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Mutations in the *Drosophila* gene *bullwinkle* cause the formation of abnormal eggshell structures and bicaudal embryos

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SUMMARY

Subcellular localization of gene products and cell migration are both critical for pattern formation during development. The *bullwinkle* gene is required in *Drosophila* for disparate aspects of these processes. In females mutant at the *bullwinkle* locus, the follicle cells that synthesize the dorsal eggshell filaments do not migrate properly, creating short, broad structures. Mosaic analyses demonstrate that wild-type BULLWINKLE function is required in the germ line for these migrations. Since the mRNA for *gurken*, the putative ligand that signals dorsal follicle cell fate, is correctly localized in *bullwinkle* mutants, we conclude that our *bullwinkle* alleles do not affect the dorsoventral polarity of the oocyte and thus must be affecting the follicle cell migrations in some other way. In addition, the embryos that develop from *bullwinkle* mothers are bicaudal. A KINESIN:β-GALACTOSIDASE fusion protein is correctly localized to the posterior pole of *bullwinkle* oocytes during stage 9. Thus, the microtubule structure of the oocyte and general transport along it do not appear to be disrupted prior to cytoplasmic streaming. Unlike other bicaudal mutants, *oskar* mRNA is localized correctly to the posterior pole of the oocyte at stage 10. By early embryogenesis, however, some *oskar* mRNA is mislocalized to the anterior pole. Consistent with the mislocalization of *oskar* mRNA, a fraction of the VASA protein and *nanos* mRNA are also mislocalized to the anterior pole of *bullwinkle* embryos. Mislocalization of *nanos* mRNA to the anterior is dependent on functional VASA protein. Although the mirror-image segmentation defects appear to result from the action of the posterior group genes, germ cells are not formed at the anterior pole. The bicaudal phenotype is also germ-line dependent for *bullwinkle*. We suspect that BULLWINKLE interacts with the cytoskeleton and extracellular matrix and is necessary for gene product localization and cell migration during oogenesis after stage 10a.

Key words: bicaudal, eggshell defects, maternal effect, RNA localization, cell migration, cytoskeleton, *Drosophila*, *bullwinkle*

INTRODUCTION

The *Drosophila* egg chamber is composed of a cyst of sixteen interconnected, germ-line cells surrounded by somatically derived follicle cells. Of the sixteen germ-line cells, one becomes the oocyte and the remaining fifteen function as nurse cells. The nurse cells provide the oocyte with RNA and proteins to be used by the embryo during its development, while the follicle cells synthesize various layers and structures of the eggshell (reviewed by Spradling, 1993). A small number of RNAs and proteins are specifically transported from the nurse cells to the oocyte early in development, while the majority are non-specifically deposited into the oocyte during the latter half of oogenesis (reviewed by Mahajan-Miklos and Cooley, 1994).

Products deposited in the egg during oogenesis determine embryonic polarity. Genetic analyses of dorsoventral mutations indicate that a signal from the germ-line cells to dorsal follicle cells establishes the dorsoventral axis of the egg (Schüpbach, 1987). This event regulates the subsequent migration of the dorsal follicle cells and their synthesis of the dorsal eggshell appendages. In addition, reception of the germ-line signal by dorsal follicle cells regulates the production of a later signal that determines the ventral axis of the embryo (Schüpbach, 1987). Thus, mutations early in the pathway affect the dorsoventral polarity of both the eggshell and the embryo, while downstream mutations affect either the eggshell or the embryo. *cappuccino* (*capu*), *spire* (*spir*), *squid* (*sqd*), *orb* and *fs(1)K10* are necessary for the correct localization of *gurken* (*grk*) mRNA, which encodes the presumptive signal from the oocyte to the follicle cells (Neuman-Silberberg and Schüpbach, 1993; Christerson and McKearin, 1994). TORPEDO (TOP), the *Drosophila* homologue of the mammalian EGF receptor, is thought to receive the 'dorsal' signal (Schüpbach, 1987; Price et al., 1989; and Schejter and Shilo, 1989), which is then transduced through the *Ras* pathway to determine dorsal follicle cell fate (Brand and Perrimon, 1994; Berg and Schnorr, unpublished data).

Mutations that disrupt the dorsoventral polarity of the oocyte alter the shape of the eggshell. The dorsal and ventral sides of the eggshell have two major morphological differences: the eggshell is shorter on the dorsal side, and the filaments are located dorsally just lateral to the midline (Fig. 1). These differences in eggshell structure result from differences in the

pattern of migration of follicle cells on the dorsal and ventral sides of the eggs. At mid-oogenesis, the dorsal anterior follicle cells, which have received the GURKEN signal, migrate into two circles. These cells secrete chorion proteins centripetally to form the base of the eggshell filaments. When the base is finished, a layer of follicle cells migrates past the previous cells and forms another circle of eggshell protein. The migration continues in this fashion, forming a hollow cylinder of chorion, until the follicle cells spread out to form the flat paddle structures at the end (King, 1970; King and Koch, 1963). In contrast, the ventral follicle cells simply expand to maintain an epithelial sheet around the oocyte at the time that the nurse cells deposit their contents into the oocyte. Dorsalizing mutations cause the ventral side to resemble the dorsal side, resulting in the formation of short eggs with an entire ring of eggshell filaments. Conversely, ventralizing mutations cause the eggs to be much longer and have a single dorsal appendage or no dorsal appendages (Schüpbach, 1987; Manseau and Schüpbach, 1989; Kelley, 1993; Wieschaus et al., 1978; Christerson and McKearin, 1994).

Genes required maternally for anterior/posterior polarity have also been identified. Two morphogens are important for proper anterior/posterior development: BICOID (BCD), the anterior morphogen, and NANOS (NOS), the posterior morphogen. Both morphogens are localized as RNA via their 3′UTRs to their respective poles during oogenesis (Macdonald and Struhl, 1988; Wang and Lehmann, 1991; Gavis and Lehmann, 1992). Localization of the *bcd* message to the anterior pole requires *exuperantia*, *swallow* and *staufen* (for review see Driever, 1993). Localization of the *nos* message to the posterior pole requires the products of the *staufen*, *mago nashi*, *oskar* (*osk*), *vasa*, *valois* and *tudor* genes, which are known collectively as the posterior group genes (reviewed by St. Johnston, 1993). *osk* mRNA, VASA, VALOIS and TUDOR proteins, and *nos* mRNA are localized in a step-wise manner to the posterior pole, and localization of each depends upon the correct localization of all previous products (Ephrussi et al., 1991; Lehmann and Nüsslein-Volhard, 1991). *osk* mRNA is localized via its 3′ UTR. If the coding region of *osk* is fused to the *bcd* 3′ UTR, *osk* mRNA is localized to the anterior pole (Ephrussi and Lehmann, 1992). Upon translation, the mislocalized OSK protein then recruits other posterior group gene products, including *nos* mRNA, and a bicaudal embryo is formed due to translational repression of *bcd* RNA (Wharton and Struhl, 1991). Mutations in several genes, including *bicaudal*, *Bicaudal-C* (*Bic-C*) and *Bicaudal-D* (*Bic-D*), also cause the formation of bicaudal embryos (Bull, 1966; Mohler and Wieschaus, 1986).

