

A Sensitized PiggyBac-Based Screen for Regulators of Border Cell Migration in *Drosophila*

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ABSTRACT

Migration of border cells during *Drosophila melanogaster* oogenesis is a good model system for investigating the genetic requirements for cell migration *in vivo*. We present a sensitized loss-of-function screen used to identify new genes required in border cells for their migration. Chromosomes bearing FRTs on all four major autosomal arms were mutagenized by insertions of the transposable element PiggyBac, allowing multiple parallel clonal screens and easy identification of the mutated gene. For border cells, we analyzed homozygous mutant clones positively marked with *lacZ* and sensitized by expression of dominant-negative PVR, the guidance receptor. We identified new alleles of genes already known to be required for border cell migration, including *aop/yan*, *DIAP1*, and *taiman* as well as a conserved Slbo-regulated enhancer downstream of *shg/DE-cadherin*. Mutations in genes not previously described to be required in border cells were also uncovered: *hrp48*, *vir*, *rme-8*, *kismet*, and *puckered*. *puckered* was unique in that the migration defects were observed only when PVR signaling was reduced. We present evidence that an excess of JNK signaling is deleterious for migration in the absence of PVR activity at least in part through Fos transcriptional activity and possibly through antagonistic effects on DIAP1.

CELL migrations are essential events that occur during embryonic development and throughout the life of animals. Many detailed studies in tissue culture cells have identified and characterized molecular components involved in the migration of individual cultured cells. Less is known about the cellular and molecular mechanisms acting *in vivo*. A number of characterized cell migrations have attracted attention and have been developed as models to study cell migration *in vivo*. Well-known examples are neural crest cells in vertebrates, lateral line primordium in zebrafish, germ cells in both vertebrates and *Drosophila*, as well as tracheal cells and border cells in *Drosophila*.

Due to the sophisticated genetics tools in *Drosophila*, including the ability to easily generate clones of mutant cells, border cells have become a powerful system for studying cell migration *in vivo*. Border cells are a group of ~8 cells that arise from the follicular epithelium of the egg chamber. The follicular epithelium is a monolayer of somatic cells that covers the germline-derived cells, the 15 nurse cells, and the oocyte. Border cells delaminate from the anterior part of this epithelium and migrate first posteriorly between the nurse cells toward the oocyte, and then dorsally along the border between the nurse cells and the oocyte (MONTELL *et al.* 1992).

Migrating upon other cells, border cells use the cell–cell adhesion molecule DE–cadherin, encoded by the *shotgun* (*shg*) locus, for substrate adhesion (NIEWIADOMSKA *et al.* 1999). Thus, expression of *shg* is required both in border cells and in the germline cells for migration to occur. Localization of DE–cadherin protein within border cells as well as protein turnover appears to be regulated at multiple levels and by different factors: the nonconventional myosin VI, the polarity proteins Par3 and Par6, as well as the transcriptional regulators Taiman and *aop/yan* (BAI *et al.* 2000; GEISBRECHT and MONTELL 2002; PINHEIRO and MONTELL 2004; SCHÖBER *et al.* 2005). Also, sequences within the C-terminal tail of DE–cadherin are specifically required for border cell migration (PACQUELET and RORTH 2005).

Loss-of-function screens have previously been performed and have identified genes whose function is required for border cells to migrate properly (MONTELL *et al.* 1992; LIU and MONTELL 1999). Among them, the transcription regulators Slbo, Taiman, Jing, and Stat92E are all expressed in border cells and required for migration (MONTELL *et al.* 1992; BAI *et al.* 2000; LIU and MONTELL 2001; SILVER and MONTELL 2001; BECCARI *et al.* 2002). Slbo was shown to upregulate DE–cadherin mRNA levels in border cells, as well as expression of *jing* (NIEWIADOMSKA *et al.* 1999; LIU and MONTELL 2001). The transcription profiles of wild-type and *slbo* mutant border cells have recently been described (BORGHESE *et al.* 2006; WANG *et al.* 2006). These studies allowed the discovery of additional genes regulated by Slbo and

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important for border cell migration, such as *stathmin* and *kuzbanian*. However, the targets of the other transcriptional regulators are still unknown and many gaps remain in our understanding of the transcriptional changes important for border cell migration.

Through gain-of-function screens, Gurken and PVF1 were identified as guidance cues for border cells. They signal through the tyrosine kinase receptors (RTKs) EGFR and PVR (PDGF/VEGF receptor), respectively. These RTKs have redundant activity in guiding the posterior migration toward the oocyte, whereas only the EGFR pathway is essential for dorsal migration as only EGFR ligands are unequally distributed between dorsal and ventral sides (DUCHEK and RORTH 2001; DUCHEK *et al.* 2001). However, it remains unclear which pathways act downstream of these RTKs in guidance. The unconventional GTP exchange factor Myoblast city and the small GTPase Rac are important for border cell migration and act downstream of PVR in regulating actin polymerization (DUCHEK *et al.* 2001). The inhibitor of apoptosis DIAP1 is also involved in border cell migration and regulates F-actin levels downstream of, or in parallel to, Rac (GEISBRECHT and MONTELL 2004).

To identify new players required for border cell migration, we developed and conducted a loss-of-function screen for mutants affecting border cell migration. Mutagenesis in this screen was based on mobilization of a PiggyBac transposon onto double FRT-bearing chromosomes. The FRT allowed us to perform a clonal screen on lethal loci, as we could analyze the phenotype of homozygous mutant cells in an otherwise heterozygous animal. We developed a modified version of the MARCM system (LEE and LUO 2001), referred to here as MdnP (for MARCM with dominant-negative PVR). In this system, *lacZ* was used to allow direct visualization of mutant border cells, even if they were rare. In addition, the MdnP system allowed us to sensitize the mutant border cells by perturbing their guidance with a dominant-negative form of PVR (dnPVR). Here, we report the results obtained from this screen and analysis of some of the uncovered mutants.

MATERIALS AND METHODS

***Drosophila melanogaster* stocks:** *P{neoFRT}FRT40A*, *P{neoFRT}FRT42D* (VEGH and BASLER 2003) was obtained from K. Basler. *P{neoFRT}FRT80*, *P{neoFRT}FRT82B* was generated by recombination and tested for neo^r and functionality of both FRTs. These double-FRT chromosomes were isogenized and tested before the generation of the screen stocks.

P{w⁺, ovo^{D1}}, P{neoFRT}FRT80 and *P{neoFRT}FRT42D*, *P{w⁺, ovo^{D1}}* chromosomes were generated as follows: *w¹¹¹⁸; P{neoFRT}FRT80/P{w⁺, ovo^{D1}}, P{FRT(w^{bs})}2A* and *w¹¹¹⁸; P{neoFRT}FRT42D/P{FRT(w^{bs})}G13, P{w⁺, ovo^{D1}}* second instar larvae were X-ray irradiated at a dose of 1000 rad (4 mA, 100 kV, 3'18'', Philips MG102) to induce mitotic recombination, including in the germline. Adult male progeny were crossed to *eyFLP, w¹¹¹⁸; P{neoFRT}FRT42D* or *eyFLP, w¹¹¹⁸; P{neoFRT}FRT80* virgins.

Appearance of mosaic eye color in progeny allowed identification of the likely correct recombinant chromosomes, subsequently tested for female sterility and generation of germline clones.

The PiggyBac (PBac) transposase chromosome (HORN and WIMMER 2000) was obtained from E. Wimmer and was recombined with the *If* dominant marker: *hsFLP²²* and *UAS β -lacZ* were recombined on a *w¹¹¹⁸* X chromosome, and *slbo-Gal4* (RORTH *et al.* 1998) and *UAS-dnPVR* (DUCHEK *et al.* 2001) were recombined on the second and the third chromosome. The *Puf1¹⁶²⁴* mutant and *EPg11235* (*EP-Puf1*) are described in DUCHEK *et al.* (2001), and *kek-LacZ^{15A6}* (MUSACCHIO and PERRIMON 1996) was obtained from N. Perrimon. *UAS-DIAP1* (WANG *et al.* 1999) and *puc²⁶⁹* (RING and MARTINEZ ARIAS 1993) were obtained from S. Cohen. *UAS-hep^{ACT}* (WEBER *et al.* 2000) was obtained from M. Mlodzik. *UAS-fosRNAi* and *UAS-junRNAi* were kind gifts from D. Bohmann. *puc^{A251}* was obtained from L. Raftery. *bsk²*, *jra^{A109}*, *kay¹* as well as recombinant chromosomes with *tub-Gal80* and *FRT40A*, *FRT42D*, *FRT80*, or *FRT82B* were obtained from the Bloomington Stock Center.

