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In vivo RNAi screen identifies candidate signaling genes required for collective cell migration in *Drosophila* ovary

LUO Jun, ZUO JunTao, WU Jing, WAN Ping, KANG Di, XIANG Cong, ZHU Hong & CHEN Jiong*

Model Animal Research Center, and MOE Key Laboratory of Model Animals for Disease Study, Nanjing University, Nanjing 210061, China

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Collective migration of loosely or closely associated cell groups is prevalent in animal development, physiological events, and cancer metastasis. However, our understanding of the mechanisms of collective cell migration is incomplete. *Drosophila* border cells provide a powerful *in vivo* genetic model to study collective migration and identify essential genes for this process. Using border cell-specific RNAi-silencing in *Drosophila*, we knocked down 360 conserved signaling transduction genes in adult flies to identify essential pathways and genes for border cell migration. We uncovered a plethora of signaling genes, a large proportion of which had not been reported for border cells, including *Rack1* (*Receptor of activated C kinase*) and *brk* (*brinker*), *mad* (*mother against dpp*), and *sax* (*saxophone*), which encode three components of TGF-β signaling. The RNAi knock down phenotype was validated by clonal analysis of *Rack1* mutants. Our data suggest that inhibition of Src activity by Rack1 may be important for border cell migration and cluster cohesion maintenance. Lastly, results from our screen not only would shed light on signaling pathways involved in collective migration during embryogenesis and organogenesis in general, but also could help our understanding for the functions of conserved human genes involved in cancer metastasis.

***Drosophila*, border cell migration, signaling pathway, TGF-β, Brk, Rack1, Src42A, Src64B**

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Cell migration is critical for embryonic development, adult wound healing, and immune system function. Understanding the mechanism of cell migration under physiological and developmental conditions can help better understand the underlying cause of abnormal cell migration under pathological conditions such as tumor metastasis and inflammation. While some cells move singly, others migrate collectively as groups [1]. During gastrulation in the embryo and in epithelial sheet migration during wound healing, cells often migrate collectively. Collective migration has also been observed during tumor metastasis [1−3]. During collective migration, how individual cells receive and integrate outside signals to coordinate group migration is poorly understood. Studies in the *Drosophila* border cell migration have provided insight into the mechanisms of collective migration [2,4]. Border cell cluster is a specialized group of cells that migrate during oogenesis [4,5]. The *Drosophila* ovary contains strings of developing egg chambers. Each egg chamber is composed of an oocyte and 15 nurse cells surrounded by a monolayer of follicle cell epithelium [4,6]. Border cells are first specified and selected out from the anterior follicle cell epithelium at early stage 9 of oogenesis. The polar cells, which are a specialized pair of follicle cells at the anterior end of the egg chamber, recruit 4–8 cells surrounding the polar cells to become border cell cluster. Border cells detach from the follicle cell epithelium and migrate between the nurse cells over ~150 μm and reach the oocyte border by stage 10 (Figure 1A).

*Corresponding author (email: chenjiong@nju.edu.cn)