A few genes are required in both the dorsoventral and the anterior/posterior pathways. Mutations in *capu* and *spire* induce premature cytoplasmic streaming in the oocyte (Theurkauf, 1994), preventing the localization of *grk* mRNA and the posterior group gene products and thereby causing the loss of ventral and posterior structures, respectively (Manseau and Schüpbach, 1989). These defects are general localization defects and do not cause the formation of bicaudal embryos.

bullwinkle (*bwk*) is unusual in that it affects several of these processes. Mutations in *bwk* cause both the formation of abnormal eggshell filaments and bicaudal embryos. We present genetic and cell biological analyses of *bwk.*

MATERIALS AND METHODS

Fly stocks

Canton-S and *mwh red* flies were used as wild-type controls. The bwk^{8482} and *PZ5650* lines were created in a *P*[*lacZ*, ry^+] mutagenesis screen (described in Karpen and Spradling, 1992). $Df(3R)Dl^{BX12}$ spans 91F1-2 to 92D3-6 and was obtained from the Bloomington stock center. The bwk^{151} line was created by Rick Kelley (1993). The $chic^{7886}$ stock was provided by Lynn Cooley. The qua^{HM14}, grk^{HG21} and $vasa^{PD23}$ stocks were provided by Trudi Schüpbach. The $vasa^{LYG2}$ stock was created by Lin Yue (Yue, Berg and Spradling, unpublished data). *KZ32* was created by Clark et al. (1994), and the *P*[ovo^{D1}] insertion line was created by Chou et al. (1993). osk^{301} was provided by Ruth Lehmann.

Detecting β-GALACTOSIDASE expression in ovaries

P[*lacZ*, ry^+] lines

Ovaries were fixed and stained according to Cooley et al. (1992). The ovarioles and individual egg chambers were mounted in 65% glycerol and examined with a Nikon microphot FXA using differential interference contrast optics.

KIN:β-GAL lines

Ovaries were fixed and stained according to Clark et al. (1994), except that fixation was carried out in 0.5% glutaraldehyde (SIGMA, EM grade) and the staining solution contained 0.75% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside).

Excision screen

ry^{506} *Sb P*[ry^+, *Δ2-3*]/TM6 females (Robertson et al., 1988) were crossed to ry^{506} bwk^{8482} males. Fifty ry^{506} bwk^{8482}/ry^{506} *Sb P*[ry^+, *Δ2-3*] males were selected from the progeny and crossed to ry^{506}/TM3, ry^{RK} *Sb* females in individual vials. In the next generation, one rosy eyed (from loss of ry^+ in the *PZ* element) ry^{506} bwk^{8482*}/TM3, ry^{RK} *Sb* male was chosen from each vial and crossed to females from the original ry^{506} bwk^{8482}/TM3, ry^{RK} *Sb* stock to test fertility and to establish lines.

cDNA in situ hybridization

cDNA in situ hybridizations to ovaries were carried out essentially according to Ephrussi et al. (1991), and to embryos as per Tautz and Pfeifle (1989). Digoxigenin probes were prepared using Boehringer Mannheim DNA labeling and detection kit. We used a *gurken* cDNA (provided by Shira Neuman-Silberberg and Trudi Schüpbach), a *bicoid* cDNA (provided by Markus Noll), an *oskar* cDNA (provided by Ruth Lehmann) and a *nano*s cDNA (provided by Paul Macdonald) to prepare our DNA probes.

Cuticle preparations

Cuticle preparations were carried out according to Wieschaus and Nüsslein-Volhard (1986), except the embryos were mounted in 4:1:1 Hoyer's : lactic acid : ddH_2O and incubation times were adjusted accordingly.

Table 1. $bullwinkle^{8482}$ excision lines

Class	Number of lines	Phenotype
Fertile female	31 (65%)	Both eggshell and embryo phenotypes are wild type
Sterile female	12 (25%)	Eggshells have bwk phenotype
Abnormal wings	1 (2%)	Line is semi-lethal, wings have extra veins, eggshells have bwk phenotype
Lethal	4 (8%)	Either late larval lethal or late pupal lethal

Embryo antibody staining

Embryos were fixed and devitellinized according to Ashburner (1989) (protocol 96), except that after rinsing in methanol, the embryos were placed in 100% ethanol for storage at −20°C. After storage the embryos were rinsed in methanol. Endogenous peroxidase activity was removed by treating with 0.3% H_2O_2 in methanol for 30 minutes. The embryos were rehydrated by washing in 75% methanol in PBTr (PBS + 0.1% Triton X-100), 50% methanol in PBTr, 25% methanol in PBTr, and then in PBTr. The blocking and antibody incubations were carried out according to Ashburner (1989), except that the blocking solution was 5% normal goat serum in PBTr. The anti-VASA antibody, a gift from Paul Lasko, was diluted 1:1000. Anti-rabbit antibody from the Vector *elite* ABC kit was used as the secondary antibody. Subsequent steps were carried out according to the *elite* ABC kit (Vector). The embryos were mounted in 75% glycerol/25% PBS.

ovo^{D1} germ-line clones

mwh red control females, *bwk*^{8482}/TM3 females, and *bwk*^{151}/TM3 females were crossed to *P[ovo*^{D1}*]/Ki* males to generate larvae of the correct genotypes. The first instar larvae (24 to 48 hours old) were irradiated with a Picker 805D X-ray machine at a constant dose of 1000 rads (70Kv, 3mA, 0.5 mm aluminum filter for 2.2 minutes), as described by Chou et al. (1993). Adult flies transheterozygous for the gene of interest and *ovo*^{D1} were selected by the presence of wild-type bristles. *bwk/P[ovo*^{D1}*]* females were mated to *bwk*^{151} males to facilitate progeny testing. The females were allowed to lay eggs for 6-7 days. Any eggs laid were scored for eggshell phenotype and viable embryos. If any eggs were laid in the vial, all females within the vial were dissected. Some females in vials where no eggs were laid were also dissected.

Pole cell transplantation

p^{p} *osk*^{301} *sbd bwk*^{8482} */ p*^{p} *osk*^{301} females were crossed to *cv-c sbd bwk*^{151} males to generate host embryos. Females homozygous for *osk*^{301} were used so that the host embryos would lack pole cells (Lehmann and Nüsslein-Volhard, 1986). Donor embryos were Canton-S. All embryos were collected for 1-2 hours at 25°C on apple juice agar plates, dechorionated in 50% bleach and rinsed in 0.5% NaCl/0.03% Triton X-100. Cellularized embryos were lined up on an agar block and transferred to a sticky coverslip. Host and donor embryos were placed on separate coverslips to allow different dehydration times and to facilitate removal of the donor embryos. (Sticky coverslips were made by shaking ~1 meter of Scotch 3M double stick tape in 10 ml of heptane for 1 hour, centrifuging and dipping coverslips into the sticky heptane.) The embryos were dehydrated by placing in a container with drierite: host embryos for 11-14 minutes, donor embryos for 4 minutes. After dehydration, Halocarbon oil series 200 (Halocarbon Products, NJ) was used to cover the embryos. Pole cells were transferred using a Narishige NU-202 3 way control micromanipulator and model IM-6 syringe. The injection tubing was filled with Halocarbon oil series 27 (Halocarbon Products, NJ). After pole cell transplantation, host embryos were transferred on their coverslips to apple juice agar plates and allowed to hatch at 20°C. Larvae were transferred to fresh food and placed at 25°C. Surviving *bwk*^{151}*/bwk*^{8482} females were crossed to males and allowed to lay eggs. Any female that failed to lay eggs after 4-6 days was dissected in *Drosophila* Ringer's solution. Our pole cell transplantation technique is based on Van Deusen (1976), with modifications by Seigfried Roth, Trudi Schüpbach and ourselves.

RESULTS

We obtained *bwk*^{8482} (*bullwinkle*), a female sterile mutation at 92D, from a large scale *P*[*lacZ, ry*$^{+}$] (*PZ*) mutagenesis screen carried out in Allan Spradling's laboratory (described in Karpen and Spradling, 1992). Mutations in *bwk* affect the proper formation of the *Drosophila* eggshell and embryo. Flies that carry *bwk*^{8482} in *trans* to a deficiency for the region (*Df(3R) Dl*^{BX12}) are also sterile and have similar eggshell defects. In addition, these flies have blistered wings, indicating that *bwk* is required for proper wing morphogenesis and that *bwk*^{8482} is not a null allele.