The genotypes of the MdnP stocks are *w¹¹¹⁸, hsFLP, UAS-lacZ; tub-Gal80, FRT40; slbo-Gal4, UAS-dnPVR/TM3, Ser* (MdnP40), *w¹¹¹⁸, hsFLP, UAS-lacZ; FRT42, tub-Gal80; slbo-Gal4, UAS-dnPVR/TM3, Ser* (MdnP42), *w¹¹¹⁸, hsFLP, UAS-lacZ; slbo-Gal4, UAS-dnPVR/CyO; tub-Gal80, FRT80* (MdnP80), and *w¹¹¹⁸, hsFLP, UAS-lacZ; slbo-Gal4, UAS-dnPVR/CyO; FRT82, tub-Gal80* (MdnP80).

We initially attempted to recover new PBac insertions based on GFP expression in late embryos/early larvae using a fluorescently activated embryo sorter, but had a very low frequency of positives. We assumed that GFP expression was inefficient as also reflected in the recovered lines (see Table 1) and instead used *white* expression to identify new insertions.

All fly cultures were performed at 25°. Heat shocks were performed for 1 hr at 37°, either on larvae (L2–L3) or on adult females.

Molecular mapping of novel PBac insertions: DNA was prepared using the DNAeasy kit (QIAGEN, Chatsworth, CA), and inverse PCR and DNA sequencing were performed as described in HACKER *et al.* (2003).

Generation of constructs: *PiggyBac* vector: A *NheI* site was cloned in place of the *PstI* site in pPBac{3xP3-EGFPaf} (HORN and WIMMER 2000). A linker containing the splice acceptor site, three stop codons, and the start site was cloned into *NheI/NcoI* (5'-GCTAGCCTTCTCTTACAGGTCGAATTGATGTGATGCTCGAGCCCATGG-3'). A linker containing the splice donor site and the *white* 3'-end was cloned into *AvrII/NgoMI* (5'-CCTAGGCTTCGGGCCCCGACGCAAGGAGTAGAAGGTAAGTAGCCCG-3'). *Mini-white* from pCasper3 (*XbaI/ApaI*) was then cloned into *ApaI/AvrII*.

1.3 LacZ: Genomic DNA was amplified with the oligonucleotides 5'-CAAAGCATGCTTGCTAGCAAGAAAC-3' and 5'-AGGCAAACCTTCTAACTGAAAAGGTG-3'. The PCR product was cloned into pCR-TOPO (Invitrogen, San Diego) and subcloned at the *EcoRI* site into P{CaSpeR-AUG-betaGal}.

X-gal staining: To monitor *lacZ* expression, ovaries were dissected in PBS, fixed in 0.5% glutaraldehyde in PBS for 10 min, rinsed in PBS-0.1% Triton (PT), and stained in 10 mM NaH₂PO₄/Na₂HPO₄ (pH 7.2), 150 mM NaCl, 1 mM MgCl₂, 3.1 mM K₄(FeII(CN)₆), 3.1 mM K₃(FeIII(CN)₆), 0.3% Triton X-100, and 0.2% X-Gal at room temperature.

Immunocytochemistry: Ovaries were dissected in Grace's medium or PBS, fixed in 4% paraformaldehyde for 20 min at room temperature, rinsed in PBS+ 0.1% Triton X-100 (PT), blocked and incubated overnight with the primary antibody in PT + 5% normal goat serum, rinsed in PT, incubated 2 hr with secondary antibody in PT, rinsed in PT and PBS, and

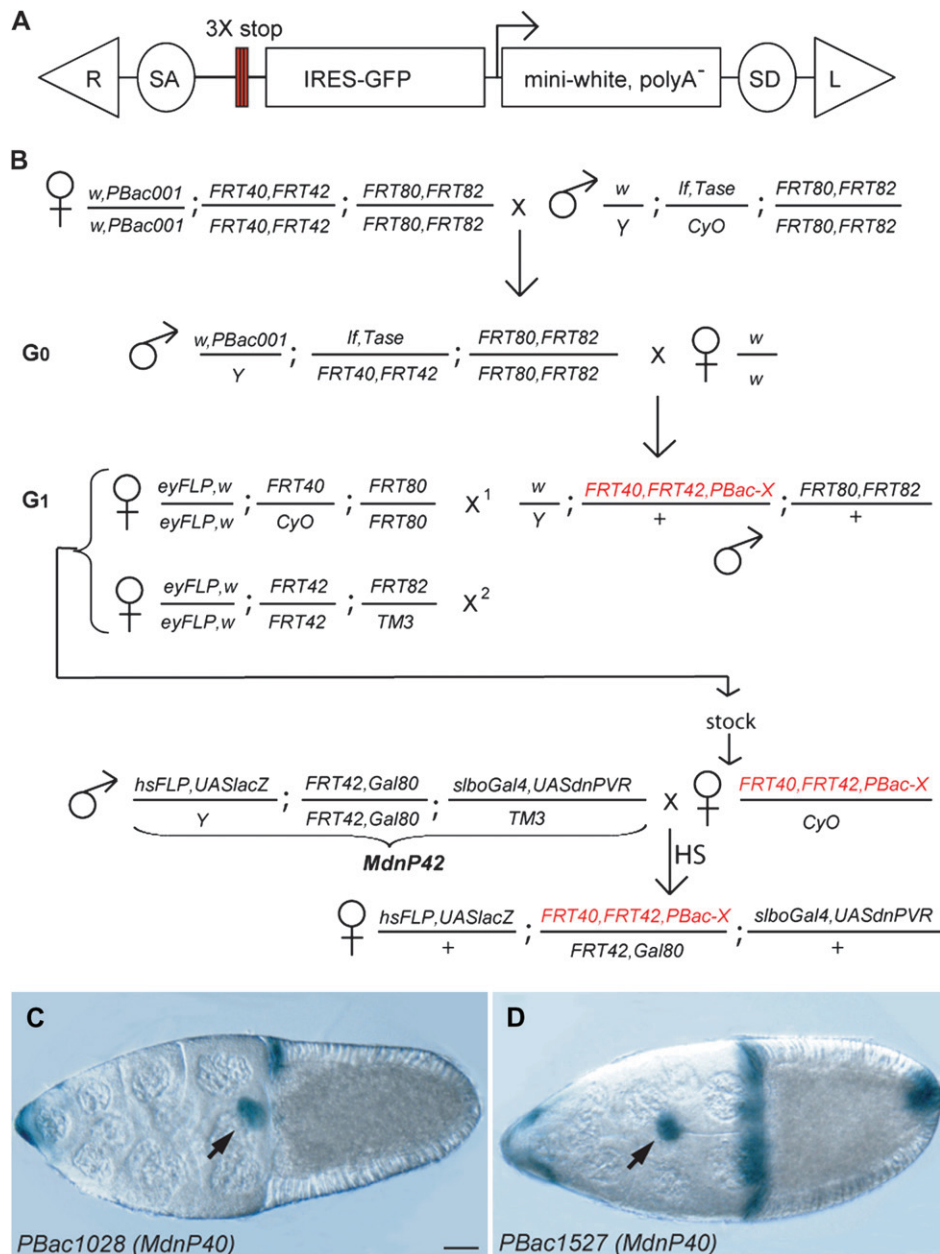


FIGURE 1.—The PiggyBac screen. (A) The PiggyBac vector used as a mutagen. R and L are the right and left feet of the PBac transposon. SA and SD are the splice acceptor and splice donor. The three stop codons, one in each frame, are represented as red bars. (B) The crossing scheme. *PBac001* indicates the jump starter. *Tase* indicates the piggyBac transposase. G0, generation of new insertion. G1, mapping. The new mutated chromosome, carrying a *w*⁺ insertion, is represented in red. *FRT40, FRT42* is indicated as an example but it could also be *FRT80, FRT82*, as the crosses are identical. X1 and X2 are the two successive crosses done with single males, progeny of G0, to map the insertion to a chromosome arm. In the MdnP cross, virgins from the *PBac* stocks are crossed with MdnP males. The genotype of the MdnP42 male is shown as example for a *PBac* insertion distal to *FRT42*. Heat shock (HS) is applied to the progeny, either at larval stage or at adult stage. Females are dissected and ovaries are stained with X-Gal. An example of insertion with a border cell migration delay phenotype is shown in D; compare to an insertion with no phenotype (C). (C and D) X-gal-stained stage 10 egg chambers from females of the genotype *hsFLP, UAS-lacZ/+; PBac1028, FRT40, FRT42/tub-Gal80, FRT40; slbo-Gal4, UASdnPVR/+* (C) and *hsFLP, UAS-lacZ/+; PBac1527, FRT40, FRT42/tub-Gal80, FRT40; slbo-Gal4, UASdnPVR/+* (D). Arrow indicates the border cell cluster. Bar, 20 μ m. Here and in all figure panels, anterior is to the left.

mounted for microscopy. Confocal images were collected sequentially on a Leica TCS-NT microscope.

