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Original Article

Blood vessel/epicardial substance (bves) expression, essential for embryonic development, is down regulated by Grk/EFGR signalling

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ABSTRACT The *Pop1/Bves* (blood vessel/epicardial substance) gene is a member of the *popeye* gene family recently identified in various species. It encodes a potential transmembrane glycoprotein and is a cell adhesion molecule present in skeletal and cardiac muscle and epithelia. We isolated the *Drosophila* homologue of Bves *(DmBves)* and found, using *in situ* hybridisation to RNA in ovaries, that *bves* is expressed in all follicular epithelial cells surrounding the oocyte at stage 10, except those in very posterior and anterior-dorsal regions adjacent to the oocyte. We show that the repression of *bves* expression in anterior-dorsal follicle cells is regulated by the Grk/EGFR signalling pathway. *Bves* is also expressed in nurse cells during oogenesis and its transcripts are then translocated into the oocyte. Expression of *bves* antisense RNA during oogenesis causes reduced viability in the resulting embryos. There is a failure in the migration of pole cells from the posterior towards the antero-dorsal side of the embryo, probably resulting from abnormal germband extension and we suggest that *bves* is essential for normal embryonic development.

KEY WORDS: *bves, Grk signalling, Drosophila, oogenesis, embryonic development*

Introduction

During early *Drosophila* oogenesis, one germ line stem cell generates 16 cystocytes by dividing four times. Only one of these cystocytes is determined to be an oocyte and the rest become nurse cells (Telfer, 1975). Many of the RNAs and proteins important for early embryogenesis are synthesized in the nurse cells and then transported into the oocyte at various stages in its development (Spradling, 1993). These include *bicoid* RNA and Dorsal protein essential for the establishment of the embryonic axis; and *osk* and *nanos* RNA needed for determination of germ cells (Riechmann and Ephrussi, 2001**).** The development of a polarized egg requires intercellular communication between the oocyte and somatic follicle cells surrounding it. The transcripts of *gurken* (*grk*), a Transforming Growth Factor (TGF)-α homologue are translocated to the posterior of the oocyte which transmits the Grk signal to the adjacent posterior follicle cells (Gonzalez-Reyes, *et al.,* 1995). Epidermal Growth Factor Receptor (EGFR) encoded by the *torpedo* (*top*) gene is then activated via the Ras signalling pathway and the follicle cells located at the posterior of the oocyte adopt a posterior follicle cell fate. The posterior follicle cells then signal back to the oocyte, mediated by the *Protein kinase A* (*PKA*), *merlin* and *Laminin A* genes and this leads to the reorganization of microtubules and results in a relocalization of *grk* RNAs to the anterior-dorsal region of the oocyte (Deng and Ruohola-Baker, 2000). This is crucial for the establishment of the anterior/posterior axis of the oocyte and embryo. The oocyte then sends the Grk signal to the adjacent follicle cells which adopt an anterior-dorsal fate, again via the EGFR and Ras pathways. These anterior-dorsal follicle cells will later mainly secrete and model the dorsal appendages, thus their signal is key to establishing the dorsal/ventral axis of the egg. The remaining ventral follicle cells later set up a ventral signal which is required to activate Dorsal protein to generate the dorsal-ventral axis of the future embryo.

Abbreviations used in this paper: bves, blood vessel/epicardial substance gene; EGFR, epidermal growth factor repceptor; Grk, gurken; PKA, protein kinase A; pop, popeye gene.

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During embryonic gastrulation, a series of morphogenetic events occur, including germ band extension. It is initiated by proctodeal invagination and followed by movement of the posterior midgut primordium from the posterior along the anterior-dorsal side. Pole cells move with the posterior midgut primordium and the pole cells later leave the midgut by traversing its epithelium to find the somatic gonadal precursor cells (Warrior, 1994, Jaglarz and Howard, 1994). Later germ band retraction begins and as a consequence, the yolk sac is pushed to the dorsal side of embryo, being only covered by the amnioserosa. Morphological analysis of germ band extension has helped to identify many genes involved in the process, including genes affecting dorsal-ventral polarity, but few genes have been found that affect germ band retraction (Costa, *et al.,* 1993).

Three *popeye* genes encoding putative transmembrane proteins have been recently identified, *Pop1*, *Pop2* and *Pop3* in human and mouse and *Pop1* and *Pop3* in chicken (Andree, *et al.,* 2000; Reese and Bader, 1999). *Pop1* generates four different transcripts (*Pop1A* to *Pop1D*). The *Bves* (blood vessel/epicardial substance) gene was the first member of the *popeye* gene family to be found and is identical to *Pop1A*. The strong conservation of *pop* genes in various species suggests that they may have an important conserved function. However, homozygous mice lacking pop1 were fertile and had a normal life span without any apparent phenotype except defects in skeletal muscle regeneration (Andree, *et al.,* 2002).

To better understand the possible role of *popeye* genes, the expression of *Pop1* has been investigated in different animals. High levels of *Pop1* transcripts were detected in both cardiac and skeletal muscle of human, mouse, chicken and frog by Northern blots and by *in situ* hybridisation to RNA (Andree, *et al.,* 2000, Hitz *et al.,* 2002 and Reese and Bader, 1999). The expression of the *Pop1* gene in the muscles was further confirmed by Pop1-lacZ expression during mouse development (Andree, *et al.,* 2002). However, staining with antibody against Bves (Pop1) has shown wider expression in variety of tissues (Andree, *et al.,* 2000, Reese, *et al.,* 1999, Osler and Bader**,** 2004, Vasavada, 2004 and DiAngelo, *et al.,* 2001). Bves protein was originally observed in the epicardial and smooth muscle of coronary arteries in the chicken (and hence called blood vessel/epicardial substance) (Reese, *et al.,* 1999) and has subsequently been observed in cardiac and skeletal muscle, as well as additional epithelial cells in both mouse and chicken (Andree, *et al.,* 2000, Vasavada, 2004 and Wada *et al.,* 2001). More recently chicken Bves protein was found to be expressed in epithelia of all three germ layers early in development, as well as in epithelial tissues during organogenesis, specifically the developing epidermis, the gut endoderm and the epicardium of the heart (Osler and Bader**,** 2004)**.** Using human corneal epithelial cells, Bves staining was observed at the epithelial surface (Ripley *et al.,* 2004).