To analyze these phenotypes more thoroughly, we generated new alleles by transposase-induced excision (Table 1). Excision of the *PZ* element reverts both eggshell and embryo phenotypes to wild type, indicating that the *PZ* insertion is

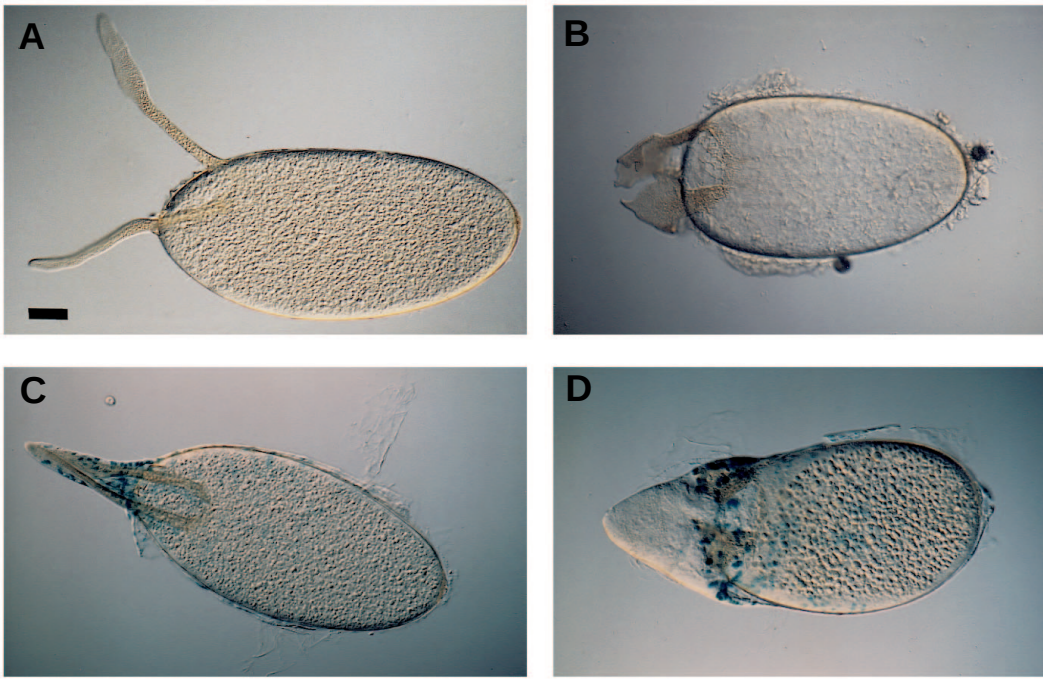

Fig. 1. Abnormal dorsal filaments in *bwk* egg chambers are due to altered follicle cell migration. Anterior is to the left, dorsal is facing out of the page, unless otherwise noted. (A) Wild-type stage 14 egg chamber, dorsal is up. (B) Stage 14 egg chamber from a *bwk*^{8482} mother. The dorsal filaments are shorter and broader than wild-type dorsal filaments. The eggs are shorter and rounder due to the slightly dumpless nature of *bwk*. (C) Wild-type stage 14 egg chamber showing the *PZ5650* enhancer trap pattern. The follicle cell nuclei stain over the dorsal filaments. (D) Alterations in the *PZ5650* enhancer trap pattern in a *bwk*^{151} background. The stained follicle cells do not migrate out as far over the nurse cells, creating short, broad dorsal filaments. Bar, 20 μm.

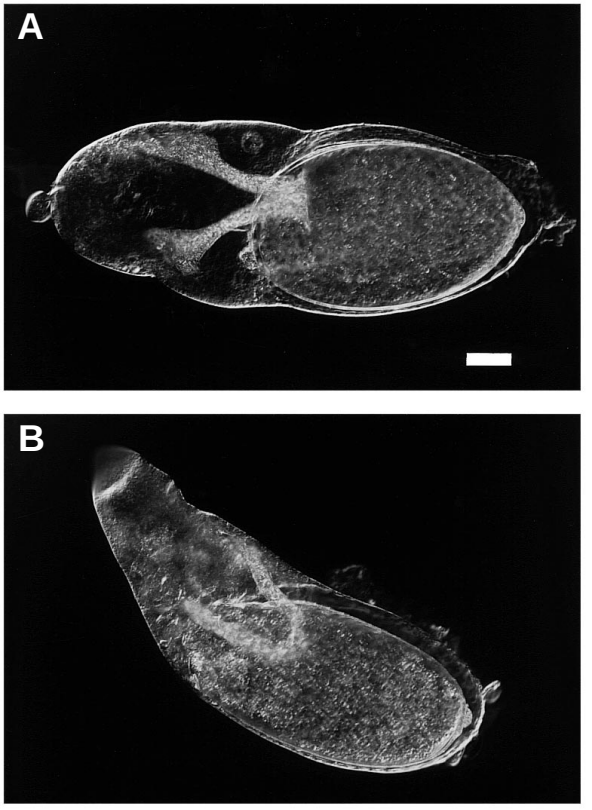

Fig. 2. Partially dumpless mutants have less severe dorsal filament defects than *bwk* eggshells. Anterior is to the left, dorsal is facing out of the page. (A) Stage 14 egg chamber from a *chic*^{7886} mother. The dorsal filaments are formed on top of the remaining nurse cells. Note that although the filaments are broader than wild-type filaments, they are longer than *bwk* filaments. The *chic*^{7886} filaments are also fused at the base. (B) Stage 14 egg chamber from a *qua*^{HM14} mother. The dorsal filaments are short, but are not nearly as broad as *bwk* filaments. Bar, 20 μm.

responsible for the *bwk*^{8482} phenotype. In addition to obtaining several lines with phenotypes similar to the starting allele, we recovered five lines with additional phenotypes (Table 1). In one excision line, which retains the *bwk*^{8482} egg phenotype, the wings of homozygous flies curve down and have extra veins. Four lethal excision alleles were also obtained. In three of the lethal lines, homozygotes die as pharate adults. The most severe lethal mutation, a deletion of at least 10 kb, is a late larval lethal with some escapers that die as pupae (data not shown). We also obtained a *P*[*ry*$^{+}$] insertion allele of *bwk*, *bwk*^{151}, which was generated by Rick Kelley. *bwk*^{151} has eggshell and embryo defects similar to *bwk*^{8482}.

Eggshell defects

Females homozygous for *bwk*^{8482} lay few eggs, which often appear deflated shortly after being laid. *bwk* eggs are shorter than wild-type eggs, being slightly 'dumpless' due to the incomplete transfer of nurse cell contents into the oocyte at stage 11. In addition, *bwk* eggs have a number of anterior eggshell defects (Fig. 1B). The dorsal eggshell filaments are short and broad and have ragged edges and thin chorion, sometimes resembling moose antlers, hence the name *bull-winkle*. The operculum is weak and yolk often streams from the anterior of the egg upon dissection. Females homozygous for *bwk*^{151} lay slightly more eggs, which have similar eggshell defects.

The eggshell phenotype of *bwk* mutants suggests that the follicle cells do not migrate properly as they form the dorsal eggshell filaments. In order to examine the follicle cell migrations more closely, we labeled these cells to observe their movements. We took advantage of line *PZ5650*, an enhancer trap line that marks the follicle cells that form the dorsal filaments, providing information on their number and placement (Gillespie and Berg, unpublished, and Kelley, 1993). During stage 10, *PZ5650* egg chambers express *lacZ* in two patches of follicle cells just dorsal to the oocyte nucleus (data not shown). During stages 11-14, these follicle cells continue to express *lacZ* as they migrate anteriorly to form the dorsal filaments (Fig. 1C). Mutations that affect the dorsoventral axis of the egg also alter the staining pattern of *PZ5650*. For example, the dorsalizing mutation *sqd* causes ventral and lateral follicle cells to contribute to the formation of the 'dorsal' filaments. This change in follicle cell fate in *sqd* egg chambers can be seen in a *PZ5650* background: during stage 10, the *lacZ* expression is expanded from the two dorsal patches to a broad ring that encircles the oocyte (Kelley, 1993). Both the number and position of the follicle cells expressing *lacZ* change in a *sqd* background, reflecting the change in fate.

