ovaries when the 3'-UTR was removed. Quantification of FLAG-DAZL expression revealed that its expression increased 20-fold in Dazl 3F; Flp at P0 (Fig 3E). The results of western blotting were also supported by immunostaining. Both total- and FLAG-DAZL expression was strongly observed in Dazl 3F; Flp ovaries (Fig 3F and S3 Fig), whereas their expression levels in WT and Dazl 3F ovaries were comparable with those in Dazl cKO ovaries. Furthermore, strong DAZL expression was observed in all stages of follicular oocytes in Dazl 3F; Flp ovaries (S3 Fig). These data indicate that DAZL is post-transcriptionally suppressed in a 3'-UTR-dependent manner in follicular oocytes.

**Role of DAZL suppression in female reproduction**

To investigate the role of 3'-UTR-dependent DAZL suppression in female reproduction, we crossed BAC transgenic females with WT males when female mice reached 6 weeks old. Each pair was kept in a breeding cage until female mice became 30 weeks old, and the number of pups delivered during this period was counted. We found that BAC transgenic females were fertile regardless of the presence or absence of the Dazl 3' -UTR (Fig 4A). The number of total pups was slightly lower by in Dazl3F females (39.8±5.5, n = 5) compared with control females (53.1±9.1, n = 7). Interestingly, Dazl 3F; Flp females produced less than half the normal number of pups (18.6±11.3, n = 5). As 3FLAG-DAZL protein rescued the germless phenotype in Dazl 3F/- mice [26], it is unlikely that the observed litter size reduction was caused by the expression of 3FLAG-DAZL. Thus, these results suggest that DAZL overexpression results in litter size reduction. We next analyzed the number of deliveries and the number of pups in each delivery. The number of pups in each delivery was fewer by Dazl 3F and Dazl 3F; Flp mice (Fig...
Role of post-transcriptional suppression of Dazl in oocytes

A

B

C

D

E

F

DAZL+FLAG+FOXL2

Control

Dazl/F,F:Gdf9/Cre

Dazl 3F

Dazl 3F,Flp
4B), but the number of deliveries was not significantly different among genotypes (Fig 4C). These results suggest that the reduced female fecundity was due to defects during follicular development, fertilization, or zygote development after fertilization, but not to the shortened reproductive lifespan.

**Excess DAZL is deleterious for pre-implantation development**

To determine the cause of the litter size reduction in the DAZL overexpressing females, we examined the development of oocytes, fertilization, and pre-implantation development. Histological analysis revealed that BAC transgenic ovaries did not have significantly different numbers of primordial, primary, secondary or antral follicles compared with WT ovaries (Fig 5A and 5B). We next asked whether ovulation normally occurs by counting the number of one-cell embryos ovulated. However, the number was not significantly different among the genotypes (Fig 5C). These data suggest that folliculogenesis and subsequent ovulation proceeds normally even in BAC transgenic females. Thus, to examine whether these ovulated eggs developed normally, we measured the proportion of blastocysts by flushing E3.5 embryos from oviducts. We cultured the collected embryos for a further two days and then counted the embryos because the different timing of sexual behavior in each mouse pair influences the progression of early embryonic development (Fig 5D). We found that only 56.1% of embryos derived from Dazl 3F;Flp females developed into blastocysts, whereas more than 97.3 and 97.1% embryos derived from control and Dazl 3F females became blastocysts, respectively. The development of the remaining 43.9% of Dazl 3F;Flp embryos stopped at the 1-cell to morula stages. These observations were reproduced in 1-cell culture experiments, in which development was specifically disrupted in embryos from Dazl 3F;Flp females (S4A Fig). Statistical analysis revealed that development was arrested during 1- to 4-cell and 8-cell to blastocyst stages in embryos from Dazl3F;Flp mother (S4B Fig). Furthermore, the spindle morphology was normal in Dazl3F;Flp oocytes (S2 Fig). These results indicate that the reduction of pups in Dazl 3F;Flp females was due to defective pre-implantation development. As strong DAZL expression was observed in Dazl 3F;Flp until the MII oocyte stage but decreased in 1-cell embryos and was no longer detectable in 2-cell embryos (S5 Fig), it is likely that abnormal expression of DAZL in oocytes causes the defective pre-implantation development.