Study of pop1/Bves at the molecular and cellular level sheds light on how it is involved in cell adhesion and movement. Bves is a membrane protein with three predicted transmembrane helices and two asparagine-linked glycosylation sites within the amino terminus (Andree, *et al.,* 2000 and Wada *et al.,* 2001**).** Membrane topology of the cell surface shows Bves is composed of a glycosylated extracellular amino terminus and a cytoplasmic carboxyl terminus (Knight *et al.,* 2003). Bves accumulates in a perinuclear region in disassociated epicardial cells until cells make contact, at which point Bves is trafficked to the cell membrane (Wada *et al.,* 2001). Bves accumulated at points of cell/cell contact, such as filopodia or cell borders and can confer adhesive behaviour to L-cells after transfection. Furthermore, Bves antibodies inhibited epithelial migration of vasculogenic cells from the proepicardium (Wada *et al.,* 2001). Recently, monoclonal antibodies against chicken Pop1/Bves have been used to demonstrate the presence of the protein in cardiomycytes and confirm its membrane location (Vasavada, *et al.,* 2004). All these data suggest that Pop1/Bves is involved in cell adhesion and cell movement in early embryogenesis.

In this paper, we describe the identification of a Pop1/Bves homologue in *Drosophila melanogaster* (*bves*). *bves* is expressed all epithelial follicle cells surrounding the oocyte except those in anterior-dorsal and very posterior regions. Its repression in anterior-dorsal epithelial cells is due to Grk/EGFR signalling. *bves* is also expressed in the nurse cells and its transcripts are transported into the oocyte. Expression of *bves* antisense RNA during oogenesis caused the failure of migration of pole cells from the posterior towards the anterior-dorsal side of embryo as well as embryonic death. This study provides evidence that Bves is required for cell migration and is essential for embryonic development in *Drosophila*.

Results

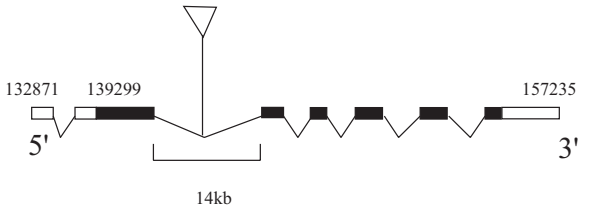
Isolation and molecular characterisation of the bves *gene*

The *bves* gene was identified as part of a GAL4/UAS enhancer trap scheme to find genes involved in the generation of dorsal ventral polarity during *Drosophila* oogenesis. When line C648, a GAL4 line, is crossed with a UAS-lacZ line, staining is detected in a large patch of follicle cells at stage 10 of oogenesis (Fig. 2A). Cloning of the target gene was achieved by plasmid rescue (Bellen *et al.,* 1989). The cDNA isolated was named *bves* (*Drosophila melanogaster bves*) as its vertebrate homologue is called blood vessel/epicardial substance (*Bves*). Two EST clones (GM07524 and LD22978) were found that were similar to *bves* cDNA. GM07524 and LD22978 have inserts of 2.6kb and 5.5kb respectively. These two EST clones were sequenced and revealed that LD22978 has an insert containing two genes. The 5' fragment of 2.6kb has a similar sequence to GM07528. The rest of the sequence of LD22978 is from another putative gene named *CG5208*, which is located on chromosome 3. We assumed EST GM07524 to be a complete cDNA and we submitted the *bves* DNA sequence we obtained to genebank (Accession No: AF247183, Oct. 3rd, 2000). The sequence is not therefore presented again in this paper.

To learn more about the function of the *bves* gene, we need to know the developmental expression profile of its transcript and this was determined by northern blotting. The results revealed that *bves* transcripts are present at various developmental stages (Fig. 1C). The transcript of *bves* was found to be 4kb long, which was not consistent with the *bves* cDNA (2.6kb) and the length of the transcript predicted from EST GM07524. This problem was resolved by the latest *Drosophila* genome annotation, which predicts that the *bves* cDNA is 3.9kb long from EST RE59473. This is consistent with the transcript slice we observed by Northern blots.

Comparing the *Drosophila* genomic sequence with the *bves* cDNA sequence established that the *bves* gene is 24kb with 7 exons (Fig 1). It is located on chromosome X at position 19E7. There is a large intron which is approximately 14kb between exon 2 and exon 3. The P-element insertion in the original line (C648) was mapped by sequencing the rescued genomic DNA generated by plasmid rescue and we showed that it is inserted in the middle of the large intron at basepair 146195 (Fig. 1A). The *bves* gene encodes a single ORF and is translated from 680bp to 1924bp, producing a peptide of 414 amino acids. The amino acid sequence of *bves* was used to search the NCBI database. Three similar genes were found in mouse and humans called *popeye*, *Pop1* to *Pop3* and two in chicken, *Bves* and *Pop3*. In the *Drosophila* genome, there is one homologue of the *popeye* gene. We named the *Drosophila* homologue *bves*. There is approximately 28% identity and 50% similarity at the protein sequence level between *bves* and its homologues. Later, homologues of *bves* were found in the mosquito (Ensembl, European Bioinformatics Institute 2004). The amino acid sequences show high identity (59%) and similarity (71%) between these two insects. Like other Pop1/Bves homologues, the predicted bves sequence contains protein that has three transmembrane domains and one glycosylation site at the amino terminus. It could therefore function an adhesion molecule in a similar way to its homologues. The predicted protein sequences

A



B

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human       1 ------------------------------------------------------------------------------------------
mouse       1 ------------------------------------------------------------------------------------------
chicken     1 KQGTRAPLS LRPADIRHQTTASRRGSTAPPPPRGTPRPHRAAPQPPGRAGGACALRPLAVGPGLAPPAARRRLLPILVTAAAG GRGGTGV
Dmbves      1 ------------------------------------------ MPSTAGSAAGVGMGALINSAGSSASSVMGIGMGRAAATG AGAPGSS
mosquito    1 ------------------------------------------------------------ ------------LGAQGRNGHNGSSSN GSIGSNG