To examine the follicle cell migration in *bwk* egg chambers, we crossed *bwk*^{151} to *PZ5650*. During stage 10, egg chambers of *PZ5650*; *bwk*^{151} females express *lacZ* in two patches of follicle cells on the dorsal side of the oocyte, identical to *PZ5650* alone (data not shown). In later stages, however, the position of the staining follicle cells is altered in *PZ5650*; *bwk*^{151} egg chambers. The follicle cells expressing *lacZ* do not migrate out as far, remaining over the short broad *bwk* dorsal eggshell filaments (Fig. 1D). These clearly marked follicle cells indicate that the migration that forms the dorsal appendages is abnormal in *bwk*^{151}. While the migration pattern of the follicle cells is altered in *bwk* egg chambers, the number of follicle cells expressing *lacZ* appears to be the same in *bwk* and wild-type egg chambers, suggesting that their cell fate is not altered.

bwk filament defects are not due to the dumpless phenotype

The slightly dumpless nature of *bwk* egg chambers presents a possible mechanism to explain the disruption in follicle cell migration. Mutations in 'dumpless' genes, such as *chickadee* (*chic*), *singed* (*sn*) and *quail* (*qua*) (reviewed in Mahajan-Miklos and Cooley, 1994), cause the formation of small eggs with abnormal dorsal filaments. Other investigators (Schüpbach and Wieschaus, 1991) have proposed that the residual nurse cell material present in these mutants acts as a barrier that inhibits the normal follicle cell migration.

We examined eggshells from two weakly dumpless lines, *chic*^{7886} and *qua*^{HM14} to compare their dorsal filament defects to those of *bwk*. Eggs from *chic*^{7886} females vary in the amount of cytoplasm that is dumped into the oocyte. The eggs range from being more dumpless than *bwk* eggs to being equivalently dumpless. Superficially, the filaments share some similarities; the *chic*^{7886} filaments are slightly shortened and broadened. The length of the filaments tends to correspond to egg length: the shorter the egg is, the shorter and broader the dorsal

filaments are. In general, however, even *chic*^{7886} eggs that are more dumpless than *bwk* have longer dorsal filaments (Fig. 2A). In addition, the bases of *chic*^{7886} dorsal filaments are closer together than wild-type filament bases, while *bwk* filament bases are further apart. *qua*^{HM14} eggshells show the same range of phenotypes as *chic*^{7886} eggshells (Fig. 2B), and compare similarly to *bwk* eggshells: eggs that are equivalently dumpless have much longer dorsal filaments that only slightly resemble *bwk* filaments. Thus the slightly dumpless nature of *bwk* eggs is insufficient to explain the dorsal filament defects.

bwk^{8482} does not affect the dorsoventral polarity within the oocyte

Although our migration studies suggested that *bwk* affects follicle cell movement and not follicle cell fate, *bwk* eggs do have characteristics of those that are slightly dorsalized: the eggs are short and round and the dorsal eggshell appendages are broader than wild type. We therefore employed several approaches to determine if our *bwk* alleles affect the dorsoventral pathway. First, we constructed double mutants between *bwk*^{8482} and *gurken* (*grk*^{HG21}), a ventralizing mutation. Second, we examined localized *grk* mRNA in a *bwk*^{8482}/*bwk*^{151} background. Finally, we examined the dorsoventral polarity of embryos produced by *bwk* mothers.

grk^{HG21} is a ventralizing mutation: the eggs are longer and less rounded than wild type, and a single dorsal eggshell filament is formed on the dorsal midline (Schüpbach, 1987) (Fig. 3A). Egg chambers from *grk*^{HG21}; *bwk*^{8482} females also have single dorsal filaments, but they are short and ragged, resembling *bwk* filaments (Fig. 3B). The follicle cells initiate filament formation according to the dorsoventral pattern dictated by *grk*^{HG21}, but continue synthesis in a bwk pattern. These studies suggest that the wild-type *bwk* gene product is needed to define the shape of the dorsal eggshell filaments after their placement has been determined by the dorsoventral pathway.

It is possible that subtle changes in the dorsoventral polarity would have been missed by the double mutant analysis. We therefore examined the localization pattern of *grk* mRNA to determine whether there were any changes in the D/V patterning within the oocyte. In wild-type egg chambers, *grk* mRNA is localized to the dorsal anterior corner of the oocyte during stage 10 (Neuman-Silberberg and Schüpbach, 1993, see Fig. 3c). Strong dorsalizing mutations such as *K10* (Wieschaus et al., 1978) cause *grk* mRNA to be extended ventrally along the entire anterior margin of the oocyte (Neuman-Silberberg and Schüpbach, 1993). If *bwk*^{8482} slightly dorsalizes the oocyte, we would expect the *grk* mRNA localization to extend ventrally. We performed cDNA in situ hybridization to *grk* mRNA in wild-type and *bwk*^{8482}/*bwk*^{151} backgrounds and found that the two patterns were indistinguishable (Fig. 3D). This result supports our previous conclusion that our *P* insertion alleles of *bwk* do not slightly dorsalize the oocyte. It remains possible, however, that wild-type BWK function is required for correct translation or localization of the GRK protein.

Finally, we examined the polarity of embryos from *bwk*^{8482} mothers. Previously characterized mutations that dorsalize the eggshell also dorsalize the embryo. Dorsalized embryos have reduced ventral dentical belts and are twisted (reviewed by Chasan and Anderson, 1993). Cuticle preparations of embryos from both *bwk*^{8482} mothers and *bwk*^{151} mothers reveal no dorsoventral defects (Fig. 4B). Surprisingly, the embryos have severe anterior/posterior defects; they are bicaudal (Table 2).

Nature of bwk anterior/posterior defects

The number of eggs that develop from *bwk*^{8482}/*bwk*^{151} mothers varies from 5 to 35% of eggs laid. Of these, almost all form cuticle, 86% of which are bicaudal. These embryos lack head structures, thoracic segments and most of the abdominal segments: the posterior abdominal segments and the telson are reflected in mirror-image symmetry in the anterior (Fig. 4B). Most of the bicaudal embryos have two and a half abdominal segments reflected across the midline (Table 2). The bicaudal phenotype suggests that *bwk* is important for determining or maintaining anterior/posterior polarity during oogenesis.

