

Identification of mutations that cause cell migration defects in mosaic clones

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SUMMARY

Cell movement is an important feature of animal development, wound healing and tumor metastasis; however, the mechanisms underlying cell motility remain to be elucidated. To further our understanding, it would be useful to identify all of the proteins that are essential for a cell to migrate, yet such information is not currently available for any cell type. We have carried out a screen for mutations affecting border cell migration in *Drosophila*. Mutations that cause defects in mosaic clones were identified, so that genes that are also required for viability could be detected. From 6000 mutagenized lines, 20 mutations on chromosome 2R were isolated that cause defects in border cell position. One of the mutations was dominant while all of the recessive mutations appeared to be homozygous lethal. This lethality was used to place the

mutations into 16 complementation groups. Many of the mutations failed to complement cytologically characterized deficiencies, allowing their rapid mapping. Mutations in three loci altered expression of a marker gene in the border cells, whereas the remaining mutations did not. One mutation, which caused production of supernumerary border cells, was found to disrupt the *costal-2* locus, indicating a role for Hedgehog signaling in border cell development. This screen identified many new loci required for border cell migration and our results suggest that this is a useful approach for elucidating the mechanisms involved in cell motility.

Key words: Cell migration, Mutant, Mosaic analysis, *Drosophila*

INTRODUCTION

Cell migration is a dramatic and essential feature of the development of multicellular organisms. Much of what is known of the mechanisms contributing to cell motility has emerged from the study of cultured cells. The current view is that cell migration includes extension of pseudopods, formation of stable cell-substratum attachments and retraction of the trailing edge. Biochemical dissection of the process has implicated the actin cytoskeleton, cell adhesion molecules and myosin motors in generating the necessary forces to propel cells forward (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996; Hynes and Lander, 1992). However, one limitation of a biochemical approach in cell culture is that it is difficult to identify the factors that control the timing and direction of cell migration; it is also difficult to test the in vivo significance of specific molecules.

A genetic screen for mutants displaying migration defects has the potential to identify many components of the migration machinery as well as the genes that regulate the expression and activities of the basic machinery. Genetic approaches to the study of cell migration have been pursued in the nematode, *Caenorhabditis elegans* and in the fruitfly, *Drosophila melanogaster*. In *C. elegans*, numerous mutants with cell migration defects have been described and cloning the genes corresponding to these mutations has provided fascinating

insights into factors that guide cells and axons. For example the *unc-6* and *unc-5* genes encode an extracellular ligand and its receptor, respectively, which act together to guide both cell migration and axon pathfinding in the developing worm (Hedgecock, 1987; Hedgecock et al., 1990; Ishi and Hedgecock, 1992; Leung-Hagesteijn et al., 1992). In addition, several transcription factors and signaling molecules have been found to be required for proper migration of specific cells (Harris et al., 1996).

One of the model systems for studying cell migration in *Drosophila* is the migration of border cells during oogenesis (King, 1970). The *Drosophila* ovary consists of egg chambers, each of which is composed of 15 nurse cells and one oocyte, surrounded by a monolayer epithelium of 1100 somatic follicle cells. The follicle cells are found initially in a uniform cuboidal monolayer. However, during stage 9 of oogenesis, these cells reorganize so that >90% of the cells change into a columnar shape and move into the posterior half of the egg chamber, in contact with the oocyte. Most of the remaining follicle cells become thin and flat and stretch to cover the nurse cells. However, six to ten follicle cells remain rounded at the anterior tip of the egg chamber and subsequently migrate through the middle of the nurse cell cluster. These follicle cells stop at the border between the nurse cells and oocyte and have been named border cells (King, 1970; Spradling, 1993; Fig. 1).

The border cells appear to undergo a fairly typical epithelial-to-mesenchymal transition, much like that of neural crest cells exiting the neural tube (Bronner-Fraser, 1993). The border cells extend cytoplasmic processes in between two nurse cells (Fig. 1). Actin filaments fill the cytoplasmic extensions and can be visualized with rhodamine-phalloidin staining (Murphy and Montell, 1996) (Fig. 1). The border cells migrate approximately 150 μm and the complete migration takes about 5 hours. The cells migrate directly towards the oocyte, passing several nurse cell junctions, without any lateral deviation from their pathway through the center of the egg chamber (Spradling, 1993). The border cells even migrate to the oocyte in abnormal egg chambers in which the oocyte is mispositioned (Lee et al., 1996), suggesting that the border cells can sense the position of the oocyte and are guided to it.

Several genes are known to be involved in border cell migration. For example, the gene *slbo*, which encodes a transcription factor of the C/EBP family, appears to coordinate terminal differentiation of the border cells with migration (Montell et al., 1992). The FGF receptor homologue *Breathless* (Murphy et al., 1995) appears to be a downstream target of SLBO. Non-muscle myosin II (Edwards and Kiehart, 1996) and the GTPase Rac (Murphy and Montell, 1996) also function in border cell migration. However many questions remain concerning the mechanism of border cell migration.

One limitation of the genetic screens for cell migration mutants has been that mutations that cause lethality during early development could not be identified. In addition, pleiotropic effects of mutations in genes coding for cell adhesion molecules or other widely expressed genes would be likely to mask their cell migration defects. One way to overcome the potential problem of pleiotropy in analyzing border cell migration is to use mosaic analysis: that is, to generate clones of cells in the adult, that are homozygous for loss-of-function mutations and screen for cell migration defects.

In this study, we utilized the FRT/FLP system to generate follicle cell clones and screened chromosome 2R for mutations affecting border cell migration. 20 mutations were identified that caused defects in border cell position. These mutations fell into 16 complementation groups. Some of the mutations altered expression of marker genes whereas others did not. One of the mutations caused defects in border cell number as well as position, and this defect was due to mutation of the *costal-2* locus, which functions in Hedgehog signaling.

MATERIALS AND METHODS

Genetic strains, mutagenesis and establishment of balanced lines

Genetic markers and balancer chromosomes have been described previously (Lindsley and Zimm, 1992). Fly stocks and crosses were grown on standard medium at 25°C unless otherwise indicated. FRT lines and FLP lines were kind gifts from Joseph B. Duffy (University of Indiana). The FRT line has a P-element with the FRT sequence inserted and is located at 42B on 2R (designated *FRT^{2R}*, also known as line G13). The FLP line has a UAS-FLP transgene and a transgene expressing GAL-4, both inserted on the 3rd

chromosome and combined with the FRT at 42B (designated *FRT^{2R};T155 UF*).

dp,PZ6356,FRT^{2R} males were treated with 25 mM EMS (Sigma Chemical) in 5% sucrose for 18-24 hours using standard methods (Grigiatti et al., 1986). Mutagenized males were mated with *ScO/CyO* virgin females. Single male progeny with the genotype of *dp, PZ6356 FRT^{2R},*/ScO* or *CyO* (where the asterisk indicates the mutagenized chromosome) were mated with *FRT^{2R};T155UF* virgins. Ovaries from two females with the genotype of *dp,PZ6356,FRT^{2R},*/FRT^{2R}; T155UF/+* were dissected, stained for β -galactosidase (β -gal) activity (as described below) and analyzed for any defects in border cell migration. If a phenotype was observed, males of the genotype *dp,PZ6356, FRT^{2R},*/FRT^{2R}; T155UF/+* were mated with *ScO/CyO;ry* virgin females and balanced stocks were established. Potential mutants from the primary screen were rescreened by crossing them back to *FRT^{2R}; T155UF* line, and more ovaries were examined to determine whether the phenotype was reproducible.