human       1 ---------------------------------- MNYTESSPLRESTAIGFTPELESIIPVPSNKTTCENWREIHHLVFHVANICFAVG
mouse       1 ---------------------------------- MNSTESIPLAQSTVAGFTSELESLTPVPSNETTCENWREIHHLVFHVANVCFAVG
chicken    91 GRGLPASGAAKSGGPCCPFVWETLSKVPIGGTGTGFFKM DTTAISPLTPLGVIPDLKNAISVPFNETACENWKEIHHLVFHVANICFAAG
Dmbves     47 GSGADAS---------------------- AAGTLIAQSIAGTSAASSGTITWDNNGTLRSINPGDWSIEQCLGP-HHLYFQLGWAFLFLA
mosquito   23 SNGNSGS---------------------- SNGESTSYRATKKAPQEGNGVHHDNEHTIRMLIDWNWPKCFSFEPKEHLYYHIGHALFLVA

human      56 LVIPTTLHLHMIFLRGMLTLGCTLYIVWATLYRCALDIMIWNSVFLGVNILHLSYLLYKKRPVKIEKELSGMYRRLFEPLRVPPDLFRRL
mouse      56 LLIPTTLHLHMILLRVMLSLGCTLYVVWATLYRCALDVMIWNSVFLGINILHLSYLLYKKRPVKIEKELGGVYHRLFEPLRVPPDLFRRL
chicken   181 LVIPTTLNLHMIFLRGLLTVGCALFIIWATLYRCALDIMIWNSVFLVVNLLHFIYLVYKRRPIKIEKELSSLYKRMFEPLHVPPELFQRL
Dmbves    114 FLAP-HGPYGALWMRAMLLIGCLMMGMHGYLVAEAPDVILWSGMGLFVNFIYLVVVLCRLRPVRFEQEIEAVYLALFQPLHVTRHQFKKV
mosquito   91 FLAP-NIPCGFLWLRCAAIAGCILMVLWGWFVACSLDAVVWFSLFLLVNAFYVVVLLCKLRPVKFEKEIESVYVALFKPLRVSRHQFKKV

human     146 TGQFCMIQTLKKGQTYAAEDKTSVDDRLSILLKGKMKVSYRGHFLHNIYPCAFIDSPEFRSTQMHKGEKFQVTIIADDNCRFLCWSRERL
mouse     146 TGQFCMIQTLKRGQVYATEDKTSVDDRLSILLKGRMKVSYRGHFLHNIYPCAFIDSPEFRSTQMHKGEKFQVTIVADDNCRFLCWSRERL
chicken   271 TGQFCNIQTLKTGQAYAAEDKTSVDDRLSILLKGKMKVSYRGHFLHNIYPCAFIDSPEFRSTQMNRGEKFQVTIIADDNCKFLCWSRERL
Dmbves    203 LNCMKVIRALKYQEVYAQEKVTKVDS-LSLVLSGKLVVSQHQRALHIVFPHQFLDSPEWFG--VSTDDYFQVSIMAMEESRVLIWHRDKL
mosquito  180 LNCMKIVRPLKYQEIYVQEKITKVDS-LSLVLSGKLVVSQNQKALHIVFPHQFLDSPEWFG--VSTDDYFQVSIMAMEESRVLIWHRDKL

human     236 TYFLESEPFLYEIFRYLIGKDITNKLYSLNDPTLNDKKAKKLEHQLSLCTQISMLEMRNSIASSSDSDDGLHQFLRGTSSMSSLHVS--S
mouse     236 TYFLESEPFLYEIFRYLIGKDITNKLYSLNDPTLNDKKVKKLEPQMSLCTQISMLEMRNSITSSSDGEDGLHHFLRGSSSTASLPMS--S
chicken   361 TYFLETEPFLYEIFKYLIGKDITNKLYSLNDPTLNDKASKKIDRQPSLCSQLSVMQMRNSVARSSDSEDGLQMFLRGTSSSSSLRPGRTS
Dmbves    290 KLSIMAEPFLQTVFDHILGRDVVKKLMQVTQVSESIASNGFLPSGGYAEDAEDKPMLILKKS--VDVGHGLTALINRQLQDEHVPLLGRT
mosquito  267 KLSIMAEPFLQAVFDHILGRDVVKKLMQVTQVSETMAQSNGCIASGYGDDSEDKPMLVMKSKGIDNGGHGLTALINRQLQDETLPLLPTV

human     324 PHQRASAKMKPIEEGAEDDDDVFEPASPNTLKVHQLP
mouse     324 PQQRASAKMKPIEEGVEDDDEVFVSP--DALKVHQLP
chicken   451 PYLRTSAKMKPIEESVEDD--VFEAPSAEKLELQRLP
Dmbves    378 YKQQQQQQQLLLLQQQLEQQRVQEATISANNIEQSAI
mosquito  357 ALNSCKALIEKVYHVVEVD------------------
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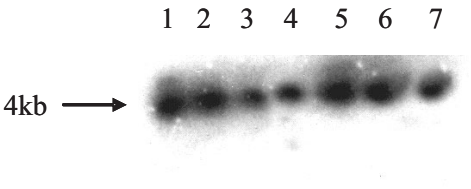
C



The *bves* gene. (A) *The intron/exon structure of the* bves *gene. The boxes are exons. Black represents the coding regions. The triangle indicates that the P-element is inserted between exon 2 and exon 3. The numbers in the figure correspond to the base pairs in the genome.* **(B)** *Alignment of protein sequences encoded by the* bves *gene and the mouse, chicken, mosquito and human (Bves) homologues. Amino acids shaded in black are identical and those shaded in grey are similar in these five sequences.* **(C)** *Total RNAs were extracted from different developmental stages. Lane 1, ovaries; Lane 2, 0-4 h embryos; Lane 3, 6-14 h embryos; Lane 4, first instar larvae; Lane 5, second instar larvae; Lane 6, third instar larvae; Lane 7, pupae.* bves *DNA was used as the probe. 20 μg total RNA of each sample was loaded on the gel.*

