Developmental Biology 377 (2013) 9–20

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Spatiotemporal control of Cindr at ring canals during incomplete cytokinesis in the *Drosophila* male germline

Åsmund H. Eikenes [a,b], Andreas Brech [a,b], Harald Stenmark [a,b], Kaisa Haglund [a,b,*]

[a] Department of Biochemistry, Institute for Cancer Research, Oslo University Hospital, Montebello, Oslo, Norway
[b] Centre for Cancer Biomedicine, Faculty of Medicine, University of Oslo, Montebello, Oslo, Norway

ARTICLE INFO

Article history:
Received 30 October 2012
Received in revised form
25 February 2013
Accepted 26 February 2013
Available online 7 March 2013

Keywords:
Drosophila spermatogenesis
Ring canal
Cytokinesis
Cindr

ABSTRACT

During male and female gametogenesis in species ranging from insects to mammals, germ cell cyst formation by incomplete cytokinesis involves the stabilization of cleavage furrows and the formation of stable intercellular bridges called ring canals. Accurate regulation of incomplete cytokinesis is required for both female and male fertility in *Drosophila melanogaster*. Nevertheless, the molecular mechanisms controlling complete versus incomplete cytokinesis are largely unknown. Here, we show that the scaffold protein Cindr is a novel component of both mitotic and meiotic ring canals during *Drosophila* spermatogenesis. Strikingly, unlike other male germline ring canal components, including Anillin and Pavarotti, Cindr and contractile ring F-actin dissociate from mitotic ring canals and translocate to the fusome upon completion of the mitotic germ cell divisions. We provide evidence that the loss of Cindr from mitotic ring canals is coordinated by signals that mediate the transition from germ cell mitosis to differentiation. Interestingly, Cindr loss from ring canals coincides with completion of the mitotic germ cell divisions in both *Drosophila* females and males, thus marking a common step of gametogenesis. We also show that Cindr co-localizes with Anillin at mitotic and meiotic ring canals and is recruited to the contractile ring by Anillin during male germ cell meiotic cytokinesis. Taken together, our analyses reveal a key step of incomplete cytokinesis at the endpoint of the mitotic germ cell divisions in *D. melanogaster*.

Introduction

The process of germ cell development in *Drosophila melanogaster* has emerged as a powerful system to study a variety of biological aspects such as stem cell biology, cell cycle control, division and differentiation as well as cancer development (Fuller and Spradling, 2007; Huynh and Johnston, 2004; Januschke and Gonzalez, 2008; Spradling et al., 2011).

Both female and male germ cells develop as clusters of cells interconnected in syncytia in *Drosophila*. Each female and male germ stem cell (GSC) divides asymmetrically to give rise to another GSC and a daughter cell, which in turn undergoes four mitotic divisions by incomplete cytokinesis to give rise to a cluster of 16 interconnected cells (Pepling et al., 1999). A germline-specific organelle, the fusome, branches throughout the interconnected cells during each of the mitotic divisions, thus aiding in cluster synchronization and organization (de Cuevas and Spradling, 1998; Lin et al., 1994; Snapp et al., 2004). During oogenesis, one of the cells in the 16-cell cluster will polarize and become specified as the oocyte, before the cluster is enveloped by somatic follicle cells and buds off from the germarium as an egg chamber (Horne-Badovinac and Bilder, 2005; Huynh and Johnston, 2004). During spermatogenesis, the 16-cell cluster will enlarge and differentiate before undergoing two successive rounds of meiosis by incomplete cytokinesis, giving rise to a total number of 64 interconnected haploid spermatids (Fig. 1A) (Fuller, 1993; Hime et al., 1996). The two meiotic male germ cell divisions provide an excellent model for cytological, molecular and functional analysis of cytokinesis *in vivo* (Giansanti et al., 2001; Gonzalez and Glover, 1993). Studies in this system have greatly contributed to the understanding of mitotic spindle regulation, plasma membrane composition, contractile ring formation and membrane trafficking during cytokinesis (Adams et al., 1998; Brill et al., 2000; Cenci et al., 1994; Dyer et al., 2007; Giansanti et al., 2004; Goldbach et al., 2010).