Since anterior/posterior polarity is established by RNA

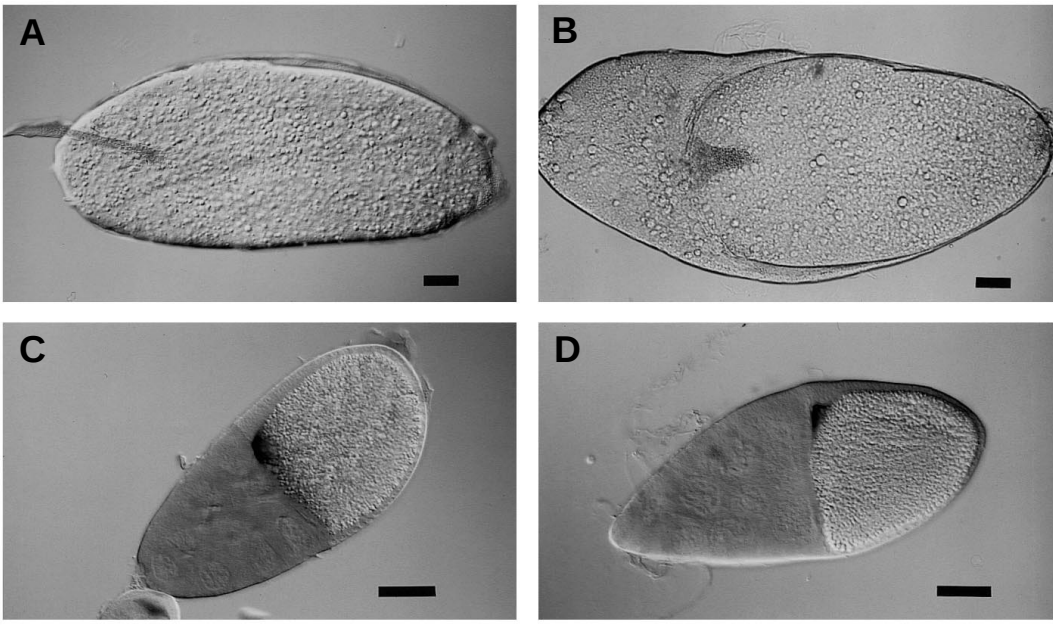

Fig. 3. *bwk* affects the structure of the dorsal filaments independently of the dorsoventral polarity pathway. Anterior is to the left. (A,B) Dorsal is facing out of the page. (A) Stage 14 egg chamber from a *grk*^{HG21} mother. A single dorsal filament is formed on the dorsal midline. (B) Stage 14 egg chamber from a *grk*^{HG21}; *bwk*^{8482} mother. A single *bwk*-like filament is formed on the dorsal midline. (C,D) In situ hybridization to the *grk* message in stage 10 egg chambers; dorsal is up. (C) Wild type. (D) *bwk*^{151}/*bwk*^{8482}: *grk* mRNA localization is normal. Bar, 20 μm.

localized via a microtubule network (Pokrywka and Stephenson, 1991; Clark et al., 1994), we examined the microtubules and general transport along them in *bwk* oocytes by using a KINESIN:β-GALACTOSIDASE fusion protein (KIN:β-GAL) reporter construct (Clark et al., 1994). The KINESIN HEAVY CHAIN is a plus end directed microtubule motor. Fusion to β-GALACTOSIDASE allows visualization of the KINESIN localization with X-gal staining. In wild-type stage 8 and 9 egg chambers, KIN:β-GAL localizes to the posterior end of the oocyte (Fig. 5A). In later stages the localization is lost, presumably due to the cytoplasmic streaming that begins at stage 10b (Clark, et al., 1994; Theurkauf et al., 1992). We examined KIN:β-GAL localization in bwk^{8482} egg chambers. The enhancer trap pattern of bwk^{8482} is observed in the nurse cells at stage 10 and the follicle cells that synthesize the dorsal filaments at stages 10-13, and thus can be easily distinguished

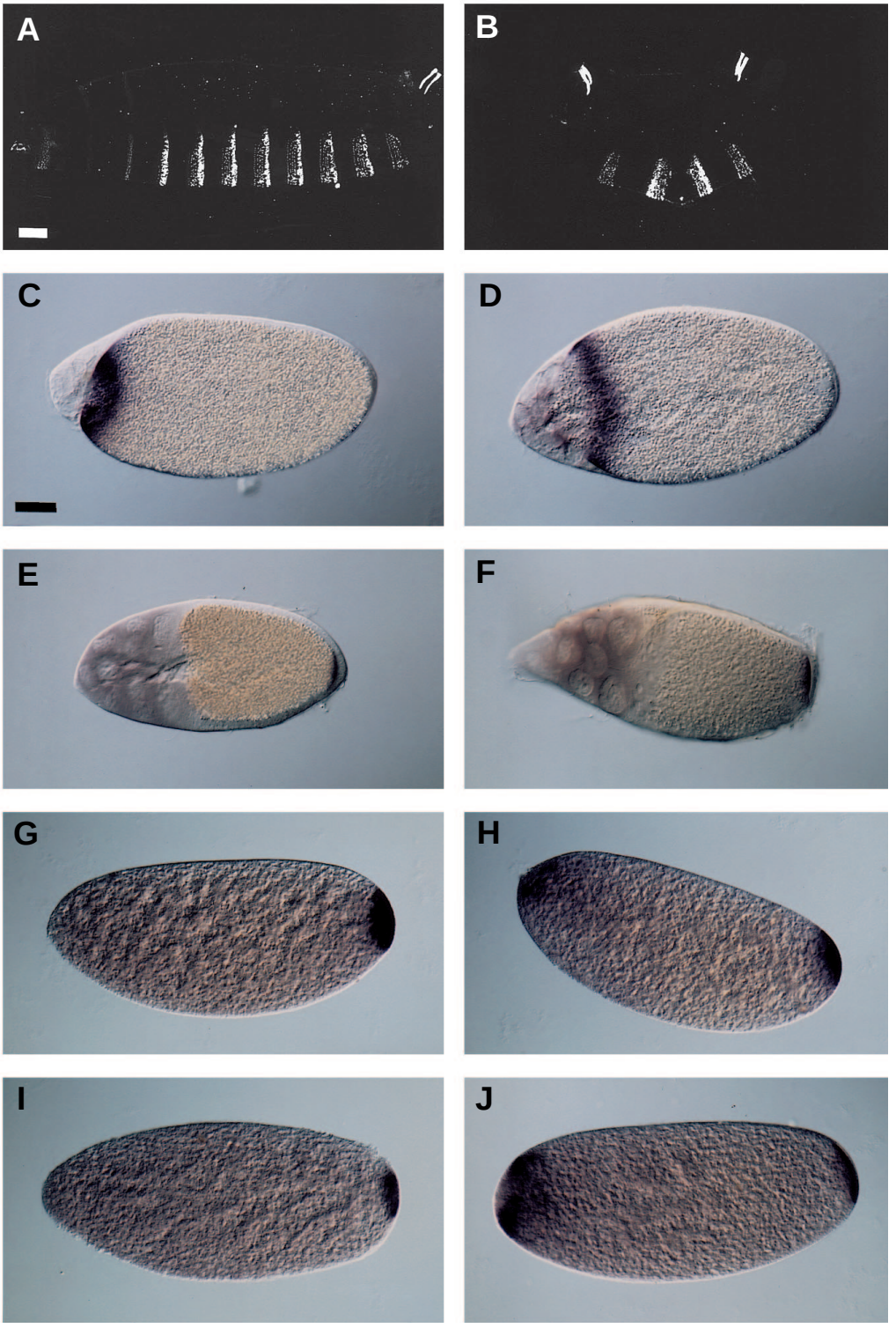

Fig. 4. *bwk* bicaudal embryos are caused by a localization defect late in oogenesis. Anterior is to the left, dorsal is up. (A) Wild-type cuticle. (B) Cuticle of an embryo from a bwk^{151}/bwk^{8482} mother showing two posterior ends in mirror-image symmetry. The head structures, thoracic segments and most of the abdominal segments have been replaced by additional posterior abdominal segments and a second telson. Note that the ventral dentical bands are not reduced. (C-J) In situ hybridization to wild-type and bwk^{151}/bwk^{8482} egg chambers and embryos. (C) *bcd* is localized to the anterior pole in wild-type egg chambers at stage 11. (D) In *bwk* stage 11 egg chambers, *bcd* mRNA is present and is correctly localized to the anterior pole. (E) By stage 10, *osk* is localized to the posterior pole in wild-type egg chambers. (F) *osk* is correctly localized to the posterior pole in *bwk* oocytes at stage 10. (G) The *osk* message remains localized to the posterior pole in early embryogenesis in wild-type embryos. (H) In *bwk* embryos, a portion of the *osk* message is mislocalized to the anterior pole. (I) The *nos* message is located at the posterior pole during early embryogenesis in wild type. (J) Some of the *nos* message is mislocalized to the anterior pole in *bwk* embryos. Bar, 20 μm.

