Follicle separation during *Drosophila* oogenesis requires the activity of the Kinesin II-associated polypeptide Kap in germline cells

Ralf Pflanz*, Annette Peter*[†], Ulrich Schäfer & Herbert Jäckle⁺

Max-Planck-Institut für biophysikalische Chemie, Abteilung Molekulare Entwicklungsbiologie, Göttingen, Germany

Cellular localization of organelles, protein complexes and single mRNAs depends on the directed transport along microtubule tracks, a process mediated by ATP-driven molecular motor proteins of the dynein and kinesin superfamilies. Kinesin II is a heterotrimeric protein complex composed of two motor subunits and a unique nonmotor Kinesin-associated protein (Kap). Kap was shown to transport both particulate cargo, as axoneme components in rafts, and membrane-bounded organelles such as melanosomes. *Drosophila* Kinesin II was shown to be essential for the axonal transport of choline acetyltransferase in a specific set of neurons. We have generated *Kap* mutants and show that gene activity is not only required for neuronal function but also for separation of follicles during early oogenesis. The data suggest that Kap participates in the transport of signalling components required for instructive interactions between germline and soma cells.

Keywords: *Drosophila*; Kinesin II; oogenesis; signalling; cellular transport

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INTRODUCTION

Asymmetric localization of cellular components, such as organelles, vesicles and mitochondria, depends on active transport along microtubule tracks by ATP-driven molecular motors of the dynein and kinesin protein superfamilies (reviewed in Goldstein & Yang, 2000). Members of the Kinesin I and the Kinesin II/Kif3 families function in the transport of organelles as well as soluble cellular components, and have a major role in axonal transport from neuronal cell bodies to nerve termini in vertebrates, *Caenorhabditis elegans* and *Drosophila* (reviewed in Goldstein & Yang, 2000; Hirokawa, 2000).

In *Drosophila*, Kinesin II forms a heterotrimeric complex (reviewed in Cole, 1999; Hirokawa 2000) composed of two motor subunits, KLP68D and KLP64D, and a singular nonmotor protein, the Kinesin-associated protein 3 (Kap3), which acts as adaptor and regulator of the complex (Ray *et al*, 1999). Recent studies on KLP64D mutants suggest that Kinesin II is specifically required for the axonal transport of choline acetyltransferase in a subgroup of neurons (Ray *et al*, 1999). A similar phenotype was reported for Kap mutants (Sarpal *et al*, 2003), suggesting that Kap/Kinesin II complexes are only required for neural function. However, in mice, humans and rats as well as in sea urchins and algae, Kinesin II was shown to be necessary for the formation and maintenance of cilia/axoneme structures (reviewed in Rosenbaum *et al*, 1999). In some organisms, a third motor protein, Kif3C, has been identified. Kif3C was proposed to be part of distinct Kinesin II complexes, which act in a spatiotemporal and/or cell-specific fashion (reviewed in Cole, 1999). We identified a Kif3C homologue in the *Drosophila* genome (CG17461; FlyBase, 2002; see supplementary information online), suggesting that the previous studies may not have revealed all aspects of Kinesin II-dependent Kap function in the fly (Ray *et al*, 1999; Sarpal *et al*, 2003). To test this possibility, we generated *Kap* lack-of-function mutants. Here we report that in addition to the recently described neural function, Kap activity is also required in germ cells for proper follicle separation during oogenesis. The results suggest that Kap participates in signalling necessary for the establishment of follicle-separating stalk cells.

RESULTS AND DISCUSSION

Generation and molecular analysis of *Kap* mutants

To assess Kinesin II requirement, we generated mutations affecting the single non-motor component *Kap* of *Drosophila* (Sarpal & Ray, 2002; own observation). The organization of the gene (Fig 1) and the conserved domain structure of the protein as revealed by comparison of Kap with human, mouse and sea urchin homologues are shown (supplementary Fig 1 online). We recovered a semilethal mutation, *l(1)G0396*, in which a *P{lacW}* element (Peter *et al*, 2002) was inserted in the first intron of the *Kap* gene (Fig 1A). In all, 95% of hemizygous *l(1)G0396* males develop into pupae and die as pharate adults; about 5% hatch and show a