Complementation, deficiency mapping and cleaning up

Mutant lines were crossed to each other to determine allelism. Failure to produce viable transheterozygous adults was taken as an indication of allelism. The deficiency kit for 2R was obtained from the Bloomington stock center. This deficiency kit includes a collection of 37 deficiencies and represents approximately 70% of 2R with little redundancy. Each mutant line was crossed to all of the deficiencies and scored for transheterozygous lethality. In order to increase our confidence that lethality and the border cell migration defect were caused by the same mutation, we selected the lines that failed to complement one or more deficiencies, to outcross to an unmutagenized *PZ6356, FRT^{2R}* chromosome. By allowing recombination to take place, unwanted second mutations could be recombined away from the mutation of interest. Between 17 and 39 recombinants for each line were analyzed for border cell migration defects in mosaic clones and for homozygous lethality. In no case was the border cell migration defect separated from lethality and none of the viable recombinant lines exhibited border cell migration defects. Prior to recombination, three mutants 11B9, 17E1 and 3A2 failed to complement two non-overlapping deficiencies on 2R; after the recombination analysis only one of the deficiencies failed to complement the recombinant chromosomes that retained the border cell migration defect. Thus the recombination analysis successfully separated unwanted lethal mutations on 2R in each of these three lines. In one case, 18E4, the mutant phenotype was not observed in any of the outcrossed lines, suggesting that the defect might have been a synthetic phenotype due to two mutations that were sufficiently far apart to recombine at high frequency. For the other mutations analyzed, every recombinant chromosome that retained the border cell migration phenotype failed to complement the same 2R deficiency that had been initially identified. Although the number of lines that definitively retained the phenotype was small for each mutation analyzed (the median number was 4 and the average was 3.8), the lethality uniformly co-segregated with the border cell migration phenotype, suggesting that, in most cases, the border cell migration defect was caused by a lethal mutation. It is likely that more recombinant lines retained the border cell migration mutation than were identified as phenotypic in the analysis, since the mosaic clone frequency varied (see discussion below concerning saturation).

Although we used an EMS concentration of 25 mM and we screened 6000 mutagenized lines, the small number of non-complementing mutations indicated a low degree of saturation. It is likely that several factors contributed to this phenomenon. We discovered through the course of the screen that the mosaic clone frequency was temperature-dependent, most likely due to temperature sensitivity of the GAL4/UAS system. We observed a higher frequency of phenotypic egg chambers when the flies were

incubated at 29°C from the pharate adult stage through the 5th day posteclosion, prior to dissection of ovaries. In addition, we found that expression of GFP in T155; UAS-GFP flies was somewhat variable, as well as mosaic so that some germaria exhibited greater expression than others.

β -galactosidase activity, phalloidin and antibody staining

For β -galactosidase activity staining, female flies were dissected in Schneider's medium plus 10% calf serum. Ovaries were fixed at 25°C in 8% glutaraldehyde in buffer B (100 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$, 450 mM KCl, 150 mM NaCl, 20 mM MgCl_2), or 6% formaldehyde in 0.17 \times buffer B, and stained with 0.2% X-gal in staining solution (10 mM PO_4 -buffer pH 7.2, 150 mM NaCl, 1 mM MgCl_2 , 3 mM $\text{K}_4[\text{FeII}(\text{CN})_6]$, 3 mM $\text{K}_3[\text{FeIII}(\text{CN})_6]$, 0.3% Triton X-100) overnight. Egg chambers were examined under a dissecting microscope at a magnification of 25 \times .

For rhodamine-phalloidin staining, ovaries were hand dissected in Schneider's medium plus 10% calf serum, individual egg chambers were removed from the ovariole sheath and fixed in 200 μl 3.7% formaldehyde in 0.1 M phosphate buffer (pH 7) containing 0.5% NP40/600 ml heptane for 20 minutes and washed in NP40 wash buffer (50 mM Tris 7.4, 150 mM NaCl, 0.5% NP40, 1 mg/ml BSA, 0.02% azide). 1 μl of rhodamine-phalloidin in methanol (Molecular Probes) was air-dried for 20 minutes in a fume hood or for 1 minute in a speed vac and resuspended in 200 μl NP40 wash buffer. Egg chambers were incubated in this solution for 2 hours at room temperature in the dark and then rinsed with NP40 wash buffer. Egg chambers were mounted in vectorshield (Vector Laboratory Inc, Burlingame, CA) and viewed using a laser scanning confocal microscope.

For antibody staining, egg chambers were dissected and fixed in the same manner as for rhodamine-phalloidin staining. After washing in NP40 wash buffer, egg chambers were blocked for 2 hours at room temperature in block solution (NP40 wash buffer plus 20% BSA). Then egg chambers were incubated in primary antibody overnight at 4°C. Anti-cadherin antibody was a kind gift of Tadashi Uemura and was diluted 1:20 in block solution. Anti-FasIII antibody (a kind gift of Nipam Patel, University of Chicago) was not diluted. A 1 hour wash was followed by incubation in fluorescein-conjugated secondary antibody (Vector Laboratories, Burlingame, CA) at a dilution of 1:200 for 2 hours at room temperature. After washing in NP40 wash buffer for 1 hour, egg chambers were mounted in Vectashield.

Plasmid rescue

Plasmid rescue of DNA flanking 1(2)k16101 was performed as follows: 3 μg of genomic DNA from 1(2)k16101/CyO flies was digested with *EcoRI* or *BglII*, phenol extracted and ethanol precipitated. Following resuspension in TE (10 mM Tris, 1 mM EDTA), the DNA was ligated in a 200 μl reaction with 1 U of T4 DNA ligase. The DNA was ethanol precipitated, resuspended in TE and 1 μl used for transformation. DNA prepared from ampicillin-resistant colonies was sequenced using a sequencing facility.

RESULTS

A screen for border cell migration mutants by producing mosaic clones in the follicle cells

The second chromosome has previously been screened extensively for female-sterile mutations (Schupbach and Wieschaus, 1991); however, none of these caused a border cell migration defect (D. J. M., unpublished data). One potential reason for the paucity of such defects among female-sterile mutants is that mutations that perturb border cell migration might also cause lethality. In fact, all of the genes previously implicated in border cell migration are either known to cause lethality during early development (*slbo*, *btl*, *zip*) (Montell et al., 1992; Murphy et al., 1995; Edwards and Kiehart, 1996), or are likely to do so (*DRac1*). In order to circumvent this limitation, we used tissue-specific expression of the site-specific recombinase FLP to create mosaic clones of lethal mutations in the follicle cells of the ovary (Golic and Lindquist, 1989; Xu and Rubin, 1993; Duffy et al., 1998). We chose chromosome 2R for the initial screen because the *slbo* locus resides at 60C1,2 on 2R (Montell et al., 1992), providing a positive control for the efficacy of the approach.