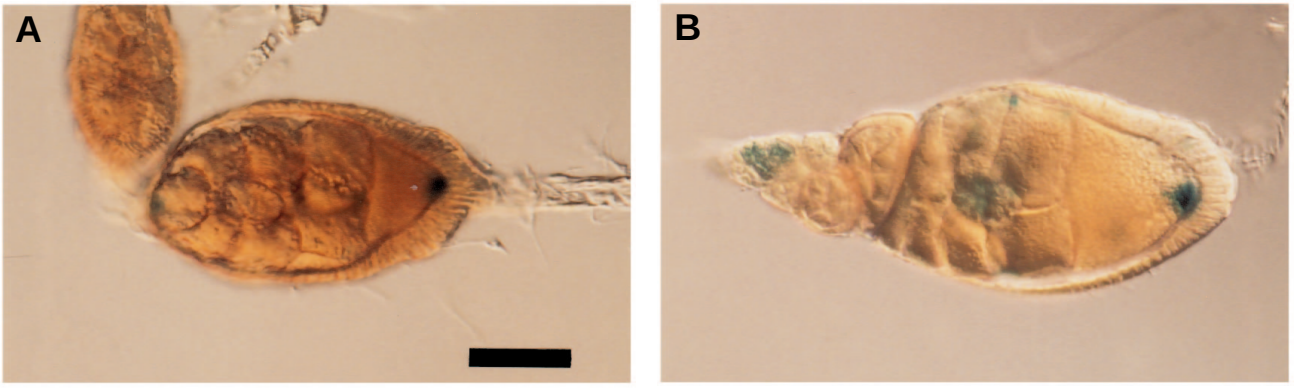

Fig. 5. Localization of KIN:β-GAL. (A) Posterior localization of KIN:β-GAL in a wild-type stage 9 egg chamber. (B) KIN:β-GAL is correctly localized to the posterior pole in *bwk*^{8482} egg chambers at stage 9. Bar, 20 μm.

from the KIN:β-GAL pattern in the oocyte. In *bwk*^{8482} oocytes, KIN:β-GAL correctly localizes to the posterior of the oocyte, indicating that general transport along the microtubules is functioning properly at least until stage 10b (Fig. 5B).

We also examined the localization of specific molecules necessary for establishing anterior/posterior polarity in the embryo. Anterior/posterior polarity is determined by *bicoid* (*bcd*), the anterior morphogen and *nanos* (*nos*), the posterior morphogen, which are localized initially as RNA to their respective poles. Posterior localization of *nos* depends on posterior localization of *osk* RNA, its subsequent translation into protein and the localization of VASA protein (reviewed by St. Johnston, 1993). We performed cDNA in situ hybridization to *bcd*, *osk* and *nos* mRNA in egg chambers and/or embryos from *bwk*^{8482}/*bwk*^{151} females in order to examine any changes in their localization pattern. *bcd* is localized as a ring at the anterior of wild-type oocytes and appears to be similarly localized in *bwk* oocytes (Fig. 4C,D). At stages 10 and 11, *osk* is correctly localized to the posterior pole in *bwk* oocytes (Fig. 4F). By early embryogenesis, however, some *osk* is mislocalized to the anterior pole in embryos from *bwk* mothers, although most of the *osk* mRNA is localized to the posterior pole (Fig. 4H). The amount of *osk* at the anterior pole of *bwk* embryos varies, as indicated by faint to very strong staining.

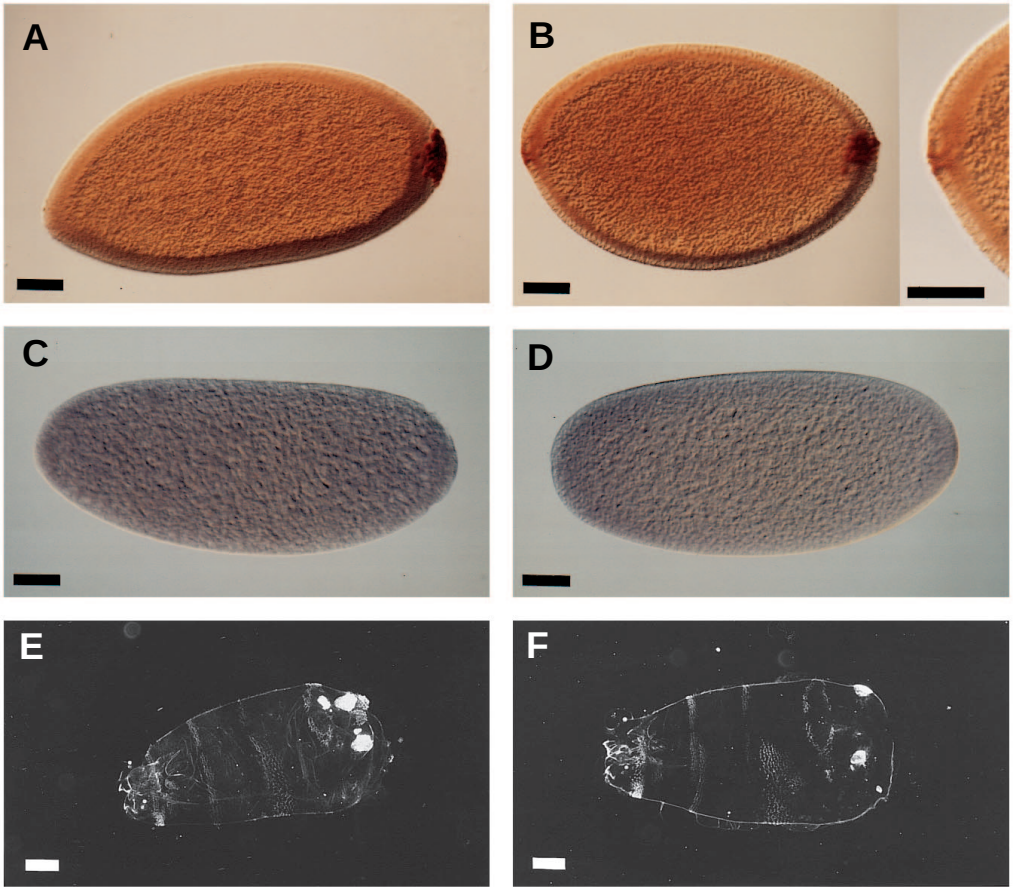

Fig. 6. Role of VASA in *bwk* embryo formation. Anterior is to the left. (A,B) α-VASA antibody staining in cellularized embryos. (A) VASA protein is localized to the pole cells at the posterior pole in wild-type embryos. (B) In embryos from *bwk*^{151}/*bwk*^{8482} mothers, the majority of VASA is localized to the posterior in the pole cells. A small amount of VASA protein is detected at the anterior pole in approximately 30% of *bwk* embryos, but no anterior pole cells are formed. Inset: higher magnification of the anterior pole. (C,D) cDNA in situ hybridization to *nos* mRNA. (C) *nos* mRNA is not localized in embryos from *vasa*^{PD23}/*vasa*^{LYG2} mothers. (D) A very small amount of *nos* mRNA is seen at one pole in embryos from vasa^{PD23}/*vasa*^{LYG2} ; *bwk*^{8482}/*bwk*^{151} mothers. (E) Cuticle pattern showing the loss of abdominal segments in embryos derived from *vasa*^{PD23}/*vasa*^{LYG2} females. (F) Cuticle pattern showing the failure to generate bicaudal structures in embryos from *vasa*^{PD23}/*vasa*^{LYG2}; *bwk*^{8482}/*bwk*^{151} females. Bar, 20 μm.

Table 2. *bullwinkle* embryo* defects

Class	Percent†	Phenotype			
		Number of segments duplicated:			
		0-2	2.5	3-4	uneven‡
Bicaudal	86.3%	11.7%	65.9%	2.8%	5.8%
		Head skeleton			
		None	Partial	Whole	
Anterior defects§	13.3%	6.0%	7.0%	0.2%	
Hatching larvae	0.4%	No segmental defects			

*Embryos were collected from bwk^{151}/bwk^{8482} mothers. 1946 eggs were laid. 497 of these eggs developed and made cuticle.

†Percentages were calculated from the total number of embryos that formed cuticle.

‡Uneven: embryos that were not perfect mirror images.