Max-Planck-Institut für biophysikalische Chemie, Abteilung Molekulare Entwicklungsbiologie, 37070 Göttingen, Germany

*These authors contributed equally to this work

[†]Present address: Institut für Zellbiologie, Universität Bremen, Postfach 330440, 28334 Bremen, Germany

⁺Corresponding author. Tel: +49 551 201 1482; Fax: +49 551 201 1755; E-mail: hjaeckl@gwdg.de

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Fig 1 | Structure and expression of the *Kap* (CG11759) transcription unit (region 10B of X chromosome; FlyBase, 2002; Sarpal & Ray, 2002). (A) Genomic organization. The *Kap* transcript contains nine exons (black bars: coding region; white boxes: untranslated sequences). Position of the *L(1)G0396* P-element insertion is marked. The sequence of *Kap* is shown in supplementary Fig 1 online. (B–E) Expression patterns in a *Drosophila* follicle (B) and embryos (C–E) as revealed by whole-mount *in situ* hybridization of a *Kap*-specific RNA probe; orientation: anterior left and dorsal up. Note strong *Kap* expression in nurse cells (stage 9) (B), a ubiquitous distribution of maternal transcripts in a stage 2 embryo (C), zygotic expression in ectodermal and mesodermal derivatives (stage 12 embryo) (D) and the enrichment of transcripts in the central and peripheral nervous systems in older embryos (E; details in Sarpal & Ray, 2002).

paralytic phenotype as described for *KLP64D* (Ray et al, 1999). Precise excisions of the P-element (Bellen et al, 1989) resulted in wild-type flies, indicating that the P insertion is the cause of the mutation. We also obtained imprecise excisions (Bellen et al, 1989), which represent hypomorphic *Kap* alleles, which have small internal deletions in the protein coding sequence. In addition, we obtained alleles such as *Kap*⁸⁹, which lack the promoter and the first exon of the gene (Fig 1A). Homozygous *Kap*⁸⁹ mutants fail to express *Kap* as revealed by *in situ* hybridization. In addition, their phenotype was as strong as the phenotype of transheterozygous *Kap*⁸⁹/*Df(1)v-N48* mutant individuals (data not shown). These findings indicate that *Kap*⁸⁹ is a null mutation. *Kap*⁸⁹ and the hypomorphic mutants could be rescued by *Kap* activity that was derived from an *Actin5C* enhancer-driven *Kap* cDNA transgene, confirming that the mutations affect only the *Kap* gene (supplementary Fig 2 online).

To determine the sites of *Kap* expression in the organism, we performed RNA *in situ* hybridization on staged ovaries and embryos. During oogenesis, *Kap* expression is observed in nurse cells from where transcripts are transported into the growing oocyte (Fig 1B). The transcripts remain ubiquitously distributed in

eggs and embryos until the blastoderm stage (Fig 1C). Zygotic *Kap* expression is initiated during gastrulation in both ectoderm and mesoderm (Fig 1D) and is subsequently enriched in neurons (Fig 1E). Based on the strong maternal expression of the gene, we asked whether *Kap* also has a role during oogenesis in addition to its recently reported function in the nervous system (Sarpal et al, 2003).

Lack of *Kap* activity affects oogenesis

To assess the function of maternal *Kap* activity (Fig 1C), we generated homozygous *Kap* mutant germline clones using the *FLP/ovo*^{D1} system (Chou & Perrimon, 1992). Females with homozygous *Kap*⁸⁹ mutant germ lines are sterile, as germline mutant follicles degenerate after they reached stage 6 of oogenesis (100%; *n* > 1,000; staging according to Spradling, 1993). Up to this stage, mutant follicles lacking *Kap* contain more cells than wild-type follicles. The supernumerary cells are either only nurse cells (type I follicles) or both nurse cells and oocytes (type II follicles). Type II follicles have multiple oocytes and a corresponding number (ratio 1:15) of extra nurse cells. Of all follicles scored (*n* > 1,000; more than 300 individuals examined), 50% showed mixed type I/II follicles. Of the remaining follicles, few (≤ 5%) showed either type I or type II follicles only.