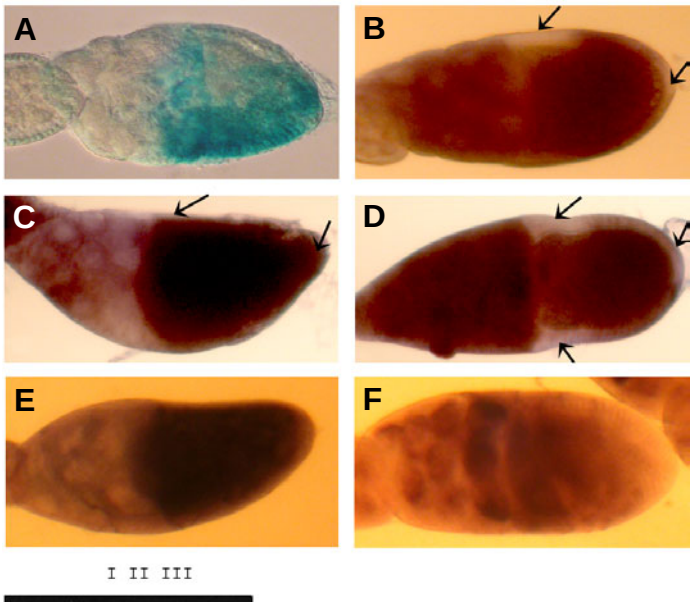


Fig. 2. β-galactosidase staining and RNA *in situ* hybridisaton to RNA in the ovary. **(A)** *β-galactosidase staining in the ovary. A large patch of follicle cells was stained.* **(B)** In situ *hybridization to the wild type ovary, showing expression in nurse cells and all follicle cells except in the anterior-dorsal and posterior regions (arrows).* **(C)** *Expression of* bves *in* gurken *mutant ovaries. Arrows indicate that* bves *is expressed in all follicle cells.* **(D)** *Expression of* bves *in* fs(1) K10 *mutant ovaries. Arrows indicate that* bves *is not expressed in a stripe along the anterior region of the oocyte.* **(E)** *Expression of* bves *antisense RNA in PCas-hs-*bves*-AS (2) ovaries.* **(F)** *Expression of* bves *antisense RNA in PCas-hs-*bves*- AS (40) ovaries. Anterior is to the left and dorsal is at the top.* **(G)** *RT-PCR to detect* bves *RNA in ovaries. I, RNA from wild type ovaries; II, RNA from heatshocked PCas-hs-*bves*- AS (40) ovaries; III, RNA from PCas-hs-*bves*-AS (2) ovaries.*

of Pop1/Bves were aligned between *bves*, the chicken, mouse, mosquito and human homologues and this is shown in Fig. 1B.

The repression of DmBves *expression in some follicle cells is caused by Grk/EGFR signalling during oogenesis*

To learn more about the *bves* gene and its role in oogenesis and embryogenesis, *in situ* hybridisation to bves transcripts using *bves* cDNA as a probe was carried out using wild type ovaries. A similar, but not identical, expression pattern in the follicle cells was observed when comparing the expression pattern of a reporter gene driven by the GAL4 insert in line C648. Differences are probably because the insert containing GAL4 only responds to some of the regulatory elements that affect the expression of the native gene. Expression was observed in the nurse cells from stage 5 onwards. Transient expression was seen in all follicle cells surrounding the oocyte at stage10 of oogenesis, except those located at the anterior-dorsal and posterior region (Fig. 2B). There was no detectable expression in follicle cells at any stages before or after stage10 of oogenesis, this expression is therefore very transient and the mRNA seems to be short-lived. During oogenesis *grk* signals to the follicle cells in the posterior region at stage 6 and signals again to the follicle cells in the anterior-dorsal region at stage 10 to determine their fates. It is therefore possible that Grk signals to an unknown gene and that the products of this gene may repress *bves* expression in these groups of follicle cells. Although Grk is located in the anterior-dorsal region at stage 10, it is possible that the products of the unknown gene remain in the posterior follicle cells for some time and repress *bves* expression.

To investigate if the bves expression pattern is dependent upon the Grk/EGFR pathway, *in situ* hybridisation to the ovaries of a number of mutants that affect the Grk/EGFR signalling pathway was carried out. In the ovaries of the *grk* homozygous female mutant, follicle cells do not receive the Grk signal either in the posterior or anterior-dorsal region and as a result, the expression of those genes downstream of Grk/EFFR signalling pathway is affected. Using *bves* as a probe for RNA *in situ* hybridization in the *grk* mutant ovaries, we found that *bves* was expressed in all follicle cells surrounding the oocyte (Fig. 2C), indicating that *bves* expression is negatively regulated by Grk/EGFR signalling. In *fs(1) K10* mutant ovaries, *grk* transcripts diffuse from their normal anterior-dorsal location towards anterior-ventral positions within the oocyte, therefore all anterior follicle cells receive the Grk signal. *In situ* hybridisation to *fs(1) K10* mutant ovaries with *bves* as a probe revealed that *bves* was not expressed at all in any anterior follicle cells (Fig. 2D). This data taken together suggests that the Grk/EGFR signalling pathway is used to establish the specific expression pattern of the *bves* gene in follicle cells surrounding the oocyte during oogenesis. *In situ* hybridisation was also carried out using wild type embryos, *bves* transcripts are detected in the embryo but there are no regions or tissues with higher expression (data not shown). Since developmental northerns show that *bves* RNAs is present levels in 0-4 hour and 6-14 hour embryos (Fig. 1C) It seems likely that *bves* mRNA distributed uniformly in the embryo. It will be crucial in future, once antibodies to *Drosophila* BVES are available to investigate the protein distribution, which may well move tissue variations or locations in specific regions within cells.

Functional studies

There are no *bves* mutant lines available. To investigate the biological function of *bves*, P-element mobilisation was carried out on fly line C648 in the hope of generating mutants of the *bves* gene. 316 balanced lines were established. Among them 156 were red eyed lines in which the P-element was relocated and 160 were white eyed lines in which the P-element had at least partially excised. Only four of the red-eyed lines were been found to be homozygous lethal. There are no homozygous lethal lines among the white eyed lines. No other visible mutants were found in either the red-eyed line or the white eyed lines, such as female steriles, or abnormal phenotypes of the adult flies. The reason why there are no mutant flies amongst the white eyed lines may be that the P-element is inserted in the middle of a large intron. The P-element may have removed some flanking genomic DNA when excising imprecisely, but not enough genomic DNA to affect gene expression. 4 red-eyed fly lines which are homozygous lethal were obtained. Southern hybridisation was used to check if the P-element is really inserted in the genome of these mutant flies, using pbluescript DNA which is contained within the pGawB insect as probe. It was found that the P-element is present in all three lines. To determine the position of the P insertion, plasmid rescue for these three lines with P-element insertions was carried out and the