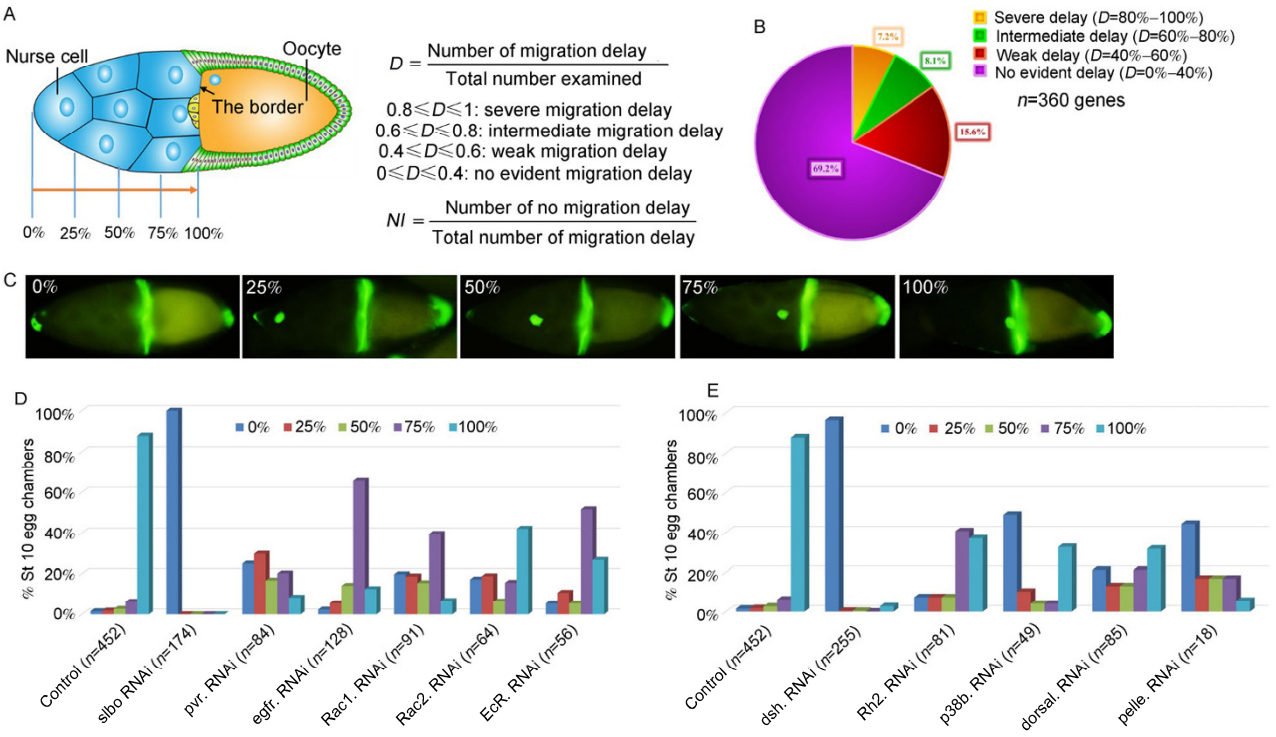

Figure 1 Overview of the screen. A, A diagram of border cell migration, the method of quantification of border cell migration delay and the classification of delay phenotype. The extent of migration for all stage 10 egg chambers examined was categorized as 0% (no migration), 25%, 50%, 75%, or 100% (reaching the border) for all quantitative analysis of border cell migration. B, Pie chart results from the RNAi screen. C, Typical samples from 0%, 25%, 50%, 75% and 100% border cell migration categories. D, Quantification of border cell migration for RNAi of selected genes. These genes have been reported to be required for border cell migration [7–12]. E, Quantification of border cell migration for RNAi of previously unreported signaling genes.

So far, at least five known signaling pathways have been extensively studied and shown to be required for different aspects of border cell migration. Steroid hormone signaling pathway coordinates the timing of migration [12,13]. Ecdysone activates the heterodimer of the nuclear hormone receptor ecdysone receptor (EcR) and ultraspiracle (USP), both of which are necessary for border cell migration [12]. JAK/STAT signaling pathway specifies border cell identity and promotes expression of migratory genes [14–17]. The cytokine in JAK/STAT signaling, unpaired (Upd), is secreted by the two polar cells. Only 4–8 cells closest to the polar cells differentiate to border cells, thus the number of border cells is controlled by STAT levels. PDGF and VEGF-related receptor (PVR) and epidermal growth factor receptor (EGFR) signalings guide the chemotactic movement of border cells to the oocyte, in response to gradients of extracellular guidance factors including PVF and EGFR ligands [8–10,18]. The guidance function of PVR is redundant with that of EGFR. Jun amino-terminal kinase (JNK) signaling pathway regulates cell adhesion between border cells to maintain the cluster during migration [19,20]. Down-regulation of JNK signaling causes the cohesive cluster of border cells to dissociate [19]. The GTP exchange factor (GEF) Myoblast city and the small GTPase Rac are required for border cell migration and they act downstream of PVR [9]. Recently, Hedgehog signaling has been reported to interact with the small GTPase Rac and the polarity protein Par1 in border cell migration, but the regulating mechanism is unknown [21]. Though these five signaling pathways have been reported to regulate border cell migration, many gaps remain in our understanding of signaling regulation. Whether other signaling pathways contribute to border cell migration and how all of these signals are integrated to regulate border cell migration are largely unknown.

The TGF-β signaling plays major roles in many developmental processes in *Drosophila*, but its roles in border cell migration are not reported. The major ligand of the TGF-β signaling in *Drosophila* is Decapentaplegic (Dpp) [22]. In the *Drosophila* ovary, Dpp is expressed in the anterior 20–30 follicle cells from stage 8 egg chambers. In late stage egg chambers, Dpp is expressed in all anterior follicle cells, including the stretched cells, the border cells, and the centripetal cells. Dpp protein forms a gradient from anterior to posterior in the follicle cells surrounding the oocyte [23]. Brinker is the target protein of the *Drosophila* Dpp morphogen signaling pathway. In *Drosophila*, two mechanisms have been proposed in the activation of Dpp target genes. One is through Dpp-dependent receptor activation which induces phosphorylation of Mad. Then Mad forms a heteromeric complex with Medea (Med) in the nucleus and functions as an activator [24]. The other one is through inhibit-

ing Brinker (Brk), a transcriptional repressor [25–27]. In fly wing development, Dpp is expressed in a narrow stripe across the anterior/posterior (A/P) boundary and Brk's expression pattern complements with that of the Dpp gradient. Brk is repressed by Dpp through a Schnurri-Mad-Med repressing complex [28]. On the other hand, Brk is also able to repress TGF-β target gene transcription by binding to Mad's binding sites and competing with Mad [29,30]. Lastly, Brk can recruit transcriptional co-repressors C-terminal binding protein (CtBP) and Groucho (Gro) via CtBP- and Gro-interaction motifs (CiM and GiM) [31,32].

PKC signaling pathway is conserved from yeast to human. It modulates integrins and chemokine response, and regulates cell adhesion, migration, differentiation and proliferation [33]. The PKC anchoring protein Rack1 (receptor for activated C kinase 1) has been described to play a role in diverse processes based on cell culture studies, including cell adhesion, migration [34–38], apoptosis, cell survival [39–42], cell growth [43], and protein translation [44–47]. Diverse protein-binding partners involved in key signaling pathways, such as PKC, Src, Integrin, IGF-I receptor, PP2A and FAK, are reported to bind to Rack1 *in vitro* [35,48–54]. The loss-of-function phenotypes of *Rack1* in *Drosophila* have been reported and they included reduction of the size of ovary and fewer germ cells per egg chamber in the *Rack1* mutants, suggesting that Rack1 may function in cell division [55]. But the *in vivo* function of Rack1 in cell migration is unknown.

Src proteins couple RTKs (receptor tyrosine kinases), integrin and GPCR (G-protein coupled receptor) to intracellular signaling pathways and regulate cell division and cell motility [56]. Interaction between Rack1 and Src has been implicated in the regulation of cell proliferation and migration in cell culture studies [36,43]. Rack1 has been reported to bind to Src [50,57,58], which in turn phosphorylate Rack1 itself, resulting in eventual negative regulation of Src activity [36,57,59]. Src activation promotes cell cycle entry, cytoskeletal rearrangements, and alterations in cell adhesion [60]. Activated Src induces the EMT (epithelium to mesenchyme transition) process through tyrosine phosphorylation of adherens junction components (the cadherin/catenin system) or acting by phosphorylating protein regulators of adherens junction [61]. The *Drosophila* genome contains two Src family kinases, Src42A and Src64B, which are functionally similar to their mammalian counterparts [62,63]. Src42A is the closest relative of vertebrate Src in *Drosophila* and functions redundantly to Src64B, and it is found to genetically interact with *D*E-cadherin and Armadillo [64]. In *Drosophila* oogenesis, strong Src42A expression is evident in invading and migrating border cells [64]. However, the function of Src in border cell migration is unknown.