The supernumerary cells in type I follicles (Fig 2C–G) suggest that the mutant germline cells have undergone more than the normal four rounds of mitotic divisions. To confirm this proposal, we inspected the number of ring channels and the fusome of the germline mutant ovaries. Both structures derive from an incomplete separation of daughter cells after the division, resulting in a maximum of four ring channels in the case of the wild-type oocyte (reviewed in Spradling, 1993) and in a branched fusome structure that interconnects the germ cells of a follicle (de Cuevas & Spradling, 1998). We found *Kap* mutant germ cells that have more than four ring channels (Fig 2C,G) and fusomes connecting up to about 50 cells (supplementary Fig 3 and movie online). This indicates that at least one extra round of mitotic divisions occurs in *Kap* mutant follicles. The use of oocyte markers, such as *gurken* (*grk*) and *oskar* (*osk*) mRNA (reviewed in Riechmann & Ephrussi, 2001), identified either one oocyte or multiple oocytes in ectopic locations within the follicles (Fig 3C–F). Collectively, these findings indicate that the determination and the initial stages of oocyte differentiation do not depend on *Kap* activity, whereas the cellular processes underlying the formation of individual follicles and regulation of germ cell proliferation do.

Kinesin I has previously been shown to participate in the transport of distinct mRNA species and associated protein to the posterior pole of the *Drosophila* oocyte (Brendza et al, 2000; Cha et al, 2002). To assess the integrity of this type of microtubule-dependent transport in *Kap*⁸⁹ mutant follicles, we examined the expression and localization of the oo18 RNA-binding protein (Orb). Orb is necessary for the directed transport and localized translation of *grk* and *osk* mRNA, the axial polarity determinants of the growing oocyte (Chang et al, 1999, 2001). We found that the expression of Orb is initiated normally, although it is not maintained subsequently (Fig 3B). The oocyte determination factor Bic-D (Wharton & Struhl, 1989) and β -tubulin are expressed normally, and *oskar* RNA is normally expressed and transported in *Kap*⁸⁹ mutant follicles (data not shown). Thus, cytoskeletal structures and the transport of *oskar* mRNA into the growing

Fig 2 | Morphological analysis of *Kap* mutant germline clones showing actin in red and DNA in green (A–I). (A) Wild-type follicle (stage 9A) showing highly polyploid nurse cells and the single-layered follicle epithelium. At this stage, the epithelial cells move towards posterior (right side). The position of border cells (out of focal plane; asterisk) is indicated. (B) Hypomorphic mutant showing two fused follicles with a double row of follicle cells (arrowhead) between the germ cell clusters. Follicle cells behave otherwise as in one individual follicle; both the surrounding epithelium and border cells (other focal plane, asterisk) move normally. Note two prospective oocytes as identified by nuclear morphology, massive subcortical actin and yolk accumulation. (C,D) Two compound ovarioles of lack-of-function mutant ovaries. Note again the follicle fusion. (E,F) Examples of follicles with supernumerary germ cells in a single layer of follicle cells. Note two oocyte nuclei (arrowheads in E) and an incorrect number of ring channels (insets: examples with three and five instead of four ring channels, respectively). Note also the normal morphology of the anterior most nurse cells shown in (F) and the altered size and shape in more posterior positions; also, no oocyte was found. (G–I) Mutant follicle surrounded by a single layer of follicle cells. Single plane overview shows 31 nurse cells (G; enlarged in H) and a single oocyte nucleus (arrowhead in H). This oocyte has five ring channels (1–5) as revealed by an overlay of nine successive focal planes, indicating at least five mitotic divisions of the germ cells. (J,K) Clonal analysis showing stalk formation by *Kap* mutant cells (marked by the absence of GFP staining in green; DNA staining: red). (J) Early stalk development (stage 1 connected to stage 3 follicle; *Kap*-deficient cells are marked by an arrowhead). (K) A mature stalk where differentiated stalk cells (arrowheads) and the polar cells (arrows) lack *Kap*. Scale bar in (A) (representative for A,B,D–F), 50 μ m. Other scale bars are 20 μ m (C,G) and 5 μ m (H–K). See text for further details.