Primary antibodies were rat anti-DE-cadherin (Oda *et al.* 1994), rabbit anti- β -galactosidase (Cappel), and rat anti-Slbo (P. ROTH, unpublished results). Fluorescent secondary antibodies were from Jackson Immunoresearch; rhodamin-phalloidin and DAPI were from Molecular Probes (Eugene, OR).

RESULTS

Mutagenesis and screening strategy: Flies were mutagenized by transposition of a PBac transposon modified to include a gene-trap cassette (Figure 1A). A splice acceptor and three stop signals were introduced to increase the chance of severely disrupting a gene upon insertion. They were followed by a modified version of

an existing gene-trap *P* element (LUKACSOVICH *et al.* 2001), including internal ribosome entry site (IRES)-GFP and a *mini-white* gene with its own promoter but lacking a 3'-UTR and natural stop signal. Upon insertion of the transposon in a transcribed region, a GFP protein might be translated from the IRES, and the mRNA encoding the *white* gene product may be stabilized if there is an endogenous poly-adenylation signal downstream. Thus, the insertions giving GFP and/or *white* expression should have a high probability of being located in transcribed regions and of severely mutating the genes in which they are inserted. The starter line had a PBac insertion on the X chromosome (*PBac001*) and second and third chromosomes carrying FRTs on both sides of the centromere (Figure 1B). *PBac001* was recovered due to its GFP expression, but was phenotypically *w*⁻. By

TABLE 1
Summary of the selected lines

PBac line	Insertion site	Gene	Molecular function	PBac orientation relative to gene	GFP expression	References (border cells)
PBac 79	2R:18871317	<i>virilizer</i>	RNA binding	Sense, in intron	–	
PBac456	2L:6919366	<i>hrp48</i>	RNA binding	Sense, in 5'-UTR	+	
PBac1527	2L:6922912	<i>hrp48</i>	RNA binding	Antisense, in intron	–	
PBac104	2L:2178018	<i>anterior</i>	Transcription factor	Sense, in intron	–	SCHOBER <i>et al.</i> (2005)
		<i>open/yan</i>				
PBac378	2L:2176647	<i>anterior</i>	Transcription factor	Antisense, in promoter	–	SCHOBER <i>et al.</i> (2005)
		<i>open/yan</i>				
PBac911	2L:247422	<i>kismet</i>	Elongation factor	Sense, in intron	–	
PBac5201	2L:242721	<i>kismet</i>	Elongation factor	Antisense, in intron	–	
PBac1916	2R:4682442	<i>rme-8</i>	Chaperone	Sense, in intron	–	
PBac2148	3L:16013380	<i>DIAP1</i>	Ubiquitin–protein ligase	Sense, in intron	+	GEISBRECHT and MONTELL (2004)
PBac3840	2L:9202838	<i>taiman</i>	Transcription co-activator	Sense, in intron	–	BAI <i>et al.</i> (2000)
PBac4936	2L:9204904	<i>taiman</i>	Transcription co-activator	Antisense, in intron	–	BAI <i>et al.</i> (2000)
PBac4624	2L:9206379	<i>taiman</i>	Transcription co-activator	Antisense, in intron	–	BAI <i>et al.</i> (2000)
PBac4354	2R:16549897	<i>shg</i>	Cell–cell adhesion	8 kb downstream of <i>shg</i>	–	NIEWIADOMSKA <i>et al.</i> (1999)
PBac3929	3R:3933469	<i>puckered</i>	Phosphatase	Antisense, in 5'-UTR	–	
PBac4251 ^a	3R:16954630	<i>slimb</i>	Ubiquitin–protein ligase	Sense, in intron	–	MUZZOPAPPA <i>et al.</i> (2005)

The insertion site (second column) gives the chromosome arm location, and the first base from the left foot of the PiggyBac. The PiggyBac orientation relative to the gene (fifth column) is relative to that shown in Figure 1A.

^aClones recovered only with adult heat shock.

mobilizing this PBac in *w⁻* mutant flies, we could therefore easily identify new insertions on the basis of the appearance of colored eyes (Figure 1B, chromosome bearing new PBac-X insertion in red). New PBac insertions were mapped to a chromosome arm by testcrosses with flies expressing FLPase in the eye primordium (*eyFLP*) and carrying specific FRT chromosomes, looking for mosaic eyes (Figure 1B). Temporary stocks were generated and used in several screens: a growth screen using the *eyFLP* system (THOMPSON *et al.* 2005, screen not described in article), a maternal effect screen using the *ovd^p* system, and the border cell migration screen using the MdnP system described in detail below. Overall, this type of screen allows mutagenesis of all four major autosomal arms at once, direct clonal screens with the new insertions, and, if a phenotype is seen, rapid identification of the affected gene.

Overview of the PBac screen: From a small pilot screen, we estimated that ~20% (7 of 32) of new PBac insertions were lethal. This frequency was similar to that obtained in the generation of other PBac collections (THIBAUT *et al.* 2004; S. THIBAUT, personal communication). The lethality was primarily due to the PBac insertion since precise excision reverted the lethality in most cases (6 of 7 lines tested). The jump efficiency (defined as the percentage of crosses with the *white* positive insertion recovered using one male carrying the PBac starter and the PBac transposase and two virgins per cross) of our starter PBac was 21%. In the screen, we

generated 5908 new jumps and recovered 3298 lines (56%) where the PBac could be clearly mapped to a chromosome arm. Partial recovery was expected due to a combination of death of the PBac carrier male before completion of mapping crosses and many “unmappable” insertions: insertions proximal to the FRTs, insertions on the fourth chromosome, and multiple insertions. With an expected lethal hit frequency of 20%, testing ~3300 lines corresponded to ~660 lethal insertions, not a saturating screen.

The border cell screen: To identify genes important for border cell migration, all mapped PBac insertions were analyzed in homozygous mutant border cell clones using the MdnP system. With the MdnP system (Figure 1B, bottom), homozygous mutant border cells were marked with expression of *lacZ*, which allowed easy identification of mutant clones (Figure 1, C and D), even if they were rare. In addition, homozygous mutant cells specifically expressed a dominant-negative form of the guidance receptor PVR (*dnPVR*), which induces a weak phenotype on its own but was expected to sensitize border cell migration to further perturbations. In the initial screen where we recovered mutant clones after larval heat shock, 3182 of the lines tested had no detectable migration phenotype, 14 exhibited a migration phenotype (Table 1), and we could not recover any clones for 102 lines (3%). The genes in this last category could be important for cell viability or for functionality of the follicular stem cells and thus not allow recovery of

mutant clones 7–10 days after clone induction as would be required after larval heat shock. We therefore generated clones with these PBac insertions by heat-shocking adult females and analyzing them 3–4 days afterward. This treatment revealed one more insertion (PBac4251) with a border cell migration phenotype and an additional 75 PBac insertions with no apparent effect in homozygous mutant border cells; for 26 PBac insertions, homozygous mutant clones could still not be obtained.

