The highly conserved LAMMER/CLK2 protein kinases prevent germ cell overproliferation in Drosophila

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ABSTRACT

Germ cells undergo proper mitotic amplification before entering meiosis. The mitosis/meiosis switch drives the germ cells to leave the potential stem cell pool and become terminally differentiated. This important process is tightly controlled in the spermatogenesis of all animals. However, a unifying mechanism has yet to be unraveled. Drosophila spermatogenesis is an ideal system to dissect the regulatory program of the mitosis/meiosis switch. The timely accumulation of the pro-differentiation factor Bam has been shown to be central in this process. In a Drosophila genetic screen, we discovered that the mutations in Doa, a gene encoding a member of the highly conserved LAMMER/Cdc2-like kinase (CLK) family, cell-autonomously induced the germ cell overproliferation due to the failed transition from mitosis to meiosis. Additional Bam expression in Doa mutant germline promoted the differentiation from the mitotic to the meiotic state. Remarkably, the human or murine CLK2 could prevent the germline overproliferation and even restore the fertility of Doa mutant flies. Such rescuing activity of Doa or its human homolog requires a conserved residue in their predicted kinase catalytic domain. We propose that LAMMER/Cdc2-like kinase, represented by Doa and its mammalian homolog CLK2, is a critical and conserved component in the regulatory program of the mitosis-to-meiosis switch.

Introduction

The mitosis-to-meiosis decision is a form of differentiation unique to the germ cell development. Mitosis of germline stem cell (GSC) renewal and subsequent cell amplification sustains the continuous gamete production, whereas meiosis of the amplified germ cells reduces the chromosome number in half and launches the gamete maturation program. Additionally, while the mitotic-amplifying cells are able to de-differentiate towards the GSCs (Brawley and Matunis, 2004; Kai and Spradling, 2004), meiotic program drives them to leave the potential stem cell pool and become terminally differentiated. Drosophila spermatogenesis provides an easy-to-track lineage system to address the questions regarding the control of mitosis/meiosis transition. Unlike that in oogenesis where this transition is complicated by the oocyte specification, all spermatogenic germ cells derived from one lineage clone enter meiosis uniformly and synchronously [for review see (Fuller, 1998)]. Thus, it is more straightforward to dissect this process in the male germline than in the female one.

In Drosophila testis, the germ cells of mitotic or meiotic state are represented by the transit-amplifying spermatogonia or the dramatically enlarged spermatocytes, respectively (Fuller, 1998; Insco et al., 2009). Discovered as genes disrupted in the over-amplifying spermatogonia, bam and bgcn encode proteins that play a central role in the switch program from mitosis to meiosis in Drosophila (Gateff, 1982; Gonczy et al., 1997; McKearin and Spradling, 1990; Ohlstein et al., 2000). Bam possessing no apparent conserved domain and Bgcn being predicted as RNA-binding, their molecular functions remain largely unknown (McKearin and Spradling, 1990; Ohlstein et al., 2000). One clue came from the discoveries in the regulation of GSC determination in Drosophila ovaries, where Bam–Bgcn complex inhibits Nanos translation by Bam's direct interaction with Pumilio (Kim et al., 2010; Li et al., 2009).

Bam is normally expressed in a stereotyped pattern during spermatogonial amplification, that is, it accumulates during 4- to 8-cell stages then quickly declines at late 16-cell stage (Gonczy et al., 1997; Insco et al., 2009). bam dosage seems to set the time at which spermatogonial cells enter meiosis without obviously affecting the division rate of the transit amplification. Consequently, increasing or decreasing Bam levels drove the meiotic entry one mitotic cycle earlier or later than normal, respectively (Insco et al., 2009). Then how is Bam protein level tuned in such a fixed pattern to ensure the accurate timing of meiotic entry?

Transcriptionally, bam is under the negative control of BMP/Dpp-Gbb signaling which is the strongest around the apex of the testis (Kawase et al., 2004; Shivdasani and Ingham, 2003). This is

consistent with the gross expression pattern of Bam if the BMP source is solely at the apical tip. Nonetheless, the abrupt decline of Bam protein level at the 16-cell stage while the 16-cell cysts being positioned randomly from the apex implies the potential post-transcriptional regulation of Bam. In germline stem cells, Bam transcripts are targeted by *microRNA7* whose expression is downregulated by a nuclear factor Mael (Pek et al., 2009). Ectopic expression of HOW, an RNA-binding protein, delays Bam accumulation in spermatogonial cells (Monk et al., 2010). Moreover, Bam protein contains a PEST signal which is critical for Bam's proper turnover and thus the accurate timing of meiotic entry (Insco et al., 2009). Still, what monitors the Bam protein level and coordinates it with mitosis/meiosis decision remains to be explored.

There is plenty of genetic and histological evidence implicating the cross-species conservation of the spermatogenic program (Shah et al., 2010; White-Cooper and Bausek, 2010). Murine DAZL (Deleted in azoospermia-like) protein is an intrinsic factor required for meiotic entry (Lin et al., 2008). The DAZ family proteins have been demonstrated highly conserved as evidenced by the functional replacement assay, where the human BOULE (a DAZ family member) reversed the meiotic arrest of fly *boule* mutants (Xu et al., 2003). These observations illustrate that Drosophila spermatogenesis is an ideal experimental system to explore the functions of the mammalian proteins acting in the mitosis/meiosis control.