The crosses required to generate mosaic clones in the follicle cells of mutations on 2R are shown in Fig. 2. In order to screen the second chromosome, it was necessary to utilize a third chromosome FLP line, *T155GAL4,UAS-FLP*, which had not been well characterized with respect to clone frequency in the follicle cells (Duffy et al., 1998). The somatic cell expression of *T155GAL4*, early in oogenesis, is shown in Fig. 3A,B. We examined the frequency and size of mosaic clones produced by *T155GAL4,UAS-FLP*, using an enhancer trap line on 2R, *PZ2970*, as a marker since it drives β -galactosidase (β -gal) expression in all follicle cells (Fig. 3C). *PZ2970* was recombined onto the *FRT*^{2R} chromosome and then crossed to

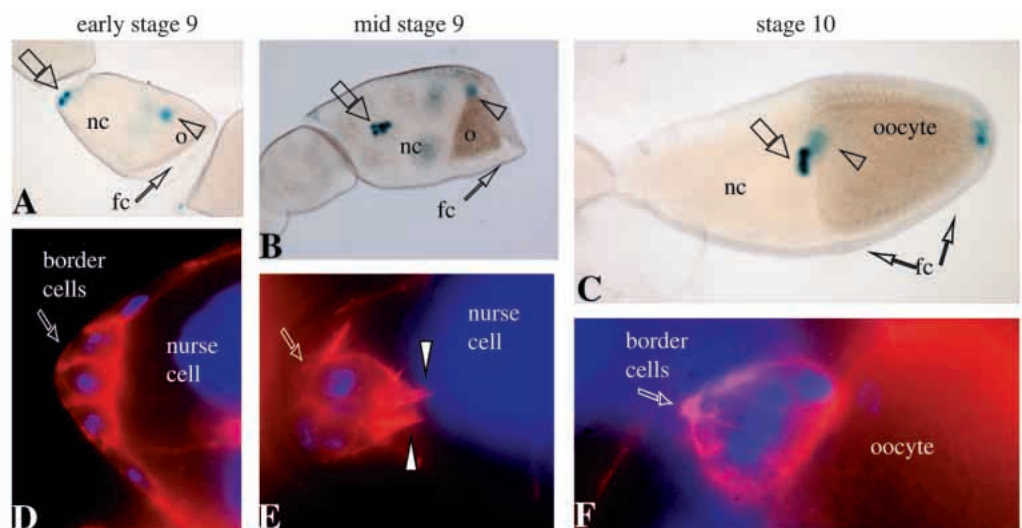


Fig. 1. Wild-type border cell migration. (A-C) β -galactosidase staining of egg chambers from enhancer trap line *PZ6356*. Wide arrows indicate border cells. Thin arrows indicate columnar follicle cells (fc). Open arrowheads indicate the oocyte nucleus. o, oocyte; nc, nurse cells. (A) Early stage 9, border cells prior to migration. (B) At mid-stage 9, the border cells have migrated half-way toward the oocyte. (C) At stage 10, the cells stop at the border between nurse cells and oocyte and align with the oocyte nucleus. (D-F) Rhodamine-phalloidin (red) and DAPI (blue) double labeling of border cells (white arrows) in early stage 9 (D), mid-stage 9 (E) and stage 10 (F) egg chambers. White arrowheads indicate the filopodia in E.

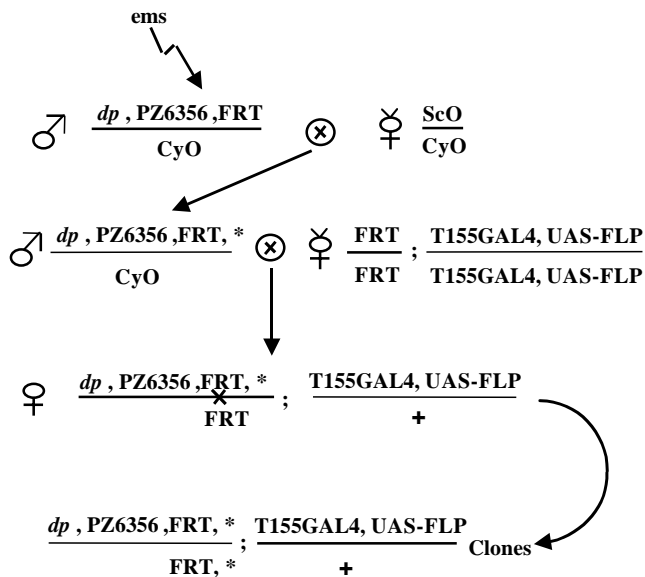


Fig. 2. Scheme for isolating mutants affecting border cell migration in mosaic clones. The * indicates the mutagenized chromosome. The X indicates a mitotic recombination event between the two FRT sites, which occurs in follicle cell precursors due to expression of FLP from the follicle cell GAL4 (*T155GAL4*) and UAS-FLP transgenes that are present on the third chromosome. See text for details.

FRT^{2R}; T155GAL4, UAS-FLP. Approximately 80% of the egg chambers examined ($n=70$) contained patches of follicle cells that did not express β -gal (Fig. 3D), indicating that they had lost the *PZ2970* P-element through recombination. The clones were typically large, comprising approximately 300-700 cells. In contrast, no germline clones were detected in experiments

using a germline marker (Y. Uehara and D. J. M., unpublished observations).

To determine the frequency of clones that included the border cells, we generated clones of an enhancer trap line, *PZ3050*, which drives β -gal expression in the border cells and centripetal follicle cells (Fig. 3E). Approximately 20% of egg chambers were β -gal negative (Fig. 3E,F). We also analyzed clones using a *slbo* null mutation, which is homozygous lethal. The null mutation does not have a *lacZ* marker associated with it; therefore to visualize the border cells, we recombined an enhancer trap line, *PZ6356*, onto the *FRT^{2R}, slbo* chromosome. The *PZ6356* enhancer trap, which is an insertion on 2L, exhibits a high level of β -gal expression in border cells and the oocyte (Tinker et al., 1998 and Fig. 1), but does not cause a detectable phenotype even when homozygous. There is a reduction in the level of β -gal activity from the *PZ6356* enhancer trap in a *slbo* mutant background compared to wild-type, indicating that expression from this line is *slbo*-dependent (R. Tinker and D. J. M., unpublished data). When we crossed the *PZ6356, FRT^{2R}, slbo* flies with *FRT^{2R}; T155GAL4, UAS-FLP*, we found that border cell migration was impaired and *PZ6356* expression was reduced, in 10-20% of stage 10 egg chambers examined ($n=100$), depending on the experiment. As a negative control, several hundred egg chambers from unmutagenized *PZ6356, FRT^{2R}* females were examined and none of them showed any defects in border cell migration. Furthermore, several hundred egg chambers from a line of the following genotype *PZ6356, FRT^{2R}, m/FRT^{2R}; T155GAL4, UAS-FLP/+*, where *m* indicates a single, irrelevant lethal mutation, were examined and no border cell migration defect was detected. These experiments confirmed that there was no detectable background frequency of border cell migration defects in this genotype and that clones that include the border cells could be obtained in 10-20% of egg chambers examined.