TABLE 1

SURVIVAL OF EMBRYOS LAID BY HEATSHOCKED FEMALES TO DRIVE ANTISENSE EXPRESSION

Time after heatshock	Fly lines	Total embryos	Dead embryos	Larvae hatched	Survival rate
First day	OrR - control	361	38	323	89%
	PCas-hs-*bves*-AS (2)	309	160	149	48%
	PCas-hs-*bves*-AS (40)	135	72	63	47%
Second day	OrR - control	150	15	135	90%
	PCas-hs-*bves*-AS (2)	164	61	103	63%
	PCas-hs-*bves*-AS (40)	53	22	31	59%
Third day	OrR - control	69	3	66	96%
	PCas-hs-*bves*-AS (2)	123	49	74	60%
	PCas-hs-*bves*-AS (40)	76	47	29	38%

The table shows survival of embryos laid on each day following induction of antisense BVES in the ovaries of the mother. The eggs laid first will have had antisense bves RNA present for a shorter period of oocyte development than eggs laid later.

rescued DNA fragments sequenced. The results revealed that the P-element had hopped to positions unfortunately upstream or downstream of *Bves* gene. Thus there was no homozygous lethal mutant resulting from P insertion in the *bves* gene, so either the gene is non-essential or redundant or we failed to damage any of the coding regions in any of the excision lines or P-hop lines.

Since no mutants were obtained we generated transgenic fly lines in which bves RNA antisense expression is induced by heatshock to try to establish potential functions of the gene by the antisense RNA knocking down bves transcript levels. To analyse the expression of *bves* antisense RNA induced by heatshock in the different transgenic lines the ovaries of the heatshocked flies were collected one hour after the last heatshock. *In situ* hybridisation to RNA in these ovaries was carried out using the *bves* sense RNA as a probe. Strong expression of *bves* antisense RNA were observed in two fly lines. The expression level and pattern of *bves* antisense RNA strand depends upon the chromosomal position of the insert (Karpen, G. H. 1994, Simmons, M.J. *et al.,* 1996). In PCas-hs- *bves* -AS line 40, *bves* antisense RNA was expressed strongly in the nurse cells and weakly in follicle cells (Fig. 2F). In the PCas-hs-*bves*-AS line 2, *bves* antisense RNA was expressed the other way around, strongly in the follicle cells and weakly in nurse cells (Fig. 2E). The different expression patterns of these two lines is probably related to the position of the P-element insert in the genome since expression patterns will be influenced by nearby enhancers. To see if the heatshocked induced antisense RNA really reduced the endogenous *bves* RNA levels, *in situ* hybridisation to the heatshocked ovaries of these two fly lines by using bves antisense RNA as a probe. There is no RNA detected by *in situ* (data not shown). RT-PCR is more sensitive and was also the reference used to detect *bves* RNA in the heatshocked ovaries of those two lines. We found that *bves* RNA still existed but at a much lower level in comparison with that observed in the wild type ovaries (Fig. 2G). Thus line 40 reduced nurse cell expression and line 2 the follicle cell expression more. Levels in all cells of the ovary are below the levels detectable by *in situ* hybridisation, but some transcripts remain, thus this approach is not leading to a null level of bves.

To investigate the function of *bves* in oogenesis, the PCas-hs-*bves*-AS line 40 and PCas-hs-*bves*-AS line 2 were heatshocked and the ovaries were observed. There were no defects detectable in the ovaries thus the eggs laid by heatshocked flies were investigated. They all had normal egg-shells and dorsal appendages. These results were not expected as one might have predicted there would be defects in oogenesis or the morphology of the egg/embryo with the expression patterns observed. It is possible that insufficient antisense RNA was produced in time to affect the phenotype, however, the RT-PCR suggests a significant reduction in the RNA levels. We found that embryos laid by heatshocked wild type females had a much higher survival rate than those laid by *bves* antisense RNA lines (Table 1). This suggests embryos were not killed by heatshock of the mothers, but by expression *bves* antisense RNA, leading to reduced levels of native *bves* RNA during oogenesis. Embryos laid by heatshocked flies (hs embryos) develop normally from stage 1 to 6, (stages of Campos-Ortega and Hartenstein, 1985; Roberts, 1998). In the wild-type embryos at stage 7, the cephalic furrow has deepened and the posterior midgut has carried the pole cells to the dorsal side of the embryo (Fig. 3B). But posterior midgut invagination did not occur properly in almost 10% of the embryos laid by females expressing *bves* antisense RNA and sometimes pole cells were left behind at the posterior end (Fig. 3D). At stage 8, in wild-type embryos the posterior midgut invagination reaches the head region along the dorsal side (Fig. 3C) but in the embryos laid by h-s antisense females, sometimes pole cells were still observed in the posterior region (Fig. 3E) and in 20% of the embryos the gut opening fails to reach the head region, resulting in a hole in the middle of the embryo (Fig. 3F). These embryos failed to develop further and a three-layered germ band was never formed. The lethality of the embryos laid by hs females reached a peak between 18 –24 hours after heatshock. At the peak 40-50% embryos died during the embryo stage. Another 2-3% embryos laid by hs females could survive to the larval stage, but they had a dark spot inside the body and eventually all died (Table 1). The rest of the embryos survived

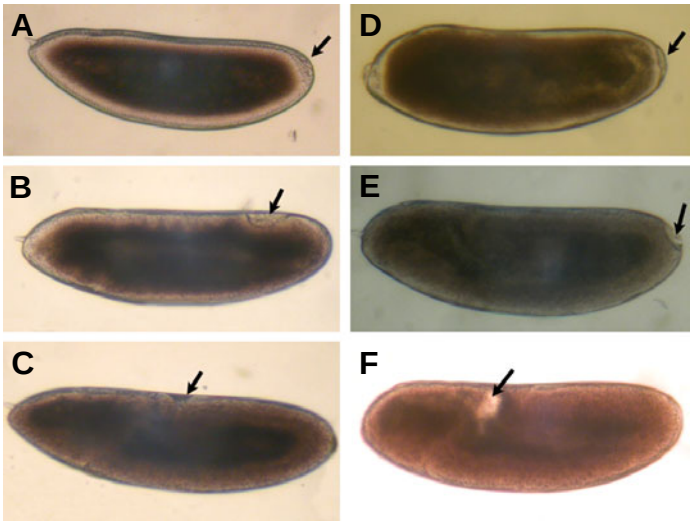


Fig. 3. Abnormal embryogenesis in eggs laid by mothers in which antisense *bves* is expressed during oogenesis. (A-C) *Wild type embryos;* **(D-F)** *embryos laid by heatshocked female.* **(A)** *Arrow indicates pole cells in the posterior region at stage 5.* **(B)** *Arrow indicates posterior midgut invagination at stage 7.* **(C)** *Arrow indicates posterior midgut invagination at the anterior at stage 8.* **(D)** *Arrow indicates that pole cells remain at the posterior during stage 6-7.* **(E)** *Arrow indicates that pole cells are still at the posterior during stage 7-8.* **(F)** *Arrow indicates that there is a hole in the middle of the embryo.*

to produce normal adult flies. Survival was lower in line 40, which affects nurse cell expression to a greater extent, but the phenotypes were similar in both lines.

