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jing: a downstream target of *slbo* required for developmental control of border cell migration

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SUMMARY

Epithelial to mesenchymal transitions and cell migration are important features of embryonic development and tumor metastasis. We are employing a systematic genetic approach to study the border cells in the *Drosophila* ovary, as a simple model for these cellular behaviors. Previously we found that expression of the basic-region/leucine zipper transcription factor, C/EBP, is required for the border cells to initiate their migration. Here we report the identification of a second nuclear factor, named JING (which means 'still'), that is required for initiation of border cell migration. The *jing* locus was identified in a screen for mutations that cause border cell migration defects in mosaic clones. The *jing* mutant phenotype resembles that of *slbo* mutations, which disrupt the *Drosophila* C/EBP gene, but is distinct from other classes of border cell migration mutants. Expression of a *jing-lacZ* reporter in border cells requires C/EBP. Moreover, expression of *jing* from a heat-inducible promoter rescues the border cell migration defects of hypomorphic *slbo* mutants. The JING protein is most closely related to a mouse protein, AEBP2, which was identified on the basis of its ability to bind a small regulatory sequence within the adipocyte AP2 gene to which mammalian C/EBP also binds. We propose that the need to coordinate cell differentiation with nutritional status may be the link between mammalian adipocytes and *Drosophila* border cells that led to the conservation of C/EBP and AEBP2.

Key words: *jing*, C/EBP, Border cell migration, Oogenesis, Epithelium, Cell-cell adhesion, *Drosophila melanogaster*

INTRODUCTION

Epithelial to mesenchymal transitions are prominent features of mammalian gastrulation and neural crest development, and are essential for the proper development of many organs and tissues. Moreover, most cancers derive from epithelial cells, and metastasis involves departure of these cells from their epithelium of origin and invasion of the surrounding tissue. It is known that epithelial to mesenchymal transitions involve alterations in gene expression, cell-cell adhesion and actin organization, however, our understanding of this process at a mechanistic level remains incomplete (reviewed in Thiery and Chopin, 1999).

The border cells in the *Drosophila* ovary form a group of six to ten cells that originate from the anterior pole of the follicular epithelium (Fig. 1A). During oogenesis the border cells delaminate from this epithelium and invade the neighboring cluster of germline cells. The border cells migrate approximately 150 μm over the course of 5-6 hours, stopping when they reach the anterior border of the oocyte.

A number of mutants have been identified that exhibit defects in border cell migration (reviewed in Montell, 1999). The first such mutation identified was *slow border cells* (*slbo*) (Montell et al., 1992), a locus that encodes the *Drosophila* homolog of the CCAAT enhancer binding protein (C/EBP). Although null mutations in the *slbo* locus cause lethality in late embryonic or early larval life, P-element insertions into the 5′ region of the gene lead to female sterility. The ovarian phenotype is quite specific; the only apparent defect is delay or failure of border cell migration. The *Drosophila* C/EBP protein is expressed in the border cells just prior to their migration, consistent with a requirement for C/EBP for the initiation of border cell migration.

One critical downstream target of C/EBP is *shotgun* (*shg*), the gene that encodes *Drosophila* E-cadherin (Niewiadomska et al., 1999), which is a homophilic cell-cell adhesion molecule. *D*E-cadherin expression is elevated during border cell migration, and the increase in expression is C/EBP dependent. E-cadherin is expressed on the surfaces of all of the cells of the egg chamber, and normal border cell migration depends upon both nurse cell and border cell *D*E-cadherin (Niewiadomska et al., 1999). *Shg* is not the only relevant downstream target of C/EBP however, as elevating *shg* expression using a transgene cannot rescue the *slbo* migration defects, even mild defects associated with hypomorphic alleles.

Regulation of the actin cytoskeleton accompanies both epithelial to mesenchymal transitions in general, and border cell migration in particular. The Rho family GTPases are well known for their ability to stimulate dramatic reorganization of the actin cytoskeleton, and one of the Rho family members,

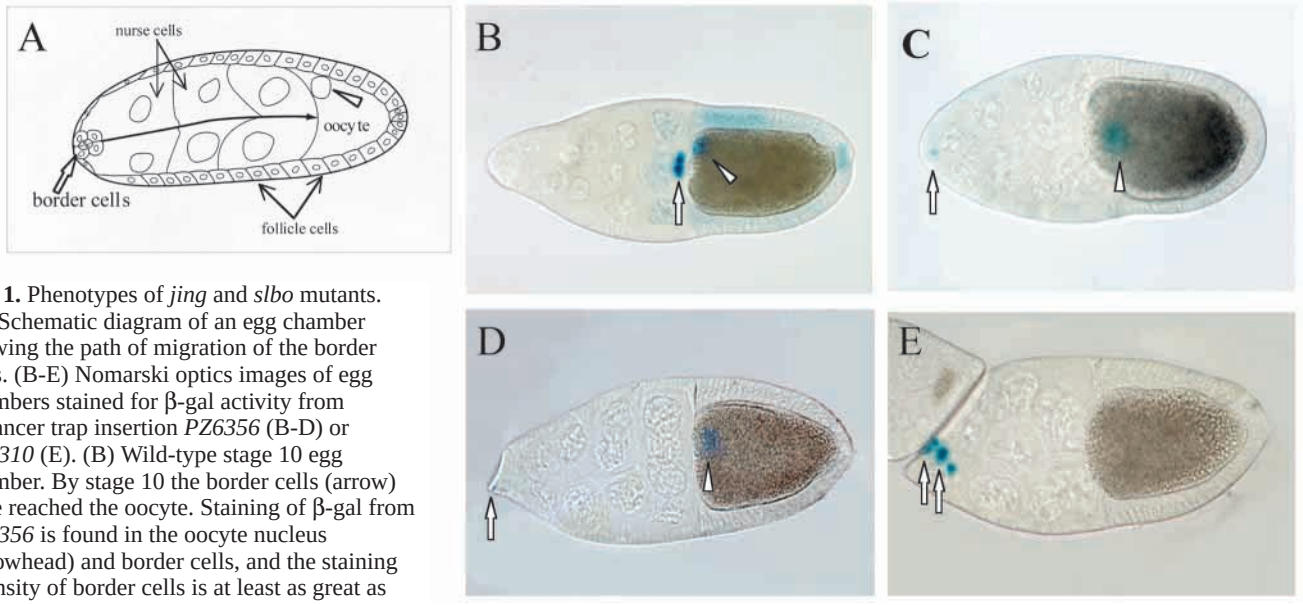

Fig. 1. Phenotypes of *jing* and *slbo* mutants. (A) Schematic diagram of an egg chamber showing the path of migration of the border cells. (B-E) Nomarski optics images of egg chambers stained for β-gal activity from enhancer trap insertion *PZ6356* (B-D) or *PZ1310* (E). (B) Wild-type stage 10 egg chamber. By stage 10 the border cells (arrow) have reached the oocyte. Staining of β-gal from *PZ6356* is found in the oocyte nucleus (arrowhead) and border cells, and the staining intensity of border cells is at least as great as that of oocyte nucleus. (C) In egg chambers containing homozygous mutant $jing^{22F3}$ clones, the border cells fail to migrate, and staining of *PZ6356* in the border cells is reduced. (D) In the $slbo^{1}$ mutant, failure of border cell migration and reduction in *PZ6356* staining are also observed. In C and D only one border cell stains although additional cells are present, as shown in E. (E) In the $slbo^{1}$; $jing^{22F3}$ double mutant background, the border cells also fail to migrate. β-gal expression from *PZ1310* (also known as $slbo^{1}$; Montell et al., 1992) is found in border cells, demonstrating that the cells are still present. The oocyte nucleus does not stain in *PZ1310*.

Rac, is critical for border cell migration (Murphy and Montell, 1996). Non-muscle myosin II is also necessary for border cell migration (Edwards and Kiehart, 1996).