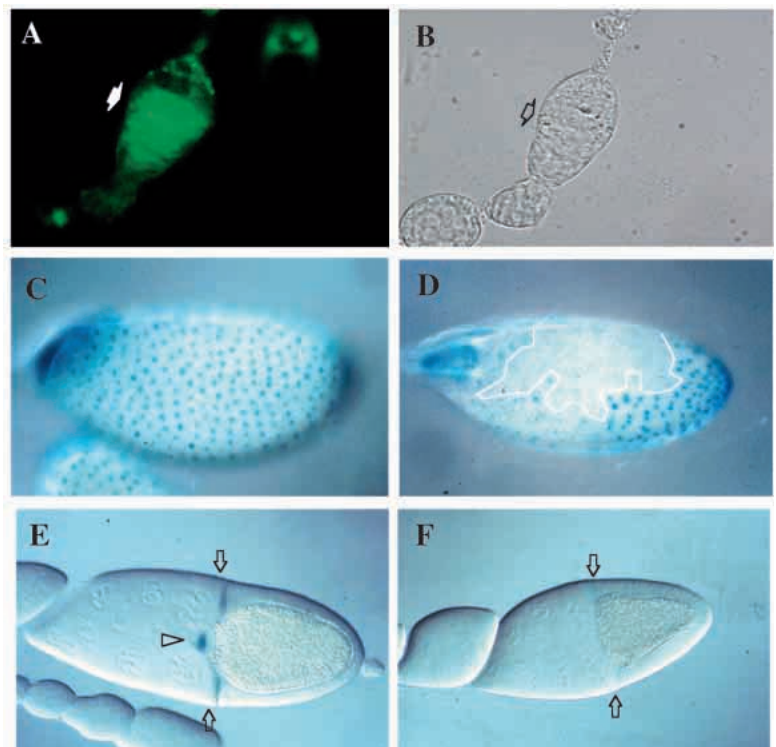


Fig. 3. Follicle cell clones produced by the FRT/FLP system. (A) Expression pattern of the *T155GAL4* line visualized in a germarium from a *UAS-GFP;T155* female fly. (B) The same germarium viewed using Nomarski optics. The arrows indicate region II of the germarium, where the follicle cell precursors reside. (C) β -gal staining of a late stage egg chamber from enhancer trap line *PZ2970/CyO*. Staining is detected uniformly in all of the follicle cell nuclei. (D) β -gal staining of an egg chamber from *FRT^{2R}, PZ2970/FRT^{2R}; T155GAL4, UAS-FLP*. The white line indicates the border of a mutant clone. (E) β -gal staining of a wild-type *PZ3050/CyO* egg chamber. Border cells (arrowhead) and centripetal follicle cells (arrows) are stained. (F) An egg chamber from *FRT^{2R}, PZ3050/FRT^{2R}; T155GAL4, UAS-FLP*. The lack of staining indicates that the border cells and most of the centripetal cells have lost the *PZ3050* P-element by recombination.

We used the scheme outlined in Fig. 2 to screen approximately 6000 ethyl methane sulfonate (EMS) mutagenized lines. Ovaries from females of the genotype *PZ6356,FRT^{2R},*/FRT^{2R}; T155GAL4,UAS-FLP/+*, where * indicates the mutagenized chromosome, were scored for defects in border cell migration. 100 lines selected from the primary screen were rescreened by crossing them back to the *FRT^{2R}; T155GAL4,UAS-FLP* line, dissecting and staining additional ovaries to confirm that the phenotype was reproducible and heritable. Following the secondary screen, we further characterized 20 lines whose phenotypes were reproduced at least twice. One of the 20 lines failed to complement *slbo^{e7b}*, indicating that this line was a new allele of *slbo*.

Phenotypic analysis, complementation and mapping

The 20 mutant lines from the secondary screen were placed into classes based on phenotypic similarity. Normal border cell migration initiates in stage 9, at the same time that the outer follicle cells rearrange (Fig. 1) and the border cells complete their migration by stage 10 (Figs 1C, 4). In class I, which was composed of five alleles including the new allele of *slbo*, the border cells failed to initiate migration, remaining at or near the anterior tip of the egg chamber even in stage 10. In addition, expression of the enhancer trap marker *PZ6356* was reduced in all but one or two of the border cells, whereas staining in the oocyte nucleus was not affected (Fig. 4). These results suggested that class I mutants caused migration defects by regulating, directly or indirectly, the expression of other genes. The phenotype of class II mutants was characterized by border cell migration failure, without any change in *PZ6356* expression (Fig. 4). The three mutations that made up class III caused a mild delay of border cell migration without any change in *PZ6356* expression (Fig. 4). In wild-type egg chambers, the border cells always complete their migration by early stage 10. Egg chambers in which border cells had migrated only halfway through the nurse cells at stage 10 were considered to exhibit mild delay.

Classes IV and V contained one allele each. The phenotype of class IV was the production of extra border cells. While most of the extra border cells migrated in between the nurse cells to some extent, the extra border cells were often ectopically located. Class V egg chambers exhibited several defects, which included mislocalization of the oocyte nucleus (Fig. 4), degenerating nurse cells and defects in the final position of the border cells. In the class V mutant, the oocyte nucleus position was random and the border cells were frequently located near the surface of the egg chamber. Normally border cells would be found either near the center of the egg chamber or juxtaposed with the oocyte nucleus towards the dorsal side. These defects did not always occur in the same egg chamber, however, and we found several examples of egg chambers with oocyte nucleus position defects without border cell defects and vice versa.

Complementation analysis was conducted by scoring for transheterozygous lethality and border cell migration defects (see Materials and Methods for details). The 20 alleles fell into 16 complementation groups (Table 1). Mutants that failed to complement for lethality also featured distinctive similarities with respect to their border cell migration phenotypes. For example, 22F3, 47H6 and 31E6 all exhibited reduced *PZ6356* staining, a feature of only five of the 20 mutants. 9B9 and 2D8, which failed to complement each other for lethality, comprised two of the three mutants that displayed a mild migration defect.