Incomplete cytokinesis during mitotic and meiotic germ cell divisions is accomplished by cleavage furrow arrest and the consequent formation of stable intercellular bridges, termed ring canals (Haglund et al., 2011; Hime et al., 1996; Robinson and Cooley, 1996). Studies of both the *Drosophila* female and male germlines show that ring canals are composed of components of the cleavage furrow, with similarities and key differences between the tissues (Hime et al., 1996; Robinson and Cooley, 1996). Cleavage furrow arrest during both the mitotic and meiotic *Drosophila* male germ cell divisions is

* Corresponding author at: Department of Biochemistry, University of Oslo Montebello, Institute for Cancer Research, Oslo University Hospital and Centre for Cancer Biomedicine, Montebello, 0379 Oslo, Norway.
E-mail address: Kaisa.Haglund@rr-research.no (K. Haglund).

0012-1606/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ydbio.2013.02.021

accompanied by the appearance of phosphotyrosine epitopes at the ring canal walls and subsequent loss of F-actin from the contractile ring (Hime et al., 1996; Robinson and Cooley, 1997). The mature male germline ring canals contain components of the arrested cleavage furrow, including the scaffold protein Anillin, at least three Septins (Septin 1, 2 and Peanut), the kinesin-like protein Pavarotti and the glycoprotein Mucin-D (Carmena et al., 1998; Giansanti et al., 1999; Hime et al., 1996; Kramerova and Kramerov, 1999).

Recent evidence from work in the *Drosophila* female germline indicates that the endpoint of the fourth mitotic germ cell division in the germarium coincides with maximal cleavage furrow constriction, establishing a framework for understanding incomplete cytokinesis and ring canal biogenesis (Ong and Tan, 2010). Ring canals formed during the mitotic divisions in both males and females appear to contain a group of core components, suggesting a common molecular process underlying their formation. However, the regulation of complete versus incomplete cytokinesis and the spatiotemporal control of the ring canal components remain unclear.

Here we have addressed the spatiotemporal regulation of incomplete cytokinesis and ring canal biogenesis by studying the scaffold protein Cindr, a recently identified regulator of incomplete cytokinesis in somatic epithelia and the female germline, as well as a regulator of the actin cytoskeleton in the developing pupal eye (Haglund et al., 2010; Johnson et al., 2011, 2008). We find that Cindr is a novel component of mitotic and meiotic germline ring canals during *Drosophila* spermatogenesis. Importantly, we identify a striking temporal regulation of Cindr at the transition from mitosis to differentiation of primary spermatocytes that involves loss of Cindr and F-actin from mitotic ring canals. Our analyses reveal similarities between the mitotic germ cell divisions in the female and male germlines that point toward a common regulation of incomplete cytokinesis during *Drosophila* gametogenesis.

Materials and methods

Drosophila stocks

Fly crosses were performed at 25 °C unless noted otherwise. Fly lines used were: w^{1118} and $bam^{\Delta 86}$ (the Bloomington Drosophila Stock Center), GFP trap CA06686 (Cindr isoform PC) and GFP trap CC01626 (Zipper, *Drosophila* Myosin II heavy chain) (FlyTrap GFP ProteinTrap Database, http://flytrap.med.yale.edu/), GFP-tagged Cindr isoforms *UAS-GFP-Cindr-PC, UAS-GFP-Cindr-PD, UAS-GFP-Cindr-PB* and *UAS-GFP-Cindr-ΔPB* (gifts from Johnson et al. (2008)), *Bam-GAL4* (gift from Julie Brill), $bam^{\Delta PEST}$ (gift from Dennis McKearin), *Ubi-GFP-Pavarotti* (gift from David Glover), *Ubi-Asterless-YFP; Ubi-Tubulin-GFP* (gift from Cayetano González) (Januschke and Gonzalez, 2010), control and *UAS-anillin-IR* (Developmental Biology: (lines 60000 and 33465 from the Vienna Drosophila RNAi Center) (Dietzl et al [Nature 448, 151-156, 12 Jul], 2007)), and *UAS-cindr-IR*$^{3.63+76}$*; UAS-cindr-IR*$^{2.21+23}$ (referred to as *UAS-cindr-IR*) (Haglund et al., 2010; Johnson et al., 2008).