In a genetic screen searching for the factors involved in the differentiation/proliferation of the male germline, we isolated a mutant that in a cell-autonomous manner caused spermatogonial accumulation, indicative of a block to meiosis. We mapped the responsible mutation to a known locus, *Doa*, which encodes a LAMMER/CLK2 protein kinase of a highly conserved protein family whose developmental functions are unclear (Fig. 1) (Yun et al., 1994). We found that Doa acted genetically upstream of Bam in the mitosis/meiosis transition. Remarkably, the human and murine homologs of Doa restored the fertility of *Doa* mutant flies.

Materials and methods

Fly genetics

Doa^Dem (Rabinow et al., 1993), *Doa^HD* (Rabinow and Birchler, 1989), and *UAS-Doa-PJ* (*UAS-Doa69KD-long3′UTR*) flies (Kpebe and Rabinow, 2008b) were gifts from Leonard Rabinow; *Doa^z8* was an EMS allele generated in our lab; *bam^bg*, *bamGAL4VP16* (Chen and McKearin, 2003) and *UAS-bam-GFP* flies (Chen and McKearin, 2003) were gifts from Dahua Chen; *esg-lacZ* flies (Kiger et al., 2000) were from Richard Mann; *UAS-nlsGFP* flies were from Joaquim Culi; *nosGAL4* (#4442), *nosGAL4VP16* (#4937), *FRT82B ubiGFP* (#5628), and 3R deficiency flies were from Bloomington Drosophila Stock Center; *vasaGAL4* was generated by cloning the ~2.6 kb genomic fragment of *vasa* (Sano et al., 2002) into the *pC3G4* vector at *StuI-BamHI* sites. All crosses were raised at 25 °C.

Transgenic flies

The cDNA of *Doa-PS* (AT11333) and *Doa-PK* (LD31161) were obtained from Berkeley Drosophila Genome Project; the cDNA of *humanCLK2* (GeneBank BC014067.2) and *mouseCLK2* (GeneBank BC015080.1) were ordered from OriGene Technologies, Inc.. The cDNA of *Doa-PS*, *3flag-Doa-PS*, *3flag-Doa-PS^{1–138}*, *3flag-Doa-PS^{139–511}*, *3flag-Doa-PS^{K193A}*, *3flag-Doa-PS^{C275Y}*, *Doa-PK*, *humanCLK2*, *humanCLK2^{K192A}*, *humanCLK2^{C274Y}* and *mouseCLK2* were cloned into the *pUAST* vector. The 3flag sequences are: ATGGACTACAAAGACCA TGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGAC AAGCTT.
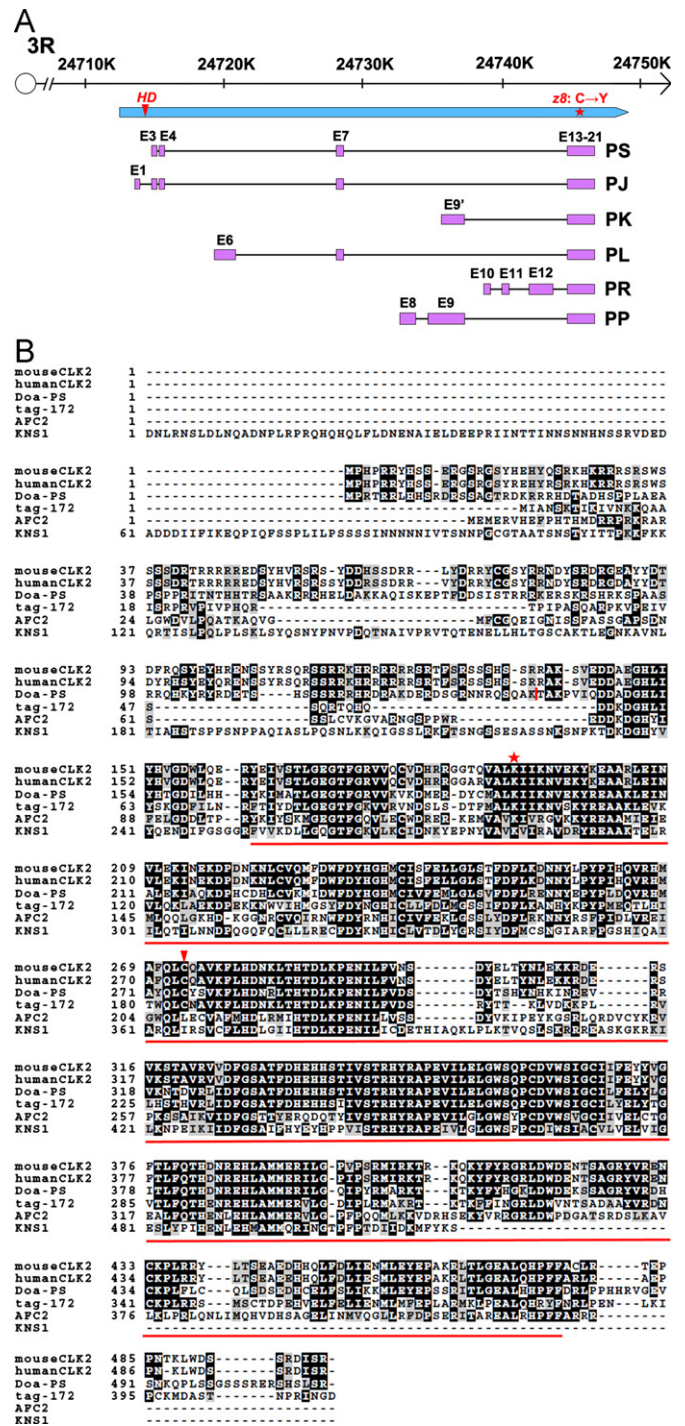