Taken together these data suggest that border cell migration is a good model system for the study of epithelial to mesenchymal transitions and cell motility, but they also indicate that many of the genes required for border cell migration mutate to a lethal, rather than female sterile phenotype. Null mutations in *slbo* are lethal, and it was only possible to identify female sterile alleles because the ovarian enhancers for the gene reside 5′ of the transcribed region, whereas embryonic enhancers are located 3′ of the transcribed sequences. Thus P-element insertions into the 5′ region specifically disrupt ovarian expression of C/EBP. Mutations disrupting *D*E-cadherin or myosin expression are also lethal. Therefore we undertook a screen for mutations that cause border cell migration defects in mosaic clones (Liu and Montell, 1999). This approach allows us to identify newly induced mutations that affect border cell migration, even if the null phenotype associated with the mutation is lethal. Twenty mutations that fell into 16 complementation groups, including one new *slbo* allele, were identified. Five of the mutations, including *slbo*, cause defects in border cell migration, accompanied by loss of expression of a border cell marker known as *PZ6356*.

One complementation group, which caused border cell migration defects and loss of PZ6356 expression, consisted of three alleles, and we have named this locus *jing*, which means ‘still’ in Chinese. The *jing* locus encodes a nuclear protein containing three Zinc finger motifs, which is most related to mouse AEBP2. The AEBP2 protein binds to a small regulatory region in the adipocyte P2 gene, to which C/EBP also binds. The *jing* gene is expressed in border cells and nurse cells within the ovary, and its border cell expression depends upon *slbo*. Expression of *jing* from a heat-inducible transgene rescues not only the *jing* mutant phenotype but also the migration defects associated with hypomorphic *slbo* alleles.

MATERIALS AND METHODS

Drosophila genetics

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. The *slbo* alleles used in this study are described by Montell et al. (1992). *PZ1310*, which is also known as $slbo^{1}$, is a weakly fertile allele that is due to insertion of a PZ enhancer trap element 223 bp upstream of the most 5′ *slbo* transcription start site. The null allele, $slbo^{e7b}$, is a deletion of the *slbo* locus (and only the *slbo* locus) derived by imprecise excision of $slbo^{1}$. $fs(2)slbo^{ry7}$ contains a $rosy^{+}$ P-element insertion 88 bp upstream of the most 5′ *slbo* transcription start site and $fs(2)slbo^{ry8}$ contains a $rosy^{+}$ P-element insertion 152 bp upstream of the most 5′ *slbo* transcription start site. These alleles are stronger than $slbo^{1}$ but are not null. $fs(2)slbo^{LY6}$ is an insertion of a $rosy^{+}$ P element in between the two *slbo* transcription start sites (Montell et al., 1992). Three EMS-induced *jing* alleles, referred to as 47H6, 22F3 and 31E6, were obtained from a screen of 6,000 mutagenized second chromosomes (Liu and Montell, 1999). All three alleles were lethal in combination with Df(2R)ST1 (42B03-05;043E15-18) and complemented Df(2R)pk78s [042C01-07;043F05-08] and Df(2R)cn9 [042E;044C], thus placing the *jing* locus in the 42B3-5 to 42C1-7 region. 22F3 and 47H6 were also lethal in combination with three allelic P elements that mapped to 42B2,3. In FlyBase, this locus is now listed as l(2)01094 and 10 P-element induced alleles have been assigned to this complementation group (01094, 07111, 07112, k02002, k03404, k05311, k10314, k10323, k16705, k02102a, rH623). The 31E6 allele exhibited partial complementation with the P elements, and thus appeared to be a

weaker allele. 22F3 and 47H6 produced indistinguishable phenotypes. For consistency, all figures show results with the 22F3 allele.

In order to analyze the *jing* phenotype in egg chambers, male flies with the genotype of *dp, PZ6356, FRT^{2R}, jing/CyO* were mated with *FRT^{2R}; T155UF* virgins. The *FRT^{2R}* (also known as G13) line has a P element with the FRT sequence inserted at 42B on 2R. The *T155UF* line has a UAS-FLP transgene and a follicle cell GAL4 transgene, which are both inserted on the 3rd chromosome. Ovaries from females with the genotype of *dp, PZ6356, FRT^{2R}, jing/FRT^{2R}; T155UF/+* were dissected, stained for β-galactosidase (β-gal) activity, and/or with rhodamine-conjugated phalloidin and/or with the indicated antibodies and analyzed for the border cell migration phenotype. Using this FLP/FRT system, 80% of the egg chambers contain follicle cell clones including 200-800 cells, 5-20% of the egg chambers contained border cell clones. Typically 5-20% of the egg chambers of a mutant line manifested border cell migration defects. This figure varied however, as the GAL4 system is temperature sensitive and GAL4 expression itself is often mosaic. In addition, the FRT; FLP stock was not stable and had to be re-established from the separate FRT and FLP stocks approximately once every 12 months.

To generate marked clones, the wild-type chromosome arm carried enhancer trap line *PZ3050*, which is inserted on 2R and drives β-gal expression in border cells and centripetal follicle cells. The residual PZ6356 staining that was occasionally observed in *jing* mutant border cells (e.g. Fig. 1C) was much weaker, and required much longer staining times, than the expression from *PZ3050*. Therefore in experiments in which both elements were present, the incubation in staining solution was carried out for 4 hours to be certain that all β-gal activity that was observed was due to the PZ3050 insertion. In other experiments, a recombinant *FRT, jing* chromosome was used that lacked the PZ6356 marker. To test for cell autonomy, female flies of the genotype *PZ6356, FRT^{2R}, jing/FRT^{2R}, PZ3050; T155UF* or of the genotype *FRT^{2R}, jing/FRT^{2R}, PZ3050; T155UF* were examined.

In complementation tests, failure to produce viable transheterozygous adults was taken as an indication of allelism. In order to increase our confidence that lethality and the border cell migration defect were caused by the same mutation, we outcrossed *22F3* and *47H6* to unmutagenized *PZ6356, FRT^{2R}* flies and to *FRT^{2R}*. By allowing recombination to take place, unwanted second mutations could be recombined away from the mutation of interest. Between 30 and 45 recombinants for each line were analyzed for border cell migration defects in mosaic clones and for homozygous lethality over the deficiency and P-element allele. In no case was the border cell migration defect separated from lethality, and none of the viable recombinant lines exhibited border cell migration defects.

β-gal activity, phalloidin and antibody staining

For β-gal activity staining, female flies were dissected in Schneider's medium plus 10% fetal calf serum. Ovaries were fixed for 10 minutes at room temperature in 6% formaldehyde in 0.17× buffer B (100 mM KH_2PO_4/K_2HPO_4, pH 6.8, 450 mM KCl, 150 mM NaCl, 20 mM $MgCl_2$), and stained with 0.2% X-gal in staining solution (10 mM phosphate buffer pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$, 3 mM $K_4[Fe(CN)_6]$, 3 mM $K_3[Fe(CN)_6]$, 0.3% Triton X-100) for various times between 30 minutes to overnight, depending on the desired intensity of staining. Egg chambers were then washed briefly in PBT (PBS+0.1% Tween 20). For double and triple labeling, egg chambers were then incubated in rhodamine phalloidin and/or DAPI (see below), washed again and then equilibrated in Vectashield (Vector Laboratory Inc, Burlingham, CA) prior to mounting on microscope slides.

For rhodamine-phalloidin staining, egg chambers were dissected free from the ovariole sheath in Schneider's medium plus 10% fetal calf serum, and collagenase treated for 2 minutes in EBR containing 3 mg/ml collagenase and 0.1 mg/ml BSA, and then fixed in 6% formaldehyde in 0.17× buffer B. One μl of rhodamine-phalloidin in methanol (Molecular Probes) was air-dried for 30 minutes in a fume hood and resuspended in 200 μl PBT. Egg chambers were incubated in this solution for 2 hours at room temperature in the dark. After rinsing with PBT, egg chambers were mounted in Vectashield. DAPI staining (0.5 μg/ml in PBT for 2 hours at room temperature) did not require removal from the ovariole sheath or collagenase treatment.