Loss-of-function screens, including EMS induced mutagenesis, had been performed in the past, and they identified many of the important genes required for border cell migration, such as Slbo, Pvf1, Apontic, Par1, Taiman, Jing, Psidin, Stat92E, Puckered, and Sec3 [7,12,14,15,65–69]. To bypass early lethality (earlier requirement of the genes essential for the survival of embryos and larva), these screens were often clonally (mosaic) based and utilized FRT/FLP technique. However, one of the drawbacks of this approach is that mosaic clones encompassing entire border cell clusters are not always easily obtained. Furthermore, it is time-consuming to map EMS-induced mutation and to clone the affected genes. Alternatively, tissue-specific RNAi is a fast and efficient way to perform a loss-of-function screen in *Drosophila*. Here, we performed an *in vivo* RNAi screen for candidate genes in major signaling pathways required for border cell migration. Overall, we isolated 111 candidate genes required for border cell migration from this screen. Among them, *Rack1* and three TGF-β signaling components, *sax*, *mad* and *brk* were characterized in more details.

1 Materials and methods

1.1 *Drosophila* genetics

Flies were cultured following standard procedures at 25°C except for RNAi experiments at 29°C. Most of the RNAi stocks were obtained from Japan NIG-fly stock center and some were obtained from Vienna *Drosophila* Resource Center (VDRC). Other strains were obtained from the Bloomington *Drosophila* Stock Center. To generate *UAS-Rack1.GFP* transgenic line, we subcloned a full-length cDNA of the *Rack1* gene into a modified pUAST-attB vector with C-terminal GFP tagging. The resulting *UAS-Rack1.GFP* construct was injected into embryos according to standard procedures. Mutant *FRT* clones were induced using *hs-FLP*. Flies were heat shocked for 1 h per day at 37°C for 3 d before eclosion, then fed with yeast and dissected 2–3 d after the last heat shock. *slbo-Gal4* specifically drives expression in border cells, and has been extensively used by many labs to over-express genes in the border cells [70]. *UAS-Dicer2* was used along with *UAS-Candidate gene RNAi* to enhance RNAi efficiency. Culture temperature of 29°C was used to enhance the activity of the UAS/Gal4 system without affecting survival. Lastly, *UAS-GFP* was used to mark the border cell clusters.

1.2 Screening system

The scheme of the screening was described below. Crossing of virgin females (*UAS-Dcr2*;*slbo-Gal4*,*UAS-GFP/CyO*) with 2–3 males from *UAS-RNAi* lines were set at 25°C. *UAS-Dcr2*;*slbo-Gal4*,*UAS-GFP/CyO* was outcrossed to w1118 and used as controls. Two to three days post eclosion, female adult flies from F_1 generation were shifted to 29°C to enhance Gal4 activity and RNAi expression. After 3 d, ovaries of the female flies were dissected in PBS, fixed in 7% paraformaldehyde for 10 min at room temperature, rinsed in PBS+0.3% Triton X-100 and PBS. Fixed ovaries

were manually dissociated in 80% glycerol. *UAS-GFP* fluorescence was used to visualize border cells in dissociated ovaries. Analysis of border cell migration was performed with an Olympus BX51 fluorescent microscope.

The quantification and categories of the phenotype are described below. The extent of migration for all stage 10 egg chambers examined was categorized as 0% (no migration), 25%, 50%, 75%, or 100% (reaching the border) for quantitative analysis of border cell migration (Figure 1A and C). The *D* value is calculated as the number of border cell migration delay egg chambers divided by the total number of examined egg chambers. The *D* value between 0.4 and 0.6 was considered weak migration delay phenotype; the *D* value between 0.6 and 0.8 was the intermediate phenotype; the *D* value between 0.8 and 1 was considered the severe phenotype (Figure 1A). NI was calculated as the number of stage 10 egg chambers exhibiting border cell non-invasion divided by the total number of stage 10 egg chambers exhibiting migration delay. If the $NI \geqslant 0.4$, we classified the genes into the invasion defective group.

1.3 Immunohistochemistry and microscopy

Ovary dissection was carried out in phosphate-buffered saline (PBS) and then fixed in Devitellinizing buffer (7% formaldehyde) and heptane (Sigma) mixture (1:6) for 10 min. After washes in PBS, ovaries were incubated in blocking solution (PBT, 10% goat serum) for 30 min and then stained overnight at 4°C. Primary antibodies and their concentrations were as follows: rat anti-*D*Ecad (DCAD2, 1:50, DSHB). After washes in PBT, ovaries were incubated with secondary antibodies (Jackson ImmunoResearch) for 2 h at room temperature. F-actin was labeled by rhodamine phalloidin (1:100; Sigma). DNA was labeled by DAPI (1:1000; Sigma). Confocal images were obtained using a Leica TCS SP5 II or an Olympus FV1000 confocal microscope.

2 Results

2.1 Overview of the RNAi screen

To identify novel candidate signaling molecules for border cell migration, we conducted a border cell-specific RNAi silencing screen of signaling pathway genes that we identified from the GO (Gene Ontology) Term list in the FlyBase website (http://flybase.org) (Table S1 in Supporting Information).