**Discussion**

In this study, we demonstrated that DAZL expression is post-transcriptionally suppressed in a 3'-UTR-dependent manner in postnatal oocytes. Although DAZL has been thought to function in postnatal oocytes, our data suggest that DAZL is not required for postnatal oocyte development. Supporting this idea, analysis of conditional Dazl knockout mice revealed that...
DAZL is dispensable for postnatal oocyte development. Furthermore, excess DAZL expression results in litter size reduction. These data indicate that post-transcriptional regulation of Dazl plays a crucial role in normal female reproduction.
Fig 5. Defective pre-implantation development is a cause of the litter size reduction. (A) PAS staining of control, Dazl 3F, and Dazl 3F;Flp ovaries at 5 weeks after birth. PrF, PF, SF, and AF are same as in Fig 2C. Scale bars, 100 μm. (B) Follicle counting analysis for control (n = 15), Dazl 3F (n = 6), and Dazl 3F;Flp (n = 6) using ovarian sections at 5 weeks after birth. Error bars represent S.D. ns, no significant difference among control, Dazl 3F, and Dazl 3F;Flp. (C) The average number of ovulated eggs from control (n = 15), Dazl 3F (n = 7), and Dazl 3F;Flp (n = 7) females. Error bars, S.D. ns, no significant difference among control, Dazl 3F, and Dazl 3F;Flp (Tukey HSD test). (D) Analysis of pre-implantation development in BAC transgenic females. (Left) E3.5 embryos from control, Dazl 3F, and Dazl 3F;Flp females. Yellow arrowheads indicate abnormal...
It was previously reported that DAZL was expressed in growing oocytes [16], but a later study stated that DAZL was not detectable in the postnatal ovary [22]. Therefore, it has been unclear whether DAZL plays a role in follicular oocytes. Our results answered this question; DAZL expression is suppressed in follicular oocytes and is dispensable for oogenesis after birth. Interestingly, this suppression coincides with the formation of primordial follicles. As Dazl mRNA was continuously expressed in oocytes regardless of developmental stage, it is likely that post-transcriptional gene regulatory mechanisms are altered between cystic oocytes and follicular oocytes. Importantly, DAZL suppression requires its 3'-UTR, suggesting the presence of some mechanisms regulating DAZL expression in postnatal oocytes. In general, post-transcriptional regulation is conducted by microRNAs and RNA-binding proteins [27]. However, it was reported that the function of microRNA is globally suppressed in oocytes and early embryonic development [28]. Thus, it is possible that Dazl expression is regulated by some RNA-binding proteins (RBPs). One possible candidate RBP for DAZL suppression is CPEB1, a mammalian ortholog of Xenopus CPEB. CPEB acts as both a translational activator and suppressor of its target mRNAs depending on its phosphorylation state [29,30]. CPEB1 is expressed in postnatal oocytes and promotes the translation of Dazl in MII oocytes[20], thus it may suppress Dazl in follicular oocytes. Further expression and functional analyses, including the phosphorylation state, of CPEB1 are required to address this question.

Our oocyte-specific Dazl KO females exhibited no ovarian developmental defect and the MII oocytes had no spindle abnormalities. Furthermore, the Dazl cKO females produced normal numbers of pups. These observations were inconsistent with Chen and colleague’s results that DAZL depletion in MII oocytes results in defective spindle formation in meiosis II [20]. One possible explanation for this contradiction is the method of gene depletion. We used the Cre-loxP system for Dazl cKO in vivo, whereas Chen et al. used morpholino knock-down in MII oocytes. A recent zebrafish report found that approximately 80% of phenotypes induced by morpholino do not correlate with mutant phenotypes induced by ZFN, TALEN or CRISPER/Cas9; therefore, the above-mentioned knock-down phenotype may have emerged due to indirect effects [31]. Alternatively, it is possible that some system that compensates for DAZL function works in Dazl -cKO MII oocytes because a previous study reported that the activation of a compensation system rescued deleterious mutations, which was not observed after translational or transcriptional knockdown [32]. Further analysis is required to evaluate the contribution of RNA-binding proteins for the progression of meiosis II.