A summary of the 15 lines showing a border cell phenotype, the insertion site of the PBac, and the gene that they affect is given in Table 1. To complement the MdnP approach, all 15 lines were subsequently tested for border cell migration defects in standard clonal analysis without dnPVR expression, and 13 of 15 (all but PBac378 and PBac3929) displayed comparable phenotypes in such clones. PBac378 is an insertion in *yan/aop* and had a very weak phenotype in our screen. PBac104 also mutates *yan/aop* and, similarly to previously described alleles, it has a border cell migration phenotype on its own (SCHÖBER *et al.* 2005). Of the 15 lines, 5 additional lines had the PBac inserted in a gene previously found to affect border cell migration (PBac3840, PBac4624, and PBac4936 in *taiman*, PBac2148 in *DIAP1*, and PBac4251 in *slimb*), and two genes were represented by two independent insertions each (PBac456 and PBac1527 in *hrp48* and PBac911 and PBac5201 in *kismet*). Further analysis of the two lines PBac4354 (a *shg* regulatory mutant) and PBac3929 (*puckered*) is described below. The two remaining lines were not investigated in more detail (PBac79 in *virilizer*, PBac1916 in *rme-8*).

A border cell enhancer for *shg*: PBac4354 is a homozygous viable mutant in which the PBac is not inserted within a gene, but 8 kb downstream of the *shg* locus (Figure 2A). As *shg*, which encodes DE-cadherin, is required in border cells for their migration (NIEWIADOMSKA *et al.* 1999), we considered that PBac4354 might disrupt some regulatory sequences required for *shg* expression in border cells. In agreement with this idea, a clear reduction in DE-cadherin levels was observed in PBac4354 homozygous mutant border cells (Figure 2C). In addition, the migration phenotype could be rescued by expressing *shg* cDNA in PBac4354 mutant border cells (Figure 2B). To characterize this apparent regulatory mutant, we analyzed the genomic region surrounding the PBac4354 insertion site (Figure 2A). Within a region of ~1.3 kb, sequence analysis revealed the presence of multiple consensus binding sites for the transcription factor Slbo, known to regulate *shg* mRNA level in border cells (NIEWIADOMSKA *et al.* 1999). This region also showed stretches of high sequence similarity to a *D. pseudoobscura* genomic region downstream of its *shg* ortholog, suggesting that it is a conserved enhancer (supplemental Figure 1 at <http://www.genetics.org/supplemental/>). We generated transgenic flies expressing *lacZ* under the control of this potential enhancer (*1.3-lacZ*). In stage 10 egg chambers, β -galactosidase expression was de-

tected in the border cells and centripetal cells (Figure 2D, middle), similar to the expression pattern of Slbo (Figure 2D, right). In *slbo* mutant border cells, expression of the *1.3-lacZ* transgene was severely reduced (Figure 2E, mutant cells marked by the presence of GFP). Thus we have identified a conserved, Slbo-responsive enhancer for the *shg* gene. The insertion of PBac4354 seems to disrupt the regulatory properties of the enhancer, perhaps by interfering with its ability to interact with the promoter of the *shg* gene. DE-cadherin is expressed in all cells of the follicular epithelium as well as in the germline, raising the question of why a border-cell-specific enhancer for *shg* would be functionally important. Specific transcriptional upregulation mediated by this enhancer may be advantageous for ensuring that the cells can retain strong DE-cadherin-dependent adhesion while having faster turnover of DE-cadherin protein during the dynamic process of migration.

***puckered* is required in border cells with low PVR signaling:** PBac3929 was the only insertion that exhibited a prominent phenotype in the sensitized background of the screen but no detectable phenotype in standard clonal analysis without dominant-negative PVR (Figure 3, A and B). PBac3929 mutates *puckered* (*puc*), as confirmed by complementation test. *puckered* encodes a dual-specificity phosphatase that acts on the Drosophila Jun kinase (JNK) basket (*bsk*) and thereby negatively regulates the activity of the JNK signaling pathway (MARTIN-BLANCO *et al.* 1998). Gain of function of JNK signaling can also be achieved by expressing a constitutively active form of hemipterous (*hep^{ACT}*), an activating kinase for *bsk* (GLISE *et al.* 1995; SLUSS *et al.* 1996; WEBER *et al.* 2000). *hep^{ACT}* had no significant effect when expressed on its own in border cells (Figure 3D). However, like *puc^{PBac3929}* loss of function, *hep^{ACT}* had a strong effect on border cell migration when coexpressed with *dnPVR* (Figure 3D). The effect of *hep^{ACT}* was stronger than that of *puc^{PBac3929}* loss of function, possibly reflecting that *puc^{PBac3929}* is not a complete loss-of-function allele. The PVR receptor is activated in border cells by its ligand PVF1 (DUCHEK *et al.* 2001). As with expression of *dnPVR* in border cells, loss of function of *Pvf1* had only mild effect on migration by itself, but seriously impaired border cell migration when combined with activated JNK signaling (Figure 3E). Thus elevated JNK signaling is deleterious for migration of border cells with reduced or absent PVR signaling.

For posterior migration of border cells, guidance is mediated by the redundant activity of PVR and EGFR. Loss of activity of a single receptor does not severely affect border cell migration, while perturbing the activity of both at the same time disrupts border cell migration (DUCHEK *et al.* 2001). The observation that activated JNK signaling specifically affected border cell migration in the background of reduced PVR signaling suggested that it might disrupt the guidance activity of EGFR. EGFR signaling in the border cells is essential for their