Fig 3 | Characterization of *Kap* germline mutant ovaries. (A) Anti-Orb antibody staining of wild-type ovaries. Note initial Orb expression in wild-type germ cells (germarium region 2), the subsequent accumulation in the two pre-oocytes (region 2A; arrow) in the adjacent germ cell cluster and the final concentration in one posterior cell of the next cell cluster (region 2B; asterisk in A). (B) Anti-Orb antibody staining of *Kap* mutant ovaries. Note that initial Orb expression and accumulation (arrow) is normal, but is not maintained in the adjacent and older germ cell cluster (asterisk). (C,D) *In situ* hybridization showing that *grk* RNA is expressed in one (C) or two (D) germline cells, which correspond to young oocytes in *Kap* mutant ovaries. Older follicles show no detectable *grk* transcripts (C; arrow). (E,F) *osk* RNA appears in region 2 of d*Kap* mutants (E), but fades out (F; arrow). (G,H) A101lacZ expression in wild-type (G) and *Kap* mutant follicles (H). (I,J) *unpaired* expression in the polar cells (J; wild type) cannot be detected in *Kap* mutants (arrow; K).

oocyte appear not to be directly affected by the lack of *Kap* activity.

Lack of *Kap* activity affects follicle separation

Separation of follicles during early oogenesis involves the formation of a characteristic intervening stalk structure (Spradling, 1993). This structure derives from a small group of precursor cells (located in germarium region 2B), which develop into polar follicle cells and the stalk cells (TwoRoger et al, 1999; Besse & Pret, 2003). Their proper development depends on cell–cell communication events between the germline and soma cells as shown by Notch/Delta, JAK/STAT and Hedgehog signalling mutants, which develop fused follicles and lack morphologically distinct stalks (Grammont & Irvine, 2001; Lopez-Schier & St Johnston, 2001; Besse et al, 2002; McGregor et al, 2002). As

such a phenotype was also observed with the *Kap* mutant ovaries (Fig 2C), we asked whether polar follicle cells, which can be visualized by *A101-lacZ* expression (Fig 3G; Ruohola et al, 1991), and stalk cells are formed. In *Kap* mutant follicles, supernumerary *A101-lacZ* expressing (Fig 3H) polar follicle cells were observed, whereas follicle-separating stalk cells were absent. Identical results were obtained by anti-FasIII antibody staining, a different marker for polar follicle cells (Ruohola et al, 1991; not shown). These observations suggest that stalk precursor cells fail to differentiate, remain in the follicle epithelium and express the molecular characteristics of follicle cells as has been observed in *Notch* mutants (Grammont & Irvine, 2001). Clonal analysis showed that stalk cells are formed properly in the absence of *Kap* activity in the somatic epithelial cells (Fig 2J,K). Thus, *Kap* activity has a cell autonomous effect on germline cell proliferation, and the absence of *Kap* in germ cells also affects somatic precursor cells in a non-cell autonomous manner.

The non-autonomous *Kap* effect on follicle cell determination could be explained by a requirement for *Kap* for the transport and/or localization of components that are needed to signal cell fate to the surrounding somatic precursor cells. We therefore examined *Kap* mutant ovaries for the expression of Delta, which activates *Notch* signalling (Portin, 2002), *hedgehog* (Forbes et al, 1996) and the JAK/STAT-activating ligand *unpaired* (Harrison et al, 1998). *Delta* and *hedgehog* expressions were not affected (data not shown), whereas *unpaired* failed to be expressed in polar follicle cells of *Kap* germline mutant ovaries (Fig 3I,J). This observation suggests that lack of *Kap* activity interferes with the signalling-mediated crosstalk between germline and somatic cells.

Recent studies on Kap/Kinesin II complexes showed that *Kap* is required for neural function (Ray et al, 1999; Sarpal et al, 2003). Our results provide evidence that *Kap* activity in germline cells is required for the proper differentiation of a distinct group of epithelial cells, the stalk cells, which separate individual follicles during early oogenesis. We found that the absence of *Kap* in germ cells prevents somatic target cells from differentiating into proper polar follicle cells, because they fail to express the ligand *Unpaired*. *Unpaired* activates JAK/STAT signalling (Harrison et al, 1998) and thereby induces stalk cell fate in the respective precursor cells (McGregor et al, 2002). We are currently investigating the details of the germline cell/polar follicle cell/stalk cell signalling cascade to address the cellular mechanism of these interactions.

