Apoptosis controls the speed of looping morphogenesis in Drosophila male terminalia

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SUMMARY
In metazoan development, the precise mechanisms that regulate the completion of morphogenesis according to a developmental timetable remain elusive. The Drosophila male terminalia is an asymmetric looping organ; the internal genitalia (spermiduct) loops dextrally around the hindgut. Mutants for apoptotic signaling have an orientation defect of their male terminalia, indicating that apoptosis contributes to the looping morphogenesis. However, the physiological roles of apoptosis in the looping morphogenesis of male terminalia have been unclear. Here, we show the role of apoptosis in the organogenesis of male terminalia using time-lapse imaging. In normal flies, genitalia rotation accelerated as development proceeded, and completed a full 360° rotation. This acceleration was impaired when the activity of caspases or JNK or PVF/PVR signaling was reduced. Acceleration was induced by two distinct subcompartments of the A8 segment that formed a ring shape and surrounded the male genitalia: the inner ring rotated with the genitalia and the outer ring rotated later, functioning as a ‘moving walkway’ to accelerate the inner ring rotation. A quantitative analysis combining the use of a FRET-based indicator for caspase activation with single-cell tracking showed that the timing and degree of apoptosis correlated with the movement of the outer ring, and upregulation of the apoptotic signal increased the speed of genital rotation. Therefore, apoptosis coordinates the outer ring movement that drives the acceleration of genitalia rotation, thereby enabling the complete morphogenesis of male genitalia within a limited developmental time frame.

KEY WORDS: Caspase, Apoptosis, In vivo imaging, Drosophila

INTRODUCTION
During animal development, dynamic cell behaviors are precisely orchestrated to accurately complete morphogenesis. However, the mechanisms that determine precisely how cell behaviors regulate morphogenesis according to the developmental timetable are still uncharacterized. Programmed cell death or apoptosis not only functions in sculpting and deleting structures in developing animals, but also it plays dynamic roles in coordinating organ morphogenesis (Stenn and Paus, 2001; Toyama et al., 2008). The Drosophila male terminalia is an asymmetric looping organ; the internal genitalia (spermiduct) loops dextrally around the hindgut. During the maturation of the internal genitalia, the male terminalia rotates 360° clockwise (Gleichauf, 1936). The orientation defect of adult male terminalia is thought to occur when this rotation is incomplete (Adam et al., 2003). Apoptosis is thought to contribute to the completion of genitalia rotation, because an orientation defect of the adult male terminalia is observed in mutants of the apoptotic gene; drICE (Muro et al., 2006) (Ice – FlyBase), the Drosophila ortholog of caspase 3; and dronc (Krieser et al., 2007) (Nc – FlyBase), the Drosophila ortholog of caspase 9. Caspase family proteases are the central executioners for the genetically encoded apoptosis in animals (Degterev et al., 2003). However, the physiological roles of apoptosis in completing the morphogenesis of male terminalia remain to be elucidated.

We herein used live in vivo imaging to determine the dynamics of the looping morphogenesis and spatiotemporal apoptosis during male genitalia development. Our observations suggest that apoptosis drives the acceleration of rotation, enabling the complete genitalia morphogenesis to occur within the developmental timetable.

MATERIALS AND METHODS

Fly stocks and temporal gene expression
Flies were raised on standard Drosophila medium at 25°C. The following fly strains were used in this study: en-GAL4, UAS-mCD8-EGFP, UAS-lacZ, Histone2Av (His2Av)-mRFP, tub-GAL80ts (Bloomington Stock Center); AbdB-GAL4DN (de Navas et al., 2006); UAS-p35 (Zhou et al., 1997); UAS-PVR DN (Duchek et al., 2001); UAS-JNK DN (Adachi-Yamada et al., 1999); UAS-SCAT3 (Takemoto et al., 2007; Kamata et al., 2005; Kuranaga et al., 2006); and UAS-Histone2B (H2B)-ECFP, UAS-nls-SCAT3 (Koto et al., 2009).

Using the TARGET system, we bred flies at the permissive temperature (18°C) of GAL80ts until the time when the head of the pupae had just everted, to suppress the activity of GAL4. After head eversion, the flies were moved to the restrictive temperature (29°C) of GAL80ts (McGuire et al., 2003) for 12 hours. Time-lapse imaging using a stereomicroscope (M205FA, Leica) was performed at 22°C after the heat shock.