For antibody staining, egg chambers were dissected, collagenase treated and fixed in 200 μl 3.7% formaldehyde in 0.1 M phosphate buffer (pH 7) containing 0.5% NP40 and 600 μl heptane for 20 minutes. After washing in NP40 wash buffer (50 mM Tris 7.4, 150 mM NaCl, 0.5% NP40, 1 mg/ml BSA, 0.02% azide), egg chambers were blocked for two hours at room temperature in blocking solution (NP40 wash buffer plus 20% BSA). Then egg chambers were incubated in primary antibody overnight at 4°C. After a 1-hour wash in NP40 buffer, egg chambers were incubated in fluorescein-conjugated secondary antibody (Vector Laboratories, Burlingham, CA) at a dilution of 1:200 for 2 hours at room temperature. For double or triple labeling, rhodamine phalloidin and/or DAPI were added to the incubation in secondary antibody. Egg chambers were mounted in Vectashield after another 1-hour wash in NP40 wash buffer. Anti-*D*E-cadherin antibody was a kind gift from Tadashi Uemura and was diluted 1:20 in block solution. Anti-FASIII antibody (a kind gift from Nipam Patel, University of Chicago) was not diluted.

Cloning and sequencing

Plasmid rescue of DNA flanking P elements *l(2)k02002, l(2)k03404, l(2)rH623* was performed as follows: 3 μg of genomic DNA was digested with *Xba*I, or *Eco*RI, or *Bgl*II, 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 unit of T4 DNA ligase. The DNA was ethanol precipitated, resuspended in TE, and 1 μl used for transformation. DNA was prepared from ampicillin- or kanamycin- (for PZ insertions) resistant colonies.

The 2.5 kb fragment flanking the *l(2)k03404* insertion was random primed, radiolabeled, and used to isolate clones from a 9- to 12-hour embryonic cDNA library (a gift from K. Zinn, Caltech). Multiple cDNAs as well as the genomic clone were sequenced to generate a transcript map. The longest cDNA was used to probe the northern blot of mRNA prepared from 0- to 24-hour embryos. The *Drosophila* EST was obtained from Research Genetics. DNA sequencing was performed by a sequencing facility. In Gadfly this sequence is referred to as CG9403.

Rescue experiments

The full length *jing* cDNA was cloned into the pCaSpeR-hs vector (a gift from Carl Thummel). This P[hs-jing] construct was co-injected into *w^{1118}* embryos with pπ27.1 (wings clipped) according to standard procedures (Spradling, 1986 #515). A single transgenic insertion line was recovered, which contained the *hs-jing* P element inserted on the X chromosome. To test for rescue of the *jing* phenotype, two to 5-day old female flies of the genotype *P[hs-jing]; FRT^{2R}, jing/FRT^{2R}, PZ3050; T155UF/+* were fed wet yeast for 1 day at 18°C, and then heat shocked in a 37°C water bath for 1 hour. After incubation at 18°C for 20 hours on wet yeast, the flies were dissected and ovaries were stained for various markers. To test for rescue of the *slbo* phenotype, females of the following genotypes were tested. Three combinations of *slbo* alleles were examined *P[hs-jing]; slbo^{1}/slbo^{1}*, *P[hs-jing]; slbo^{1}/slbo^{ry7}* and *P[hs-jing]; slbo^{1}/slbo^{e7b}*. Rescue was observed for the former two genotypes but not for the latter.

The *hs-slbo* transgene used has been described by Murphy et al. (1995). P[hs-slbo] rescues border cell migration in all *slbo* allelic combinations tested, when flies are heat shocked for 1 hour at 37°C and allowed to incubate overnight at 18°C.

Preparation of anti-JING antisera and affinity purification

The portion of the *jing* cDNA coding for the zinc finger domain was cloned into the *E. coli* expression vector PGEX (Pharmacia Biotechnologies) by PCR-mediated addition of an *Xba*I site and *Xho*I

site, in order to generate a GST fusion protein. The expression vector was transformed into BL21 cells, and after being induced with IPTG, the cells were pelleted. The pellet was suspended in PBS with proteinase inhibitor, and the cells were sonicated and centrifuged. The fusion protein was soluble and was present in the supernatant. The supernatant was coupled to GST beads (Pharmacia Biotechnologies) and washed with PBS. The GST beads, which were coupled with the fusion protein, were boiled in Laemmli sample buffer and run on SDS-polyacrylamide gels, and the band containing the fusion protein was cut from the gel. Rabbits were injected and bled at Cocalico Biologicals Inc. These antisera recognized the denatured *E. coli*-expressed GST-JING in western blot analysis.

For affinity purification, the portion of the *jing* cDNA coding for the zinc finger motifs was subcloned into pMAL-c2 (New England BioLabs) to fuse the zinc finger domain with the maltose binding protein (MBP). The MBP fusion protein was purified using amylose resin (New England Biolab) and coupled to the cyanogen bromide-activated Sepharose 4B gel (Pharmacia Biotech) and the gel was packed into a column. The antiserum was passed over the column. After extensive washing using PBS, bound antibodies were eluted with glycine (pH 2.5). After neutralizing with Tris buffer (pH 9.0), the eluate was dialyzed and stabilized with 5% normal goat serum. The anti-JING antibody was used at a dilution of 1:200.

RESULTS

jing, a new locus required for border cell migration

In a screen of 6,000 lines carrying new ethylmethane sulfonate (EMS) induced mutations, we identified 20 mutations, which fell into 16 lethal complementation groups, that caused border cell migration defects in mosaic clones (Liu and Montell, 1999). The phenotypes of the mutants in three complementation groups resembled *slbo* mutants in that the border cell migration defects were accompanied by reduction in border cell expression of β-gal from the PZ enhancer trap line known as *PZ6356* (Tinker et al., 1998). One of these mutations failed to complement the *slbo* null. Of the remaining four mutations, three (22F3, 31E6 and 47H6) were allelic and failed to complement the same overlapping deficiencies (Liu and Montell, 1999). Thus these three mutations defined a new locus required for border cell migration, which we named '*jing*' (Fig. 1B-D). Despite the lack of staining for the PZ6356 marker, the border cells were still present in the mutant egg chambers, as detected by Nomarski optics imaging, rhodamine phalloidin staining and DAPI staining (not shown) as well as by staining for the *slbo-lacZ* reporter from enhancer trap line *PZ1310* which is also known as *slbo*^{1} (Fig. 1E).

The similarity between the *jing* and *slbo* mutant phenotypes suggested that *jing* might function in a common pathway with *slbo*. However, it did not appear that *jing* was an upstream factor required for *slbo* expression, since no reduction in β-gal expression from the *slbo* enhancer trap was detected in *jing* mutant border cells (Fig. 1E).

Using GAL4-mediated expression of FLP, mosaic clones were generated exclusively within the follicle cell epithelium (Liu and Montell, 1999), therefore it was clear that *jing* function was required in follicle cells rather than germline cells. However, in order to rule out the possibility that the migration defects were an indirect consequence of defects in other follicle cells, we determined the cell autonomy of *jing* within the follicle epithelium. To do this, the wild-type chromosome arm was marked with an enhancer trap line PZ3050, which expressed β-gal in border cells (Fig. 2A). In every mosaic egg chamber in which all of the border cells were β-gal positive, they migrated normally (Fig. 2A,B, $n>20$). In every mosaic egg chamber in which the entire border cell cluster failed to stain for β-gal activity, and therefore was homozygous mutant, migration failed (Fig. 2C, $n>20$). In these cases the location of the border cell cluster was determined by staining for filamentous actin and Fasciclin III (Fig. 2D), a cell adhesion molecule that is enriched in two cells within the border cell cluster. Thus, *jing* function was autonomous to the border cells.

Border cell clusters composed of a mixture of heterozygous and homozygous cells, exhibited several different types of behavior. Clusters containing a single β-gal-positive (and therefore wild-type) cell failed to migrate, though the wild-type cell typically moved to the front of the cluster (Fig. 2E,F) ($n=10$). Clusters containing a single mutant cell migrated normally (not shown, $n>10$). Clusters containing two or more wild-type cells frequently split into two groups ($n=6$), with the heterozygous cells detaching from the mutant cells and migrating partway towards the oocyte (shown schematically in Fig. 2G). In other instances ($n=6$) mixed clusters stayed together as one group and migrated partway (Fig. 2H). Finally, in two cases the border cell cluster became very extended, with the wild-type cells migrating in a line and the mutant cells trailing behind (Fig. 2I).