§Embryos that contained some normal posterior segments, but had variable anterior defects.

Unfortunately, the in situ hybridization technique is not effective in stages 12 through 14 of oogenesis. Consistent with the mislocalization of *osk*, some *nos* mRNA is also mislocalized to the anterior pole in *bwk* embryos (Fig. 4J). Presumably the *nos* mRNA is translated into protein, which directs abdomen formation at the anterior pole.

VASA is also normally required during oogenesis for the localization of the *nos* message (Wang et al., 1994). We wished to determine whether the normal stepwise process was functioning to localize *nos* mRNA at the anterior or whether there was a general defect in RNA localization. We therefore examined VASA protein localization in embryos from bwk^{8482}/bwk^{151} females. In cellularized embryos, some VASA was detected at the anterior pole of approximately 30% of *bwk* embryos (Fig. 6B). Since the immunocytochemical analysis on VASA protein was inconclusive, we tested whether VASA is required for the formation of *bwk* bicaudal embryos by examining embryos from $vasa^{PD23}/vasa^{LYG2}$; bwk^{8482}/bwk^{151} mothers. $vasa^{-}$ females produce embryos that lack abdominal segments (Fig. 6E) (Schüpbach and Wieschaus, 1986) due to the absence of localized NOS protein. In situ hybridization to embryos from $vasa^{PD23}/vasa^{LYG2}$; bwk^{8482}/bwk^{151} mothers revealed that the amount of *nos* message at the anterior pole is greatly reduced (Fig. 6D). These embryos have either a uniform distribution of the *nos* message or a small amount of *nos* at one pole, indicating that VASA is required for the mislocalization of *nos* mRNA to the anterior pole in *bwk* embryos. As expected from these RNA studies, cuticle preparations of $vasa^{PD23}/vasa^{LYG2}$; bwk^{8482}/bwk^{151} embryos resemble *vasa* embryos in that they form head structures and lack abdominal segments (Fig. 6F). We occasionally observe head skeletal defects, possibly due to the small amount of *nos* message detected in the anterior of these embryos. Our *vasa*; *bwk* studies demonstrate that the mislocalization of the *nos* message in *bwk* oocytes depends on a previously mislocalized posterior group gene product.

Since the mechanism that establishes the posterior abdominal segments also establishes the pole cells, or future germ cells, we examined embryos from bwk^{8482}/bwk^{151} mothers to determine whether pole cells are also formed at the anterior end. Pole cells are easy to identify because they are the first cells to form in the syncitial blastoderm, they have a distinctive round shape, and they are specifically labeled by antibodies to VASA. We examined pole cell formation in *bwk* embryos using α-VASA antibodies and found that pole cells form only at the posterior end (Fig. 6B, inset) despite low levels of VASA detected at the anterior pole in approximately 30% of the embryos. Lack of pole cell formation at the anterior pole is not surprising because *Bic-D* mutants also fail to form pole cells at the anterior pole despite the presence of *osk* mRNA (Kim-Ha et al., 1991; Ephrussi et al., 1991). Overexpression studies of *osk* demonstrate that a lower threshold of *osk* is required for abdomen formation than is needed for pole cell formation (Smith et al., 1992). Presumably enough *osk* is present at the anterior of *bwk* embryos to drive the formation of an abdomen, but not pole cells.

bullwinkle is required in the germ line, not in the follicle cells

The enhancer trap pattern and mutant phenotypes of bwk^{8482} suggest that the wild-type *bwk* gene product might be required in both the follicle cells and the germ line: in the follicle cells, for proper cell migration and, in the germ line, to localize *osk* and *nos* mRNA correctly. We examined the tissue requirement for *bwk* function through two different types of mosaic analysis.

In the first approach, we used the 'dominant female sterile' technique to generate germ-line clones (Chou et al., 1993). This method takes advantage of autosomal insertions of $P[ovo^{D1}]$ that allow the formation of germ-line clones with X-ray irradiation. ovo^{D1} is a dominant female sterile mutation that is germ-line specific and blocks the formation of late stage egg chambers. A late stage egg chamber can be formed only if a germ-line clone lacking ovo^{D1} is created. Mitotic recombination in a female transheterozygous for ovo^{D1} and *bwk* can create two sister cells, such that one is homozygous for ovo^{D1} and the other is homozygous for *bwk* (Fig. 7A). In such a female, the eggs produced would have *bwk*/+ follicle cells and *bwk* nurse cells and oocyte. Production of a wild-type egg would indicate that the wild-type *bwk* gene product is not required in the germ cells. Conversely, production of a *bwk* egg would indicate that the wild-type *bwk* gene product is required in the germ line.

Mitotic recombination was induced in bwk^{8482}/ovo^{D1}, bwk^{151}/ovo^{D1} and control *mwh red*/ovo^{D1} females (see Table 3). 1.2% of the *mwh red* transheterozygous females laid wild-type eggs, confirming that mitotic recombination in the germ line was occurring at a measurable frequency. 0.8% of the bwk^{8482}/ ovo^{D1} females and 1.4% of the bwk^{151}/ovo^{D1} females laid eggs with *bwk* eggshells, indicating that wild-type *bwk* function is required in the germ line. None of the *bwk* eggs laid by either line hatched. Dissection of many irradiated

Table 3. Germline mosaic analysis: irradiated female flies

Genotype	No. scored	Laying eggs	Phenotype	
			Wild-type eggs	*bwk* eggs
mwh red/ovo^{D1}	400	5 (1.2%)	100%	0%
bwk^{8482}/ovo^{D1}	500	4 (0.8%)	0%	100%
bwk^{151}/ovo^{D1}	290	5 (1.7%)	20%*	80%

*The germ line of this fly was heterozygous for bwk^{151} and thus these eggs may be disregarded.

Table 4. Pole cell transplants

Embryos injected	Adult flies	*bwk* females*	*bwk* females with pole cells		Phenotype
510	139 (27%)	34	10 (29%)	female pole cells: 4 male† pole cells: 6	wild-type eggshell wild-type embryo tumorous ovaries

*Females homozygous for *bwk* have the following genotype: osk^{301} bwk^{8482} *sbd*/+ bwk^{151} *sbd*.
†Presumed to be male pole cells by the tumorous ovary phenotype.

bwk^{151}/ovo^{D1} females revealed several females that were producing *bwk* eggs but were not laying them. In contrast, one irradiated bwk^{151}/ovo^{D1} female laid wild-type eggs that hatched wild-type larvae. Wild-type eggs can be generated by recombination between *bwk* and ovo^{D1} (Fig. 7B). Fortunately such an event is detectable by examining the phenotype of progeny from the female in question. The germ line of this female was tested and found to be heterozygous for bwk^{151}; the eggs from this female may therefore be disregarded.

In the second approach, we generated the reverse mosaic combination, bwk^{8482}/bwk^{151} soma and wild-type germ line, by pole cell transplantation. Donor pole cells were taken from Canton-S (wild-type) embryos and transferred to *bwk* host embryos that lacked pole cells of their own due to an osk^{301} mutation in the mothers. Four surviving bwk^{8482}/bwk^{151} females laid wild-type eggs and these hatched wild-type larvae, indicating that the wild-type *bwk* gene product is not required in the follicle cells for proper formation of the eggshell or for proper formation of the embryo (see Table 4). These results are consistent with the germ-line requirement for *bwk* found in the ovo^{D1} mosaic analysis.

DISCUSSION

Mutations in *bwk* affect several developmental processes. To elucidate the function of the wild-type gene product, we examined two of the mutant phenotypes, the dorsal/anterior defects of the eggshell and the anterior/posterior defects of embryos from *bwk* mothers.