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Fig. 1. In vivo imaging and quantitative analysis of genitalia rotation. (A) An image (left) and schematic drawing (right) of the male genitalia of His2Av-mRFP flies at 24 hours APF. Each segment is highlighted in a different color: A8 (green), A9 (orange) and A10 (yellow). (B,D,E) Time-lapse series of genitalia rotation in (B) His2Av-mRFP/+ (C) en-GAL4 UAS-H2B-ECFP/+ and (E) en-GAL4 UAS-H2B-ECFP/UAS-p35 flies are shown. Ventral is towards the top in all figures. (C) Image (left) and schematic drawing (right) of genitalia in en-GAL4 UAS-H2B-ECFP/+ at 24 hours APF. The posterior region of the A8 segment is highlighted in green. (F,G) Scanning electron micrograph of the adult male genitalia of en-GAL4/UAS-lacZ (F) and en-GAL4/UAS-p35 (G). (H) The genitalia angle (θ) in control (black) and p35-expressing flies (red) was measured every 30 minutes, and the mean angle is shown. Error bars indicate s.d. (control, n=10; p35, n=8). (I) Velocity (V=dθ/dt) and (J) acceleration rate (a=dV/dt) were quantified by measuring θ and V as a function of time t in control (black) and p35-expressing flies (red). The initiation of genitalia rotation (>1 hour) in p35-expressing flies was similar to control flies (indicated by the gray area). Genotypes of the control flies were as follows: en-GAL4 UAS-mCD8-GFP/+, en-GAL4 UAS-H2B-ECFP/+ and en-GAL4 UAS-NLS-ECFP-venus+. (K) The mean genitalia angle (θ) in flies expressing dominant-negative JNK (JNK-DN; blue) and dominant-negative PVR (PVR-DN; green) were plotted every 30 minutes. Error bars indicate s.d. (JNK-DN, n=12; PVR-DN, n=11). θcontrol and θp35 in Fig. 1H are represented for reference as black and red lines, respectively. (L) The average velocity (V=dθ/dt) was quantified by measuring θ as a function of time t in control (black), p35 (red), JNK-DN (blue) and PVR-DN (green) flies (mean±s.d.) (**P<0.01, *P<0.05).
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**RESULTS AND DISCUSSION**

To visualize the genitalia rotation in living animals, we first used the His2Av-mRFP *Drosophila* line whose nuclei are ubiquitously marked by a fluorescent protein (Pandey et al., 2005). The genital disc is a compound disc comprised of cells from three different embryonic segments: A8 (male eighth tergite), A9 (male primordium) and A10 (anal). During metamorphosis, the genital disc is partially everted, exposing its apical surface, and adopts a circular shape (Fig. 1A) (Keisman et al., 2001). Our results captured the male genitalia undergoing a 360° clockwise rotation (Fig. 1B; see Movie 1 in the supplementary material). Inhibiting apoptosis by expressing the baculovirus pan-caspase inhibitor p35 (Fig. 1B; see Movie 1 in the supplementary material). Inhibiting apoptosis by expressing the baculovirus pan-caspase inhibitor p35 driven by *en*GAL4 (*en*-GAL4), which is expressed in the posterior compartment of each segment, results in genital mis-orientation at the adult stage (Macias et al., 2004).

In flies expressing nuclear fluorescent protein driven by *en*-GAL4, we observed that the posterior part of the A8 segment (A8p) formed a ring of cells surrounding the A9-A10 part of the disc (Fig. 1C). First, we recorded the images at a low resolution (10× objective lens) to measure the rotation speed accurately in control and p35-expressing flies, because long-term time-lapse imaging at a high resolution can cause photodamage, and thus alter pupal development. Most of the cells in the A8p that seem to disappear at the end of Movies 2 and 3 in the supplementary material actually moved out of the plane of focus. In our imaging results, the rotation started around 24 hours APF (after puparium formation) and stopped about 12 hours later (12 hours 5 minutes±58 minutes; n=10) (Fig. 1D; see Movie 2 in the supplementary material). To confirm whether the mis-oriented genital phenotype in the caspase-inhibited flies was caused by incomplete rotation, we observed the rotation in flies expressing p35 under the *en*-GAL4 driver. In the p35-expressing flies, the rotation began, but it stopped before it was complete, after about 12 hours (12 hours 8 minutes±1 hour 27 minutes; n=8), i.e. with the same timing as in control flies (Fig. 1E; see Movie 3 in the supplementary material). This suggested that the reduced caspase activation in A8p prevented the genitalia from completing the rotation, resulting in mis-oriented adult genitalia (Fig. 1F,G).