At the time of normal border cell migration, expression of *Drosophila* E-cadherin (*D*E-cadherin) increases within the border cells, and *Drosophila* C/EBP is required for this elevation of *D*E-cadherin expression (Niewiadomska et al., 1999). *Drosophila* β-catenin, known as Armadillo (ARM), colocalizes with *D*E-cadherin in both wild-type and mutant egg chambers (Niewiadomska et al., 1999). To determine whether *jing* function was also required for proper accumulation of *D*E-cadherin and ARM, egg chambers containing *jing* mutant border cells were stained with antibodies against *D*E-cadherin or ARM, and the staining was compared to wild-type and *slbo* mutant border cells. In wild-type border cell clusters, staining for *D*E-cadherin and ARM is strongest in the central cells known as polar cells, which express FASIII (Fig. 3A,D,G; also known as FAS3, FlyBase) and in the junctions between border cells. The staining is somewhat less intense and punctate in appearance at the interfaces between border cells and nurse cells. In *slbo* mutant clusters, *D*E-cadherin and ARM staining is only detected in the central polar cells (Niewiadomska et al., 1999; and Fig. 3B,E,H). Border cells mutant for *jing* exhibited normal expression of both *D*E-cadherin and ARM (Fig. 3F,I). Thus *jing* function, unlike *slbo*, was not required for either *D*E-cadherin or ARM expression. In all cases FASIII staining was normal, indicating normal polar cell fate (Fig. 3A-C).

Molecular cloning of the *jing* locus

Deficiency mapping indicated that the *jing* locus was located at 42B2-3;42C. We tested all of the lethal P-element insertions within this interval and found three allelic P elements that failed to complement the *jing* alleles, though *jing*^{31E6} showed partial complementation with the P elements, indicating that it might be a weak allele. DNA flanking each of the three elements was cloned by plasmid rescue and sequenced. The sequences, combined with genomic Southern blotting (not shown), demonstrated that the three P elements were located

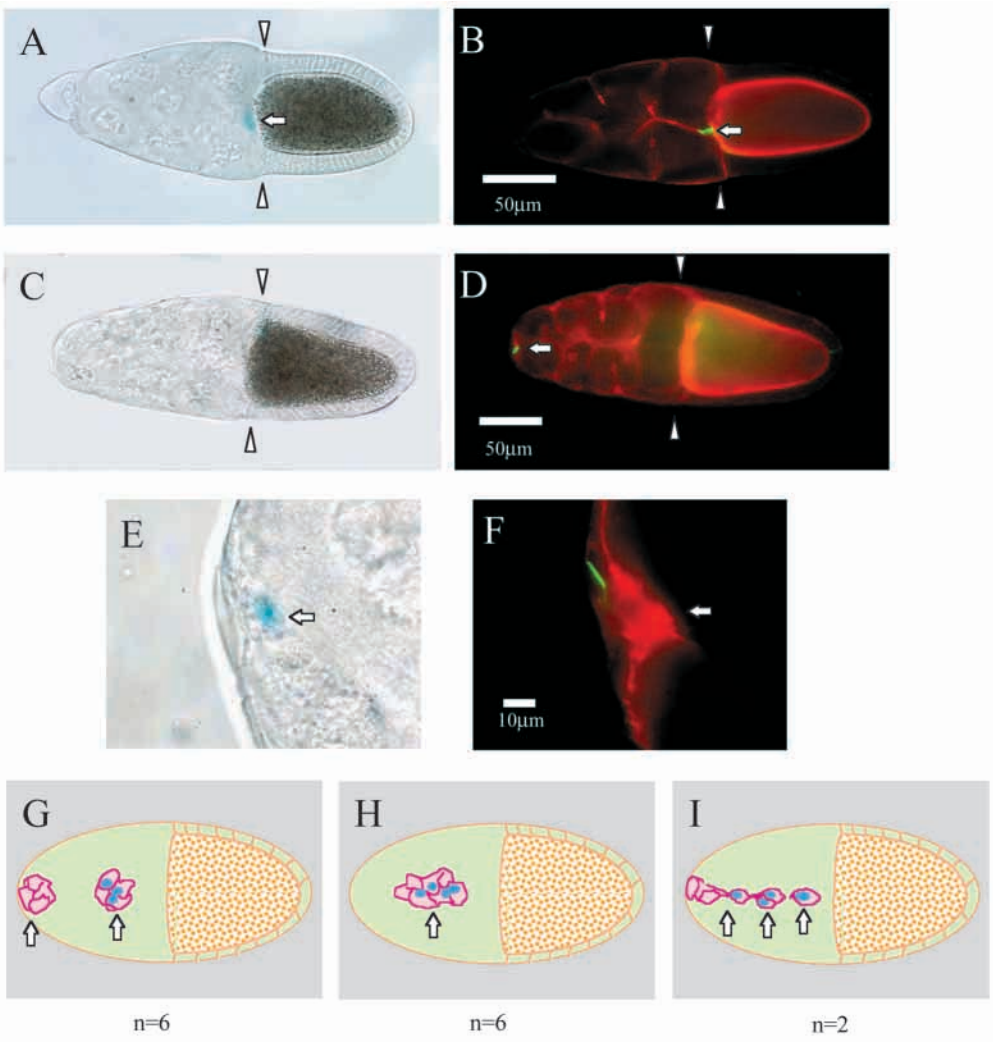

Fig. 2. Autonomy of the *jing* phenotype. All egg chambers shown are at late stage 9 or stage 10, when wild-type border cells have completed migration. *jing*^{22F3} mutant border cells were generated by crossing *dp, PZ6356, FRT*^{2R}, *jing*^{22F3}*/CyO* or *FRT*^{2R}, *jing*^{22F3}*/CyO* male flies with *FRT*^{2R}*,PZ3050/CyO;T155UF* virgins (see Materials and Methods). In this experiment *PZ3050* was used to mark the wild-type chromosome arm. Wild-type border cells show *PZ3050* β-gal staining, while border cells homozygous for *jing* lose the *PZ3050* β-gal staining. Staining for β-gal activity was carried out for 4 hours so that the slight, residual PZ6356 β-gal activity that was sometimes observed in homozygous *jing* mutant border cells would not be detected (see Materials and Methods for details). A, C, and E are Nomarski images while B, D and F are fluorescence micrographs of rhodamine-phalloidin and anti-FASIII staining of the egg chambers shown in A, C and E, respectively. (A,B) All border cells are wild type, and the border cells have finished migration. (C,D). All border cells are mutant, the border cell cluster remains at the tip of the egg chamber. Arrowheads indicate the extent of outer follicle cell rearrangement in (A-D), and arrows indicate border cells. (E,F) A border cell cluster containing one wild-type cell (arrow). The whole cluster fails to migrate, although the wild-type cell has moved to the front of the cluster. (G-I) Schematic drawings of stage 10 egg chambers. Border cell clusters composed of mixtures of wild-type and mutant cells exhibit three types of behavior. Border cells are shown outlined in red, and the blue dots indicate border cells that have retained the *lacZ* expression and therefore *jing* function. The border cells are drawn larger than their actual size. (G) Border cells split into two clusters, with most wild-type cells migrating forward, and most mutant cells not migrating (*n*=6). (H) Border cells migrating as one cluster, but delayed at stage 10 (*n*=6). (I) A scattered border cell cluster in which wild-type cells have migrated to varying extents (*n*=2). The cells were visualized with rhodamine phalloidin staining, however all of the cells were not located in the same focal plane.

within two kilobases of each other. One of the *jing* enhancer trap elements, *rH623*, exhibited expression of the β-gal reporter gene in the border cells and nurse cells in the ovary (Fig. 4A). To determine whether *jing* might be a downstream target of C/EBP, we examined the expression of *rH623* in *slbo* mutant egg chambers. Expression of β-gal in the nurse cells was unchanged, however expression of β-gal in the border cells was dramatically reduced (Fig. 4B). This was not due to absence of the border cells in the *slbo* mutant because the cells were still present, as revealed by staining for the *slbo* enhancer trap line *PZ1310*, which is also known as *slbo*^{1} (Fig. 4C,D and Montell et al., 1992).

