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The Ste20 kinase *misshapen* is essential for the invasive behaviour of ovarian epithelial cells in *Drosophila*

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Stationary-to-migratory transitions of epithelial cells have a key role in development and tumour progression. Border cell migration is a powerful system in which to investigate this transition in living organisms. Here, we identify the Ste20-like kinase *misshapen* (*msn*) as a novel regulator of border-cell migration in *Drosophila*. Expression of *msn* in border cells is independent of the transcription factor slow border cells and of inputs from all pathways that are known to control border-cell migration. The *msn* gene functions to modulate the levels and/or distribution of *Drosophila* E-cadherin to promote the invasive migratory behaviour of border cells.

INTRODUCTION

Cell migration has a key role in embryogenesis and in adult organisms. It is a complex process, the first step of which is often a stationary-to-migratory transition of epithelial cells. This transition involves changes in cell adhesion and polarity as well as the formation and extension of cellular protrusions in the direction of movement.

The border cells of the *Drosophila* ovary provide a useful system in which to study the mechanisms that control migration *in vivo* (Rorth, 2002; Montell, 2003). *Drosophila* oogenesis proceeds through 14 morphologically distinct stages (S1–S14). Each egg chamber contains one oocyte and 15 nurse cells surrounded by a monolayer of follicle cells, known as the follicular epithelium. Border cells are determined at the anterior pole of the follicular epithelium, and by S9 they delaminate and migrate posteriorly between the nurse cells until they contact the oocyte at S10 (Fig 1A–C). This migration is synchronized with the posterior movement of the remaining follicle cells. Finally, border cells migrate towards the dorsal side of the egg chamber.

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Border-cell migration is highly regulated; the migratory cluster is first specified as a group of two polar cells in a central position that, at S8, recruit 6–8 outer border cells. Once the cluster has formed, border cells acquire a motile polarity and become migratory through the reorganization of cell polarity molecules such as Fasciclin-II, Discs large (Dlg), Lethal (2) giant larvae, Par6 and Bazooka (Goode & Perrimon, 1997; Pinheiro & Montell, 2004; Szafranski & Goode, 2004). Border cells subsequently break contact with neighbouring cells, delaminate from the follicular epithelium and initiate migration as a cohesive group.

Slow border cells (s*lbo*), which codes for the *Drosophila* homologue of the CCAAT-enhancer-binding proteins (C/EBP) transcription factor, is a key regulator of border-cell migration. It is expressed in border cells before and during their migration, is essential for their motility and controls the expression of most genes required for their migration including *shotgun* (*shg*), the gene encoding *Drosophila* E-cadherin (*D*E-Cad) (Montell *et al*, 1992; Rorth *et al*, 2000). Regulation of *D*E-Cad is crucial for border cells, as a reduction or an increase in *D*E-Cad levels results in migration defects (Niewiadomska *et al*, 1999; Schober *et al*, 2005).

Four main signalling pathways are involved in border-cell determination and migration: Janus kinase/signal transducer and activator of transcription (Jak/Stat), ecdysone receptor, and the epidermal growth factor receptor (EGFR) and platelet-derived growth factor/vascular endothelial growth factor receptor (PVR) pathways. The Jak/Stat pathway is required for recruitment of outer border cells, acquisition of a motile status and migration itself (Silver & Montell, 2001; Silver *et al*, 2005). It acts both upstream and in parallel to Slbo to regulate the activity of genes essential for border-cell migration (Silver & Montell, 2001; Beccari *et al*, 2002). The timing of migration seems to be regulated by the ecdysone pathway (Bai *et al*, 2000). Finally, the EGFR and the PVR pathways act redundantly to guide border cells towards the oocyte (Duchek & Rorth, 2001; Duchek *et al*, 2001).