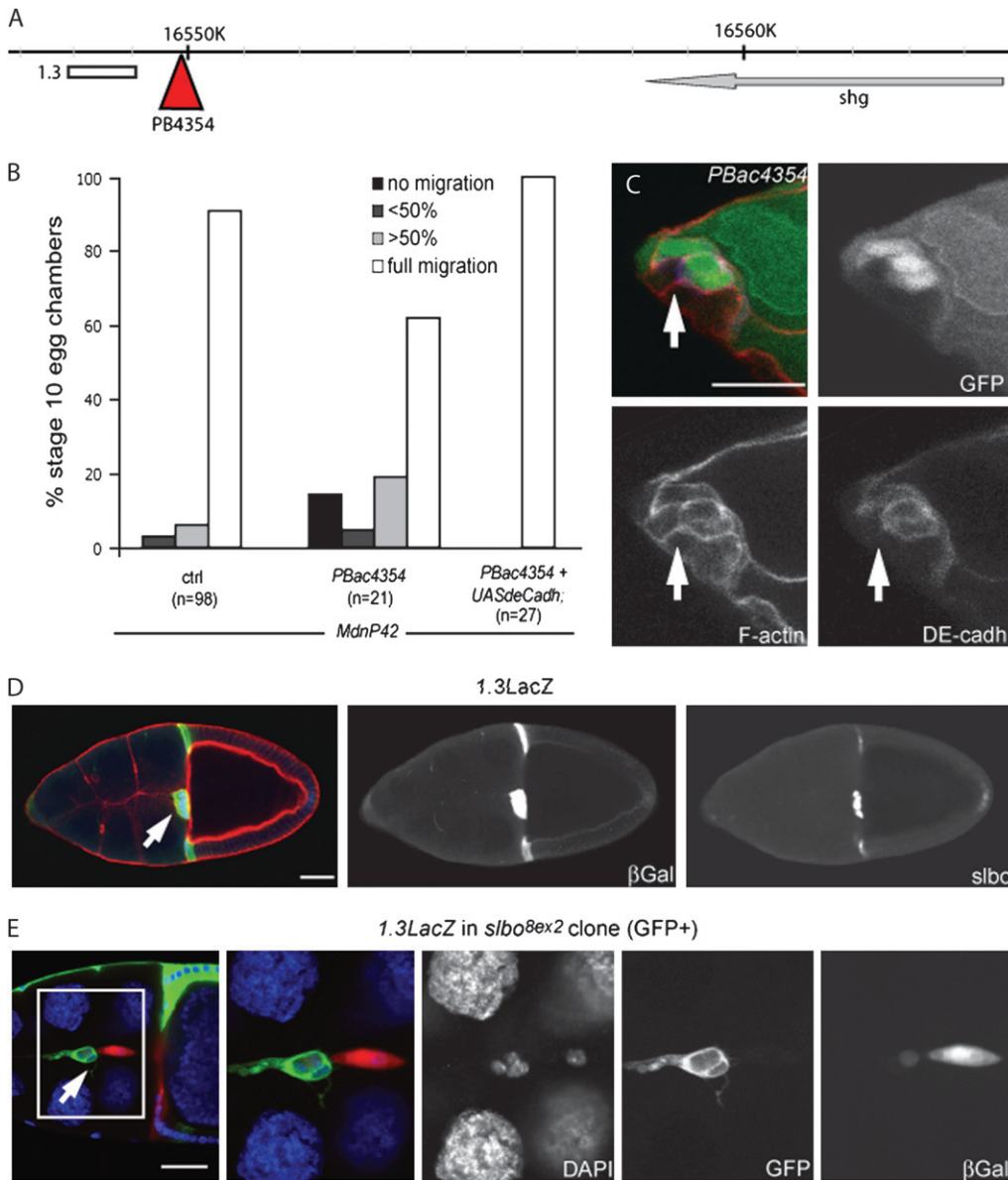


FIGURE 2.—PBac4354 identifies a *shotgun/DE-cadherin* enhancer. (A) Scheme of the genomic region around PBac4354. The PBac (red triangle) is inserted 8 kb downstream from the 3'-end of the *shg* gene. The white bar corresponds to the fragment used to generate the *1.3LacZ* construct. (B) Quantification of migration in control border cell clones, PBac4354 clones, and PBac4354 border cell clones expressing a *shg* cDNA generated with the MdnP system. The genotypes of the dissected females were *hsFLP; UAS-LacZ/+; FRT40,FRT42/FRT42,tubGal80; slbo-Gal4, UAS-dnPVR/+* (control, left) *hsFLP,UAS-LacZ/+; FRT40,FRT42, PBac4354/FRT42,tubGal80; slbo-Gal4,UAS-dnPVR/+* (PBac4354, middle) and *hsFLP,UAS-LacZ/UAS-shg; FRT40,FRT42, PBac4354/FRT42,tubGal80; slbo-Gal4,UAS-dnPVR/+* (PBac4354+ UAS-shg, right). (C) Stage 10 egg chamber from a female of the genotype *hsFLP; FRT40,FRT42,PBac4354/FRT42,UbiGFP* subjected to adult heat shock. Mutant cells are marked by the absence of GFP (top right and green in overlay). Phalloidin labels F-actin (bottom left and red in overlay) and an antibody labels DE-cadherin (bottom right and blue in overlay). The membrane staining (arrow) of DE-cadherin between two mutant border cells is strongly reduced. (D) Stage 10 egg chambers from female

carrying the *1.3LacZ* reporter, stained with phalloidin (red in overlay), anti- β -galactosidase (β -gal, middle, green in overlay), and anti-Slbo (right, blue in overlay). Left is the overlay. Arrow indicates the border cell cluster in D and E. (E) Stage 10 egg chambers from female with the *1.3LacZ* reporter and *slbo*^{8ex2} mutant clones (GFP-positive cells, green in overlay, are homozygous mutant), stained with DAPI to show nuclei (blue in overlay) and with anti- β -galactosidase (right, red in overlay) to reveal the expression of *1.3LacZ*. The region in the white box is enlarged in the right panels. The genotype is *hsFLP,UAS-GFP/+; FRT42,slbo^{8ex2}/FRT42,tubGal80; tubGal4/1.3lacZ*. (C–E) Bar, 20 μ m.

final, dorsal migration (DUCHEK and RORTH 2001). To test whether elevated JNK signaling affects EGFR guidance activity, we therefore analyzed dorsal migration of *puc*^{PBac3929} border cell clones and of *hep*^{ACT}-expressing border cells (Figure 3G). In both cases, border cell clusters did migrate dorsally. In addition, the classical MAPK/ERK signaling downstream of EGFR is not affected in *puc*^{PBac3929} clones in the dorsal follicular epithelium, as indicated by the unchanged level of expression of the *kek-lacZ* reporter (Figure 3H; MUSACCHIO and PERRIMON 1996; GHIGLIONE *et al.* 1999). These results suggested that *puc*^{PBac3929} mutant cells were not deficient in EGFR signaling.

Another feature of PVR and EGFR as guidance receptors is that uniformly activating them, for example, by mis-expressing a ligand throughout the tissue that the cells migrate through, also impairs migration. To further investigate whether the gain of JNK signaling interferes with the guidance of border cells, we analyzed the phenotype of *puc*^{PBac3929} mutant border cells (Figure 3C) and of border cells expressing *hep*^{ACT} (Figure 3F) in which PVR signaling is uniform due to overexpression of *Pvf1*. Excess of JNK signaling did slightly enhance the migration phenotype due to unlocalized PVR signaling but did not show the strong synergy seen with PVR loss of function. This contrasts with the effect of perturbing