To construct a transcript map of the region neighboring the P elements, fragments of genomic DNA flanking the sites of P-element insertion were used to probe northern blots and an embryonic cDNA library (Fig. 5). Sequencing of cDNAs isolated from the cDNA library, as well as expressed sequence tag (est) clones, indicated that alternative splicing of the locus produced at least four different classes of mRNAs. However, all of the mRNAs contained the same coding sequence as shown schematically in Fig. 5. A major transcript of 6.4 kb and a minor larger transcript hybridized to probes made from DNA flanking the P-element insertion sites (Fig. 6B).

The *jing* transcripts contained an open reading frame of 1486 amino acids (Fig. 6A). A search of the predicted protein sequence using Prosite revealed the presence of three zinc finger motifs (underlined in Fig. 6A). A BLAST search using the predicted protein sequence revealed that the JING protein was most highly related to a mouse transcription factor known as AEBP2, which is also predicted to have three zinc fingers (Fig. 6C). JING exhibited 50% amino acid identity with AEBP2 within the zinc finger motifs and 20% identity C-terminal to the zinc fingers (BLAST E value of 1e-34). After AEBP2, the most similar proteins were several members of the

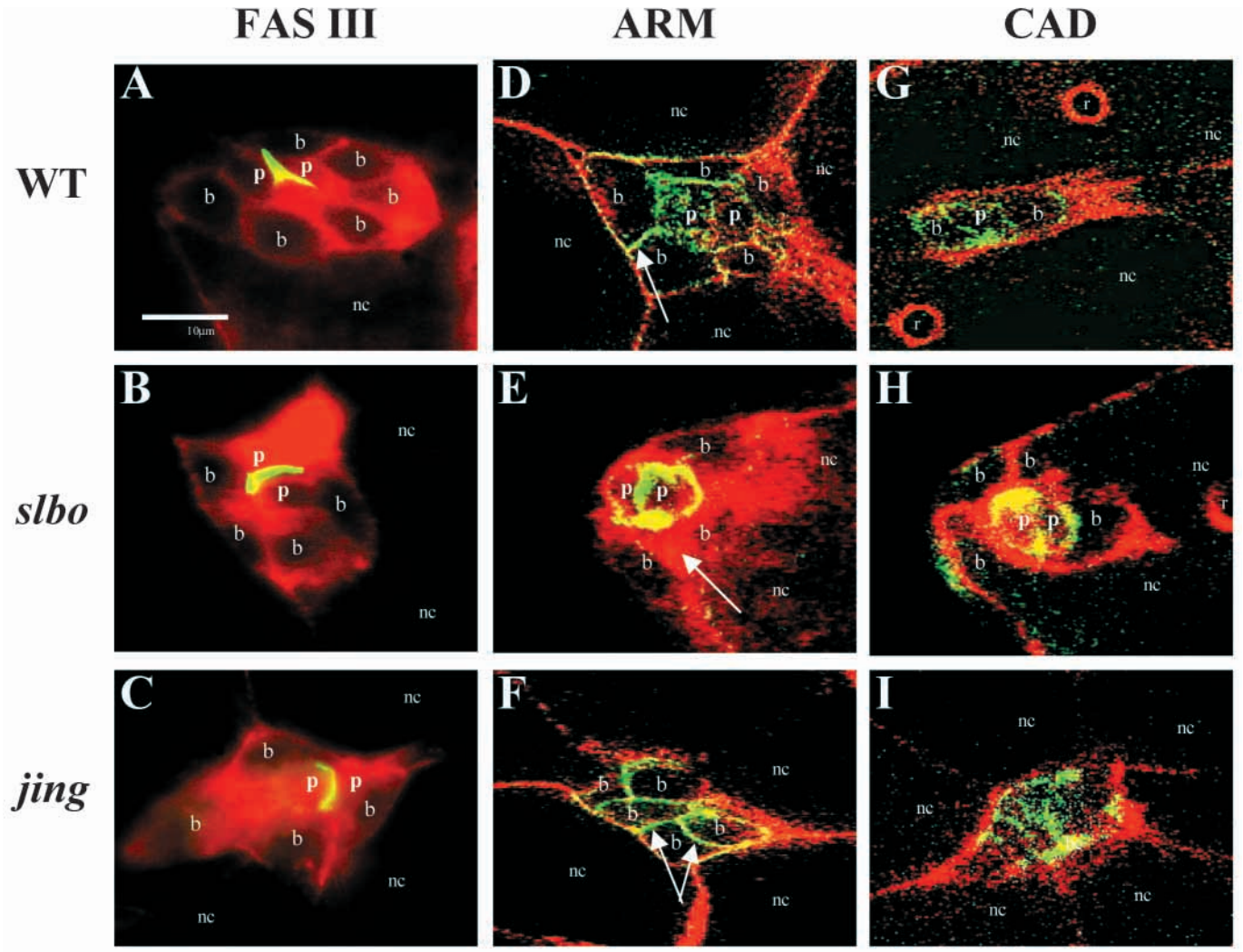

Fig. 3. FASIII, ARM and *D*E-cadherin expression in wild-type, *slbo* and *jing* mutant border cells. Confocal micrographs showing border cell clusters of the indicated genotypes labeled with rhodamine phalloidin (red) and the indicated antibodies (green). (A-C) FasIII antibody labels the interface between the two central polar cells (p) within the border cell cluster. FasIII staining appears normal in all genotypes. (D) In wild-type egg chambers ARM antibody labels the junctions in between border cells strongly (arrow), whereas the junctions between border cells and nurse cells (nc) show lighter, more punctate labeling. (E) In *slbo* mutants, the polar cells (p) are strongly labeled, however the outer border cells (b) express little or no detectable ARM. (F) In contrast, *jing* mutant border cells exhibit normal levels of ARM labeling (the polar cells are not present in this particular optical section). (G-I) Staining for *D*E-cadherin resembles that for ARM in each genotype. Each panel shows a different border cell cluster.

GLI family of zinc finger transcription factors. However GLI proteins typically contain five zinc finger motifs. The GLI family of proteins, which includes *Drosophila* CI, were only 25% identical to JING within the zinc fingers and did not exhibit homology outside of these motifs (Fig. 6C) (BLAST E value of 5e-9).

To demonstrate that the AEBP2-related protein was indeed responsible for the border cell migration defects that we observed in *jing* mosaic clones, we generated transgenic flies expressing the putative JING protein under the control of the heat inducible hsp70 promoter (*hs-jing*). When JING was expressed from the transgene, border cell migration was restored in *jing* mosaic egg chambers (Fig. 7). Partial migration was observed even in the absence of heat shock, possibly due to leaky expression from the hsp70 promoter at 25°C (Fig. 7A). Migration was complete in all stage 10 egg chambers examined (n>20), when flies were subjected to a 1-hour heat pulse and then incubated overnight at 18°C to allow migration to occur (Fig. 7B). However, heat inducible expression of SLBO was not able to rescue the *jing* migration defect (Fig. 7C). While *hs-jing* rescued the migration defect, it did not appear to provide the proper level or timing of expression to restore PZ6356 expression.

