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The axon guidance gene *lola* is required for programmed cell death in the *Drosophila* ovary

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Abstract

longitudinals-lacking (*lola*) was identified in *Drosophila* as a gene encoding several alternatively spliced transcription factors involved in axon guidance. Here we report that *lola* also plays a critical role in programmed cell death in the ovary. *lola* mutant germline clones show a high percentage of egg chambers with nurse cell nuclei persisting past stage 13, indicating a block in developmental nurse cell death. Mutants also show a disruption in the induced programmed cell death that occurs during mid-oogenesis in response to starvation. Further characterization revealed that *lola* germline clones exhibit abnormal nuclear organization which becomes increasingly severe with age. Chromatin appears diffuse and fails to condense properly or undergo DNA fragmentation in dying nurse cells. Masses of nuclear material accumulate in the ovaries of older flies containing *lola* germline clones. We propose that *lola* is necessary for complete chromatin condensation which occurs during programmed cell death in the ovary. Alleles differed in the strength of their phenotypes but interestingly, the severity of their ovarian phenotypes was independent of the strength of their neuronal phenotypes, suggesting a differential requirement for individual *lola* isoforms in the ovary and nervous system.

Keywords: Programmed cell death; Apoptosis; Oogenesis; *Drosophila*; *lola*; Germline clones; Chromatin condensation; Nurse cells

Introduction

In *Drosophila*, the process of oogenesis is divided into 14 stages based on well defined morphological characteristics (King, 1970; Spradling, 1993). A *Drosophila* ovary consists of 15–20 ovarioles, each containing several egg chambers at different stages of development. Each egg chamber is surrounded by somatically derived follicle cells and contains 15 germline-derived nurse cells in addition to the oocyte. The nurse cells serve to provide the developing oocyte with essential proteins, organelles, and other cellular components.

Programmed cell death (PCD) is known to occur during early, mid-stage, and late-stage *Drosophila* oogenesis (reviewed in McCall, 2004). In response to nutrient deprivation, germline cyst cells may undergo PCD in the beginning of oogenesis in the germarium, or entire egg chambers may die around stage seven or eight, indicating that there are checkpoints at specific stages of oogenesis. During late *Drosophila* oogenesis, as a part of normal egg chamber development, the nurse cells undergo PCD resulting in a mature oocyte surrounded by the follicle cells that make the eggshell. Cytoskeletal changes occur as nurse cell PCD initiates, allowing for the transfer of cytoplasm from the nurse cells through intracellular bridges into the oocyte, a process commonly referred to as "dumping". After the nurse cells transfer their mRNA, organelles, and proteins into the developing oocyte, the remaining nurse cell nuclei undergo chromatin condensation and DNA fragmentation (Cavaliere et al., 1998; Foley and Cooley, 1998; McCall and Steller, 1998). Eventually the nurse cell remnants are engulfed by the neighboring follicle cells (Cummings and King, 1970; Nezis et al., 2000).

Although these are well established characteristics of PCD in the ovary, it is currently unclear what role nurse cell DNA fragmentation plays, along with chromatin condensation, in the PCD process. *Drosophila* mutants lacking caspase-activated DNase (CAD) failed to undergo nucleosomal DNA fragmenta-

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tion during oogenesis, although the ovaries did not accumulate DNA, and the nurse cells appeared to complete PCD (Mukae et al., 2002). Upon induction of apoptosis, lack of DNA fragmentation was also observed in human cell lines expressing a caspase-resistant form of CAD inhibitor (ICAD), which is normally cleaved by caspases to release CAD (McIlroy et al., 1999). However, nucleosomal DNA fragmentation was still present in mice expressing a caspase-resistant ICAD (McIlroy et al., 2000). Flies containing a mutation in the lysosomal acid DNase gene, *dDNaseII*, exhibited enhanced DNA fragmentation along with an accumulation of nurse cell DNA in the ovary. In addition, flies lacking CAD and DNaseII also showed DNA accumulation (Mukae et al., 2002). These results suggest that CAD and DNaseII may be involved in a two-step process mediating DNA fragmentation in the ovary (Mukae et al., 2002) similar to the three-step process proposed for *C. elegans* (Wu et al., 2000).

Previous studies have shown that many of the genes known to play essential roles in PCD during *Drosophila* embryogenesis and organogenesis are not necessary for PCD during oogenesis (Foley and Cooley, 1998; reviewed in Baum et al., 2005). This suggests that a different set of genes may be controlling this process in the ovary. Furthermore, the checkpoint PCD pathway active in mid-oogenesis appears to require different genes than the developmental PCD pathway active in later stages (reviewed in McCall, 2004). Over-expression of the inhibitor of apoptosis protein, Diap-1, has been shown to significantly block the mid-stage checkpoint cell death pathway, but has a milder effect on late-stage nurse cell death (Peterson et al., 2003; Mazzalupo and Cooley, 2006; J. Baum and KM, unpublished). Also, mutations in the effector caspase gene *dcp-1* prevent starvation-induced PCD but not late-stage developmental PCD (Laundrie et al., 2003). In fact, relatively few genes involved in late-stage developmental PCD have been identified thus far.