Discussion

The *popeye* genes (*Pop1*, *Pop2* and *Pop3*) are members of a gene family recently identified in chicken, mouse and human (Andree, *et al.,* 2000; Reese and Bader,1999). We have identified the homologue of one member of this gene family (*bves*) in *Drosophila melanogaster*. Its expression during *Drosophila* oogenesis as well as during embryonic development has been investigated. By reducing the expression levels of *bves* during oogenesis by expression of its antisense strand we have observed abnormal epithelial movement resulting in the failure of pole cell migration and a high percentage of embryonic death.

bves encodes a putative transmembrane glycoprotein, which is well conserved in evolution. Most similar to *Pop1/Bves*. Five *Pop1/Bves* homologues including *bves* have been cloned so far from human, mouse, chicken, *Drosophila* and the mosquito (Andree, *et al.,* 2000; Reese and Bader, 1999; Ensembl, European Bioinformatics Institute,2004). The comparison of Pop1/Bves protein sequences from 5 species shows that *bves* has 59% identity and 71% similarity to the mosquito, which is much higher than the identity and similarity compared to vertebrates. Pop1/Bves proteins all have three putative transmembrane domains and one or two potential glycosylation sites. Both glycosylation of Pop1/Bves protein and its location in the cellular membrane have been shown recently, indicating that they are cell adhesion molecules.

bves is very well conserved in evolution and might therefore have an important function in development. The pop1/Bves knockout mice are viable but have impaired skeletal muscle regeneration after muscle damage by cardiotoxin (Andree, *et al.,* 2002).

bves expression is regulated by the Grk/EGFR signalling pathway. Cellular interaction between the germ line derived oocyte and somatic epithelial follicle cells surrounding the oocyte is required for the development of a mature egg (Schupbach, 1987). Grk/EGFR signalling is involved in the establishment of both the anterior- posterior and dorsal-ventral axes of egg (Gonzalez-Reyes, *et al.,* 1995). During the establishment of the dorsa-ventral axis, *grk* transcripts together with the oocyte nucleus are located in the anterior-dorsal region of the oocyte and the oocyte sends the Grk signals to adjacent follicle cells. Upon activation of the EGFR downstream signalling pathway, these follicle cells which receive the signals are determined to become dorsal cells. The follicle cells in turn are involved in specifying the dorsal-ventral axis of the subsequent egg. While we were searching for genes downstream of the Grk/EGFR signalling pathway expressed in follicle cells, using a GAL4/UAS enhancer trap system (Deng, *et al.,* 1997), we cloned the *bves* gene. A developmental Northern analysis showed that *bves* is expressed in oogenesis, embryogenesis and postembryogenesis (Fig. 1C). During oogenesis, *bves* is first activated in nurse cells from stage 5-6 onwards and then in the follicle cells at stage 10. The expression of *bves* in follicle cells is restricted to those cells which do not receive the Grk signal from oocyte. We found that *bves* was expressed in all follicle cells in *grk* mutant ovaries where there was no Grk signal from the oocyte and that *bves* was not expressed in an anterior follicle cells in *fs(1) K10* mutant ovaries where grk transcripts are no longer restricted to anterior dorsal, but are found across the entire anterior of oocyte. Thus, the expression of *bves* is downstream of the Grk/EGFR signalling pathway.

bves is also expressed in nurse cells and its transcripts are then transported into oocyte. RT-PCR has shown that *bves* RNA is highly abundant in the 0-2 hour embryo (Data not shown). Since there is no transcription by this stage, *bves* RNA must be maternally provided and could be translated and used for early embryogenesis.

Unfortunately there are no *bves* mutants available neither did we generate any lethal mutants by P-element mobilisation. Thus, in order to investigate the biological function of *bves*, its antisense strand has been expressed directed by heatshock of pCaS-hs-*bve*-AS lines. Embryos laid by these heatshocked transgenic flies showed a high percentage of embryonic death compared with wild type flies and at the peak, 40-50% of the embryos died. Another 2-3% of embryos survived to the larval stage, but eventually died. One of the most obvious mutant phenotypes is the failure of pole cell migration. At stage 7, there was no obvious posterior midgut invagination and with germ band extension, pole cells were left behind at the posterior end of embryos (Fig. 3D,E). Another mutant phenotype was a hole or gap forming between the cephalic furrow and the migrating anterior germ band (Fig. 3F).

The results from the transgenic fly antisense experiment is not consistent with that from the P-element mobilisation. No *bves* mutants were obtain by P-element mobilisation in our screen. However, it was possible we did not generate any damage to the coding sequence in any line. Searching for *bves* information in flybase (flybase.bio.indiana.edu), the piggyBac transposon, has been inserted into *bves* gene but the flies are still viable and fertile (Bloomington Drosophila Stock Center, 2004). While this result is consistent with our mutagenesis experiment it then makes it less clear why heatshocked *bves* antisense flies caused the lethality of embryos. One model is that possibly *bves* is a redundant or non-essential gene. Unfortunately, within species there can be very low levels of sequence similarly between members of the *Pop1/BVES* family (Osler, *et al.,* 2006) so it is difficult to ascertain if there is a potentially redundant gene in *Drosophila* based on sequence analysis. If the products of the *bves* gene can interact with other proteins for cell adhesion, when BVES is knocked out or reduced, perhaps another gene can fulfil the role of *bves*. Perhaps it is not possible in early embryos to compensate for the lack of maternal product as the product of *bves* or its substitute is required prior to transcription of the zygotic genome and the redundant gene product is not maternally stored or not present in sufficient quantities to enable normal embryogenesis to occur. The importance of the maternal products in this effect is also suggested by the higher lethality of embryos in the line where nurse cell expression is reduced to a greater extent than the line affecting largely follicle cell expression. Another possibility is that the piggyBac insertion simply does not knock out or down *bves* transcription and *bves* is not redundant but an essential gene for early embryogenesis.