The reduction in border cell expression of *jing-lacZ* in *slbo* mutant egg chambers suggested that *jing* might be a downstream target of *slbo*. Therefore we tested whether heat inducible expression of *jing* could restore migration in $slbo^{1}$ mutant egg chambers. Although no rescue was observed in the absence of heat shock (Fig. 7D, n>25), border cell migration was complete in all stage 10 egg chambers observed, following a one-hour heat shock and an overnight incubation at 18°C (Fig. 7E, n>25). P[hs-slbo] also rescued

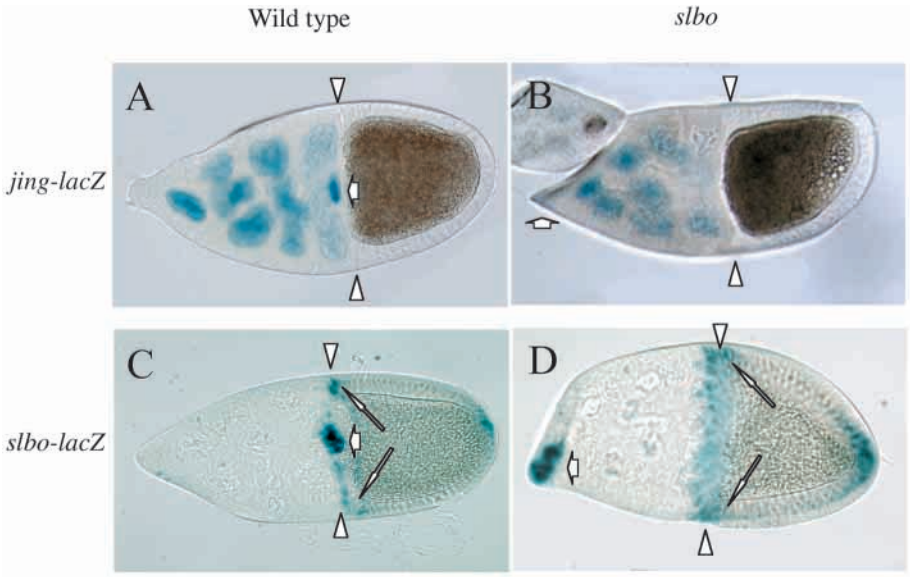

Fig. 4. Expression of a *jing-lacZ* reporter in wild-type and *slbo* egg chambers. (A,C) Wild-type egg chambers, (B,D) *slbo* mutant egg chambers. (A,B) β-gal staining of egg chambers from the *jing* enhancer trap line *l(2)rH623/CyO*. (C,D) β-gal staining from the *slbo* enhancer trap line *PZ1310*. All egg chambers are at stage 10, which is characterized by the complete outer follicle cell rearrangement (arrowheads). (A) *l(2)rH623* drives β-gal expression in border cells (arrow) and nurse cells. (B) In $slbo^{ry8}/slbo^{e7b}$ mutant egg chambers, staining of *l(2)rH623* in nurse cells is still present, while the staining in border cells is undetectable. (D) In *slbo* mutant egg chambers, the border cells are present at the tip of the chamber at stage 10 unlike the wild type in C. A $slbo^{1}$ egg chamber is shown but the same is true for all *slbo* alleles. Thick arrows indicate border cells, arrowheads indicate the extent of follicle cell rearrangement, and thin arrows indicate centripetal follicle cells.

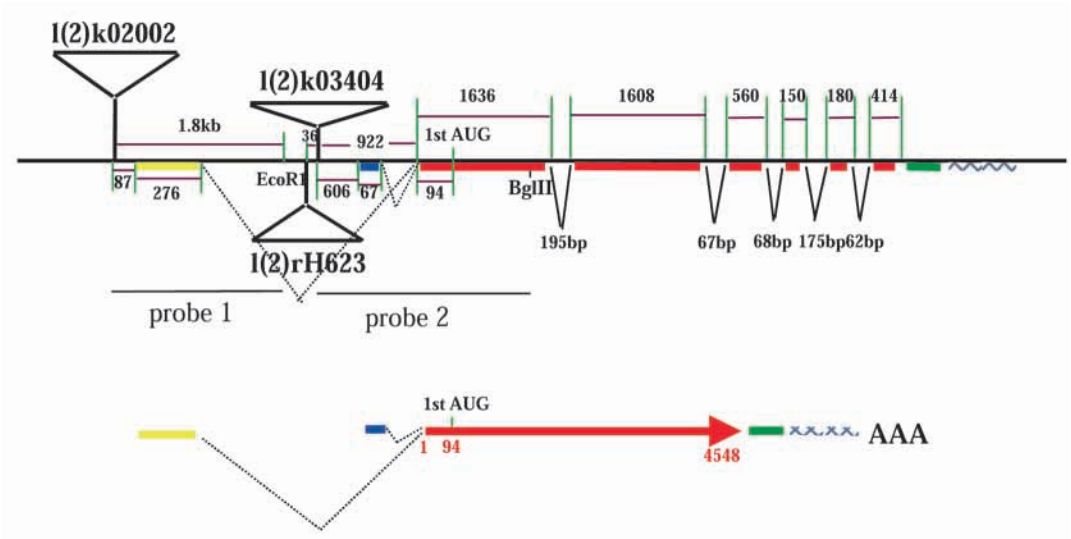

Fig. 5. Genomic map of the *jing* locus. The thick black line represents genomic DNA at the *jing* locus. The P-element insertion sites are indicated by triangles. The orientations of *l(2)k02002* and *l(2)k03404* are 5′ to the left and 3′ to the right, and *l(2)rH623* is inserted in the opposite orientation. The open reading frame is depicted by the solid red bars. Yellow and blue bars indicate two different types of 5′ untranslated regions found in cDNA clones. Green and jagged blue lines indicate alternative 3′prime untranslated regions found in cDNA clones.

the *slbo* migration defect fully, as expected (Fig. 7F, $n>25$). The P[hs-jing] transgene rescued border cell migration in two different combinations of *slbo* alleles, $slbo^{1}/slbo^{1}$ and $slbo^{1}/slbo^{ry7}$. However, expression of *jing* did not rescue border cell migration in $slbo^{1}/slbo^{e7b}$, the null allele (not shown), whereas P[hs-slbo] does rescue border cell migration in $slbo^{1}/slbo^{e7b}$ (D. Montell, unpublished observation). Therefore over-expression of *jing* was able to compensate for reduced levels of *SLBO* protein that are observed in $slbo^{1}$ and $slbo^{ry7}$, but not for the more severe reduction in SLBO protein that is found in $slbo^{1}/slbo^{e7b}$ (Montell et al., 1992).

To determine the subcellular localization of the JING protein, antibodies were generated against a GST-JING fusion protein (see Materials and Methods for details). Even following affinity purification, the antibodies did not reproducibly stain wild-type egg chambers. However specific, nuclear staining in all cells of the egg chamber was observed following heat induced expression of JING from the *hs-jing* transgene (Fig. 8B). In the absence of the *hs-jing* transgene, no specific signal was observed following heat shock (Fig. 8A).

*D*E-cadherin expression is required for border cell migration and is reduced in *slbo* mutant border cells (Niewiadomska et al., 1999, and Fig. 8C), but not in *jing* mutant border cells. Yet expression of JING was able to rescue the migration defect associated with the *slbo* hypomorph. We examined *D*E-cadherin expression in the *P[hs-jing]; slbo/slbo* egg chambers to determine whether the cells were able to migrate despite the absence of *D*E-cadherin expression or, alternatively, whether high levels of JING were able to restore *D*E-cadherin expression. We found that *D*E-cadherin expression in the border cells was restored in early stage 9 *slbo;hs-jing* egg chambers, following expression of JING (Fig. 8D), but not at later stages (not shown).