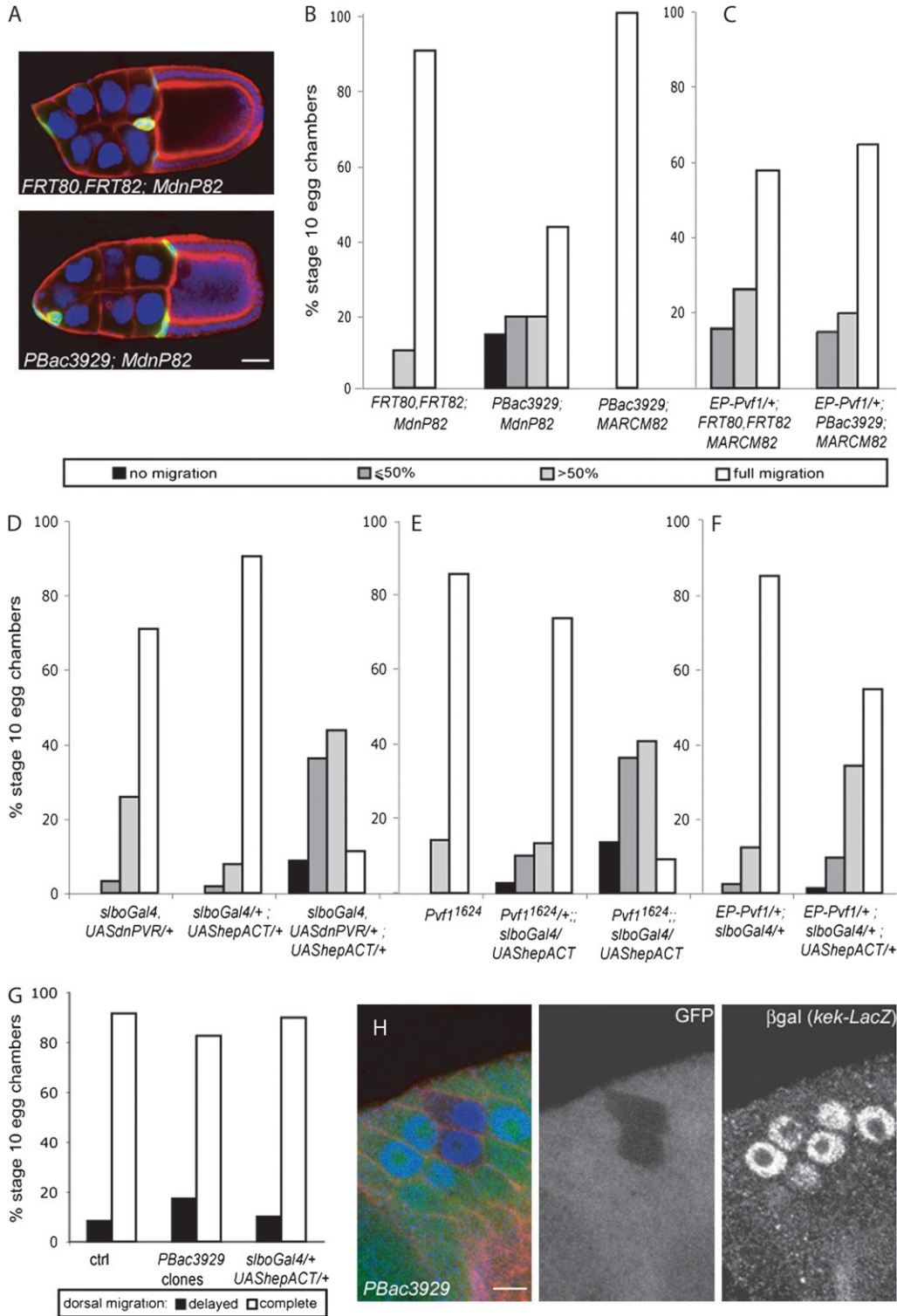


FIGURE 3.—*puc* is required in border cells with low PVR signaling. (A) Stage 10 egg chambers, stained with DAPI (blue) to show nuclei, anti- β -galactosidase antibody (green) to reveal mutant cells, and phalloidin (red) from females of the following genotypes: *hsFLP, UAS-lacZ/+; slbo-Gal4, UAS-dnPVR/+; FRT80, FRT82/tub-Gal80* (top) and *hsFLP, UAS-lacZ/+; slbo-Gal4, UAS-dnPVR/+; FRT80, FRT82, puc^{PBac3929}/FRT82, tubGal80* (bottom). Bar, 20 μ m. (B–F) Quantification of border cell migration toward the oocyte (posterior migration) in stage 10 egg chambers. (B) *puc^{PBac3929}* clones with or without *dnPVR* and control MdnP clones from females of the genotypes *hsFLP, UAS-lacZ/+; slbo-Gal4, UAS-dnPVR/+; FRT80, FRT82/tub-Gal80* (left, $n = 10$); *hsFLP, UAS-lacZ/+; slbo-Gal4, UAS-dnPVR/+; FRT80, FRT82, puc^{PBac3929}/FRT82, tub-Gal80* (middle, $n = 21$); and *hsFLP, UAS-GFP/+; tub-Gal4/+; FRT80, FRT82, puc^{PBac3929}/FRT82, tub-Gal80* (right, $n = 10$). (C) Control border cells overexpressing *Pvf1* (left; genotype *hsFLP, UAS-GFP/EP-Pvf1; tubGal4/+; FRT80, FRT82/tubGal80*, $n = 19$) and *puc^{PBac3929}* clones overexpressing *Pvf1* (right; genotype *hsFLP, UAS-GFP/EP-Pvf1; tubGal4/+; FRT80, FRT82, puc^{PBac3929}/FRT82, tubGal80*, $n = 20$). (D) Border cell clusters expressing *dnPVR*, *hep^{ACT}*, or both ($n = 93, 272, 80$). (E) Clusters expressing *hep^{ACT}* in control, *Pvf1/+*, or *Pvf1* mutant background ($n = 42, 149, 22$). (F) Clusters expressing uniform *Pvf1*, alone or with *hep^{ACT}* ($n = 203, 73$). Genotypes are indicated and in-

clude the controls in each experiment. (G) Quantification of dorsal migration in *puc^{PBac3929}* border cell clones (from *hsFLP/+; FRT80, FRT82, puc^{PBac3929}/FRT82, UbiGFP* females, $n = 23$), border cells expressing *hep^{ACT}* (*slbo-Gal4/+; UAS-hep^{ACT}/+*, $n = 20$), and the control (*slbo-gal4/+*, $n = 12$). (H) Follicular epithelium of stage 10 egg chamber from female of the genotype *hsFLP/+; kek-lacZ/+; FRT80, FRT82, puc^{PBac3929}/FRT82, UbiGFP*, stained with anti- β -galactosidase antibody (β -gal, blue in overlay) to reveal *kek-lacZ* reporter expression and phalloidin (red in overlay). *puc^{PBac3929}* mutant cells are marked by absence of GFP (green in the overlay). Dorsal is to the top. Bar, 5 μ m.

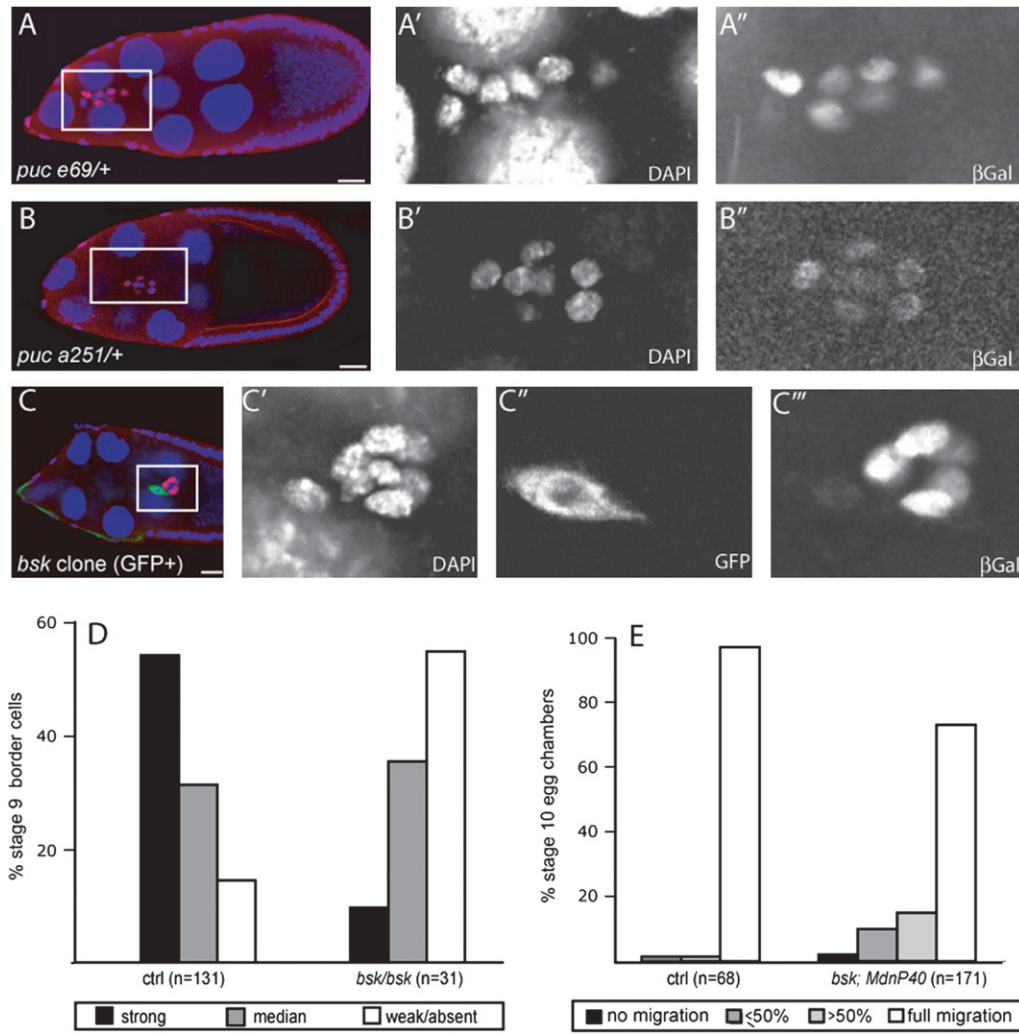


FIGURE 4.—JNK signaling in border cells. (A–C) Stage 9 egg chambers, stained with DAPI (blue nuclei in overlay) and anti- β -galactosidase antibody (β -gal, red in overlay) to reveal *puc-lacZ* reporters (*puc^{E69}* in A and C, *puc^{A251}* in B) in control flies (A and B) and *bsk²* mutant border cell clone (C; mutant cells are GFP positive and green in overlay). Females were of the following genotypes: (A) *puc^{E69}/+*, (B) *puc^{A251}/+*, and (C) *hsFLP,UAS-GFP; bsk²,FRT40/tubGal80; tubGal4/puc^{E69}*. Left panels show overlays, outlined box indicates the region enlarged in the panels to the right showing the indicated single channel images. Bar, 20 μ m. (D) Quantification of β -galactosidase staining in control cells (ctrl) and *bsk²* homozygous mutant border cells from female with genotype as in C. (E) Migration at stage 10 of *bsk²* mutant and control border cells with the MdnP system from the following genotypes: *hsFLP,UAS-lacZ/+; FRT40,FRT42/tubGal80, FRT40; silbo-Gal4,UAS-dnPVR/+* (ctrl) and *hsFLP,UAS-lacZ/+; bsk²,FRT40/tubGal80, FRT40; silbo-Gal4,UAS-dnPVR/+*.

EGFR signaling, which strongly synergizes with misexpression of *Puf1* (DUCHEK *et al.* 2001). Overall, this indicated that excess JNK signaling was deleterious for border cell migration if PVR activity was low or absent, but excess JNK signaling still allowed EGFR to function as a guidance receptor.

JNK signaling activity in the border cells: *Puc* is a negative regulator of JNK signaling, but is also a transcriptional target of the JNK pathway. For example, the expression of the *puc^{E69}* reporter, a *lacZ* insertion in the *puc* locus that reflects *puc* transcription in the dorsal epidermis of the embryo (MARTIN-BLANCO *et al.* 1998), is lost in *hep* and *bsk* mutants (GLISE *et al.* 1995; RIESGO-ESCOVAR *et al.* 1996). This negative feedback loop is also present during thorax closure in the *Drosophila* pupae (ZEITLINGER and BOHMANN 1999) and in the follicular epithelium of the ovary at late stages (SUZANNE *et al.* 2001). We asked whether such a feedback loop was present in border cells using *puc^{E69}* reporter as transcriptional readout.