The components of major signaling pathways in *Drosophila* were selected for this RNAi screen. We restricted our screen to 572 *UAS-RNAi* lines corresponding to 360 genes in a variety of major signal transduction pathways, including Wnt, TGF-β, Hh, Integrin, Toll, G protein coupled receptor (GPCR), Notch, small GTPases, stress, and apoptosis signaling pathways. About half of these genes had two corresponding RNAi lines in this screen. We used *D* (delay) value to quantify the border cell migration delay phenotype (Figure 1A). *D* value reflected the proportion of the migration delay border cell clusters. A *D* value of 1.0 means that 100% of stage 10 egg chambers examined display various extents of migration delay, which is characterized by failure of border cells reaching the border of oocyte. Of the 360 genes, we found about 70% of genes had no significant migration delay (*D* value below 0.4) when knocked down, 15% had weak migration delay phenotype ($0.4<D<0.6$), 8% had intermediate phenotype ($0.6<D<0.8$) and 7% had severe phenotype ($0.8<D<1.0$; Figure 1B). Next, we subdivided the migration delay genes into two groups by the border cell cluster's detachment or invasion ability. We used *NI* (noninvasion) value to measure the border cell cluster's detachment or invasion defect (Figure 1A). *NI* value reflected the proportion of the no migration clusters among migration delay border cell clusters. An *NI* value of 1.0 means that all the migration delayed stage 10 egg chambers display a complete lack of migration by border cells. These border cells still remained attached to the anterior end of egg chamber and appeared not to invade and initiate migration between underlying nurse cells. Therefore, we would define such a defect as a non-invasion phenotype. An *NI* value equal or greater than 0.4 means a significant invasion defect. From comparing the *D* value and the *NI* value, we found that significant invasion defects ($NI \geqslant 0.4$) were often associated with severe migration delay phenotypes ($0.8<D<1.0$), and most of the transcription regulator encoding genes isolated from this screen showed invasion defects ($NI \geqslant 0.4$) when knocked down (Table 2), suggesting that the transition from border cells' non-invasive state to the invasive (early migratory) state requires much transcriptional activity.

Overall, we uncovered 111 candidate genes required for border cell migration from this screen (Table 1). Six of these genes were previously found to affect border cell migration, including *slbo*, *pvr*, *egfr*, *Rac1*, *Rac2* and *Ecdysone receptor*, validating the efficacy of our screen. (Figure 1D). For example, 87.5% of *egfr* RNAi stage 10 egg chambers displayed border cell migration delay (Figure 1D). Of those with migration delay, three out of four stage 10 egg chambers are in 75% migration category, suggesting that the EGFR signaling mainly promotes the later stage of border cell migration, which is consistent with the previous report [8]. More importantly and interestingly, we have found for the first time that RNAi of some key components of various signaling pathways caused migration delay, such as *dsh* (*disheveled*), *Rh2* (*Rhodopsin 2*), *dorsal*, *p38b* and *pelle* (*pll*) (Figure 1E). Dishevelled (Dsh) is a key component of Wnt signaling. Dorsal and Pelle are components of Toll signaling. Dorsal is a transcriptional factor and Pelle is a Serine/Threonine protein kinase. p38b is a MAPK (mitogen-activated protein kinase). Rh2 is a G-protein coupled receptor. Furthermore, diverse receptors, transcription regulators and kinases are implicated for the first time to func-

Table 1 Summary of the selected genes and the screen result[a)]

Signaling	Wnt	Hh	TGFb	Toll	Notch	GPC[1]	Stress	Apo[2]	NT[3]	SG[4]	Other	Total
Gene tested	28	23	23	28	17	12	16	15	20	24	154	360
Gene (D≥40%)	11	7	6	11	8	8	4	4	2	6	44	111
Gene (NI≥40%)	7	4	3	6	6	2	3	3	2	1	23	60

a) 1, GPC: G protein coupled signaling; 2, Apo: apoptosis signaling; 3, NT: neurotransmitter transporter; 4, SG: small GTPase.

Table 2 Molecular function categories of the uncovered candidate genes

Class	Receptor	Transcription regulator	Kinase	Small GTPase	Ligand	mRNA binding	Protein binding	Transp orter	Other	Total
Gene (D≥40%)	18	20	11	6	6	3	15	3	29	111
Gene (NI≥40%)	6	16	8	0	2	2	6	3	17	60

tion in border cell migration (Table 2). Below we described two signaling pathways recovered from the RNAi screen, the TGF-β signaling and Rack1-mediated PKC signaling.

2.2 RNAi knockdown of *brk*, *mad* or *sax* affects border cell migration

The TGF-β signaling components, Brk (Brinker), Mad (Mothers against dpp), and Sax (Saxophone), were uncovered from the RNAi screen. Brk is a transcriptional repressor and a target protein of the *Drosophila* Dpp morphogen signaling pathway. Inhibiting Brk can activate Dpp target genes. We found that knockdown of *brk* caused very strong border cell migration defect (Figure 2C and F). In *brk* RNAi, all of the border cell clusters (D=1.0) failed to reach the border and most of them (NI=0.82) had invasion defect, suggesting that *brk* was required for border cell invasion process probably by inhibiting the expression of Dpp target genes. Furthermore, the high values of both D and NI suggest that *brk* may be required for the initial invasive stage of border cell migration. Mad is a receptor-regulated Smad (R-Smad) in *Drosophila*. In *mad* RNAi, about 60% (D=0.56) stage 10 egg chambers showed border cell migration defect (Figure 2D and F), and the NI value (0.19) did not indicate a significant invasive defect, suggesting that *mad* is required in the mid-migratory phase of the border cell migration rather than in the initial phase. We did not find that other Smads were required for border cell migration in this screen, implying that border cell migration was probably regulated by non-canonical signaling functions of Mad. Sax is a type I receptor in *Drosophila*, which can propagate the signal through phosphorylation of the Smad proteins. In *sax* RNAi, 63% (D=0.63) of stage 10 egg chambers showed border cell migration defect (Figure 2E and F), while NI value is low (0.27), suggesting that like *mad*, *sax* is also required in the mid-migratory phase of the migration process. Taken together, results from *brk*, *mad* and *sax* indicate that keeping a proper level of TGF-β signaling during different stages (invasive and migratory stages) is essential for border cell migration. Over-activation of TGF-β signaling by inhibiting Brk during the initial invasive stage and down-regulation of TGF-β signaling by Mad deficiency caused distinct migration defects.