The shared phenotypic characteristics among mutations that failed to complement for lethality suggested that the lethality

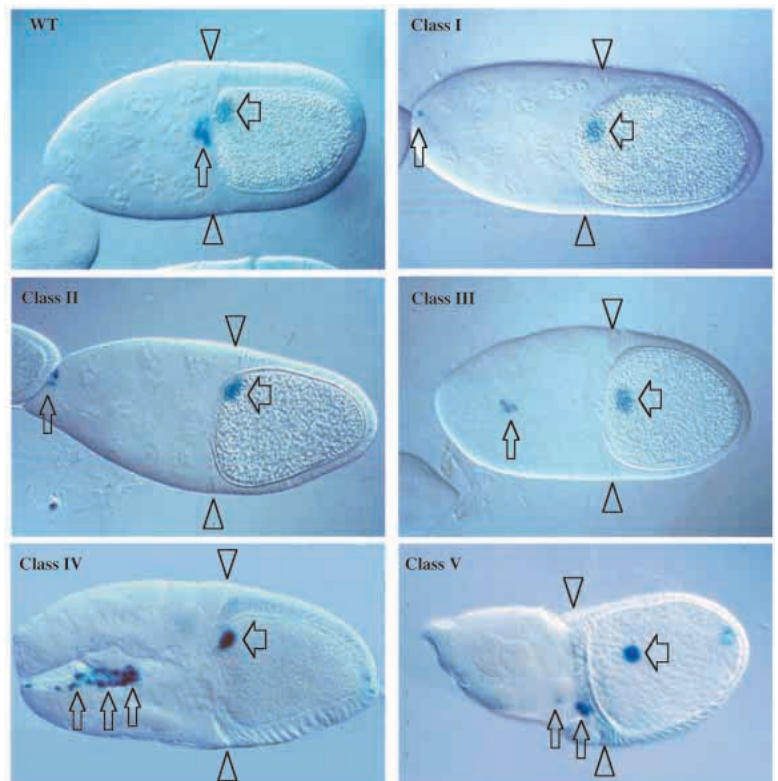


Fig. 4. Classes of mutant phenotypes. A wild-type (WT) egg chamber is shown for comparison. Note that, by early stage 10, which is characterized by complete rearrangement of the outer follicle cells and by the size of the oocyte, which fills half of the egg chamber at this stage, the border cells have invariably completed their migration. In wild-type, β -gal expression in the border cells is typically as strong as, or slightly stronger than, the oocyte nucleus staining. Class I, no migration of border cells and reduced *PZ6356* staining. One cell can be seen staining whereas additional cells are present but can only be detected at high power or by staining with DAPI or phalloidin. Class II, failure of border cell migration with no change in *PZ6356* staining. Note that, in contrast to class I, the border cell β -gal staining is as dark as the oocyte nucleus staining. Class III, mild delay of border cell migration. The apparent difference in oocyte nucleus position in this particular class III egg chamber is due to its orientation. Class IV, supernumerary border cells. Thin arrows indicate the border cells; thick arrows indicate the oocyte nucleus. Class V, mislocalized oocyte nucleus and displaced border cells. Arrowheads indicate the extent of outer follicle cell rearrangement. The displacement of the oocyte nucleus in class V is reproducible and not dependent on the orientation of the egg chamber. The class IV and class V examples exhibit somewhat stronger expression of β -gal, and a somewhat pitted nurse cell morphology, because of glutaraldehyde fixation of these egg chambers. All other egg chambers were fixed using formaldehyde (see Materials and Methods).

Table 1

Line no.	Additional alleles	Phenotypic class	Non-complementing Df Breakpoints
2D8	9B9	III	no Df
3A2	no	I	49E7;50D2
5D4	slbo	I	60C1-2
11B9	23A9	II	47D1-2;47F
12A9	no	II	no Df
17E1	no	II	49E7;50D2
17F1	no	II	no Df
18E4	no	II	44D;44F
22F3	47H6 31E6	I	42B3-5;42C1-7
27C1	no	II	56F9-17;57D11-12
31B8	no*	II	n.a.
46F4	no	II	no Df
50F7	no	II	no Df
52B7	no	IV	42E;43E
61E5	no	V	no Df
65C2	no	II	no Df

*31B8 is a dominant allele.

was caused by the same mutation that caused the border cell migration phenotype. To further increase our confidence that the lethality and border cell migration defects were caused by the same mutation, we selected several lines to outcross to an unmutagenized *PZ6356*, *FRT^{2R}* chromosome and allowed recombination to take place. In this way, unwanted second

mutations could be recombined away. Between 17 and 39 recombinants for each line were analyzed for border cell migration defects in mosaic clones and for homozygous lethality (see Materials and Methods for details). In no case was the border cell migration phenotype separated from lethality. In addition, all of the viable recombinant lines were tested for border cell migration defects and none were found, confirming that the border cell migration defects were most likely due to lethal, rather than female-sterile mutations. In order to map the mutations, each line was crossed to a collection of deficiency stocks, which represent approximately 70% of 2R (Bloomington Stock Center), and scored for transheterozygous lethality. These results are summarized in Table 1. For those mutations that failed to complement a deficiency, we examined the chromosomal region for previously identified loci that might be considered logical candidates for cell migration genes, such as loci coding for motor proteins or mutants with morphogenetic defects. However, we did not find any obvious candidates in the appropriate regions. We also tested for complementation with lethal P-elements in these regions, as described in more detail below.

Two of the class II mutant lines, 27C1 and 17E1, appeared to interact genetically since female flies transheterozygous for 27C1 and 17E1 featured border cell migration defects in greater than 50% of egg chambers. This effect was specific