Although DAZL expression is suppressed after birth, introducing the BAC transgenic allele in the Dazl+/+ background reduced litter size even in the presence of the 3'-UTR (Fig 4). As a previous study reported that Dazl dosage in females influences their litter size, and Dazl+/− females produced more pups than Dazl+/+ females [22], the slight reduction in litter size by our Dazl 3F mice may be attributed to the dosage effect. However, our histological and embryo culture experiments did not reveal any abnormalities in Dazl 3F mice. In addition, we were unable to observe obvious differences in resorption after implantation. One possible explanation is that insertion of the BAC transgene influences female reproduction.

Our results suggest that the suppression of Dazl translation in follicular oocytes is required for producing the proper number of progeny. However, why excess DAZL expression causes defective pre-implantation development remains still unclear. DAZL has been implicated in...
the positive regulation of translation [14,33,34], thus it is possible that the observed defect may be due to abnormal translational promotion. Alternatively, it is also possible that excess DAZL abnormally suppresses its target RNAs because DAZL works as a component of stress granules, cytoplasmic RNP granules involved in translational suppression or mRNA storage, in the testis [35,36]. Therefore, it is likely that suppression of DAZL expression in follicular oocytes is an important molecular mechanism for controlling proper gene expression.

**Materials and methods**

**Ethics statement**

All mouse experiments were approved by the Animal Experimentation Committee at the National Institute of Genetics (approval number 30–5) and Yokohama National University (approval number 2017–09) and conducted under the Regulations for Animal Experiments at the National Institute of Genetics, Research Organization of Information and Systems and the guideline at Yokohama National University.

**Mice**

Mice were housed in a specific-pathogen-free animal care facility at the National Institute of Genetics (NIG). All experiments were approved by the NIG Institutional Animal Care and Use Committee and the animal experimental committee at Yokohama National University. The genetic background of mice used in this study was C57BL/6N (Clea Japan), except in the DAZL expression analysis and conditional Dazl knockout mice (mixed genetic background of ICR and C57BL/6N). The BAC-carrying transgenic mouse line was generated in a previous study [26]. The BAC transgenic mice were backcrossed with C57BL/6N at least 3 times. Dazl flox mice were generated from an ES cell line produced by the Knock Out Mouse Project (KOMP, Dazl tm2a (KOMP) Wtsi).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)**

Total RNAs were isolated from whole gonads of wild-type and BAC transgenic mice at each stage by RNeasy Mini Kit (Qiagen). One hundred ng (1W to 5W) and 40 ng (E12.5 to P0) of total RNA were used for cDNA synthesis using Prime Script RT Reagent Kits with gDNA Erase according to the manufacturer’s protocol (Takara). Real time PCR was performed with KAPA SYBR FAST qPCR kits using a thermal cycle dice real time system (Takara). The obtained data was normalized by Mvh.

The following primers were used for PCR amplification:

- **Dazl**
  - Forward: 5’-CACGCCTCAGTGACTCGGAGAC-3’
  - Reverse: 5’-CGAAGCATACAGACAGTGTC-3’
- **Mvh**
  - Forward: 5’-GTTGAAGTATCTGGACATAATGACAC-3’
  - Reverse: 5’-CGAGTTGGCTACTCAAATACACTC-3’
- **G3pdh**
  - Forward: 5’-ACCACAGTCCATGCCATTAC-3’
  - Reverse: 5’-TCCCACCCCTTGGGTGTAGTA-3’
- **FLAG tagged Dazl**
  - Forward: 5’-CAGCGCTCAGTGACTCGGAGAC-3’
  - Reverse: 5’-CACCGTCATGGCTTGTAGTC-3’
- **Dazl cKO**
  - Forward: 5’-GACCTACATGCGCCTCCACCACATG-3’
  - Reverse: 5’-AACAGGCCAGCTGATATCCGATGATG-3’
Western blotting