To compare complete rotation with incomplete rotation, we calculated the rotation speed by measuring the angle (θ<sub>control</sub> and θ<sub>p35</sub>) of the A9 genitalia every 30 minutes on time-lapse images. The normal rotation was composed of at least four steps: initiation, acceleration, deceleration and stopping (Fig. 1H). We calculated the velocity of rotation V=θ/dt by measuring θ as a function of time t. At first, the genitalia rotated at an average velocity (V<sub>control</sub>) of 7.67±3.72°/hour by 1 hour after initiation, then the rotation

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**Sample preparation for time-lapse imaging and scanning electron microscopy**

Staged pupae (24 hours APF) were washed in water and mounted on a glass slide using double-sided tape. The pupal case covering the dorsal part of the abdomen was removed. A very wet filter paper was placed around the pupae to keep them hydrated. The pupae were covered with a coverslip in a small drop of water to avoid desiccation. Silicon (Shinetsu) was used to seal the chamber. In most cases, the animal survived the data acquisition and developed into an adult. Time-lapse images were captured using an SP5 confocal microscope (Leica) with a spinning disc-type confocal unit (CSU10, Yokogawa, Tokyo) equipped with the Aquacosmos/Ashura system (Hamamatsu Photonics) (Kuranaga et al., 2006). The FRET analysis was performed using the Aquacosmos (Hamamatsu Photonics) and MetaMorph software (Molecular Devices) programs. For the scanning electron microscopy, we used the VE-8800 microscope system (Keyence).

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**Fig. 2. Cellular behaviors and apoptosis in the A8p region.** (A) A time-lapse series of genitalia rotation in *en*-GAL4 UAS-nls-ECFP-venus/+. The posterior compartment is visible in this fly. Cells represented by magenta rotated with A9-A10, and cells colored green rotated later. (B) Image (left) and schematic drawing (right) of genitalia in *en*-GAL4 UAS-nls-ECFP-venus/+ at 24 hours APF. A8p was divided into two parts, A8pa and A8pp. Each part is highlighted in a different color: A8pa (green) and A8pp (magenta). (C) Caspase activity was examined by the imaging of a FRET-based probe, nls-ECFP-venus (nls-SCAT3), and is shown in pseudo-color. White circles indicate the cell that underwent apoptosis, pseudo-color gradually changed from red to blue. (D) Result of cell tracing in the A8p region. Cells that underwent apoptosis are marked by yellow dots. Magenta dots show cells located in A8pp, which moved with A9, and green dots represent cells in A8pa, which rotated later. Genotype was *en*-GAL4 UAS-nls-SCAT3/+; His2Av-mRFP/+.

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**DEVELOPMENT**
accelerated, with $V_{\text{control}}$ gradually increasing to $53.83 \pm 7.11^\circ/\text{hour}$ by 7 hours after initiation (Fig. 1I). Interestingly, in the p35-expressing flies, the rotation normally started at 24 hours APF, and the average velocity ($V_{p35}$) from the initial rotation to 1 hour later was $7.45 \pm 2.98^\circ/\text{hour}$, which was not significantly different from the normal rotation. However, the acceleration of the rotation in the p35-expressing flies was lower than normal, with $V_{p35}$ gradually increasing to $21.35 \pm 7.45^\circ/\text{hour}$ at 5.5 hours after initiation (Fig. 1I). As shown in Fig. 1J, the first peak of the acceleration rate, which was defined as the initiation of rotation, was observed in the p35-expressing flies ($\theta_{ap35}$) and was the same as in the control flies ($\theta_{acontrol}$). However, the duration of the acceleration period was shorter in the p35-expressing flies. These data suggest a relationship between apoptosis and the acceleration of genitalia rotation.

Next, we examined the signaling mechanism(s) involved in the acceleration of genitalia rotation. The inhibition of JNK (c-Jun N-terminal kinase) and PVF (platelet vascular factor) signaling in male flies has been shown to result in mis-oriented adult male terminalia, and it has been hypothesized that the PVF/PVR (PVF receptor) may affect the genitalia rotation via JNK-mediated apoptosis. Consistent with previous reports, the acceleration of genitalia rotation was significantly impaired in flies expressing dominant-negative forms of JNK (JNK-DN) and PVR (PVR-DN) (Fig. 1K,L). These data implied that caspase activation and JNK signaling contribute to driving the acceleration of genitalia rotation.