METHODS

Stocks and genetics. Wild-type strain Oregon R and mutant strains *FM6y¹dm⁺B/Dp(1;Y)y⁺, Df(1)v-N48,f⁺C(1)DXy¹f¹/Dp(1;Y)y⁺v⁺#3, P{ry⁺17.2=IaRb}neur^{A101}ry⁵⁰⁶/TM3ry^{RK}Sb¹Ser¹, y¹w^{*}v²⁴P{w⁺mW.hs=FRT(w^{hs})FRT¹⁰¹, y¹w¹¹¹⁸P{w⁺mC=Ubi-GFP.nls}X1P{w⁺mW.hs=FRT(w^{hs})9-2; Kr¹/CyO and w^{*}ovo^{D1}v²⁴P{w⁺mW.hs=FRT(w^{hs})FRT¹⁰¹/C(1)DX/Y;P{ry^{17.2}=hsFLP}38* were obtained from the Bloomington stock centre; *l(1)G0396* was derived from a lethal P-element screen (Peter et al, 2002). A total of 13 *Kap* mutants were generated by mobilizing the X chromosomal *l(1)G0396* insertion (Bellen et al, 1989). Homozygous cell clones were generated by mitotic recombination using the FLP-*ovo*^{D1} system (Chou & Perrimon, 1992). F₁ larvae of the genotype *Kap*^{mut}/*ovo*^{D1}; *FLP*³⁸/+ were heat shocked twice (38 °C; 1 h; second and third

instar stages) to induce recombination. To distinguish wild-type (green) and mutant cells, we used *Kap*^{mut}/*ubiGFPnls*; *FLP*³⁸/+ individuals. Ovaries were isolated and examined after staining with different markers (see below); the phenotypes (see text) obtained with *ovo*^{D1} and GFP marked germlines were identical. For gene symbols and alleles, see Lindsley & Zimm (1992).

Transgenic flies. Sequences corresponding to the open reading frame of *Kap* were PCR amplified (cDNA clone LP01387; FlyBase 2002) using the *Kap*-ATG-*EcoRI* (5'-TAGTCGAATTCCGACAGCAGCAGCAACAACAACCTG-OH) and *Kap*-FLAG-STOP-*XbaI* (5'-TTTCTAGATTACTTGTCATCGTCATCCTTGTAATCAGCCATCAGCAGCTCCTCG-OH) primers. The PCR product was digested (*EcoRI* and *XbaI*) and cloned into the matching sites of pAc5.1-V5-His-B (Invitrogen). The *SmaI*-*XbaI* fragment was subcloned into *SmaI*/*XbaI*-digested pC4-hs43-AUGβGal (pC4-Ac5.1-Kap-FLAG) and transformed into *Drosophila* (Rubin & Spradling, 1982).

Staining of ovaries and embryos. Embryos were fixed and devitellinized (Patel, 1994), and ovaries were prepared according to Theurkauf et al (1992). Rabbit anti-β-gal (1:5,000; Cappel), rabbit anti-GFP (Lot 71B1-3; 1:600; Molecular Probes) and mouse anti-Orb 4H8 and 6G4 (1:20, DSHB; Lantz et al, 1994) were used as primary antibodies; goat anti-mouse and anti-rabbit (Alexa 488 or 546; 1:500; Molecular Probes) were used as secondary antibodies. Filamentous actin was stained by adding 1.5 U of Alexa 488- or 633-coupled phalloidin (Molecular Probes, Leiden) during secondary antibody incubation. For DNA detection, samples were extensively treated with RNaseA (Sigma; 20 µg/ml in all buffers) and stained with 0.1 µM Sytox Green or TOTO-3 (Molecular Probes; ≥1 h), also added to the embedding medium (0.05 µM).

Whole-mount *in situ* hybridizations were performed according to Hauptmann & Gerster (2000). Digoxigenin- or fluorescein- (DIG and FITC labelling mix; Roche) labelled probes and horseradish-peroxidase-or alkaline-phosphatase-coupled anti-DIG and anti-FITC antibodies (Roche) were used in combination with nitro blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (Sigma) or Tyramide Signal Amplification System Plus-Cyanine 3 (Perkin-Elmer), respectively. Images were taken with Zeiss Axiophot and LSM microscopes.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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