Ovaries were lysed in RIPA buffer (50 mM Tris-HCl (pH8.0), 150 mM NaCl, 0.5% Sodium deoxycholate, 0.1% Sodium dodecyl sulfate, 1% NP-40) and sonicated. After removing the debris by centrifugation, lysates were dissolved in 2xSDS sample buffer, and heated. MII oocytes and 1-cell zygotes were lysed in 10μl 2xSDS sample buffer. Each sample was applied to gels for SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in 5% skim-milk in TBST (50mM Tris-HCl (pH7.5),150mM NaCl, 0.1% Tween-20) for 1 hour at room temperature (RT). Membranes were incubated with primary antibodies (Abcam, anti-rabbit DAZL antibody, 1:2000 for ovarian sample or 1:500 for MII, 1-cell and 2-cell zygote / Abcam, anti-rabbit DDX4 antibody, 1:1000/Santa Cruz, anti-mouse β-actin, 1:2000/Sigma, anti-FLAG antibody, 1:2000) diluted in 3% skim-milk in TBST or Can Get Signal immunoreaction Enhancer Solution (TOYOBO) overnight at 4˚C. After washing the membranes with TBST, membranes were incubated with anti-rabbit HRP-conjugated secondary antibody (Cell signaling, 1:5000) and anti-mouse HRP-conjugated secondary antibody (Cell signaling, 1:5000) in TBST or Can Get Signal immunoreaction Enhancer Solution, respectively, at RT for 90 min. The signals were detected by SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) and AE-9300H EZ-CAPTURE MG (ATTO). Western blotting results were quantified by Gel Analysis with ImageJ software.

Immunostaining for paraffin embedded samples

Ovaries were fixed in 4% PFA (paraformaldehyde) at 4˚C overnight and embedded in paraffin wax. Each sample was sliced at 6-μm thickness and placed on glass slides. After removing the paraffin wax and autoclaving in antigen unmasking solution/high pH (Vector Laboratories), glass slides were washed in PBST (PBS, 0.1%Tween-20) and pre-incubated in 3% skim milk in PBST blocking solution at RT for 1 hour. The slides were reacted with primary antibodies (Anti-DAZL antibody, Abcam, 1:200 / Anti-FOXL2 antibody, Abcam, 1:200/ Anti-FLAG antibody, SIGMA, 1:10000) at 4˚C overnight. Then, slides were washed with PBST and incubated with second antibodies (Alexa 488 Donkey anti-Rabbit, Life technologies, 1:1000 /Alexa 594 Donkey anti-Mouse, Life technologies, 1:1000/Alexa 594 Donkey anti-Goat, Life technologies, 1:1000 / Cy5 Donkey anti-goat, Rockland, 1:1000) at RT for 60 min. DNA was counter-stained with DAPI, and fluorescent images were obtained using confocal microscopy FV1200 (Olympus).

Immunostaining for frozen samples

Ovaries were fixed with 4% PFA (paraformaldehyde) at 4˚C overnight, which was then graded to 30% sucrose, and ovaries were then embedded in O.C.T compound (Sakura Fine tek). Each sample was sliced at 6-μm thickness. After removing the O.C.T compound, slides were incubated with 3% skim milk in PBST (PBS, 0.1% Tween-20) for 1 hour. Primary antibody reactions were performed with the following dilutions (Anti-DAZL antibody, Abcam, 1:200 / Anti-FOXL2 antibody, Abcam, 1:200) at 4˚C overnight. After washing with PBST, secondary antibody reaction was performed with the following dilutions (Alexa 488 Donkey anti-Rabbit, Life technologies, 1:400 /Alexa 594 Donkey anti-Goat, Life technologies, 1:400) at RT for 90 min. Slides then were counter-stained by DAPI at RT for 15 min. Fluorescent images were obtained by confocal microscopy FV1200 (Olympus).
Immunostaining of MII oocytes

MII oocytes were fixed with MeOH at -20°C for 3 minutes, washed with PBS-TX (0.1%TritionX, PBS), and were then incubated with blocking solution (3%BSA, 0.1%TritionX, PBS) at 4°C for 3 hours. Primary antibody reactions were performed with the following dilutions (Anti-α-tubulin antibody, Sigma, 1:1000) at 4°C overnight. After washing with PBS-TX, secondary antibody reaction was performed with the following dilutions (Alexa 488 Donkey anti-Rabbit, Life technologies, 1:1000) and DAPI at RT for 60 min. Then, oocytes were washed with PBS-TX. Fluorescent images were obtained using confocal microscopy FV1200 (Olympus).