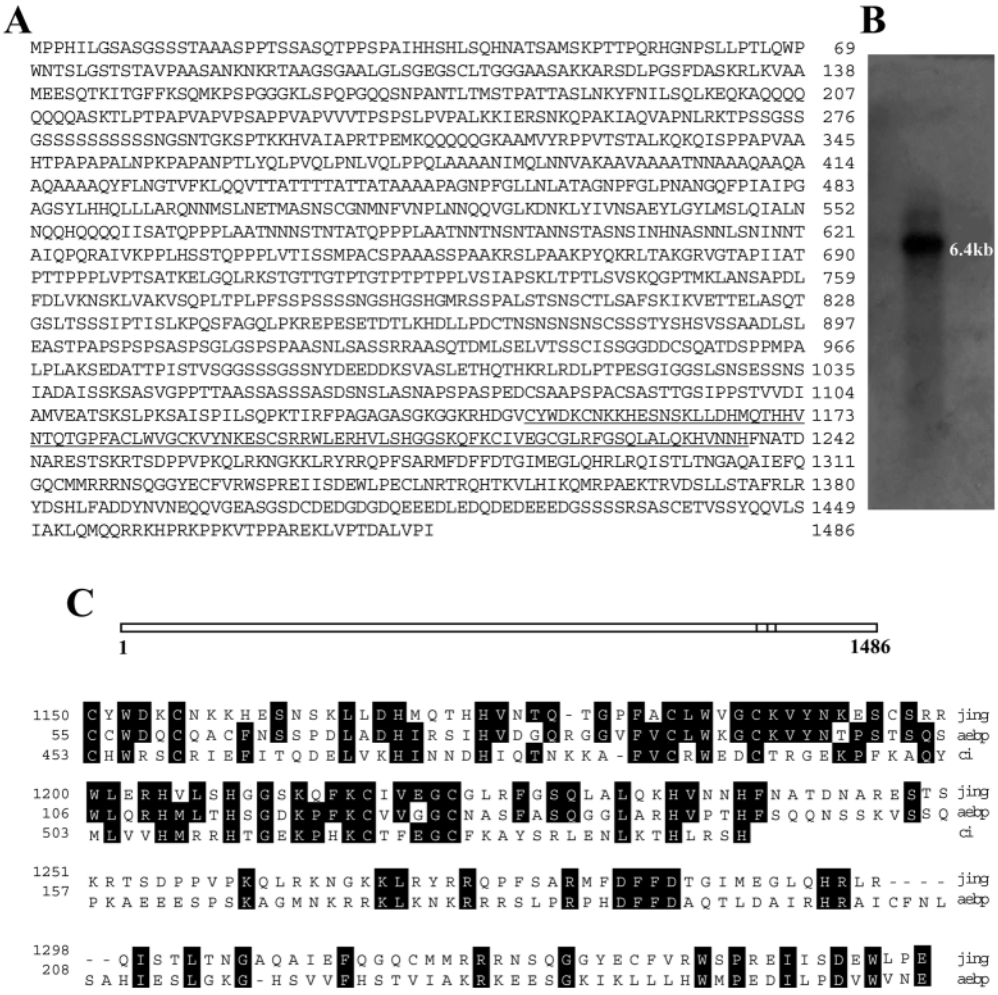

Fig. 6. JING amino acid sequence, mRNA expression and homology. The amino acid sequence is shown using the single letter code beginning with the first predicted methionine. The zinc finger motifs are underlined. (B) Northern blot of *jing* using a *jing* cDNA as a probe. (C) Amino acid sequence homology between JING, mouse AEBP-2 and *Drosophila* CI. Open horizontal bar represents the JING protein, and three small vertical bars indicate the three zinc finger motifs. Amino acid identities are shaded. CI is a more distantly related zinc finger protein, which contains five zinc fingers, three of which are shown for comparison.

DISCUSSION

jing defines an essential downstream target of *slbo*

We have identified a new locus in *Drosophila*, *jing*, which is required for initiation of border cell migration. The *jing* locus appears to function in the *slbo* pathway, based on several lines of evidence. Firstly, the phenotypes of *slbo* and *jing* are similar in that border cell migration defects are accompanied by loss of expression of the *PZ6356* marker. Secondly, expression of *jing* in the border cells depends upon wild-type *slbo* function. This regulation appears to be at a transcriptional level, since reduction in *lacZ* reporter gene expression from the *jing* enhancer trap line is evident on *slbo* mutant egg chambers. Further evidence that *jing* and *slbo* function in a common pathway is that expression of JING from a heat-inducible transgene can rescue the border cell migration defects associated with hypomorphic *slbo* alleles. This result also indicates that JING is a critical downstream target of *slbo*.

JING is likely to cooperate with SLBO in activating transcription from downstream target genes. The evidence for this is that, *in vivo*, both *jing* and *slbo* are normally required for *PZ6356* expression and over-expression of JING can compensate for reduced levels of SLBO. Moreover, the mammalian protein most related to JING, AEBP2, was identified in a screen for proteins that bind to the same enhancer element as C/EBP, in the adipose P2 gene (He et al., 1999). AEBP2 was originally reported to encode a 300 amino acid protein with transcriptional repressor activity. However, the mRNA for AEBP2 is 4 kb in length whereas the published cDNA was only 2 kb in length. Also, the AEBP2 cDNA sequence did not contain an in-frame stop codon upstream of the reported open reading frame. Therefore it is quite likely that the reported protein sequence is incomplete and represents only the C-terminal DNA binding domain of AEBP2. The protein expressed from such a truncated clone exhibits repressor activity, but the full-length protein may in fact be an activator. The JING protein is considerably longer than the reported AEBP2, and the loss of *PZ6356* expression in the *jing* mutant background would be consistent with the proposal that JING functions as an activator in vivo.

*D*E-Cadherin expression was not affected in *jing* mutant clones, though it is reduced in *slbo* mutants. DE-cadherin expression may require the presence of either *jing* or *slbo*. In *slbo* mutants, expression of a *jing-lacZ* reporter was also reduced, and DE-cadherin expression was affected. However in *jing* mutants, *slbo* expression did not appear to be reduced and DE-cadherin expression was unaffected. However DE-cadherin expression may require that some SLBO protein is present since over-expression of JING did not rescue the strong female sterile combination of *slbo* alleles (LY6/e7b) even though it did rescue

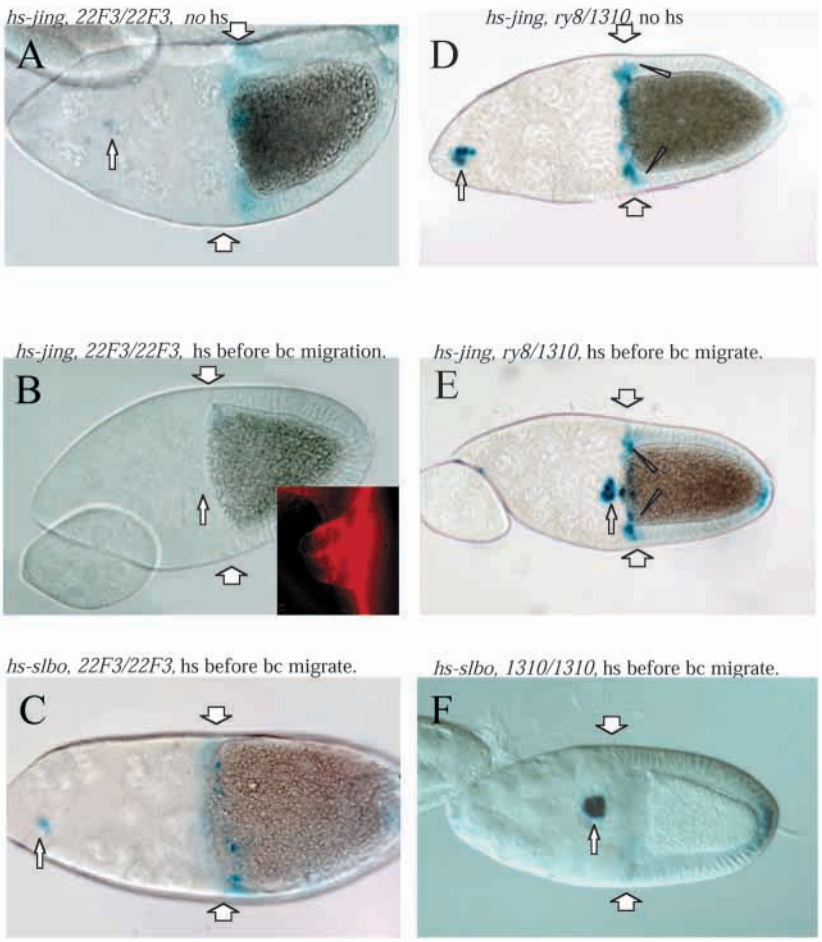

Fig. 7. Rescue of *jing* and *slbo* migration defects by *hs-jing*. Egg chambers in A-E are at late stage 9 or stage 10, when border cells should have completed migration. (A-C) *jing* mutant border cells with reduced PZ6356 expression. (A) In the presence of the *hs-jing* transgene but without heat shock, the border cells migrate a little, possibly because of the leaky expression of *hs-jing* at 25°C. (B) Following a 1-hour heat shock and 20 hours at 18°C, border cell migration appears normal. Border cells fail to express β-gal. Border cell position was assessed using rhodamine-conjugated phalloidin staining (inset). (C) Border cell migration was not rescued in *jing* mutants by heat shock *slbo* (see Materials and Methods for details), although a slight increase in β-gal expression from the *PZ6356* enhancer trap was observed. (D-F) Rescue of *slbo* migration defects by *hs-jing* and *hs-slbo*. (D) *slbo* mutant egg chamber with *hs-jing* transgene. In the absence of heat shock, border cell migration fails. (E) Following a 1-hour heat shock and 20 hours at 18°C, border cell migration was complete. (F) Heat shock *slbo* also rescued *slbo* migration defects. Thin arrows indicate border cells, thick arrows indicate extent of outer follicle cell rearrangement, arrowheads indicate centripetal cells.