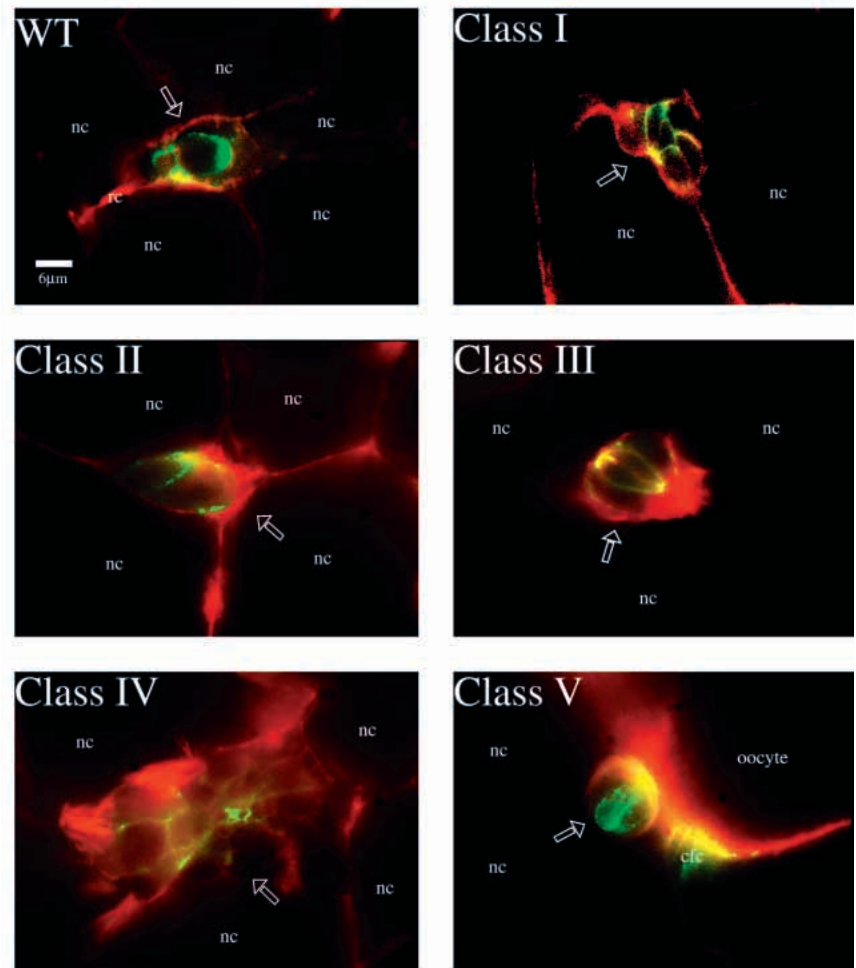


Fig. 5. Filamentous actin and cadherin distribution in border cells in wild-type and mutant egg chambers. Rhodamine-phalloidin (red) and anti-cadherin (green) staining of border cells in wild-type (WT) stage 9 and mutant stage 10 egg chambers. In all cases, phalloidin staining can be seen at the cortex of the border cells (which are indicated by the arrows) and cadherin accumulates in localized patches. The class I egg chamber shown is from the 22F3 mutant line. The class II egg chamber is from line 11B9. The class III egg chamber is from line 65C2. Class IV is 52B7 and class V is 61E5. Co-localization of cadherin and actin appears yellow. nc, nurse cell; rc, ring canal; cfc, centripetal follicle cells.

because no other transheterozygous combinations of mutations resulted in any detectable border cell migration defect. 27C1 also exhibited migration defects in combination with any one of four non-overlapping deficiencies, out of the 34 tested, including the deficiency that uncovers 17E1. Taken together these observations suggest that 27C1 exhibits dominant interactions with several loci, including 17E1.

Cadherin expression and filamentous actin distribution were unchanged in all mutants examined

To determine whether the defects in migration were due to gross morphological abnormalities or atrophy of the border cells, we compared the distribution of F-actin in wild-type and mutant border cells. Rhodamine-phalloidin staining of wild-type stage 9-10 egg chambers reveals enrichment of F-actin distribution in the cortex of border cells (Fig. 5). In addition, one or more of the border cells can frequently be seen to extend filopodia between the nurse cells. These filopodia stain brightly with rhodamine-phalloidin (Murphy and Montell, 1996; Fig. 1E). To determine if there was any detectable change in the actin cytoskeleton in the mutant border cells, rhodamine-phalloidin-stained egg chambers from each mutant line were examined. A representative example from each of the mutant classes is shown in Fig. 5. The mutant border cells exhibited normal F-actin distribution in the cortex and, in some cases, filopodia were also visible (not shown). These results indicated that actin structures in the mutants were not severely disrupted.

Since cell adhesion is presumed to be an important aspect of cell migration, we examined the expression of two cell adhesion molecules known to be expressed in border cells: DE-cadherin and Fasciclin III (FasIII). Cadherin expression is normally high in the border cells (Fig. 5). We examined cadherin expression in the phenotypic egg chambers of each of the mutant lines and found no significant change in the level or distribution of cadherin (Fig. 5) that might account for the observed cell migration defects. FasIII is expressed at highest levels in only two cells at each pole of the egg chamber, the so-called polar cells. Polar cells are specialized follicle cells, which cease division earlier than other follicle cells. The anterior polar cells invariably migrate as part of the border cell cluster (Fig. 6). FasIII expression appeared normal in all of the mutants, with the exception of 52B7 (class IV). In this mutant, abnormally large numbers of border cells were observed and the extra border cells contained additional polar cells, as discussed further below. A representative example of each of the mutant classes is shown in Fig. 6.

Mutant 52B7, an allele of *costal-2*

In order to facilitate the molecular identification of the loci identified in the screen, each of the mutants that failed to complement a deficiency was tested for complementation with the lethal P-element insertions located within the same interval. In four cases at least one lethal P-element was found that failed to complement, and the DNA flanking the element was

cloned and sequenced. In only one case was the P-element inserted within a previously identified locus. The mutant, 52B7, mapped to 42E-43E, and failed to complement a lethal P-element located at 43B, 1(2)k16101. Flanking DNA of P-element 1(2)k16101 was obtained by plasmid rescue. The sequence of the flanking DNA was 100% identical to the gene *costal-2* (*cos-2*) (Fig. 7A), which encodes a negative regulator of the Hedgehog (Hh) signaling pathway.

The phenotype of mutant 52B7 was characterized by the production of extra border cells. In some egg chambers two or more clusters of border cells were observed (Fig. 7E) whereas in other examples a single, large cluster of border cells was apparent (Figs 5, 6, class IV). Most of the clusters of border cells appeared to be capable of migrating to some extent, although the final position was not always correct (Fig. 7F).

Previous work (Forbes et al., 1996a,b) showed that ectopic expression of Hh in the ovary using a heat-shock promoter, results in ectopic polar cell formation throughout the follicle cell layer. The anterior polar cells are known to migrate as part