Histological analysis

Histological analysis was carried out by PAS (Periodic acid-Schiff) staining according to the standard protocol. Briefly, ovaries were fixed in Bouin solution, embedded in paraffin wax, and sliced at 6-μm thickness. The sections were submerged in xylene, 100%, 90%, 70% ethanol, and distilled water at RT, and stained with PAS solution. Ovarian images were obtained with an inverted microscope BX 51 and 61(Olympus). Follicle stages were counted on every 5 sections.

Litter size investigation

*Dazl*+/+, *Dazl* 3F, and *Dazl* 3F;Flp females at 6 weeks old were crossed with C57BL/6N males, and kept together until female mice reached 30 weeks old. The number of pups and deliveries was recorded. Pups were removed after counting the number and sex. Females that killed their pups were excluded from the analysis.

Collection of MII oocytes, 1-cell, 2-cell embryos and blastocysts

To obtain MII, 1-cell and 2-cell oocytes for western blotting, female mice were injected with PMSG (ASKA Pharmaceutical). Forty-eight hours after PMSG injection, mice were stimulated with hCG (ASKA Pharmaceutical) for 14 h and MII oocytes were collected. To obtain western blotting samples of 1-cell and 2-cell embryos, each female was crossed with a WT male after hCG injection. Eggs with obvious abnormalities were removed from experiments. One-cell embryos for investigation of ovulation number and pre-implantation development investigation were obtained from the ampulla of pregnant females at E0.5. Blastocysts for examining progression of early embryonic development were obtained by flushing oviducts at E3.5. Collected blastocysts were cultured for two days in KSOM medium (Ark resource).

Statistical analysis

Significance was assessed by the Student’s t-test for differences between two samples. For quantitative analyses among multiple samples, significance was assessed using one-way ANOVA followed by Tukey HSD (Honest Significant Difference) test. Asterisks in figures indicate significance: *P < 0.05, **P < 0.005.

Supporting information

S1 Fig. *Dazl*fllox/lox females recapitulate the *Dazl* knockout phenotype. (A) A photograph of *Dazl*fllox/+ and *Dazl*fllox/lox 3W ovaries. Scale bar, 1 mm. (B) PAS staining of *Dazl*fllox/+ and *Dazl*fllox/lox 3W ovaries. Note that homozygous mutants contain no oocytes, reminiscent of the previous *Dazl* knockout ovary [16]. PrF, PF, SF, and AF are the same as in Fig 2C. Scale bar, 100 μm.

(TIF)
S2 Fig. Spindle morphology in Dazl 3F; Flp and Dazl cKO M II oocytes. Immunostaining of MII oocytes of control (n = 85), Dazl<sup>fl/fl</sup>;Gdf9iCre (n = 34), Dazl 3F (n = 45) and Dazl 3F; Flp (n = 37) MII oocytes using an antibody against α-tubulin (green). DNA was counterstained with DAPI (magenta). Scale bar, 40 µm.

(TIF)

S3 Fig. DAZL expression in postnatal oocytes. Immunostaining of 5W ovaries of control, Dazl<sup>fl/fl</sup>;Gdf9iCre, Dazl 3F and Dazl 3F; Flp mice. Antibodies against for DAZL (green) and FOXL2 (magenta) were used, and DNA was counterstained with DAPI. PrF, PF, SF, and AF are same as in Fig 2C. Scale bar, 50 µm.