Fig. 1. The molecular information of *Doa* mutants. (A) The genomic organization of *Doa* locus. Only the published isoforms (Kpebe and Rabinow, 2008a) are shown and named according to FlyBase. *Doa^HD* is a *copia* retrotransposon insertion about 177 bp from the 3′ end of Exon-2 (Kpebe and Rabinow, 2008b). *z8* is a G to A mutation of the 62nd nucleotide in the Exon-17, causing an amino acid change from Cysteine to Tyrosine. (B) The protein sequence alignment of Doa-PS with other eukaryotic LAMMER kinases: KNS1 (*Saccharomyces cerevisiae*), AFC2 (*Arabidopsis thaliana*), tag-172 (*Caenorhabditis elegans*), mouseCLK2 (*Mus musculus*), and humanCLK2 (*Homo sapiens*). The alignment scores of Doa-PS with these LAMMER kinases are (respectively: identities of the full length, positives of the full length, identities of the catalytic domain, positives of the catalytic domain): KNS1 (44%, 63%, 45%, 65%), AFC2 (44%, 62%, 49%, 67%), tag-172 (61%, 76%, 63%, 79%), mouseCLK2 (71%, 83%, 74%, 85%), humanCLK2 (73%, 84%, 74%, 85%). C-terminal to the vertical bar of Doa-PS (a.a.139-511) is common to all known Doa isoforms; the residue K (star) is essential for the kinase activity in mouseCLK1~4 (Nayler et al., 1997) and the residue C (arrowhead) is mutated to Y in *z8* mutant; the underneath line indicates the catalytic domain of Doa-PS predicted by SMART software (http://smart.embl-heidelberg.de/).

Finally, the transgenic flies were generated by injecting the w^{1118} embryos.

Generation of clones

The germ cell clones were induced by nosGAL4:UAS-FLP system. The cyst cell clones were generated by the MARCM (mosaic analysis with a repressible cell marker) system (Lee and Luo, 1999). Mutant clones were induced by incubating the 1-day-old flies at 37 °C for half an hour and observations were made 5 days later.

Fertility test

To set up the cross, both the males for fertility check and w^{1118} virgins were 2-day-old. Each cross contained 1 male and 3 female virgins. The parents were discarded after 6 days. 15 days after the cross set-up, the empty pupal cases were counted as the number of progenies. 50 single males were tested for each genotype.

Immunostaining

The immunostaining protocol was described previously (Wang and Mann, 2003). Primary antibodies were used at the following dilutions: rabbit anti-Bam at 1:2000; rabbit anti-Vasa at 1:10,000 (these are from Dahua Chen); mouse anti-α-spectrin at 1:200 (3A9, DSHB); mouse anti-FasIII at 1:1000 (7G10, DSHB); guinea pig anti-

Achi at 1:4000 (Wang and Mann, 2003); mouse anti-GFP at 1:2000 (B-2, Santa Cruze); rabbit anti-STAT92E at 1:4000 (generated against polypeptides: GMADFDTITNFENF). AlexaFluor conjugated secondary antibodies were used at 1:4000 (Molecular Probes of Invitrogen). Fluorescent images were collected by OLYMPUS FV1000 Confocal microimaging system except for Fig. S2(B, B′), which was taken by Zeiss ApoTome microimaging system.

Determine the molecular information of z8

z8 (zengzhi-8) failed to complement with Bloomington stocks #430, 7688, 27361, and 27378 (deficiencies on 3R chromosome) in terms of lethality. The overlapping region is 3R(24696033~24816740), which covers 9 genes including Doa. Exon sequencing showed a G to A mutation of the 62nd nucleotide in Doa's 17th exon, causing a cysteine to tyrosine change.

Results

Doa is intrinsically required for the proper amplification of spermatogonia

The EMS-induced, clonal-analysis-based genetic screen generated a point mutation that converted a cysteine to a tyrosine in the predicted kinase catalytic domain of Doa (Doa^{z8}, Fig. 1B, arrowhead). Although this mutant is homozygous lethal at the organismal level, the mutant germline clonal cells are viable. However, two previously
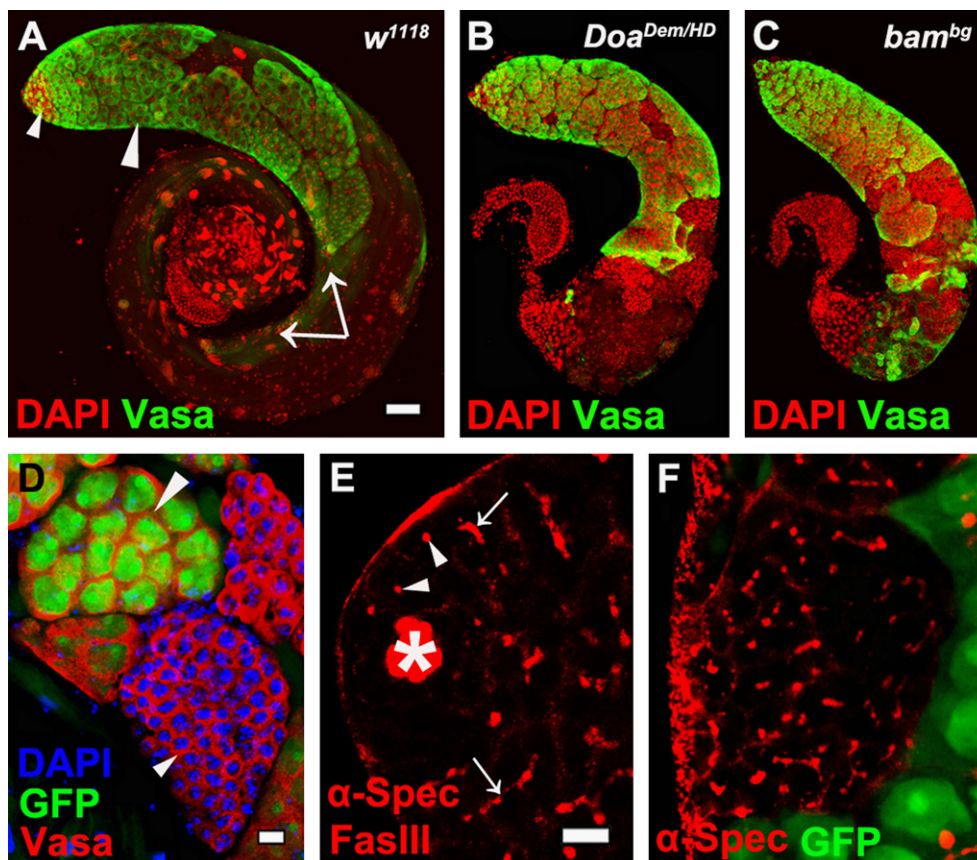