Pop1/Bves protein has been observed in epithelial tissue during organogenesis in other species (Osler and Bader, 2004; Ripley, *et al.,* 2004). Immunostaining of cardiomyocytes cultured *in vitro* confirmed the membrane localization of Pop1/Bves in cells

that participate in cell adhesion (Vasavada, *et al.,* 2004). An *in vitro* model of corneal wound healing showed that Bves staining was missing at the epithelial surface during cellular migration across the wound, but it reappeared at the points of cell contact during the reinitiation of epithelial continuity (Ripley, *et al.,* 2004). The C-terminal intracellular region of the BVES protein is likely to interact with other proteins but it lacks obvious protein-protein interaction domains (Osler, *et al.,* 2006). The tight junction component ZO-1 interacts with BVES (Osler, *et al.,* 2005). ZO-1 is a scaffold protein that interacts with a number of tight adherens and gap junction proteins (Itoh, *et al.,* 1993; 1999, Fanning, *et al.,* 1998; Barker, *et al.,* 2001) however, just how this protein interacts with BVES is unknown. From the phenotypes observed in our bves antisense RNA experiment, we suggest that bves, as a glycosilated transmembrane protein is required for posterior midgut invagination, adhesion of pole cells to the posterior midgut primordium and contact between the cephalic furrow and the leading edge of the germ band. Hence reduced protein levels, eventually caused the mutant phenotypes we have observed. The need for antibodies to follow the location of bves protein in specific cells during early embryogenesis and changes in the antisense lines is essential.

The function of bves in oogenesis remains unclear. Its expression is restricted to ventral follicle cells by GRK/EGFR signalling. We presume that bves, as an adhesion molecule might more likely be involved in the migration of those ventral follicle cells to cover oocyte than in the establishment of ventral signal essential for embryonic dorsal-ventral pattern. As no mutant phenotype using antisense was found during oogenesis, this hypothesis remains to be investigated.

The key to further progress/investigation of this potentially exciting gene product in cell adhesion and cell migration in embryogenesis in *Drosophila* will need the generation of good antibodies and mutants.

Materials and Methods

Fly stocks

Wild type flies were OrR. Embryo hosts for injection were w^{1118}. For other lines refer to papers referenced. grk^{HK} and grk^{WG} (Schüpbach, 1987), *fs(1)K10* (Wieschaus *et al.,* 1978), UAS-lacZ (Brand and Perrimon, 1993), GAL4 line C648 (Deng *et al.,* 1997). All flies were raised on standard cornmeal-yeast agar medium at 25ºC.

Library screening and DNA sequencing

5 kb of genomic DNA was rescued from GAL4 line C648. The genomic DNA was then used to screen an ovarian cDNA library (Stratagene, Îzap, a gift from Y. Jan, UCSF, USA). One clone was obtained, with an insert of 0.8 kb, which was sequenced. An expressed sequence tag (EST) database search was carried out. Two EST clones (GM07524, LD22978) were found to be similar to the cloned cDNA. These two EST clones were obtained from Berkeley *Drosophila* Genome Project (BDGP) and sequenced.

Generation of transgenic flies

A screen mobilising the P-element in the GAL4 line C648 did not generate any lethal mutants in the *bves* genes, despite generating lethal mutants in other nearby genes. It was therefore necessary to use alternative approaches to investigate the function of this gene. To construct heatshock *bves* antisense RNA lines, *bves* cDNA (GM07524) was digested with *EcoRI* and the fragments were ligated into pCaSpeRhs vectors in the antisense orientation.. The constructs were then introduced into w^{1118} flies by P-element mediated germline transformation (Spradling, 1986), resulting in the transgenic flies: pCaS-hs-*bve*-AS.

*Heatshock treatment of pCaS-hs-*bve*-AS fly lines*

Flies were cultured at 25ºC and fed yeast one day before heatshock. Flies were heatshocked for 45 minutes at 39ºC, then put in the 18ºC incubator for 30 minutes and finally the flies were heatshocked again for 45 minutes at 39ºC. The flies were moved back to 25ºC and the eggs collected and observed at 4 hours intervals.

Whole mount in situ *hybridisation and β-galactosidase staining*

Whole mount *in situ* hybridisation and β-galactosidase staining were as described by Zhao *et al.* (2000).

Observation of living embryos

Mutant and wild type embryos were dechorionated by rolling the embryos gently on double-sided sticky tape. Dechorionated embryos were transferred to a slide which had a thin strip of double sided sticky tape on it. The embryos were covered with KELF oil and observed. No defects were observed in wild type embryos.

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References

ANDREE, B., FLEIGE A., ARNOLD H. H. and BRAND T. (2002) Mouse Pop1 is required for muscle regeneration in adult skeletal muscle. *Mol Cell Biol.* 22: 1504-12.

ANDREE, B., HILLEMANN, T., KESSLER-ICEKSON, G., SCHMITT-JOHN, T., JOCKUSCH, H., ARNOLD, H.H., BRAND, T. (2000). Isolation and characterization of the novel popeye gene family expressed in skeletal muscle and heart. *Dev Biol.* 15; 223(2): 371-82.

BELLEN, H.J., O'KANE, C.J., WILSON, C., GROSSNIKLAUS, R.K. and GEHRING, W.J. (1989). P-element-mediated enhancer detection: a versatile method to study development in *Drosophila. Genes Dev*. 3: 1288-1300.

BRAND, A.H. and PERRIMON, N. (1993). Target gene expression as a means of altering cell fates and generating dominant phenotypes. *Development,* 118: 401-415.

BARKER, R.J., PRICE, R.L. and GOURDIE, R.G. (2001). Increased co-localization of connexion 43 and ZO-1 in dissociated adult myocytes. *Cell Commun Adhes.* 8: 205-208

CAMPOS-ORTEGA, J.A. and HARTENSTEIN, V. (1985). The Embryonic development of *Drosophila melanogaster*. Springer-Verlag, Berlin and Heidelberg.

COSTA, M., SWEETON, D. and WIESCHAUS, E. (1993). Gastrulation in *Drosophila*: cellular mechanisms of morphogenetic movements. In: M. Bate and A. Martinez-Arias, Editors, The development of *Drosophila* melanogaster 1, *Cold Spring Harbor Laboratory Press, New York*, pp. 425-465.