2.3 Rack1 is required for border cell cluster migration and organization

Another candidate gene that we further analyzed in details was *Rack1*. Interaction between Rack1 and Src has been implicated in the regulation of cell proliferation and migration in cell culture studies [36,43]. Rack1 has been previously reported to negatively regulate Src activity [36,57,59]. In our RNAi screen, we found knockdown of *Rack1* caused moderate delay of border cell migration (D=0.29, NI=0; Figure 3C and H), suggesting that *Rack1* was required for the migratory process of border cell cluster, rather than the early invasion and delamination stage of border cells. To validate the result from RNAi knockdown, we generated *Rack1* mutant clones in border cells. Three *Rack1* mutant alleles have been reported [55]. $Rack1^{EY128}$ is a null allele in which a P-element is inserted 53 bp upstream of the translation start site. $Rack1^{EE}$ is a hypomorphic allele which expresses S81F mutant protein at a much lower level. $Rack1^{1.8}$ is a null allele which changes glutamine 6 to a stop codon. About 30% of the mosaic border cell clusters containing $Rack1^{1.8}$, $Rack1^{EE}$ or $Rack1^{EY128}$ homozygous clones displayed migration delay (Figure 4A–C″ and E). Thus, results from both RNAi and genetic mosaic analysis indicate that *Rack1* is required for border cell migration.

In *Rack1* knockdown or *Rack1* mutant border cells, we found abnormal arrangement of border cell cluster with one or more individual cells trailing behind the main cluster

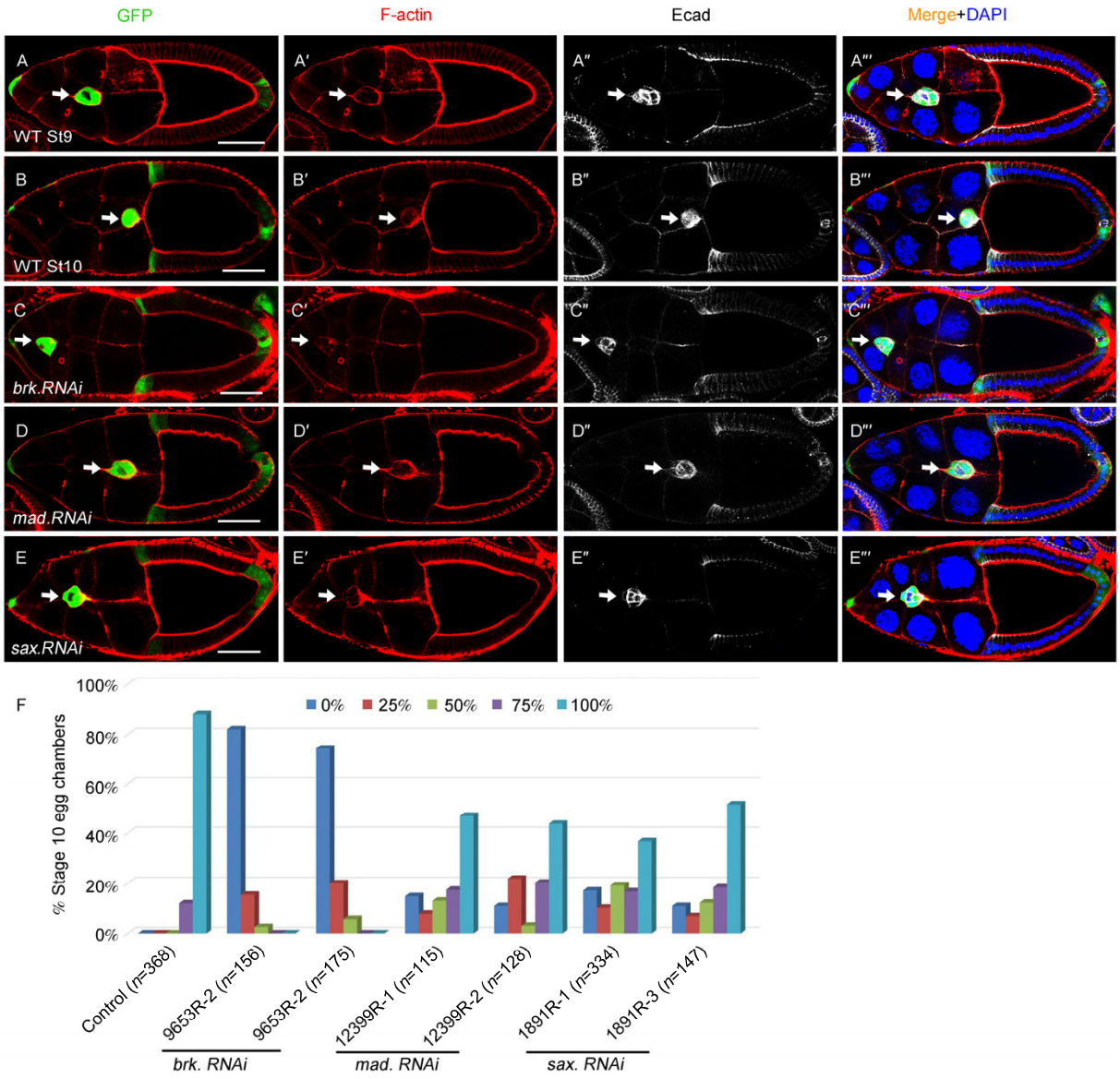

Figure 2 TGF-beta signaling components *brk*, *mad* and *sax* are candidate genes required for border cell migration. A–A‴, Wild-type mid stage 9 egg chamber showing border cell migrating through the nurse cells. B–B‴, Wild-type stage 10 egg chamber showing border cell reaching the border between nurse cells and oocyte. C–C‴, Border cell migration is severely delayed when *brk* is knocked down. D–D‴, Border cell migration is delayed when *mad* is knocked down. E–E‴, Border cell migration is delayed when *sax* is knocked down. F, Quantitation of border cell migration. The *y*-axis denotes the percentage of stage 10 egg chambers examined for each genotype that exhibits each degree of migration, as represented by the five color-coded bars for each genotype. Arrows indicate the border cells. F-actin is labeled by rhodamine phalloidin. Scale bar, 50 μm.