We first examined the expression of *puc^{E69}* in a control egg chamber (*puc^{E69}/+*) by antibody staining. We could

detect some β -galactosidase staining in migrating border cells, but the expression level was not uniform among the cells (Figure 4A). The central cells, the anterior polar cells, generally had weak staining. For the migratory outer border cells, staining could be strong, intermediate, or weak and we did not detect any obvious correlation between the staining intensity and the position of the cell in the cluster. To confirm this observation, we analyzed the expression level of β -galactosidase in another *puc* reporter, *puc^{A251}* (MARTIN-BLANCO *et al.* 1998). Although the overall expression level of the reporter was weaker than that of *puc^{E69}*, we also saw some unequal distribution of the β -galactosidase staining in *puc^{A251}/+* border cells (Figure 4B). Analysis of *puc^{E69}* expression in many border cell clusters revealed that in a control situation most of the outer border cells have a strong or intermediate level of staining (Figure 4D). To determine if the expression of *puc* depends on JNK signaling in border cells, we analyzed the expression of *puc^{E69}* in *bsk* mutant border cells. Mutant cells showed a significant reduction in staining (Figure 4, C and D), indicating that *puc* expression is positively

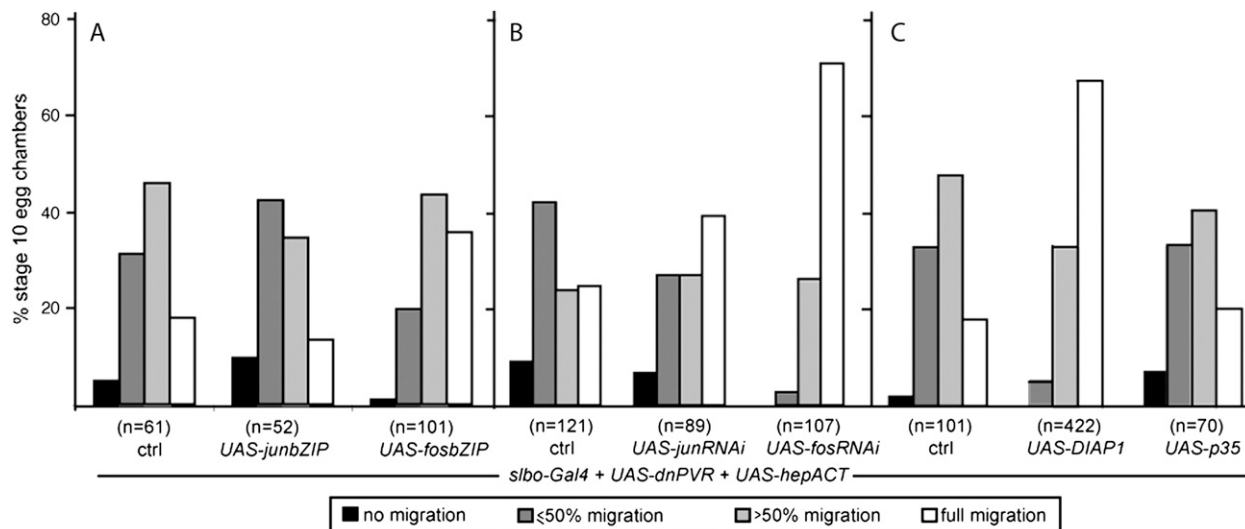


FIGURE 5.—Suppression of a high-JNK, low-PVR phenotype by decreasing Fos/Jun or increasing DIAP1 expression. (A–C) Quantification of migration at stage 10 of border cells expressing *hep^{ACT}* and *dnPVR* (ctrl in all experiments), as well as (A) dominant-negative versions of Jun (*slbo-Gal4,UAS-dnPVR/+; UAS-hep^{ACT}/UAS-junbZIP*) or Fos (*slbo-Gal4,UAS-dnPVR/+; UAS-hep^{ACT}/UAS-fosbZIP*), (B) expressing RNAi against *jun* (*UAS-junRNAi/+; slbo-Gal4,UAS-dnPVR/UAS-hep^{ACT}*) or *fos* (*UAS-fosRNAi/+; slbo-Gal4,UAS-dnPVR/UAS-hep^{ACT}*) in border cells, and (C) overexpressing *DIAP1* (*slbo-Gal4,UAS-dnPVR/+; UAS-hep^{ACT}/UAS-DIAP1*) or *p35* (*slbo-Gal4,UAS-dnPVR/+; UAS-hep^{ACT}/UAS-p35*) in border cells. The control flies in A and C had the genotype *slbo-Gal4,UAS-dnPVR/+ UAS-hep^{ACT}/+* and, in B, *slbo-Gal4,UAS-dnPVR/UAS-hepact*. Controls were done in parallel with each experiment.

regulated by the JNK pathway in border cells as in other tissues.

The expression of the *puc* reporter *per se*, as well the altered expression in *bsk* clones, indicated that the JNK pathway was somewhat active in border cells. Although *hep* mutant border cells were described to migrate properly (SUZANNE *et al.* 2001), we wondered whether JNK signaling would be required for border cell migration in the absence of PVR signaling. To address this question, we quantified the migration phenotype of *bsk* mutant clones generated with the MdnP system (Figure 4E). In this situation, some clusters were delayed, although the phenotype was less severe than that of *puc^{PBac3929}* with *dnPVR*. Thus the JNK pathway has some activity in border cells, and loss of JNK signaling affects migration if PVR signaling is also reduced. Overall, increased JNK signaling coupled with decreased/absent PVR signaling was quite deleterious for border cell migration (Figure 3, B, D, and E), whereas loss of function for both pathways (Figure 4E) or gain of function for both pathways (Figure 3, C and F) gave milder phenotypes without clear indication of synergy.

Fos and DIAP1 may mediate the *puc* phenotype in border cells: JNK is well known to stimulate the activity of the transcription factors Fos and Jun (in *Drosophila* encoded by *kayak/fos* and *jra/jun*, respectively; PEVERALI *et al.* 1996; ZEITLINGER *et al.* 1997). But JNK has also been shown to bind and phosphorylate cytoskeletal regulators such as Spire (OTTO *et al.* 2000). To test whether the effects observed upon overactivation of the JNK pathway were due to the transcriptional effects, we reduced the level of active Jun or Fos in border cells with

elevated JNK signaling and reduced PVR. This was achieved by coexpressing *hep^{ACT}* and *dnPVR* with either dominant-negative Jun or Fos (Figure 5A) or with RNA interference (RNAi) directed against *jun* or *fos* (Figure 5B). Reducing Fos activity, by either approach, suppressed the border cell migration phenotype induced by elevated JNK signaling. Reducing Jun activity had a mild or no suppressive effect. This suggested that the Fos transcriptional activity stimulated by the JNK pathway contributes to the border cell phenotype.

Although some studies have investigated the transcriptional changes resulting from a modulation of JNK signaling in embryos, larvae, or adult heads (JASPER *et al.* 2001; ETTER *et al.* 2005), no information is available in border cells. Given that we observed a *puc* phenotype only in the *dnPVR* context, we reasoned that the targets of JNK responsible for the border cell phenotype might be regulated by RTK signaling in the opposite direction. One general role of JNK signaling is to promote apoptosis, while RTK signaling generally promotes cell survival. Indeed, the pro-apoptotic factor Hid was shown to be positively regulated by the JNK pathway (MORENO *et al.* 2002) and negatively regulated by MAPK signaling (BERGMANN *et al.* 1998, 2002; KURADA and WHITE 1998). Hid is a regulator of DIAP1, a ubiquitin ligase that inhibits caspase activity and thereby inhibits apoptosis. DIAP1 is also essential for border cell migration (GEISBRECHT and MONTELL 2004) and an additional allele was recovered in this PBac screen. If the phenotype observed in border cells expressing *hep^{ACT}* and *dnPVR* was due to a misregulation of the *hid/DIAP1* pathway, one might expect that it could be suppressed by increasing *DIAP1*

levels. We tested this hypothesis by overexpressing *DIAP1* together with *hep^{ACT}* and *dnPVR* in border cells and found that this indeed improved their migration (Figure 5C). We next determined whether this effect of *DIAP1* was due to a simple suppression of apoptosis by expressing the caspase inhibitor *p35* instead. Expression of *p35* together with *hep^{ACT}* and *dnPVR* did not show any suppressive effect (Figure 5C). Also, border cells expressing *hep^{ACT}* and *dnPVR* were not visibly apoptotic. These results indicate that the suppression observed with *DIAP1* is specific and likely unrelated to its anti-apoptotic function.