Fig. 2. Doa is intrinsically required for the proper amplification of spermatogonia. (A–C) The Doa mutant testes were filled with early stage germ cells, phenocopying bam mutant. w^{1118} is a wild type control (A), which contained early stage germ cells (small arrowhead), spermatocytes (big arrowhead), and differentiating spermatids (arrows). Scale bar: 50 μM in (A) for (A–C). (D) Loss of Doa in germ cells caused early germ cell over-proliferation (the GFP negative cyst indicated by the small arrowhead), while the adjacent wild type cyst contained 16 spermatocytes (the GFP positive cyst indicated by the big arrowhead). Genotype: nosGAL4/UAS-FLP; FRT82B Doa/FRT82B ubiGFP. Scale bar: 10 μM. (E, F) The over-proliferating germ cells mutated for Doa were spermatogonia. FasIII is the hub cell marker (asterisk in E) and α-spectrin labels the fusome. (E) w^{1118}, wild type controls. The GSCs and goniablasts contain dot-like fusomes (arrowheads), whereas spermatogonia contain the branched ones (arrows). (F) The fusomes of the 'tumorous' germ cells mutated for Doa were branched. Genotype: nosGAL4/UAS-FLP; FRT82B Doa/FRT82B ubiGFP. Scale bar: 10 μM in (E) for (E, F). Doa^{Dem}, Doa^{HD}, or Doa^{z8} gave similar results and the representative images are shown in (D and F).

documented *Doa* alleles, *Doa^{Dem}* (Rabinow et al., 1993) and *Doa^{HD}* (Rabinow and Birchler, 1989), when in transheterozygous can be viable but sterile, due to the spermatogonial accumulation which was very similar to that of *bam* mutant (Fig. 2A–C). *bam* is an extensively studied gene promoting the mitosis-to-meiosis transition in Drosophila spermatogenesis (Gonczy et al., 1997; Insco et al., 2009). Like the behavior of *bam*, *Doa* is also required in the germ cells for their differentiation from spermatogonia to spermatocytes, as shown in the mutant clonal analysis (Fig. 2D). To confirm that *Doa* indeed acts cell-autonomously in the germ cells, we positively labeled the mutant somatic cells and did not observe any abnormal growth in the germ cells surrounded by these mutant somatic cells (Fig. S1).

Simply judged by the morphology and DNA staining of the mutant cell clones, they could be GSCs, gonialblasts, or spermatogonia. To determine the developmental stage of the over-amplifying germ cells, we used 3 cellular markers: spectrin reveals the differential morphologies of the cytoskeletal network in different staged germ cells (Kiger et al., 2000; Lin et al., 1994); STAT92E and esgLacZ positively label only GSCs and gonialblasts (Chen et al., 2002; Kiger et al., 2000; Wang et al., 2006). The branched spectrin-staining (Fig. 2E and F) and the absence of STAT92E or esgLacZ signals (Fig. S2) in the over-accumulating *Doa* mutant germ cells clearly indicate that these cells are spermatogonia. Thus, we demonstrate that Doa is intrinsically required for the germline differentiation from spermatogonia to spermatocytes.

Doa acts genetically upstream of the differentiation factor Bam in the transition from spermatogonia to spermatocytes