(Figure 3C–E‴). The expression pattern of the adherens junction protein E-cadherin was affected in *Rack1* knockdown border cell clusters (Figure 5). The dissociated cluster phenotype and the affected adherens junctions suggest that Rack1 functions in regulating cell adhesion between border cells, which has not been previously reported. Activated Src has been reported to induce EMT process through tyrosine phosphorylation of adherens junction components (the E-cadherin/β-catenin system) or by phosphorylating regulators of adherens junctions [61]. We found that overexpression of Src42A.CA (constitutively active form) or Src64B caused strong migration delay and abnormal arrangement (dissociated cluster) of border cells, which was similar to the *Rack1* loss-of-function phenotype (Figure 3F–H). These results suggested that Src activity was regulated by Rack1 in border cells. Our data suggest that in *Rack1* deficient border cells, Src activity was not inhibited by Rack1 and the activated Src induced reduction of cell-cell junctions between border cells, which resulted in dissociated border cells. Therefore, we hypothesize that Rack1 negatively regulates Src activity to stabilize cell-cell junctions and promotes cell migration. Lastly, we generated the *UAS-Rack1-*

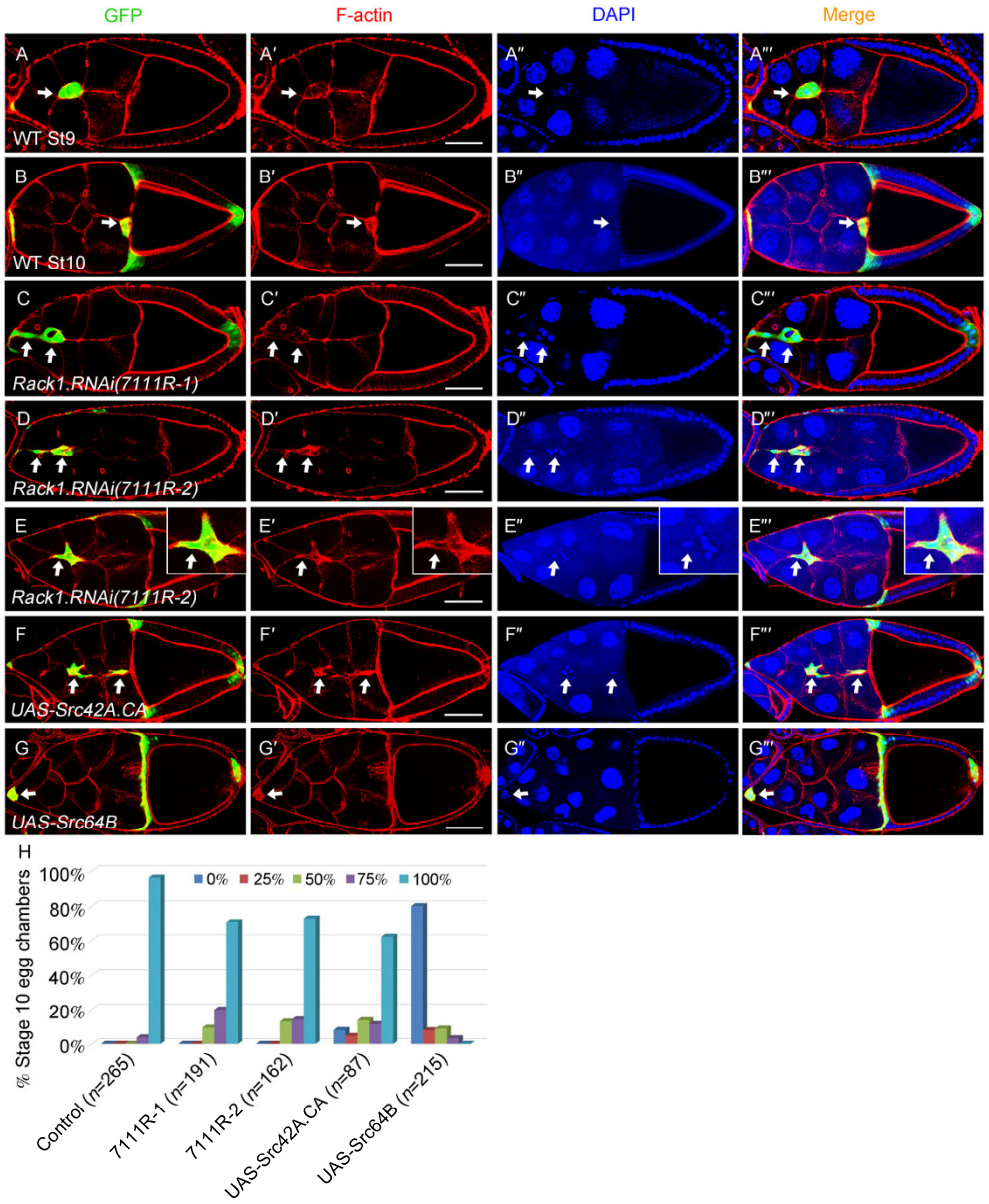

Figure 3 Rack1 is required for border cell migration and cluster cohesion. A–A‴, Wild-type stage 9 egg chamber shows border cell migration. B–B‴, Wild-type stage 10 egg chamber shows border cells reaching the border. C–E‴, Border cell cluster cohesion is disrupted when *Rack1* is knocked down. F, Border cell cluster arrangement is disrupted when *Src42A.CA* is overexpressed. G, Border cell migration is delayed when Src64B is overexpressed. H, Quantitation of border cell migration for each genotype. Arrows indicate the border cells. F-actin is labeled by rhodamine phalloidin. Scale bar, 50 μm.