(TIF)

S4 Fig. Survival rate analysis from 1-cell zygote to blastocyst stage. (A) Survival rates of pre-implantation embryos. One-cell stage zygotes (n = total number of embryos examined, number of used mothers) were collected from control (n = 120, 15), Dazl 3F (n = 59, 7) and Dazl 3F; Flp (n = 54, 7) mothers. The proportion of surviving zygotes at each stage was calculated as follows: the number of surviving zygotes out of the number of 1-cell zygotes in each experiment (mother). Error bars, S.D. Significance level of changes are indicated (Tukey HSD; ++P<0.005, *P<0.05).

(B) Statistical analysis of surviving zygotes from 1-cell zygotes to blastocysts in Dazl 3F; Flp (n = 54). The p-value for the average number of zygotes in each stage was calculated using Tukey HSD. P<0.05 is written in red.

(TIF)

S5 Fig. DAZL expression in preimplantation embryos. (A) Western blotting analysis of MII oocytes and 1- and 2-cell embryos. Both FLAG and endogenous DAZL were detected using the anti-DAZL antibody. Anti-β actin antibody was used as a loading control. Note that both FLAG and endogenous DAZL were not detectable in 2-cell embryos. Filled and open arrowheads indicate FLAG- and endogenous DAZL, respectively.

(B) Quantification of western blotting results for MII and 1-cell samples. The vertical axis represents relative DAZL expression level normalized by β actin.

(TIF)

Acknowledgments

We thank Dr. T. Chinen, Mr S. Yamamoto and Dr. D. Kitagawa for helping with the immunostaining of MII spindle and Danelle Wright for editing this manuscript.

Author Contributions

Conceptualization: Kurumi Fukuda, Yuzuru Kato.

Data curation: Kurumi Fukuda.

Formal analysis: Kurumi Fukuda.

Funding acquisition: Kurumi Fukuda, Atsushi Suzuki, Yuzuru Kato, Yumiko Saga.

Investigation: Kurumi Fukuda, Aki Masuda, Takuma Naka.

Methodology: Kurumi Fukuda.

Project administration: Yuzuru Kato, Yumiko Saga.
Resources: Kurumi Fukuda, Atsushi Suzuki, Yuzuru Kato.
Supervision: Atsushi Suzuki, Yuzuru Kato, Yumiko Saga.
Visualization: Kurumi Fukuda.
Writing – original draft: Kurumi Fukuda.
Writing – review & editing: Yuzuru Kato, Yumiko Saga.