Bam protein accumulation has been shown to set the threshold for ceasing spermatogonial proliferation and initiating the differentiation towards spermatocytes (Insco et al., 2009). Then how is Doa related to the pro-differentiation factor Bam? Can Doa expression drive the differentiation of *bam*-mutant germ cells or vice versa? We tried to determine the genetic relationship
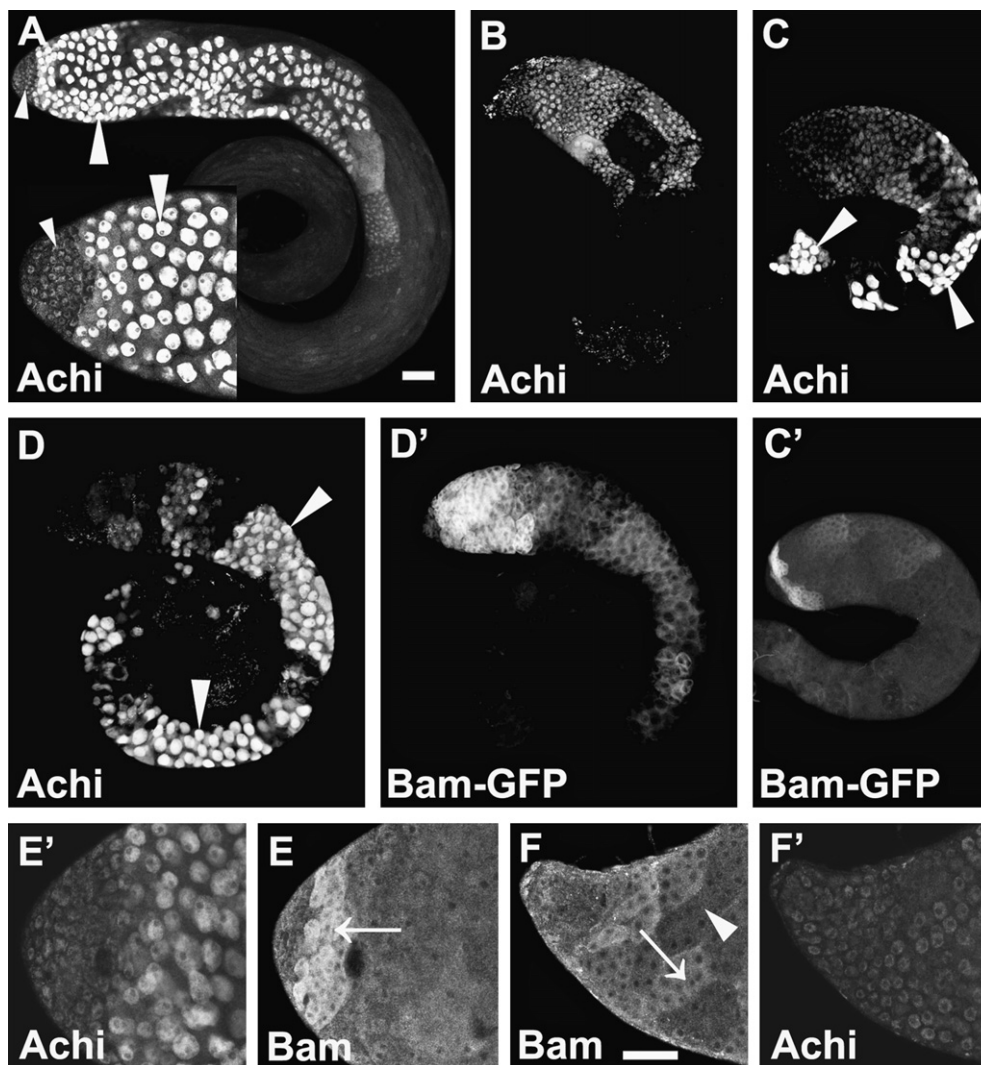

Fig. 3. *Doa* acts upstream of *bam* to promote the differentiation from spermatogonia to spermtocytes. (A–D′) Bam promoted the differentiation of *Doa* mutant spermatogonia to become spermatocytes. (A) *w^{1118}* testis. The lower left insert is a higher magnification of the testis tip. Achi is a nuclear protein weakly detected in early stage germ cells (small arrowheads) but highly in spermatocytes (big arrowheads) (Wang and Mann, 2003). (B) *Doa^{Dem/HD}* testes were filled with small early germ cells. (C–D′) Over-expression of Bam either in spermatogonia by *bamGAL4VP16* (C, C′) or in most germ cells by *vasaGAL4* (D, D′) promoted the differentiation of *Doa* mutant spermatogonia. Note the bright Achi-staining in the big germ cells (arrowheads in C and D, similar to the spermatocytes in A). Genotypes: *bamGAL4VP16/Y; UAS-bam-GFP/+; Doa^{Dem/HD}* (C, C′) and *vasaGAL4/UAS-bam-GFP; Doa^{Dem/HD}* (D, D′). Scale bar: 50 μM in (A) for (A–D′). (E–F′) Compare the apparent Bam protein levels in wild type and *Doa* mutant spematogonia. For all testes, both the immunostaining conditions and the image acquisition parameters were identical for each fluorescent channel. Achi served as an immunostaining control. (E, E′) *w^{1118}*, wild type controls. Note that Bam was expressed in spermatogonia, especially in 4- to 8-cell-stage ones (arrow in E). (F, F′) In the *Doa^{Dem/HD}* testis, Bam protein was detectable in some over-proliferating spermatogonia (arrow in F) but not in others (arrowhead in F). Scale bar: 30 μM in (F) for (E–F′).

Table 1
Over-expressing Bam in the germline promoted *Doa* mutant spermatogonia to become spermatocytes.