RNAi-mediated gene silencing in vivo

For RNAi-mediated gene silencing in the male germline, *Bam-GAL4* virgin females were crossed to *UAS-cindr-IR, UAS-anillin-IR* or wild-type males (Goldbach et al., 2010). As a genetic background control, *UAS-cindr-IR* females were crossed to wild-type males. Offspring genotypes were *Bam-GAL4/+*, *UAS-cindr-IR/+* (*UAS-cindr-IR*$^{3.63+76}$*/+; UAS-cindr-IR*$^{2.21+23}$*/+*), *Bam-GAL4/UAS-cindr-IR* (*UAS-cindr-IR*$^{3.63+76}$*/+; Bam-GAL4/UAS-cindr-IR*$^{2.21+23}$) and *UAS-anillin-IR (Bam-GAL4/UAS-anillin-IR*). The last 5 days before hatching and dissection, offspring were transferred to 29 °C for increased expression of the RNAi sequences. For Western blotting to examine RNAi efficiency, testes from 15 males were dissected, lysed, mixed with Laemmli buffer (BioRad) containing a final concentration of 0.1 M DTT and boiled before loading onto a 4–20% gradient SDS-PAGE gel (BioRad).

Phase contrast microscopy

For quantification of onion stage cells, testes squashes were performed according to established protocols (Gonzalez and Glover, 1993). Testes from 0–2 day old males were dissected in PBS, transferred to a microscope slide with a drop of PBS and carefully opened. A coverslip was carefully placed on top, and the testes were squashed by carefully removing PBS using a small piece of Whatman paper. During squashing, testes were monitored in a phase contrast microscope, and images were taken with a 40× objective.

Statistical analysis

Quantifications are based on results from at least three independent experiments. In RNAi experiments (Fig. 6), an average of 2500 cells was counted per genotype, and the results were analyzed using a two-tailed student's *t*-test. For overexpression of Cindr transgenes (Fig. S2), 1800 cells per genotype were counted and statistical analysis was performed using a paired, two-tailed Student's *t*-test.

Antibodies and immunostaining

Antibodies used were: rabbit anti-Cindr (1:1000 for IF and 1:400 for WB) and guinea pig anti-Anillin (1:50) (Haglund et al., 2010), mouse anti-α-spectrin (1:20) and mouse anti-Peanut (1:10, DSHB), mouse anti-GFP (1:200, Invitrogen) and mouse anti-α-tubulin (1:20000, Sigma). GFP-Booster (1:200) was from Clontech. Rhodamine-Phalloidin (1:400, Molecular Probes) was used to visualize F-actin. Secondary antibodies were conjugated to Alexa-488, DyLight-549 (1:500, Molecular Probes), HRP, Cy2 or Cy3 (1:500, Jackson Immunoresearch). Three different immunostaining protocols were used for optimal antigen preservation and antibody detection. Most experiments were performed using the previously described squash technique, with fixation in ice-cold methanol and acetone (Cenci et al., 1994). For optimal detection and imaging of GFP-tagged proteins and Cindr isoforms, fixation was performed with ice-cold ethanol and 3.7% formaldehyde (Gunsalus et al., 1995). Lastly, for F-actin preservation and experiments with testes from $bam^{\Delta PEST}$ and $bam^{\Delta 86}$/+ males, *in totu* staining using fixation in 3.7% formaldehyde on ice was performed (Haglund et al., 2010). Results were confirmed with several of the protocols.

Confocal microscopy

Images were acquired using a Zeiss LSM 780 confocal microscope equipped with a 63x NA 1.4 oil DIC III (Plan-Apochromat) objective. Z-stacks were obtained and maximum projections were generated using the Zen 2010 software. Adobe Photoshop and Adobe Illustrator were used to assemble figures and make schematics, respectively. Images were adjusted for clarity. Imaris (Bitplane) was used to generate volume renderings of data from confocal z-stacks.