DENG, W.M., ZHAO D., ROTHWELL, K. and BOWNES M.(1997). Analysis of P[gal4] insertion lines of Drosophila melanogaster as a route to identifying genes important in the follicle cells during oogenesis. *Mol Hum Reprod*. 3: 853-62.

DENG, W.M. and RUOHOLA-BAKER, H. (2000). Laminin A is required for follicle cell oocyte signalling that leads to establishment of the anterior-posterior axis in D*rosophila*. *Curr Biol* 10: 683–686.

DIANGELO, J.R., VASAVADA, T.K., CAIN, W. and DUNCAN, M.K. (2001). Production of monoclonal antibodies against chicken Pop1 (BVES). *Hybrid Hybridomics*. 20: 377-81.

Ensembl, European Bioinformatics Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge (2004). Anopheles Genome Sequencing consortium.

FANNING, A.S., JAMESON, B.J., JESAITIS, L.A. AND ANDERSON, J.M. (1998). The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. *J Biol Chem* 272: 29745-29753.

GONZALEZ-REYES, A., ELLIOTT, H. and, ST JOHNSTON, D. (1995). Polarization of both major body axes in *Drosophila* by *gurken-torpedo* signalling. *Nature* 375: 654–658.

HITZ, M.P., PANDUR, P., BRAND, T and KUHL, M. (2002) Cardiac specific expression of Xenopus Popeye-1. *Mech Dev.* 115: 123-6.

ITOH, M., NAGAFUCHI, A., YONEMURA, S., KITANIYASUDA, T. and TSUKITA, S. (1993). The 220-KD protein colocalizing with cadherins in non-epithelial cells is identical to ZO-1, a tight junction-associated protein in epithelial cells: cDNA cloning and immunoelectron microscopy. *J Cell Biol.* 121: 491-502.

ITOH, M., FURUSE, M., MORITA, K., KUBOTA, K., SAITOU, M. AND TSUKITA S. (1999). Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2 and ZO-3, with the COOH termini of claudins. *J Cell Biol.* 147: 1351-1363.

JAGLARZ, M. and HOWARD, K. (1994). Primordial germ cell migration in *Drosophila melanogaster* is controlled by somatic tissue. *Development* 120: 83–89.

KNIGHT, R.F., BADER, D.M. and BACKSTROM, J.R. (2003). Membrane topology of Bves/Pop1A, a cell adhesion molecule that displays dynamic changes in cellular distribution during development. *J Biol Chem.* 29: 32872-9.

KARPEN, G.H. (1994) Position-effect variegation and the new biology of heterochromatin. *Curr. Opin. Genet. Dev.* 4: 281-291.

MAHOWALD, A.P. (2001). Assembly of the Drosophila germ plasm, *Int Rev Cytol.* 203:187-213.

OSLER, M.E. and BADER, D.M. (2004). Bves expression during avian embryogenesis. *Dev Dyn.* 229: 658-67.

OSLER, M.E., CHANG, M.S. AND BADER D.M. (2005). Bves modulates epithelial integrity through an interaction at the tight junction. *J Cell Sci.* 118: 4667-4678.

OSLER, M.E., SMITH, T.K. AND BADER D.M. (2006). Bves, a member of the popeye Domain-containing Gene Family. *Dev Dyn.* 235: 586-593.

REESE, D.E., ZAVALJEVSKI, M., STREIFF, N.L. and BADER, D. (1999). Bves: A novel gene expressed during coronary blood vessel development. *Dev Biol.* 209: 159-71.

REESE, D.E. and BADER, D.M. (1999). Cloning and expression of hbves, a novel and highly conserved mRNA expressed in the developing and adult heart and skeletal muscle in the human. *Mamm. Genome.* 10: 913-5.

RIECHMANN, V. and EPHRUSSI, A. (2001). Axis formation during *Drosophila* oogenesis. *Curr. Opin. Genet. Dev.* 11: 374-383.

RIPLEY, A.N., CHANG, M.S. and BADER D.M. (2004). Bves is expressed in the epithelial components of the retina, lens and cornea. *Invest Ophthalmol Vis Sci.* 45: 2475-83.

ROBERTS, D.B. (1998). *Drosophila*, A practical approach. second edition (Oxford university press).

SCHUPBACH, T. (1987) Germ line and soma cooperate during oogenesis to establish the dorsoventral pattern of egg shell and embryo *in Drosophila melanogaster. Cell.* 49: 699-707.

SIMMONS, M.J., RAYMOND, D., GRIMES, C.D., BELINCO, C., HAAKE, B.C., JORDAN, M., LUND, C., OJALA, T.A. and PAPERMSTERET, D. (1996). Repression of Hybrid Dysgenesis in *Drosophila* melanogaster by heat shock inducible sense and antisense P-element constructs. *Genetics.* 144: 1529-44.

SPRADLING, A.C. (1986). In *Drosophila*: A Practical Approach. (ed. D.B.Robert), pp.175-197. Oxford, England: IRL Press.

SPRADLING, A.C. (1993). Developmental genetics of oogenesis. In: M. Bate and A. Martinez-Arias, Editors, The development of *Drosophila* melanogaster 1, Cold Spring Harbor Laboratory Press, New York pp. 1–70.

TELFER, W. (1975). Development and physiology of the oocyte-nurse cell syncitium. *Adv. Insect Physiol.* 11: 223–319.

VASAVADA, T.K., DIANGELO, J.R. and DUNCAN, M.K. (2004). Developmental expression of Pop1/Bves. *J Histochem Cytochem.* 52: 371-78.

WADA, A.M., REESE, D.E. and BADER, D.M. (2001). Bves: prototype of a new class of cell adhesion molecules expressed during coronary artery development. *Development.* 128: 2085-93.

WARRIOR, R. (1994). Primordial germ cell migration and the assembly of the Drosophila embryonic gonad. *Dev Biol.*, 166: 180-94.

WIESCHAUS, E., MARSH, J and GEHRING, W. (1978). *fs(1) K10*, a germline dependent female sterile mutation causing abnormal chorion morphology in *Drosophila melanogaster*. *Roux's Arch.Dev.Biol.* 184: 75-82.

ZHAO, D., CLYDE, D., BOWNES, M. (2000). Expression of *fringe* is down regulated by Gurken/Epidermal Growth Factor Receptor signalling and is required for the morphogenesis of ovarian follicle cells. *J. Cell Science* 113: 3781-3794.

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