Genotype	Testes with spermatocytes/Total
Doa^{Dem/HD}	7/67
bamGAL4VP16/Y; UAS-bam-GFP/+; Doa^{Dem/HD}	81/88
vasaGAL4/UAS-bam-GFP; Doa^{Dem/HD}	52/52

between the two factors by the mutant rescuing experiments. Achi is a nuclear protein weakly detected in early stage germ cells but strongly in spermatocytes (Wang and Mann, 2003) and can serve as a marker to better reveal the different cell stages (Fig. 3A). In the *Doa* mutant testes, the big spermatocytes highly stained by Achi were rarely observed (Fig. 3B, Table 1). Over-expression of Bam in the *Doa* mutant background using two different germline drivers (*bamGAL4VP16* or *vasaGAL4*) overcame the block at the spermatogonial stage and facilitated the differentiation to spermatocytes (Fig. 3C and D, the presence
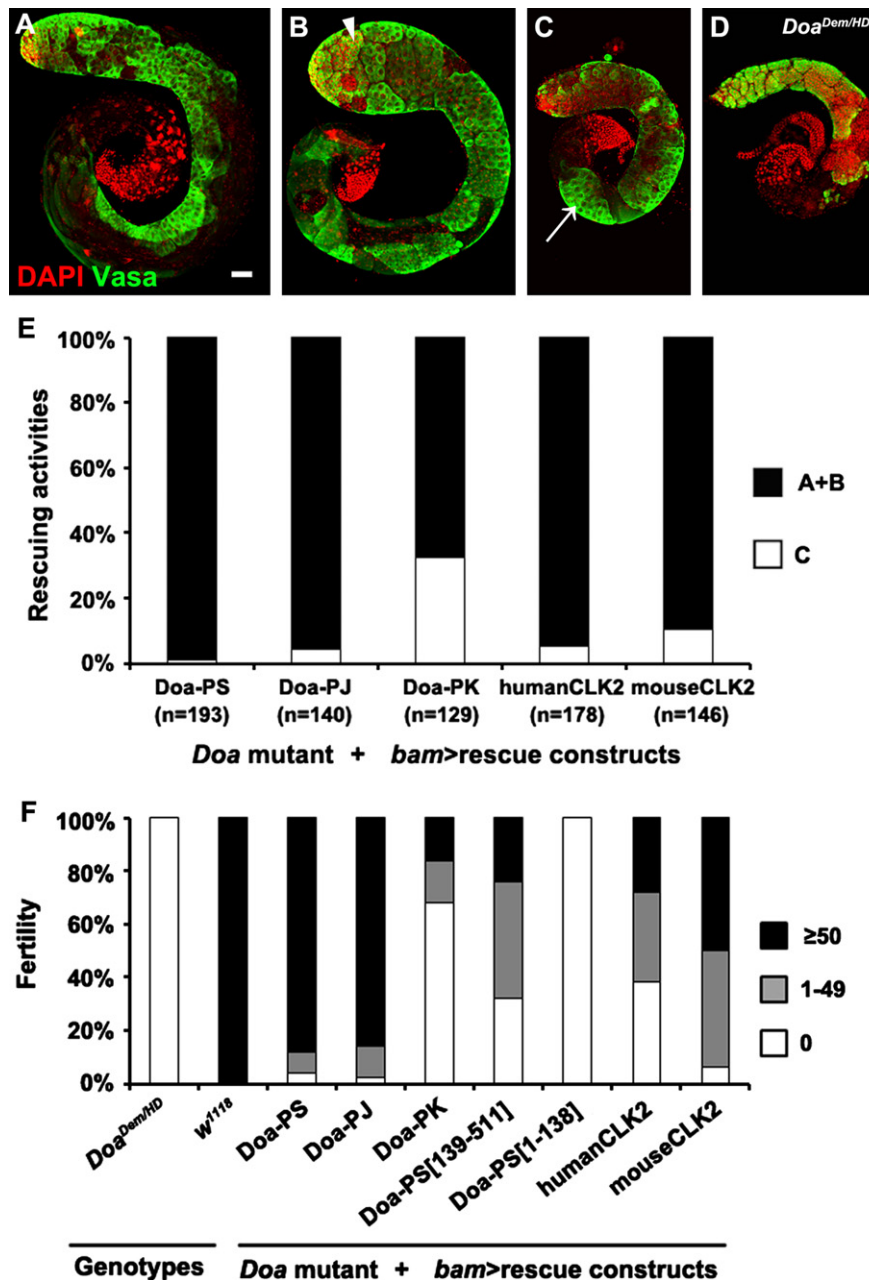

Fig. 4. Three Doa isoforms, humanCLK2, or mouseCLK2 can restore the fertility of *Doa* mutant. (A–C) The representative testes rescued to different extent: (A) similar to the wild type; (B) nearly normal but with a few germ cell tumors (arrowhead); (C) smaller than the wild type but containing many spermatocytes (arrow, large cells stained by Vasa). Scale bar: 50 μM in (A) for (A–D). (D) *Doa^{Dem/HD}* testes served as the non-rescued controls. (E, F) The rescuing activities of different Doa constructs, humanCLK2, or mouseCLK2. The targeted expression was driven by *bamGAL4VP16* (*bam* >). (E) The rescuing activities at the tissue level. 'n' is the total number of testes scored. (A+B) or (C) corresponds to the results in panel [(A) and (B)] or (C), respectively. (F) The fertility of different rescued flies. The detailed method was described in Materials and Methods. Each genotype was tested by 50 single crosses which were classified into 3 groups according to the number of progenies: ≥50, 1–49, or 0 progeny. The genotypes of "*Doa* mutant+*bam* > rescue constructs" are: *bamGAL4VP16/Y; UAS-(Doa-PS, Doa-PJ, Doa-PK, 3flag-Doa-PS^{139–511}, 3flag-Doa-PS^{1–138}, humanCLK2, or mouseCLK2)/+; Doa^{Dem/HD}*.

of big, Achi-bright cells; Table 1). Simultaneous visualization of Bam-GFP fusion proteins confirmed the extent of Bam expression which was consistent with the extensiveness of spermatocytes induction (Fig. 3C′ and D′).