References
1. Thomson TC, Fitzpatrick KE, Johnson J (2010) Intrinsic and extrinsic mechanisms of oocyte loss. Mol Hum Reprod 16: 916–927. https://doi.org/10.1093/molehr/gaq066 PMID: 20651035
2. Weill L, Belloic E, Bava FA, Mendez R (2012) Translational control by changes in poly(A) tail length: recycling mRNAs. Nat Struct Mol Biol 19: 577–585. https://doi.org/10.1038/nsmb.2311 PMID: 22664985
3. Kang MK, Han SJ (2011) Post-transcriptional and post-translational regulation during mouse oocyte maturation. BMB Rep 44: 147–157. https://doi.org/10.5483/BMBRep.2011.44.3.147 PMID: 21429291
4. Groisman I, Huang YS, Mendez R, Cao Q, Theurkauf W, et al. (2000) CPEB, maskin, and cyclin B1 mRNA at the mitotic apparatus: implications for local translational control of cell division. Cell 103: 435–447. PMID: 11081630
5. Stutz A, Conne B, Huarte J, Gubler P, Volkel V, et al. (1998) Masking, unmasking, and regulated polyadenylation cooperate in the translational control of a dormant mRNA in mouse oocytes. Genes Dev 12: 2535–2548. PMID: 9716406
6. Racki WJ, Richter JD (2006) CPEB controls oocyte growth and follicle development in the mouse. Development 133: 4527–4537. https://doi.org/10.1242/dev.02651 PMID: 17050619
7. Guzeloglu-Kayisli O, Laloti MD, Aydiner F, Sasson I, Ibay O, et al. (2012) Embryonic poly(A)-binding protein (EPAB) is required for oocyte maturation and female fertility in mice. Biochem J 446: 47–58. https://doi.org/10.1042/BJ20120467 PMID: 22621333
8. Tay J, Richter JD (2001) Germ cell differentiation and synaptonemal complex formation are disrupted in CPEB knockout mice. Dev Cell 1: 201–213. PMID: 11702780
9. Mak W, Fang C, Holden T, Dratver MB, Lin H (2016) An Important Role of Pumilio 1 in Regulating the Development of the Mammalian Female Germline. Biol Reprod 94: 134. https://doi.org/10.1095/biolreprod.110.984370
10. Medvedev S, Pan H, Schultz RM (2011) Absence of MSY2 in mouse oocytes perturbs oocyte growth and maturation, RNA stability, and the transcriptome. Biol Reprod 85: 575–583. https://doi.org/10.1095/biolreprod.111.091710 PMID: 21613634
11. Yu J, Hecht NB, Schultz RM (2002) RNA-binding properties and translation repression in vitro by germ cell-specific MSY2 protein. Biol Reprod 67: 1093–1098. PMID: 12297523
12. Fu XF, Cheng SF, Wang LQ, Yin S, De Felici M, et al. (2015) DAZ Family Proteins, Key Players for Germ Cell Development. Int J Biol Sci 11: 1226–1235. https://doi.org/10.7150/ijbs.11536 PMID: 26327816
13. Venables JP, Ruggiu M, Cooke HJ (2001) The RNA-binding specificity of the mouse Dazl protein. Nucleic Acids Res 29: 2479–2483. PMID: 11410564
14. Reynolds N, Collier B, Maratou K, Bingham V, Speed RM, et al. (2005) Dazl binds in vivo to specific transcripts and can regulate the pre-mesiotic translation of Mvh in germ cells. Hum Mol Genet 14: 3899–3909. https://doi.org/10.1093/hmg/ddi414 PMID: 16278232
15. Jenkins HT, Maikova B, Edwards TA (2011) Kinked beta-strands mediate high-affinity recognition of mRNA targets by the germ-cell regulator DAZL. Proc Natl Acad Sci U S A 108: 18266–18271. https://doi.org/10.1073/pnas.1102306108 PMID: 22021443
16. Ruggiu M, Speed R, Taggart M, McKay SJ, Kilanowski F, et al. (1997) The mouse Dazl gene encodes a cytoplasmic protein essential for gametogenesis. Nature 389: 73–77. https://doi.org/10.1038/37987 PMID: 9288969
17. Saunders PT, Turner JM, Ruggiu M, Taggart M, Burgoyne PS, et al. (2003) Absence of mDazl produces a final block on germ cell development at meiosis. Reproduction 126: 589–597. PMID: 14611631
18. Gill ME, Hu YC, Lin Y, Page DC (2011) Licensing of gametogenesis, dependent on RNA binding protein DAZL, as a gateway to sexual differentiation of fetal germ cells. Proc Natl Acad Sci U S A 108: 7443–7448. https://doi.org/10.1073/pnas.1104501108 PMID: 21504946
19. Lin Y, Gill ME, Koubova J, Page DC (2008) Germ cell-intrinsic and -extrinsic factors govern meiotic initiation in mouse embryos. Science 322: 1685–1687. https://doi.org/10.1126/science.1166340 PMID: 19074348

20. Chen J, Melton C, Suh N, Oh JS, Horner K, et al. (2011) Genome-wide analysis of translation reveals a critical role for deleted in azoospermia-like (Dazl) at the oocyte-to-zygote transition. Genes Dev 25: 755–766. https://doi.org/10.1101/gad.2028911 PMID: 21460039

21. Koubova J, Hu YC, Bhattacharyya T, Soh YQ, Gill ME, et al. (2014) Retinoic acid activates two pathways required for meiosis in mice. PLoS Genet 10: e1004541. https://doi.org/10.1371/journal.pgen.1004541 PMID: 25102060