To identify genes necessary for proper nurse cell developmental PCD, we performed a germline clone (GLC) screen of chromosome 2R. One of the genes identified through this screen was *longitudinals-lacking* (*lola*). Lola was previously identified as a transcription factor involved in axon guidance (Giniger et al., 1994; Madden et al., 1999; Crowner et al., 2002; Goeke et al., 2003). Specifically, Lola has been shown to regulate the expression of axon guidance proteins Robo and Slit (Crowner et al., 2002). Furthermore, Lola has been shown to interact with chromosomal kinase JIL-1 (Zhang et al., 2003b), suggesting that it is also involved in chromatin structure modification and its roles in development could be more broad than initially thought. The *lola* gene spans over 61 kb, contains 32 alternatively spliced exons, and is known to encode at least 20 different isoforms (Goeke et al., 2003; Ohsako et al., 2003). Transcripts for all 20 isoforms contain common exons 5–8, which encode a BTB (Bric-a-brac, Tramtrack, Broad complex) domain involved in dimerization. *lola* additionally contains four alternatively spliced 5′ exons and 24 alternatively spliced 3′ exons. The 3′ variable exons encode several unique zinc finger motifs present in 17 of the 20 isoforms (Goeke et al., 2003; Horiuchi et al., 2003; Ohsako et al., 2003). *lola* has been shown to exhibit intragenic complementation through inter-allelic *trans*-splicing events, indicating that this is a very large and complex locus (Horiuchi et al., 2003).

Here we show that *lola* is also required for proper PCD in the ovary. Through GLC analysis, we have determined that loss of *lola* results in a block in the developmental PCD which occurs late in oogenesis. Interestingly, *lola* mutants also show a delay in checkpoint PCD which occurs during the mid-stages, suggesting *lola* affects events common to both forms of PCD. Specifically we have observed a disruption in normal nurse cell nuclear organization, chromatin condensation, and DNA fragmentation that normally occur during PCD. We also show a differential requirement for individual *lola* splice forms in the ovary and in embryonic development of the nervous system. We hypothesize that Lola isoform K is specifically required for nuclear organization, chromatin condensation, and DNA fragmentation in the ovary during PCD.

Materials and methods

Drosophila strains

All fly lines were obtained from the Bloomington Stock Center unless otherwise noted. Seven deficiency lines and 35 lethal alleles were used for complementation analysis including btb^{k09901} and $lola^{00642}$. $lola^{5D2}$, $lola^{4E4}$ (Giniger et al., 1994), $lola^{ORC4}$, $lola^{ORE50}$ (Goeke et al., 2003), and $lola^{ORE120}$ (Crowner et al., 2002) were gifts from Ed Giniger. The *BB127* enhancer trap line (Schüpbach and Wieschaus, 1991) was a gift from Trudi Schüpbach. yw^{67c23} flies served as a wild-type control. Nutrient deprivation experiments were carried out as described (Peterson et al., 2003). All crosses were carried out at 25 °C on standard fly food unless otherwise noted.

Genetic manipulations

The screen from which $lola^{629}$ was isolated was carried out in the following manner. Males from two different isogenic *dp FRTG13/CyO* recombinant fly lines were subjected to 35 mM EMS in 10% sucrose overnight. These males were then crossed to y^2 *ras v* $RpII215shi^{ts}$*/FM7, l*; *Gla/CyO* virgin females, resulting in only female progeny. In the F1 generation, single non-*Gla* females were crossed with *y w hsflp BB127*; *FRTG13* ovo^D*/CyO* males containing a heat-shock inducible flippase, the *BB127* enhancer trap encoding nuclear β-galactosidase, and *FRTG13* marked with the dominant female sterile mutation ovo^D. On days 5 and 6 or as soon as third instar larvae were visible, progeny were heat shocked in a 37° water bath for 1 h. *y w hsflp BB127/FM7, l* or +; *dp FRTG13/FRTG13* ovo^D females were collected from the F2 generation and conditioned on wet yeast paste along with males. These potential GLC containing females were dissected after being conditioned 3–10 days. Siblings were used to make balanced stocks of each screen line that exhibited an oogenesis phenotype as determined through DAPI staining.

To generate GLCs of existing *lola* alleles, the *FRT* site from *FRTG13 L/CyO* flies was recombined onto *lola* stocks. Correct stocks were determined through eye color, loss of *L*, and failure to complement the original *lola* stock and other alleles.

Staining procedures

For antibody and DAPI staining, females were conditioned on wet yeast paste and ovaries were dissected, fixed, and stained as described (Verheyen and Cooley, 1994) except they were mounted in Vectashield with DAPI (Vector Labs, Burlingame, CA). For propidium iodide staining following the Cleaved Caspase-3 antibody staining procedure, fixed tissue was incubated with 600 μg/ml RNase in PBS for 2 h and stained with 1 μg/ml propidium iodide (Molecular Probes, Eugene, OR) in PBS for 30 min in the dark, washed 2 × 15 min in PBS, and mounted in Vectashield (Vector Labs, Burlingame, CA). Cleaved Caspase-3 antibody (Cell Signaling Technology, Danvers, MA) was