GFP transgenic fly to analyze the subcellular localization of Rack1. *slbo-Gal4* was used to drive its expression in border cells. We found that Rack1-GFP was enriched in cell-cell junctions, suggesting that Rack1 was probably involved in cell-cell adhesion or cell-cell communication.

3 Discussion

Here, we present an *in vivo* RNAi border cell screen for a collection of *Drosophila* signaling genes. We uncovered a plethora of signaling genes, a large proportion of which had not been previously reported for border cell migration. Among these, 18 genes encode for receptors, and they include Wnt signaling receptor *fz3*, type I TGFβ receptor *sax*, type II TGFβ receptor *wit* and G protein coupled receptors *Rh2* and *GRHR*, suggesting that border cell migration could be regulated by diverse signaling from extracellular factors. According to our data, there is not any single pathway that has all of their components isolated from the screen. Firstly, this implies that *slbo-Gal4* driven RNAi may not be sensitive enough to isolate all genes in our case. After all, the effective time window for *slbo-Ga4* expression only last for about 6 h, spanning from early stage 9 to late stage 9. Thus,

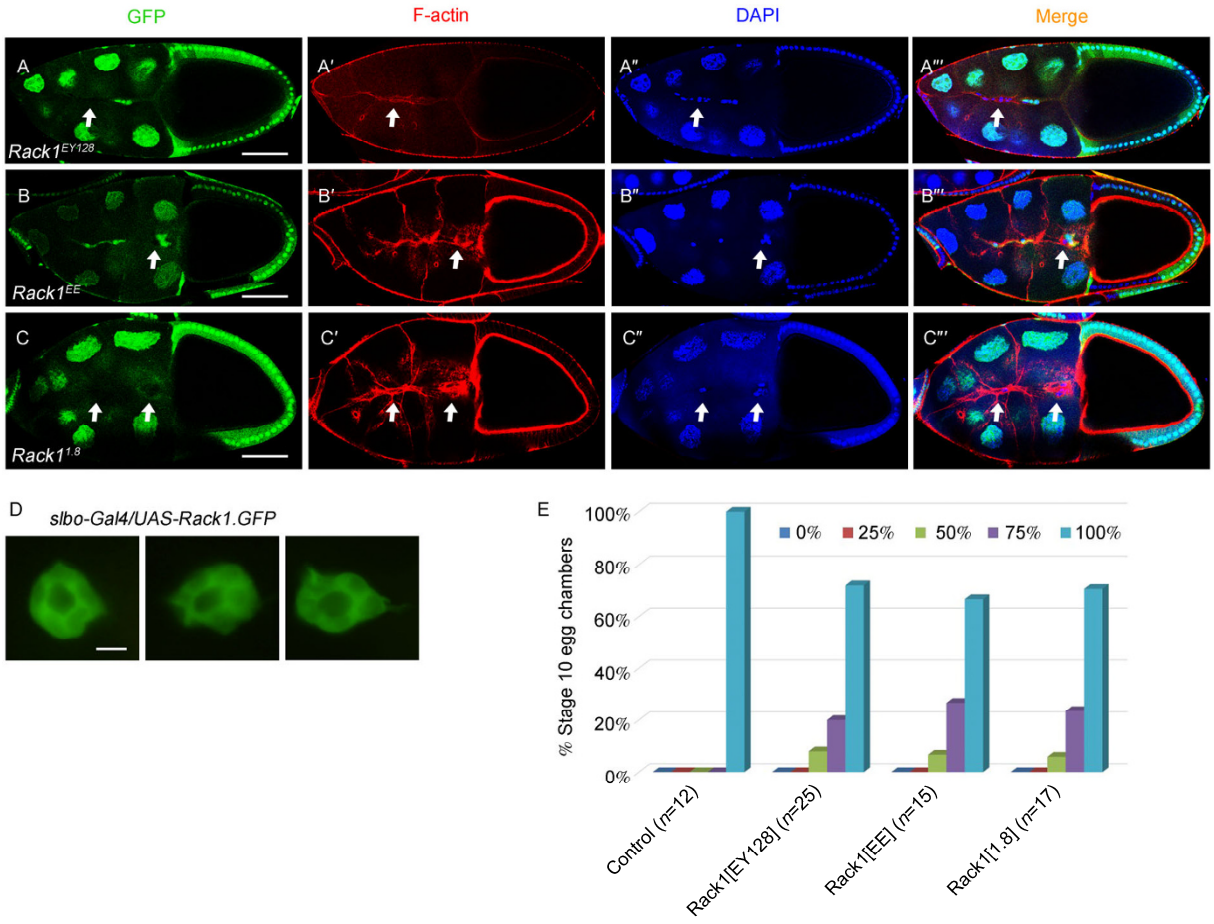

Figure 4 Validation of the *Rack1* knockdown phenotype. A–C‴, Border cell migration is delayed for border cell cluster including *Rack1EY*^{128} mutant clones (A–A‴), *Rack1*^{EE} mutant clones (B–B‴), or *Rack1*$^{1.8}$ mutant clones (C–C‴). Arrows indicate the mutant border cells. D, The expression pattern of *UAS-Rack1.GFP* in border cells. E, Quantitation of border cell migration in *Rack1* mutant clones. F-actin is labeled by rhodamine phalloidin. Mutant clones are marked by the lack of GFP. Scale bar, 50 μm in A–C‴, and 10 μm in D.

during this small time window, the level of knockdown may not be effective enough for all genes tested. Secondly, this implies that border cell migration could be regulated by novel function of individual signaling components or by the non-canonical signaling functions of these components.