To analyze how the genitalia accelerate their rotation, we traced the movement of A8p at the single-cell level. For this experiment, we performed live imaging at a high resolution (20x objective lens), which enabled the cells in A8p to be tracked at single-cell resolution. As shown in Fig. 2A, cells (magenta) that were neighbors of A9 rotated with A9, whereas cells (green) located in the anterior half of A8p rotated later than A9. Based on our imaging, we divided A8p into two sheets, named A8pa (anterior of A8p) and A8pp (posterior of A8p), as shown in Fig. 2B. We found that a part of the cells in A8p underwent apoptosis.

To observe caspase activation in living animals, we generated a FRET (fluorescence resonance energy transfer)-based indicator, SCAT3 (sensor for activated caspases based on FRET). We used a nuclear localization signal-tagged SCAT3 (nls-SCAT3; UAS-nls-ECFP-venus) (Koto et al., 2009). The nls-SCAT3 signal was clearly observed in A8p (Fig. 2C). Cells exhibiting high caspase activity were extruded into the body cavity and disappeared, consistent with their apoptotic death and engulfment by circulating hemocytes. We tracked each cell in the A8p region during the first half of the rotation and found that
At least three types of cellular behavior were observed, as shown in Fig. 2D: cells located in A8pp (magenta) moved with A9, cells underwent apoptosis (yellow) and cells located in A8pa (green) rotated later (Fig. 2D). Thus, to observe the behavior of the cells in A8pa, we used Abdominal B (AbdB) as an A8 marker. AbdB is a homeotic gene that is required for the correct development of the genital disc (Estrada et al., 2003; Gorfinkiel et al., 2003), and AbdB-GAL4 is expressed in the segment A8 (in A8a and A8p) of the genital disc during the 3rd instar larval stage (de Navas et al., 2006; Benitez et al., 2010; Rousset et al., 2010). At 24 hours APF, AbdB was expressed in A8 and formed a ring (Fig. 3A,B). We took time-lapse images, and unexpectedly found that most of the cells in the AbdB-expressing region underwent a 180° clockwise movement, suggesting that AbdB was not expressed in the A8pp region that moved 360° with A9 (Fig. 3A; see Movie 4 in the supplementary material). To determine the speed of the AbdB-expressing cells, we traced three individual cells in each fly (Nfly = 4), and calculated the value of the turning angle of the cells (θ_AbdB) (Fig. 3C). Our findings confirmed that the AbdB-expressing region moved halfway around. Although cells in the AbdB-expressing region moved only 180°, the A8pp (inner ring), which was encircled by the AbdB-expressing region (outer ring), still moved 360°. Furthermore, our imaging data indicated that the movement of the outer ring started 1-2 hours later than that of the A9 region (Fig. 3C), when the acceleration of the genitalia began to increase.

Fig. 4. Initiation of outer ring rotation correlates with apoptosis. (A) Acceleration of the rotation of the AbdB-expressing region was quantified by measuring θ_AbdB and V_AbdB as a function of time t (green line). Histogram showing the frequency of apoptosis every 10 minutes from 0 hours, when rotation started, to 8 hours. R_apoptosis was normalized to the total number of apoptotic cells in each individual. Error bars indicate s.d. (n=3 flies). (B) Data points represent the relationship between θ_AbdB and R_apoptosis for 0-3 hours using linear regression (R²=0.951). (C) The genitalia angle (θ) in lacZ-expressing flies (black) and Rpr-expressing flies (gray) was measured every 30 minutes and the mean angle is shown. Error bars indicate s.d. (lacZ, n=8; Rpr, n=8). Genotypes of flies were as follows: en-GAL4 UAS-nls-ECFP-venus/UAS-lacZ; tub-GAL80ts/+ and en-GAL4 UAS-nls-ECFP-venus/UAS-Rpr; tub-GAL80ts/+. (D) The average velocity (V = dθ/dt) was quantified by measuring θ as a function of time t in lacZ (black) and Rpr-expressing (gray) flies (mean±s.d.) (**P<0.01, *P<0.05). (E) Model of acceleration of genitalia rotation. The inner ring (magenta) rotates in concert with A9 genitalia (light gray), then the outer ring (green) that encircles inner ring begins to move, which functions like a ‘moving walkway’ to accelerate the speed of the inner ring. The initiation of outer ring movement strongly correlated with apoptosis (yellow); moreover, this movement was impaired by the inhibition of apoptosis. Therefore, the apoptosis increases the rotation of genitalia faster in the direction it is already moving, enabling the full 360° rotation to occur with the correct timing.
rotation occurred (Fig. 1H-J). These observations raise the possibility that the outer ring movement is related to the acceleration of the genitalia rotation.