DISCUSSION

Technical evaluation of the screen: In this study, we presented a piggyBac insertional mutagenesis coupled with a modified version of the MARCM system, called MdnP, to perform a clonal screen for border cell migration phenotypes. Due to the expression of β -galactosidase in mutant clones, we could easily detect and score mutant clones under the dissecting microscope after X-Gal staining. This allowed us to detect clones even when they were rare, which was the case for PBac104 (*aop*), PBac3929 (*puc*), and PBac4251 (*slmb*). The choice of piggyBac as a mutagen in this study was dictated by the need to directly mutagenize FRT-bearing chromosomes for immediate generation of clones. Although it was reported that piggyBac does not share hotspots of integration with the *P* element (HACKER *et al.* 2003; THIBAUT *et al.* 2004), all the mutants that we identified and studied affected genes that had already been targeted by one or more *P*-element insertions. This reflects the high degree of saturation with *P* elements but also indicates that on a genome level the mutagenic insertion sites of *P* elements and PBac elements are perhaps not so different. The presence of PBac “hotspots” was indicated by the finding that of the 10 genes identified as mutating to a border cell migration phenotype, three genes were hit twice and one gene hit three times. This is significant, considering that it was very far from a saturating screen with 3300 lines tested and a projected 660 (20%) lethal insertions. In total, 15 of 3300 tested lines, or ~0.5%, had border cell migration defects and 26, or 1%, were apparent cell lethals. In a clonal screen for border cell migration defects using EMS as mutagen (LIU and MONTELL 1999), 16 complementation groups were found on 2R, compared to three genes in our screen. From these considerations, it is likely that we have identified <20% of the genes that can mutate to give viable clones but border cell migration defects in this screen.

Among the mutants that we recovered in the border cell screen, most were located on the second chromosome. We think that this is an artificial bias introduced by the fact that the *Slbo-Gal4*, *UAS-dnPVR* combination

located on the third chromosome had a weaker effect on border cell migration than its counterpart on the second chromosome. It was therefore easier to score migration defects above background when using the MdnP40 and MdnP42 stocks. For example, mutants affecting migration only slightly (such as PBac911, PBac5201/*kis*, and PBac79/*vir*) might have looked like the background effects with the stronger *slbo-Gal4*, *UAS-dnPVR*. In addition, expression of Gal80 from chromosome arm 3L was not strong enough to fully block the activity of Gal4. This led to leaky β -galactosidase expression in some nonmutant cells and thus imperfect identification of mutant cells such that weak phenotypes might have been missed.

Designing the screen such that mutant border cells would also express *dnPVR* was intended to sensitize the mutant border cells to further mild perturbations. However, expression of *dnPVR* did not appear to generally increase the number of mutants recovered, as most of the mutants recovered had a comparable phenotype when tested in standard clones without *dnPVR*. The approach did allow us to uncover an allele of *puc*, which would not have been identified otherwise. Finally, we noted that, when analyzed, most of the PBac mutants were apparent hypomorphs with weaker phenotypes than those published for strong alleles. This includes PBac2148 (*DIAP1*), PBac3929 (*puc*), PBac378, and PBac104 (*yan/aop*) as well as PBac4354, the *shg* regulatory mutant.

The RNA-binding proteins *vir* and *hrp48*: Among the 10 genes identified in this screen as affecting border cell migration, 2 of them encode the RNA-binding proteins *vir* and *hrp48*. These two genes have been shown to be involved in the regulation of alternative splicing of *Ubx* mRNA (BURNETTE *et al.* 1999). We do not have any evidence that *Ubx* loss of function affects border cell migration, but *vir* and *hrp48* could also cooperatively regulate the splicing of other targets with important functions in border cells. In addition, *hrp48* has recently been shown to regulate mRNA localization/translation in the *Drosophila* oocyte (HUYNH *et al.* 2004; YANO *et al.* 2004). It could play a similar role in border cell migration, regulating mRNA localization or translation. Localization of β -actin mRNA has been described in chicken embryo fibroblasts, where it appears to be important for cell motility (KISLAUSKIS *et al.* 1997). RNAs encoding components of the Arp2/3 complex, a nucleator of actin assembly, have also been reported to be localized in these cells although through a different mechanism than the one responsible for β -actin mRNA localization (MINGLE *et al.* 2005). The components of the Arp2/3 complex are required for normal border cell motility (K. SOMOGYI and P. RORTH, unpublished results). Thus it is possible that the mRNAs of actin and/or some of the Arp2/3 components need to be localized and regulated by Hrp48 in border cells for proper migration.

A convergence of JNK and PVR signaling in border cells: *puc*^{PBac3929} exhibited a phenotype only in combination with *dnPVR*. Given the redundant activity of EGFR and PVR during border cell guidance, a simple explanation for the observed phenotype would be that loss of *puc* activity, and hence gain of JNK signaling, specifically blocked or perturbed EGFR signaling. Finding that *puc*^{PBac3929} mutant border cells migrated dorsally and that excess JNK signaling did not synergize with delocalized PVR signaling, we rejected this hypothesis. As we observed the most pronounced border cell migration defects in the context of reduced PVR signaling and excessive JNK signaling, it seemed likely that these two pathways had convergent but antagonistic effects on a protein or a process important for border cell motility.

In vertebrate cells and in *Drosophila*, convergence between the ERK and JNK MAPK pathways has been described at the level of the transcription factor AP-1, composed of a homo- or a hetero-dimer of Fos and Jun (PEVERALI *et al.* 1996; KOCKEL *et al.* 1997; LEPPA *et al.* 1998; CIAPPONI *et al.* 2001). In *Drosophila*, Fos was found to be phosphorylated on different sites by rl/ERK compared to bsk/JNK, and it was proposed to be in different activated states, depending on whether it was phosphorylated by ERK or JNK (CIAPPONI *et al.* 2001). One might imagine an antagonism of the two pathways on the basis of competing activities acting on a fixed pool of Fos protein. In such a scenario, one role of PVR/ERK signaling would be to block potentially excessive JNK/Fos transcriptional activity. The suppression of the *dnPVR* and *hep*^{ACT} border cell phenotype by *fos* RNAi or dominant negative (*fospZIP*) is in agreement with such a scenario. However, this is unlikely to be the explanation since strong enhancement of ERK signaling in border cells (overexpression of activated Raf) did not suppress the phenotype of *dnPVR* and *hep*^{ACT} (data not shown). Thus, the convergent but antagonistic effect of the pathways is more likely to happen downstream of Fos and Jun.

The suppression experiment suggests that DIAP1 may act as an important convergence point of PVR and JNK signaling in border cells. JNK signaling can downregulate the DIAP1 level whereas PVR signaling, through MAPK or other pathways, may increase the DIAP1 level or activity. Specifically, *DIAP1* overexpression has previously been shown to suppress JNK-induced apoptosis (IGAKI *et al.* 2002; MORENO *et al.* 2002). *DIAP1* overexpression also suppressed the weak border cell migration phenotype induced by *dnPVR* (GEISBRECHT and MONTELL 2004). In these two situations, DIAP1 acts as a negative regulator of the caspase DRONC. The function of DIAP1 in border cells is not related to apoptosis but rather to the control of actin assembly (GEISBRECHT and MONTELL 2004). Such nonapoptotic function of caspases is not unique to border cells but was also observed in sperm individualization (ARAMA *et al.* 2003, 2006; HUH *et al.* 2004; MURO *et al.* 2006). In addition, DIAP1

levels and activity in relation to control of the actin cytoskeleton can be regulated by different kinases (KURANAGA *et al.* 2006; OSHIMA *et al.* 2006). Our genetic results suggest that the antagonism of JNK and RTK signaling on DIAP1 function is not only relevant in the context of apoptosis, but also in the regulation of the actin cytoskeleton.

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