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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

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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.

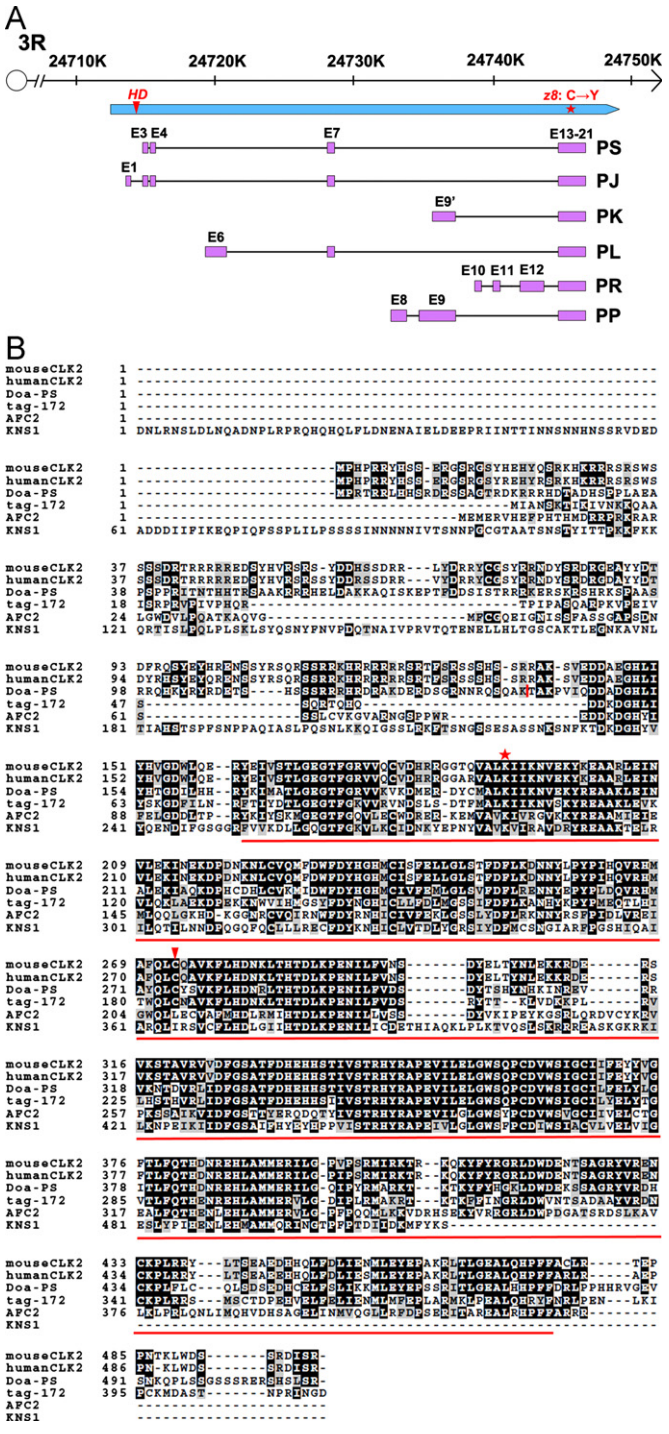

Fig. 1. The molecular information of *Doa* mutants. (A) The genomic organization of *Doa* locus. Only the published isoforms (Kpebe and Rabinow, 2008a) are shown and named according to FlyBase. *Doa^{HD}* is a *copia* retrotransposon insertion about 177 bp from the 3′ end of Exon-2 (Kpebe and Rabinow, 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/).