We therefore considered that the outer ring movement was restricted in the p35-expressing flies, resulting in an incomplete genitalia rotation of about 180°, which mimics the movement of only the inner ring. To verify this possibility, we examined the movement of the outer ring in the p35-expressing flies (en-GAL4+UAS-p35). Although the inner ring rotated normally, the rotation of the outer ring was impaired in the p35-expressing flies (Fig. 3D). We determined the turning angles by tracing cells in the p35-expressing flies and found that \( \theta_{p35 \_inner} \) increased, while the increase of \( \theta_{p35 \_outer} \) was impaired (Fig. 3E). These data suggest that the A8 segment was composed of two independently regulated rings, and when apoptosis was inhibited, the inner ring could move only 180° with no outer ring movement, resulting in incomplete genitalia rotation.

Thus, to determine whether apoptosis correlates with the outer ring movement, we quantified the apoptosis in A8pa every 10 minutes from 0-8 hours after the start of genitalia rotation. The frequency of apoptosis (Rapoptosis) was normalized to the total number of apoptotic cells in each individual. Pulsatile increases in Rapoptosis were observed, with peaks at 1, 2.5 and 4 hours after the start of genitalia rotation (Fig. 4A). To verify the participation of Rapoptosis in the initiation of outer ring movement, we calculated the acceleration rate of \( \theta_{tdbd} (a_{\theta_{tdbd}}) \) by measuring \( V_{tdbd} \) as a function of time \( t \), and compared Rapoptosis with \( a_{\theta_{tdbd}} \). The starting time of outer ring movement was characterized by the early peaks of \( a_{\theta_{tdbd}} \) (Fig. 4A). Our analysis suggested that the \( a_{\theta_{tdbd}} \) was related to the Rapoptosis because \( a_{\theta_{tdbd}} \) showed its first two peaks at about 1 and 2.5 hours after genitalia rotation started (Fig. 4A). To quantify these observations, we calculated the correlation between Rapoptosis and \( a_{\theta_{tdbd}} \). This analysis confirmed that there was a strong correlation between these parameters (R^2=0.951), because the correlation between \( a_{\theta_{tdbd}} \) and Rapoptosis is approximately linear during this time (Fig. 4B). Therefore, these data implied a possible mechanism of apoptosis that facilitates the outer ring movement.

To verify this possibility, we examined whether the upregulation of apoptotic signals induces an increase in genitalia rotation speed. Because the expression of apoptotic genes using an en-GAL4 driver, which is expressed at the embryonic stage, is lethal, we used the TARGET system to control gene expression temporarily (McGuire et al., 2003). Flies were allowed to develop at 18°C until the head of the pupae had just everted, to inhibit gene expression. The pupae were then heat-shocked at 29°C for 12 hours to induce gene expression. Live imaging was performed at 22°C, after the heat shock. At this temperature, the genitalia rotation in the control flies was slower than in control flies bred at 25°C, because a low breeding temperature affects the rate of fly development, including genitalia rotation. Therefore, it was necessary in this experiment to compare the rotation speeds at the same temperature. The expression of \( reaper \) (rpr), a pro-apoptotic gene, using the TARGET system, showed that the upregulation of apoptotic signaling significantly increased the timing of acceleration and speed of genitalia rotation (Fig. 4C,D). These observations led us to propose that the outer ring functions like a ‘moving walkway’ to accelerate the speed of the inner part of the structure, including the A9 genitalia, enabling genitalia to complete rotation within the appropriate developmental time window (Fig. 4E).

According to our observations, we found that apoptosis drives the movement of cell sheets during the morphogenesis of male terminalia. Further questions remain with regard to how apoptosis contributes to the cell sheet movement. A recent study indicated the possibility that local apoptosis acts as a brake release to regulate genitalia rotation, coupled with left-right determination (Suutanto et al., 2010). However, it has been reported that the cell shape change by apoptosis enables not only the extrusion of dying cells, but also the reorganization of the actin cytoskeleton in neighboring cells (Rosenblatt et al., 2001). Therefore, apoptosis could affect the behavior of neighboring cells, to act as a main driving force of the cell-sheet movement. Taken together, apoptosis may generally participate in the morphogenetic process of cell-sheet movement during morphogenesis.

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Competing interests statement
The authors declare no competing financial interests.

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