Our next question was whether Bam expression was affected in Doa mutant. We detected wild type bam transcripts in Doa mutant testes (data not shown). Immunostaining of Bam protein in the testes revealed none or weak to moderate levels of Bam in the mutant spermatogonia, relative to that in the wild type (Fig. 3E and F). It seems that Bam proteins did not accumulate enough in these Doa mutant cells. Supplying more Bam could facilitate the spermatogonia to overcome this barrier for the differentiation towards spermatocytes (Fig. 3C and D), though not sufficient to rescue fertility. In contrast, providing more Doa in bam mutant testes did not induce any spermatocytes (Fig. S3). Thus, we reason that Doa acts genetically upstream of Bam to mediate the differentiation from spermatogonia to spermatocytes.

The mammalian homologs of Doa restored the fertility of Doa mutant

Transheterozygous Doa mutant ($Doa^{Dem/HD}$) is viable but sterile (Figs. 2B and 4F). Germ cell-specific expression of Doa driven by bamGAL4VP16 or vasaGAL4 restored not only the proper differentiation of the germ cells (Fig. 4A–E and Table S1), but also the fertility of the flies (Fig. 4F and Table S1; the expression patterns of bamGAL4VP16 or vasaGAL4 are shown in Fig. S4). The PS isoform of Doa appeared to be the best among the three

isoforms tested. These three isoforms were selected because their transcripts were detected in the testes by RT-PCR (data not shown). The PJ isoform has 69 more amino acids at the N-terminus than the PS (the Doa sequence shown in Fig. 1A), but delivered less activities in the functional assays.

Mammalian CLKs (Cdc2-like kinases) belong to the LAMMER protein kinase family (Yun et al., 1994). Among the CLK1∼4 in the mouse genome, CLK2 shares the highest homology with Doa-PS and is very abundant in testis (Nayler et al., 1997), but its developmental function is unclear. We thus tested the functional conservation of mammalian CLK2. Amazingly, the human and mouse CLK2 could substitute for the function of its fly counterpart and restored the fertility of Doa mutant to the extent better than that of Doa-PK (Fig. 4E and F). Thus, the conservation of LAMMER kinase family from fly to mammal is not only structural, but also functional in terms of conferring Drosophila fertility.

The kinase domain of Doa or humanCLK2 is required to promote the differentiation of spermatogonia

Since Doa is predicted as a protein kinase (Lee et al., 1996), we examined whether the kinase domain is necessary for its function in spermatogenesis. Again, the assay was based on how well different protein constructs could rescue the Doa mutant ($Doa^{Dem/HD}$), specifically speaking, whether the testing constructs could drive spermatogonia to become spermatocytes (Fig. 5) or could restore the fertility (Fig. 4F, Table S1). We first made the deletion constructs to compare the rescuing activities of Doa with
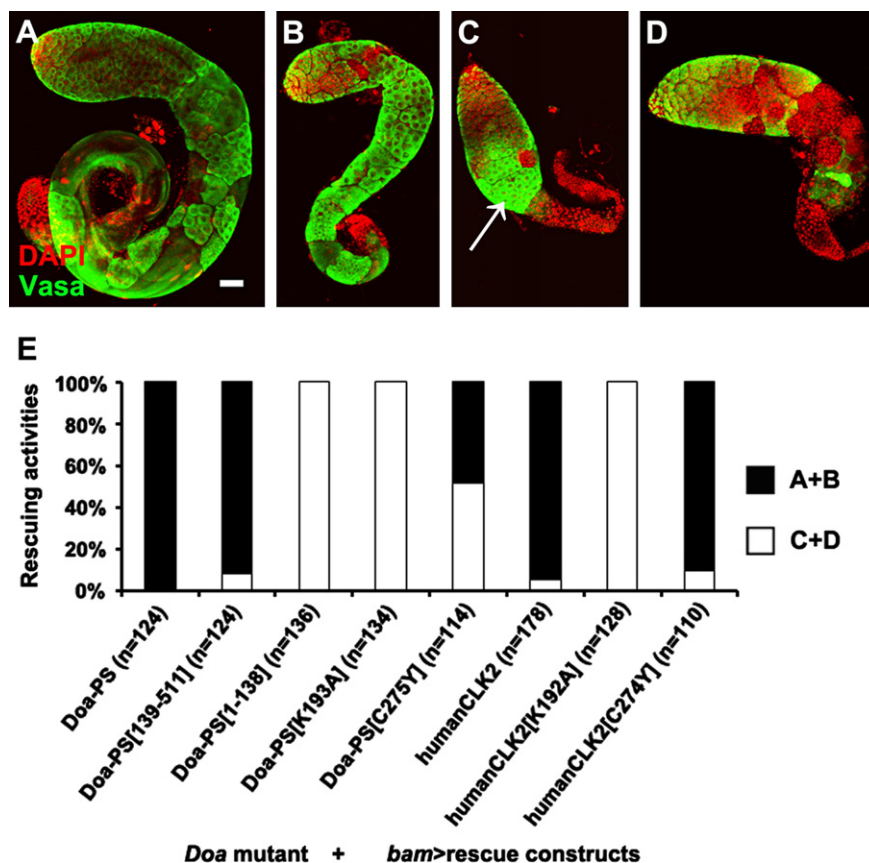