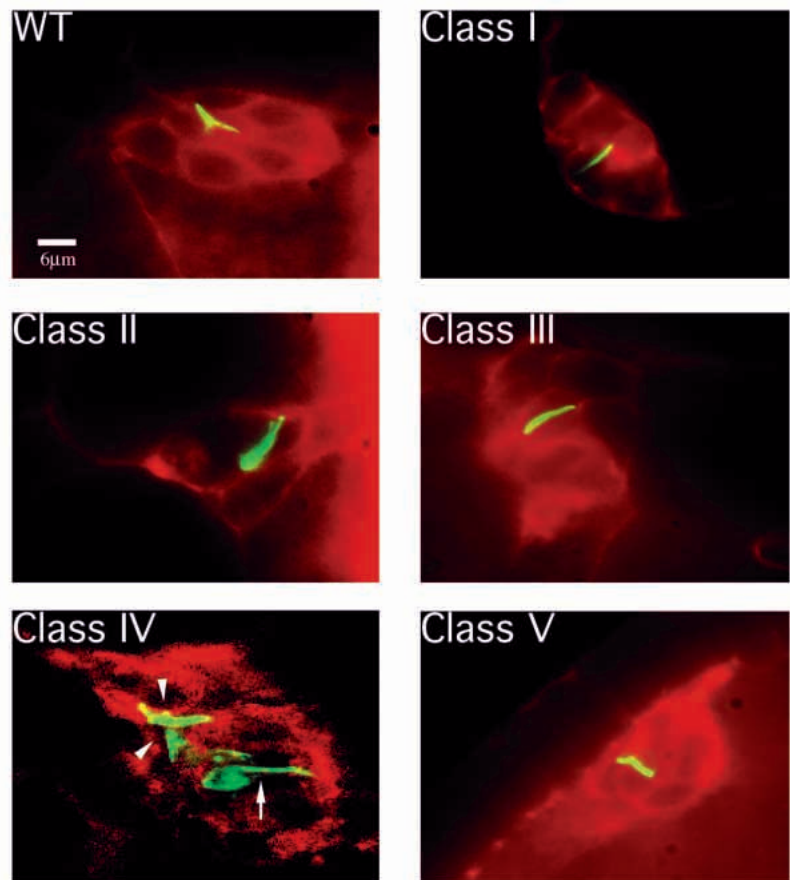
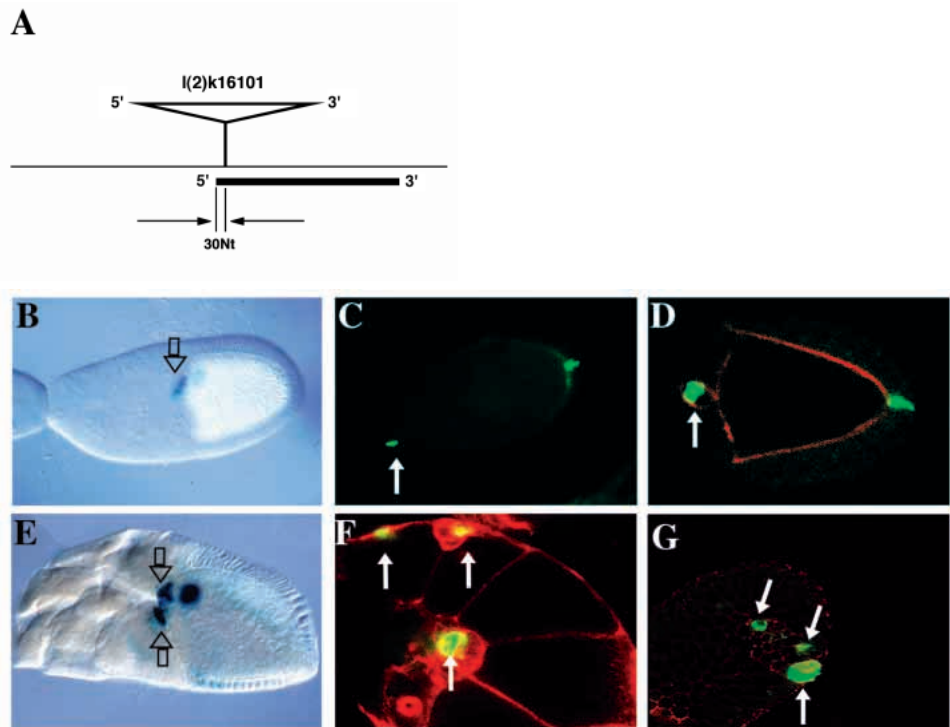


Fig. 6. Fasciclin III accumulation in wild-type and mutant border cells. Monoclonal antibody 2D5 was used to detect FasIII protein (green) and rhodamine phalloidin staining was used to detect filamentous actin (red) in wild-type (WT) stage 9 and mutant stage 10 border cells. In wild-type, FasIII accumulates to highest levels at the interface between the two polar cells where it appears as a single line. The class I egg chamber shown is a *slbo* mutant. The class II mutant shown is from line 11B9. The class III mutant is from line 65C2. The class IV mutant is 52B7. Note the multiple lines of staining, in contrast to the other mutants, some very near each other (arrowheads) and others separated by a few cells (arrow). A large cluster of border cells surrounds these polar cells. The class V mutant is 61E5.

Fig. 7. Ectopic polar cells and overproduction of border cells in 52B7, an allele of *cos-2*. (A) Mutant 52B7 failed to complement the lethal P element *l(2)k16101*, which contains a P-element insertion 30 nucleotides downstream of the 5' end of the transcribed region of the *cos-2* gene. (B) β -galactosidase staining of a wild-type egg chamber, illustrating the normal-size of the border cell cluster. (C) A wild-type, early stage 9 egg chamber stained with anti-FasIII antibody. Brightest staining is observed at the interface between the two polar cells at each end of the egg chamber. (D) A wild-type stage 10 egg chamber double stained with anti-FasIII antibody (green) and rhodamine-phalloidin (red), also showing concentrated FasIII staining in the two pairs of polar cells. In the anterior pair (arrow), the interface between the two polar cells is in the plane of the page, leading to a disc shape. (E) β -galactosidase staining of mutant 52B7, showing two neighboring clusters of border cells (arrows). (F) Anti-FasIII antibody (green) and rhodamine-phalloidin (red) double labeling of the anterior half of a stage 10 52B7 mutant egg chamber, showing multiple FasIII-positive cells (arrows). (G) Anti-FasIII antibody and rhodamine-phalloidin double labeling of the oocyte-associated follicle cells of a 52B7 mutant egg chamber, showing multiple, nearby FasIII-positive cells (arrows).



of the border cell cluster (Spradling, 1993). Therefore the extra border cells observed in the *cos-2* mutant clones might have formed as a consequence of extra polar cell differentiation. To test this hypothesis, we stained polar cells in egg chambers that contained *cos-2* mutant clones with an antibody to FasIII. In wild-type egg chambers, FasIII staining is concentrated at the membrane where the two polar cells contact each other (Fig. 7C,D). In egg chambers containing *cos-2* mutant clones, additional FasIII-positive cells were observed (Fig. 7F,G), at many different locations within the follicle cell layer. Only those ectopic polar cells anterior to the oocyte appeared to become surrounded by border cells (Fig. 7F), whereas clusters of additional rounded cells were not observed to surround the posterior polar cells (Fig. 7G). To confirm that the *cos* mutation was responsible for the observed border cell phenotype, we examined egg chambers containing mosaic clones of a previously characterized *cos-2* mutation and we also observed supernumerary polar cells (not shown). Thus, the phenotype of loss-of-function of *cos 2* in the ovary was the same as gain-of-function of Hh.

DISCUSSION

Many of the genes that are required for normal cell migration may not have been identified previously since their roles might have been obscured by pleiotropic effects of such mutations on other developmental events, or due to early lethality preventing the analysis of later cell migrations. In this study, we demonstrated that it is possible to circumvent these limitations by screening for mutations that cause cell migration defects in

mosaic clones. Further study of the new loci identified in this screen promises to provide many components of the cell migration machinery. As more and more gene sequences become available from genome project efforts, new means of specifying gene functions, such as the screen described here, will become more and more important.

Stages of border cell differentiation and migration

Based on the mutant phenotypes that we have observed, we can dissect the differentiation and migration of border cells into several steps: first, polar cell fate is determined; second, the anterior polar cells recruit border cells to surround them; third, border cells change from a stationary epithelial state into a migratory state by altering gene expression; fourth, the border cells migrate toward the oocyte; and, finally, the border cells align themselves with the oocyte nucleus.