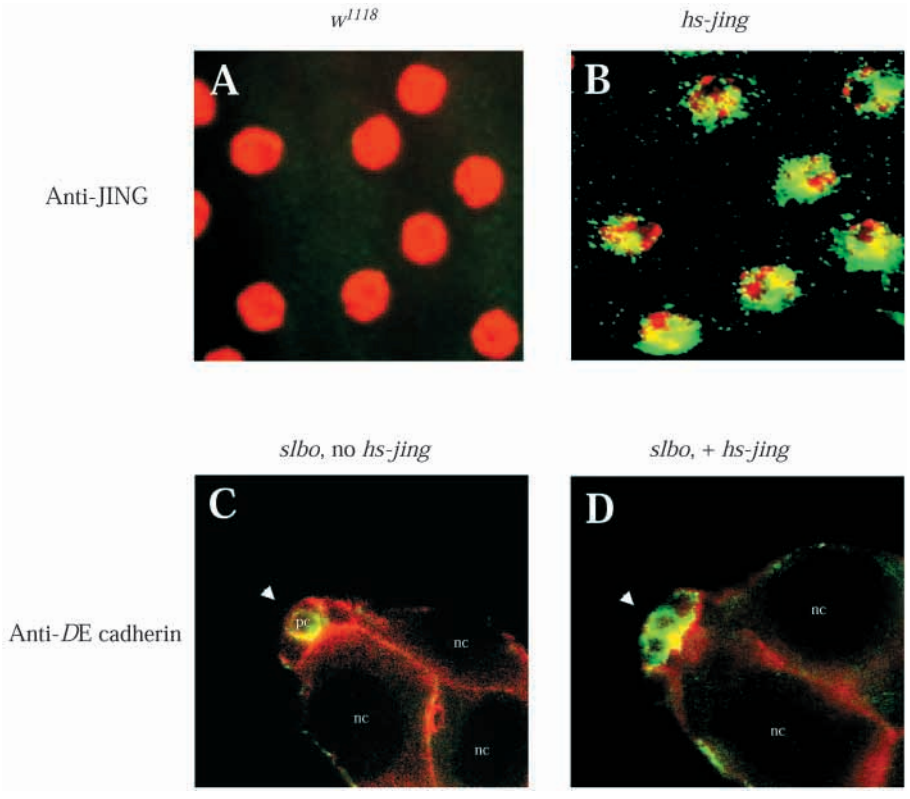

Fig. 8. Restoration of *D*E-cadherin expression in *slbo* mutants by *hs-jing*. A and B show follicle cells stained with DAPI (red) and anti-JING antibody (green) in egg chambers from females without (A) or with (B) the *hs-jing* transgene. Flies were heat shocked for 1 hour and allowed to recover at 25°C for several hours prior to dissection and staining. C and D show egg chambers labeled with rhodamine phalloidin (red) and anti-*D*E-cadherin antibody (green) from $slbo^{1}$ mutant females without (C) or with (D) the *hs-jing* transgene. *D*E-cadherin expression is significantly elevated in D as compared to C.

the weaker allele (*slbo*^{1}). This selective rescue was also observed with *hs-breathless*, which rescued the mild but not the strong female sterile *slbo* alleles (Murphy et al., 1995). To date only *hs-slbo* has been observed to rescue the border cell migration defects associated with the strongest female sterile alleles of the *slbo* locus (unpublished observation). Thus *jing* cannot completely substitute for *slbo*, consistent with the observation that there are multiple downstream targets of *slbo* with essential roles in border cell migration.

Our analysis of border cell clusters that were composed of mixtures of wild-type and homozygous mutant cells indicated that a single mutant cell can passively 'ride' along with wild-type clusters. However, a single wild-type cell did not appear to be capable of pulling multiple mutant cells. When a single wild-type cell occurred in an otherwise mutant cluster, the wild-type cell always appeared to move to the front of the cluster. Border cell clusters composed of roughly half mutant and half wild-type cells frequently split into two clusters and the wild-type cells invaded the nurse cell cluster. This behavior contrasted with the mixed-clone behavior reported for mutations in the *shotgun* (*shg*) locus, which encodes *D*E-cadherin. In this case the cells never seemed to split apart (Niewiadomska et al., 1999). The authors concluded that *D*E-cadherin could not be the only adhesion molecule responsible for holding the border cells together. Thus, there may be one or more additional adhesion molecules, whose expression is regulated by JING, that are responsible for holding the border cells together as a cluster.

Genetic control of border cell migration

It appears that the border cell epithelial-to-mesenchymal transition requires changes in gene expression, some of which are mediated by C/EBP and JING. *D*E-Cadherin is clearly one key downstream target of C/EBP, however it is not the only relevant downstream target. A thorough understanding of all of the changes required to convert a stationary epithelial cell to a migratory cell will require identification of more of the genes required for this process. It is striking that the three genes that have been identified to date in forward genetic screens for mutations that inhibit border cell migration, *slbo*, *jing* and *taiman* (Bai et al., 2000) have not been identified in any previous genetic screens. This raises the possibility that the genetic control of the epithelial-to-mesenchymal transition is significantly different from genetic control of pattern formation, eye development and other processes that have been subjected to extensive genetic analysis in *Drosophila*. However more extensive characterization of genes controlling border cell migration will be required before it is clear how different this process is.

Similarities between control of border cell migration and adipocyte differentiation

Undoubtedly many genes are required for an epithelial cell to become motile; therefore it is striking that the *jing* locus encodes a protein with such a clear functional connection to C/EBP. Evolution, it seems, has conserved functional networks of transcriptional regulators, rather than individual genes. Mammalian proteins related to JING and SLBO appear to be involved in the regulation of adipocyte differentiation, a process that bears little obvious resemblance to border cell migration. One similarity between adipocytes and border cells is that both cell types appear to coordinate their differentiation with nutritional status of the organism. In the case of adipocytes, at least two independent transcriptional regulatory pathways appear to be required (Cowherd et al., 1999; Loftus and Lane, 1997). One pathway is the C/EBP pathway, which may also require AEBP2. A second pathway requires the activity of PPARgamma, a steroid hormone receptor-like molecule that is activated by circulating fatty acids (Rocchi and Auwerx, 1999) whose levels depend upon diet. *Drosophila* ovarian development also responds to nutritional signals (Schwartz et al., 1985). Flies emerge from the pupal case with ovaries that contain only immature egg chambers. Further progression of oogenesis requires that the flies consume a rich diet. Application of lipophilic hormones, such as juvenile hormone or ecdysone, can bypass this requirement (Schwartz et al., 1985). Recently we have found that the ecdysone receptor is required in the border cells for their migration (Bai et al., 2000). Thus border cell migration, like adipocyte differentiation, requires a hormonal signal, which reflects nutritional status, to be integrated with an intrinsic developmental program. It is this latter program that appears to be mediated by the C/EBP, AEBP2 and the *Drosophila* proteins SLBO and JING.

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