Transcription regulators have been reported to be critical for border cell migration, such as Slbo, STAT, Taiman, Apontic and Abrupt [7,12,13,15,68]. From our screen, we found that knockdown of 20 transcription regulator encoding genes caused border cell migration delay and 16 of them also caused border cell cluster invasion defects, such as *brk*, *CtBP*, *CHES-1-like*, and *osa*. CHES-1-like is a checkpoint suppressor homologue and is related to Foxn2 and Foxn3, the mouse counterpart of human CHES1 [71]. Osa is a chromatin-remodeling protein and interacts with Cyclin E in *Drosophila* eye imaginal discs [72]. Most of them have not been reported to be required for border cell migration. This result suggests that transcription regulators mainly drive the detachment or invasive stage of border cell clusters.

One pathway we recovered was the Sax, Mad and Brk from TGF-β signaling. TGF-β is an inducer of EMT process [73]. In cancer progression, EMT was associated with tumor invasiveness. During border cell migration, the invasion of border cell cluster is an EMT-like process. From the RNAi screen, we found that TGF-β signaling may also contribute to the invasion of border cell clusters. However, not all TGF-β signaling pathway components are uncovered in the RNAi screen, suggesting that TGF-β signaling regulates border cell migration probably through a non-canonical fashion.

Another pathway we identified was the Rack1-Src pathway. Border cell migration analysis in *Drosophila* shows that RNAi knockdown of *Rack1* or overexpression of *Src* results in border cell migration and cluster cohesion defects. Consistently, *Rack1* loss-of-function mosaic clones in border cell clusters resulted in similar phenotypes, suggesting that the inhibition of Src activity by Rack1 in border cells is critical for migration and cluster cohesion, probably through regulating cell-cell adhesion between border cells or between border cells and nurse cells.

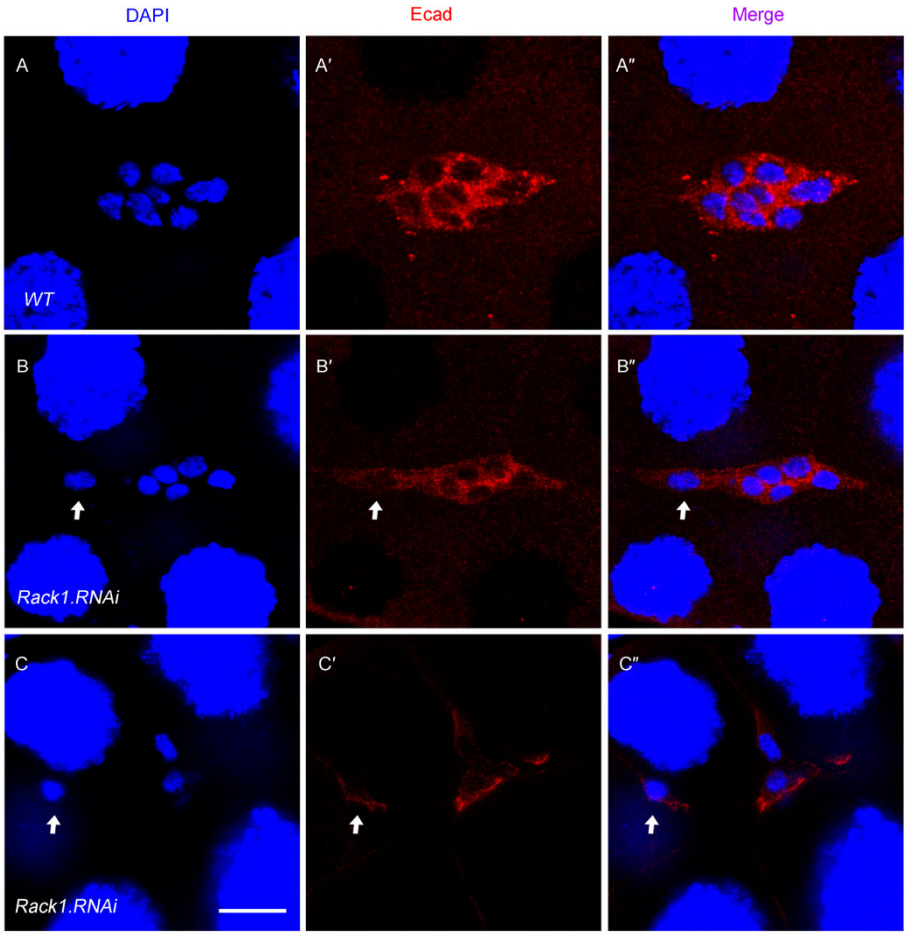

Figure 5 E-cadherin staining is affected in *Rack1* RNAi border cell clusters. A–A″, E-cadherin expression pattern in the wild-type border cell cluster. B–C″, E-cadherin level is significantly reduced in border cell cluster that is split up (B–B″) or in cluster that is still coherent (C–C″). DAPI labels the nuclei of border cells and the large nuclei of the surrounding nurse cells. Arrows indicate the split border cell in C–C″ and the border cell that lag behind in B–B″. Scale bar, 10 μm.

Lastly, we demonstrate that RNAi-based forward screen is a fast and efficient way to uncover candidate genes for border cell migration. However, all these candidate genes still need to be further validated and confirmed in the future by using loss-of-function mutations in mosaic analysis as we have done for *Rack1*.

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Supporting Information

Table S1 Primary RNAi screen results and signaling classification of the candidate genes identified by the RNAi screen.

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