Fig. 5. The kinase domain of Doa or humanCLK2 is required to promote the differentiation of spermatogonia. (A–D) The representative testes of $Doa^{Dem/HD}$ background rescued to different extent: (A) similar to the wild type; (B) most of the germline stages were present except spermatids and mature sperm; (C) spermatocytes were present (arrow); (D) similar to $Doa^{Dem/HD}$ testes. Scale bar: 50 μM in (A) for (A–D). (E) The rescuing effects of different protein constructs. 'n' is the total number of testes scored. (A+B) or (C+D) corresponds to the result in panel [(A) and (B)] or [(C) and (D)], respectively. The genotypes of "Doa mutant+bam > rescue constructs" are: bamGAL4VP16/Y; UAS-(3flag-Doa-PS, 3flag-Doa-PS$^{139–511}$, 3flag-Doa-PS$^{1–138}$, 3flag-Doa-PS^{K193A}, 3flag-Doa-PS^{C275Y}, humanCLK2, humanCLK2^{K192A}, or humanCLK2^{C274Y})/+; $Doa^{Dem/HD}$.

or without the predicted kinase domain. Not surprisingly, Doa-PS [a.a.139-511] which contains the kinase domain is absolutely required and exhibited the activity nearly as good as the full length of humanCLK2 (Figs. 4F and 5E, compare a.a.1-138 and a.a.139-511).

To pinpoint the critical residues in the kinase catalytic domain, we also made two point mutations in the PS isoform, K193A and C275Y (Fig. 1B), designed according to the previous studies in mammalian cells (Nayler et al., 1997) and Doa^{z8} from our screen, respectively. Remarkably, a single residue change (K193A) completely abolished the activity of Doa, and so did the point mutation to the same conserved residue of humanCLK2 (Fig. 5E). In comparison, C275Y reduced the Doa activity to about 50% in this assay, whereas the C274Y change in humanCLK2 did not alter much of its activity. Because the Lysine residue (K192 in CLK2) has been demonstrated critical for the kinase activities in cultured mammalian cells and in in vitro assays (Nayler et al., 1997), we thus provide evidence for both Doa and humanCLK2 that their kinase domain is essential for promoting spermatogonial differentiation consequently preventing overproliferation.

Discussion

In this study we have analyzed the Doa mutants that block the germ cell's transition/differentiation from the mitotic to the meiotic state in Drosophila spermatogenesis. Doa encodes a highly conserved kinase whose mammalian homologs can replace the functions of its fly counterpart in spermatogenesis. This kinase protein represents a new component in the regulatory network potentially conserved from fly to human, but many questions regarding the control mechanism of the mitotic/meiotic decision remain to be answered.

Does the mitotic/meiotic switch mechanism tightly couple the following two events: the exit out of mitosis and the entry into meiosis? To date, we and others in the field using many genetic manipulations have not found such a condition under which germline mitotic cycle simply stops without entering meiosis. Mitotic exit and meiotic entry are coupled at a certain point [seen in the wild type or bam dosage manipulations (Insco et al., 2009)], or the mitotic divisions run out of control (seen as spermatogonial over-proliferation in mutants such as bam, bgcn, Doa, etc.). Of course, lack of growth in cell number or size is a phenotype not as easily identified as the over-growth phenotypes. Nonetheless, we still consider spermatogonial over-amplification as a defect in mitosis/meiosis decision.

What are the downstream targets of Doa? According to its primary sequences, Doa belongs to the LAMMER protein kinase family whose founding members contain a signature stretch of amino acids "LAMMER" (Yun et al., 1994). LAMMER kinases phosphorylate SR- and SR-like proteins that are involved in sex-specific mRNA splicing (Du et al., 1998). Consistently, certain alleles of Doa caused somatic sex-transformation which could be enhanced by the mutations in the genes encoding SR- or SR-like proteins (Du et al., 1998; Kpebe and Rabinow, 2008b; Rabinow and Samson, 2010). To test whether SR- or SR-like proteins are also downstream of Doa in spermatogenesis, we did an RNAi screen of these genes and found that several of these RNAi led to spermatogonial accumulation (such as RNAi against X16, Rsf1, SRm160, etc.). However, we have not been able to confirm this observation using the null alleles.

Since Bam proteins seemed less abundant in Doa mutant spermatogonial cells than in the wild type, is Bam possibly post-translationally regulated by Doa as a kinase? It is unlikely because the addition of Bam synthesized de novo in Doa mutant worked well as the pro-differentiation factor (Fig. 3 and Table 1).

Meanwhile, as the kinase catalytic domain is required for Doa's function in the meiotic switch, it appears that Doa is indirectly involved in tuning the accumulation rate of Bam.

We have provided solid evidence illustrating that human or murine homolog of Doa, namely CLK2, could replace the function of Doa in spermatogenesis even to the extent of restoring fertility (Fig. 4). Notably, mutating a single conserved residue in the kinase catalytic domain of either Doa or CLK2 completely killed their activities in the functional rescue assay. Then, does CLK2 exert similar functions in mammalian spermatogenesis? No mouse mutants of CLK2 are available at this point. Interestingly, previous studies have shown that CLK2 transcripts are especially abundant in the testis (Nayler et al., 1997). We analyzed the expression pattern in temporal details and observed that its transcripts were enriched in the mouse testes 7-day post birth (data not shown), when spermatogonial cells are predominant in quantity (Vergouwen et al., 1993; Vergouwen et al., 1991). Although expression pattern does not necessarily correlate with function, at least mouseCLK2 transcripts are abundant in the right tissue at the right time. Eventually, whether CLK2 acts at the mitosis/meiosis switch during mammalian spermatogenesis awaits the generation of CLK2 mutant mouse model.

Acknowledgements

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.01.023.

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