22. McNeilly JR, Watson EA, White YA, Murray AA, Spears N, et al. (2011) Decreased oocyte DAZL expression in mice results in increased litter size by modulating follicle-stimulating hormone-induced follicular growth. Biol Reprod 85: 584–593. https://doi.org/10.1095/biolreprod.110.086264 PMID: 21270429

23. Pepling ME, Spradling AC (2001) Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. Dev Biol 234: 339–351. https://doi.org/10.1006/dbio.2001.0269 PMID: 11397004

24. Toyooka Y, Tsunekawa N, Takahashi Y, Matsui Y, Satoh M, et al. (2000) Expression and intracellular localization of mouse Vasa-homologue protein during germ cell development. Mech Dev 93: 139–149. PMID: 10781947

25. Lan ZJ, Xu X, Cooney AJ (2004) Differential oocyte-specific expression of Cre recombinase activity in GDF-9iCre, Zp3cre, and Mxe2Cre transgenic mice. Biol Reprod 71: 1469–1474. https://doi.org/10.1095/biolreprod.104.031757 PMID: 15215191

26. Kato Y, Katsuki T, Kokubo H, Masuda A, Saga Y (2016) Dazl is a target RNA suppressed by mammalian NANOS2 in sexually differentiating male germ cells. Nat Commun 7: 11272. https://doi.org/10.1038/ncomms11272 PMID: 27072294

27. Loffreda A, Rigamonti A, Barabino SM, Lenzken SC (2015) RNA-Binding Proteins in the Regulation of miRNA Activity: A Focus on Neuronal Functions. Biomolecules 5: 2363–2387. https://doi.org/10.3390/biom5042363 PMID: 26437437

28. Suh N, Baehner L, Moltzahn F, Melton C, Shenoy A, et al. (2010) MicroRNA function is globally suppressed in mouse oocytes and early embryos. Curr Biol 20: 271–277. https://doi.org/10.1016/j.cub.2009.12.044 PMID: 20116247

29. Radford HE, Meijer HA, de Moor CH (2008) Translational control by cytoplasmic polyadenylation in Xenopus oocytes. Biochim Biophys Acta 1779: 217–229. https://doi.org/10.1016/j.bbabio.2008.02.002 PMID: 18316045

30. Mendez R, Hake LE, Andresson T, Littlepage LE, Ruderman JV, et al. (2000) Phosphorylation of CPE binding factor by Eg2 regulates translation of c-mos mRNA. Nature 404: 302–307. https://doi.org/10.1038/35005126 PMID: 10749216

31. Kok FO, Shin M, Ni CW, Gupta A, Grosse AS, et al. (2015) Reverse genetic screening reveals poor correlation between morpholino-induced and mutant phenotypes in zebrafish. Dev Cell 32: 97–108. https://doi.org/10.1016/j.devcel.2014.11.018 PMID: 25533206

32. Rossi A, Kontarakis Z, Gerri C, Nolte H, Holper S, et al. (2015) Genetic compensation induced by deleterious mutations but not gene knockdowns. Nature 524: 230–233. https://doi.org/10.1038/nature14580 PMID: 26168398

33. Collier B, Gorgoni B, Loveridge C, Cooke HJ, Gray NK (2005) The DAZL family proteins are PABP-binding proteins that regulate translation in germ cells. EMBO J 24: 2656–2666. https://doi.org/10.1038/sj.emboj.7600738 PMID: 16001084

34. Reynolds N, Collier B, Bingham V, Gray NK, Cooke HJ (2007) Translation of the synaptonemal complex component Sycp3 is enhanced in vivo by the germ cell specific regulator Dazl. RNA 13: 974–981. https://doi.org/10.1261/rna.465507 PMID: 17526644

35. Kim B, Cooke HJ, Rhee K (2012) DAZL is essential for stress granule formation implicated in germ cell survival upon heat stress. Development 139: 568–578. https://doi.org/10.1242/dev.075846 PMID: 22223682

36. Kedersha N, Anderson P (2002) Stress granules: sites of mRNA triage that regulate mRNA stability and translatability. Biochem Soc Trans 30: 963–969. https://doi.org/10.1042/ PDT040955