Eggshell defects

In *bwk* females, the follicle cells fail to migrate properly during late stages of oogenesis, creating eggs with shortened dorsal filaments and other anterior defects. Our results indicate that BWK functions in the germ line, yet is not involved in establishing the dorsoventral polarity of the oocyte. Thus we favor a model in which *bwk* encodes a germ-line product essential for the traction or guidance of the follicle cells. Without BWK, the follicle cells migrate out only a short distance over the nurse cells, lacking the adhesion or attraction that normally permits or induces their outward movement. Later follicle cells are blocked by previous follicle cells and spread out to create shorter, broader dorsal filaments.

Bicaudal embryo phenotype

bwk embryos are bicaudal due to the mislocalization of *nos*

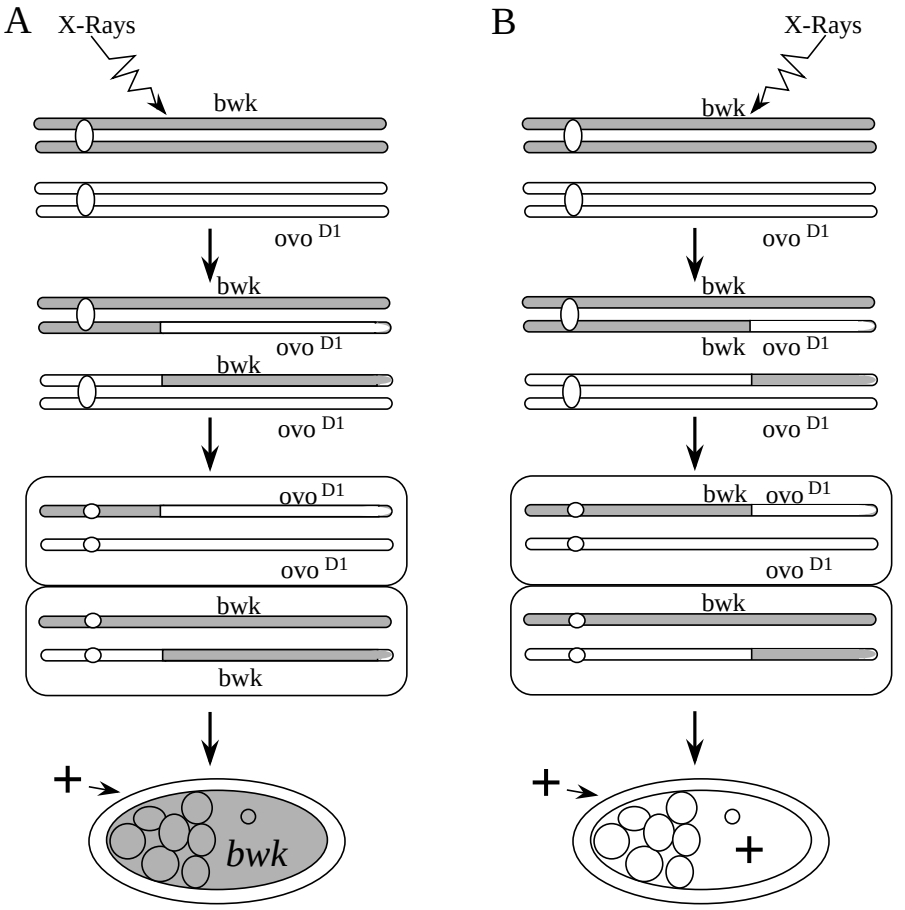

Fig. 7. Possible germ-line clones resulting from X-irradiation in bwk/ovo^{D1} cells. (A) If DNA breakage results in recombination between the centromere and *bwk*, chromosome segregation may create two sister cells such that one is homozygous for ovo^{D1} and the other is homozygous for *bwk*. If the *bwk* cell were to colonize the germ line, mosaic egg chambers (*bwk* nurse cells and oocyte surrounded by wild-type follicle cells) would be produced. (B) If recombination occurs between *bwk* and ovo^{D1}, segregation may produce a cell which does not contain ovo^{D1} and is only heterozygous for *bwk*. If this cell were to colonize the germ line, wild-type egg chambers would be produced.

mRNA during oogenesis. The *nos* message is normally localized to the posterior pole through the stepwise action of other posterior group gene products, which require a functional microtubule network (Ephrussi et al., 1991; Lehmann and Nüsslein-Volhard, 1991; Clark et al., 1994). Correct localization of KIN:β-GAL and *osk* mRNA to the posterior pole of *bw*k oocytes during stages 9 and 10, respectively, implies that the structure of the microtubules and the transport along them is normal in *bwk* egg chambers during this earlier stage of development (Clark et al., 1994). In this respect, *bwk* differs from other bicaudal mutations such as *Bic-D*, which cause *osk* mRNA to be mislocalized to the anterior pole of the oocyte during stage 10 (Ephrussi et al., 1991; Kim-Ha et al., 1991). These results imply a fundamentally different defect in *bwk* oocytes.

The microtubule structure of the egg chamber changes dramatically several times during oogenesis, most notably at stage 10b after *osk* mRNA has been localized to the posterior pole. During stages 8 through 10a, the microtubules are localized primarily at the anterior cortex of the oocyte, with a cortical gradient of microtubules emanating from the anterior to the posterior pole (Theurkauf et al., 1992). During stage 10b, subcortical microtubules form within the oocyte and cytoplasmic streaming begins, continuing through stage 12. In addition, the nurse cells begin transporting their contents into the oocyte at the end of stage 10b. That *bwk* interferes with several processes that begin after stage 10a (*osk* mRNA and other posterior group gene product localization as well as the cytoplasmic transfer from the nurse cells) suggests that BWK functions at this time. Preliminary studies show that cytoplasmic streaming occurs correctly in *bwk* egg chambers, indicating that some aspects of microtubule function are normal.

Localization of the *nos* mRNA in wild-type egg chambers occurs after stage 12 (Ephrussi et al., 1991), when cytoplasmic streaming has stopped, indicating that a new cytoskeletal architecture must be organized in order to transport *nos* message to the posterior pole. In *bwk* egg chambers, *osk* mRNA is not detected at the anterior pole until after stage 12. *bwk* may be necessary for the proper formation of this post-streaming localization system, in which case, further analysis of the bwk phenotype will be invaluable in elucidating this later process. Such a localization system may be more similar to other RNA transport systems since it is contained within a single cell.

Any models of BWK function must account for the defects in follicle cell migrations, the partially dumpless phenotype, and the mislocalization of *osk* mRNA and other posterior group gene products. The combination of these defects suggests that *bwk* may encode a transmembrane protein whose extracellular domain forms heterophilic interactions with a cell surface component on the surface of the follicle cells, and whose cytoplasmic domain interacts with the actin cytoskeleton within the germ cells. Alternatively, *bwk* could encode an extracellular matrix (ECM) molecule or integral cytoskeleton protein required for cell-cell interactions and adaptation of the cytoskeleton to the signaling process. The interaction of *bwk* need not be direct; *bwk* could encode a transcription factor required to express critical components in these processes. During the last decade, a body of knowledge has accumulated documenting the interactions of cell surface receptors with couterparts on other cells, with ECM molecules, and with multiple components of the cytoskeleton (reviewed by Otey and Burridge, 1990; Clark and Brugge, 1995). These molecules are essential in such developmental processes as axon guidance (Whitington, 1993; Harrelson, 1992), neural crest cell migration (Delannet et al., 1994; Bronner-Fraser et al., 1991), organ morphogenesis (Nelson et al., 1990) and yeast cell mating (Chenervet et al., 1994). In addition, changes in these cell surface and cytoskeletal molecules occur during metastasis of cancer cells (Zetter, 1993), wound healing (Pierschbacher et al., 1994) and aging (Yaar and Gilchrist, 1990). Most of these studies employ in vitro techniques to examine cell adhesion and the localization of products within the cell that mediate these cell-cell interactions. Studies on the *bwk* gene provide an excellent in vivo system for documenting the role of these molecules in subcellular localization, cell migration and pattern formation.

We are currently cloning the *bwk* gene to characterize it at the molecular level. Preliminary studies suggest that the gene is large, encoding a 9 kb transcript (data not shown).

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