The Hh/Cos-2 signaling pathway is involved in determining polar cell number and/or identity. The *cos-2* gene encodes a protein with homology to kinesins; however, it is not believed to possess motor activity (Robbins et al., 1997; Sisson et al., 1997). It has been shown to bind to microtubules and to the proteins Fused (FU) and Cubitus Interruptus (Ci). One model for *cos-2* function is that Cos-2 protein binds to microtubules and serves to anchor the transcription factor, coded for by the *Ci* locus, in the cytoplasm. Upon reception of a Hh signal, this complex dissociates, allowing Ci to translocate to the nucleus, where it regulates gene expression. The effect of the 52B7 *cos-2* mutation, which caused excess border cells to form as a result of excess polar cell formation, can easily be interpreted in light of this model since overexpression of Hh produces the same phenotype.

Hh signaling is known to affect both cell proliferation as well as cell fate determination (Heberlein et al., 1995) and we observed phenotypes consistent with both types of effects. Polar cells are known to cease dividing prior to the rest of the follicle cell epithelium (Spradling, 1993). In some egg chambers harboring *cos-2* mutant clones, a single, abnormally large cluster of border cells containing more than two polar cells could be seen, which might have resulted from an extra division of polar cells after their formation. In other egg chambers containing *cos-2* mutant clones, multiple, ectopic border cell clusters were present, most likely resulting from transformation of lateral follicle cells to a polar cell fate, followed by recruitment of border cells to surround them. In addition, some of the abnormal border cell clusters exhibited cell migration defects. It is not yet clear whether *cos-2* might play a direct role in cell migration, separate from its role in cell division and cell fate specification, or whether the border cell position defects were due to incomplete transformation of cell fate in these cells. In any case, the identification of a Hh pathway mutant in the current work suggests that additional components of the Hh signaling pathway might be identified by further screening using this method.

The transition of border cells from stationary epithelial cells to motile cells requires changes in gene expression, some of which are mediated by *slbo*. In this screen, we identified two additional loci that were required for normal expression of the *PZ6356* marker and which are probably involved in the promotion of invasive behavior, by altering the pattern of gene expression in the cells. The absence of effect on cadherin or FasIII expression despite the reduction in expression of the *PZ6356* marker indicates that these mutants have specific defects in gene expression, rather than wholesale failure of border cell differentiation. It is likely that these loci will turn out to encode a transcription factors, a prediction that has been confirmed by the molecular characterization of one of the loci (Y. L. and D. J. M., unpublished data).

The mutations that did not alter the expression of the *PZ6356* marker are more likely to affect genes with a more direct role in promoting cell migration. In particular, since we have previously determined that Rac activity is necessary for normal border cell migration, we expect that genes encoding proteins that modulate the activity and/or effects of the Rac GTPase might be included in this class. The identification of these gene products might be very helpful in sorting out the mechanisms by which Rac becomes activated in specific regions of the cell and in elucidating the identities of the proteins that mediate the effects of Rac activity in cell motility. The strong genetic interaction between the class II mutants *27C1* and *17E1* suggests that the products of these two loci might act in a common pathway. The observation that *27C1* interacted dominantly with several deficiencies might offer a relatively simple way to identify additional loci that play a role in this process.

After the border cells reach the oocyte, they undergo a second, smaller movement toward the dorsal side of the egg chamber and align closely with the oocyte nucleus. It is not clear what feature of the dorsal side of the oocyte the border cells associate with, though it does not appear to be the oocyte nucleus itself. In mutant egg chambers such as *grk*, in which the oocyte nucleus is mispositioned, the border cells are capable of migrating to the oocyte and remaining in roughly

the correct location (Tinker et al., 1998). The class V mutant *61E5*, was of interest because both border cell position and oocyte nucleus position were affected; however, they were not always affected in the same egg chamber. The oocyte nucleus normally moves from the posterior of the egg chamber to the anterior and future dorsal corner during stage 8 of oogenesis, in response to a signal from posterior follicle cells. This process is dependent on the Grk signal from the oocyte and the EGF receptor pathway acting in the posterior follicle cells (Gonzalez-Reyes et al., 1995). Virtually nothing is known about the signal from the follicle cells to the oocyte, which is critical for the correct placement of the oocyte nucleus. The locus mutated in *61E5* may participate in this signaling event since its function is required in the follicle cells for proper placement of the oocyte nucleus. Since the defects in border cell position were not evident until stage 11, it seems that *61E5* affects a later stage in border cell positioning than the other loci.

Cell autonomy of migration mutants

Because most of the protein components, both regulatory and structural, that are involved in cell migration, are expected to function within the migrating cell, we sought to identify mutations that cause cell autonomous defects. The mutations presented here are likely to act cell autonomously because the clones were restricted to follicle cells (Duffy et al., 1998; Y. Uehara and D. J. M., unpublished data). While it is possible that mutations affecting other follicle cells could impinge on the ability of the border cells to migrate, it is more likely that the mutations are acting cell autonomously to prevent migration. In fact, in the two cases we have examined directly, *slbo* and *22F3*, the defects are cell autonomous (Montell et al., 1992; Rorth et al., 1998; Y. L. and D. J. M., unpublished data). However, further experiments, including development of an appropriate genotypic marker, will be required to determine whether each of the other loci functions cell autonomously.

In contrast, in a recent screen for zygotic mutations that disrupt primordial germ cell (PGC) migration in *Drosophila* embryos, all of the genes were found to affect PGC migration non-cell-autonomously, and did so by affecting the development of the somatic tissues through which the germ cells migrate (Moore et al., 1998). This is probably because zygotic transcription does not begin in the PGCs until after their migration is complete (Van Doren et al., 1998). In this respect, these two screens are complementary.

Border cell migration has also been studied using a cell autonomous overexpression screen. Rørth et al. (1998) mis-expressed or over-expressed 2300 random loci specifically in border cells and found 60 loci that were capable of rescuing a hypomorphic *slbo* allele. It is not yet clear what fraction of such genes will produce a border cell migration defect as a loss-of-function phenotype and therefore to what degree the genes identified in these two screens overlap. However it is likely that there will be genes that are required for border cell migration that would not rescue a *slbo* mutant when over-expressed.

Specificity of mutations affecting border cell migration

One concern about genes identified based on the phenotype produced in mosaic clones is that the screen might identify

general factors required for cell growth, differentiation or metabolism, and the effect on cell migration might be a secondary consequence of such defects. To address this issue, we examined the morphology and differentiation state of the mutant border cells and did not detect general defects. In each case, the cells appeared normal in morphology, F-actin distribution and cadherin expression. In some of the mutants, expression of the *PZ6356* enhancer trap marker was reduced, suggesting that specific aspects of gene expression in the border cells had been affected. However the majority of the mutations did not affect border cell differentiation in any overt way. Finally, all other aspects of oogenesis, including outer follicle cell shape and arrangement, oocyte maturation and nurse cell morphology, seemed to be normal in egg chambers harboring mutant clones, with one exception. The class V mutant displayed oocyte nucleus position defects and variable degrees of nurse cell degeneration. These results indicate that most of the mutations identified in this screen affect border cell migration relatively specifically, at least within the ovary. However, since all of the border cell migration mutants appear to be homozygous lethal, there are obviously additional developmental defects associated with them. Perhaps mutations that cause general growth or metabolic defects were not detected because such mutations would be cell lethal.

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