We aimed to isolate new genes required for border-cell migration, and identified the Ste20-like kinase *misshapen* (*msn*) as an essential gene in their migration. *msn*⁻ border cells accumulate ectopic *D*E-Cad, and a reduction in *D*E-Cad function ameliorates the migration defects of *msn*-mutant border cells, we therefore propose that *msn* regulates *D*E-Cad turnover to facilitate their migration.
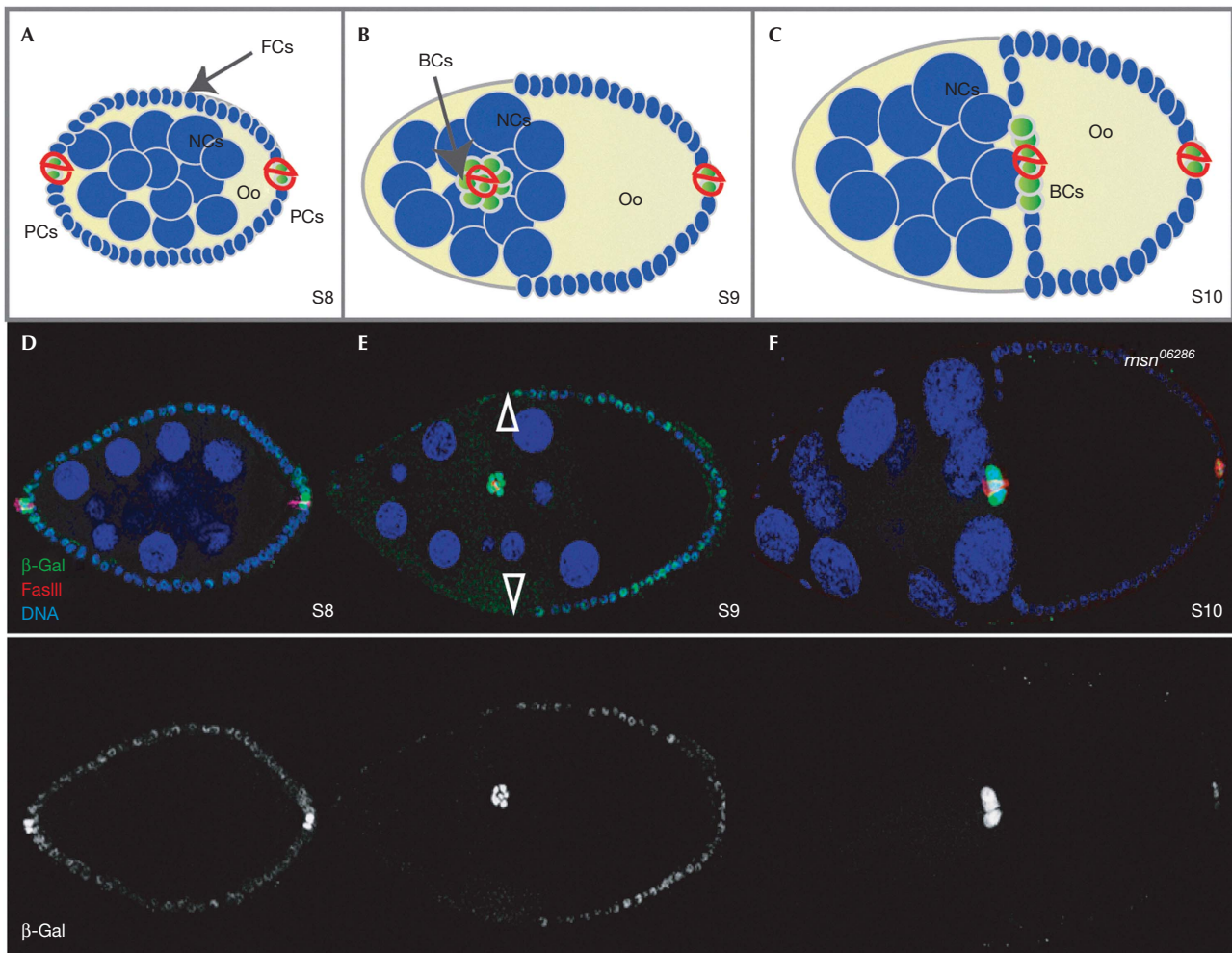
Fig 1 | Ovarian expression of *misshapen*. (**A–C**) Illustration of stage 8 (S8), S9 and S10 egg chambers. NCs, Oo, FCs, PCs (red) and BCs (green) are indicated. (**D–F**) Egg chambers from the *msn*[06286] line stained for β-gal activity (green), the PC marker Fasciclin-III (FasIII; red) and the nuclear marker TO-PRO-3 (blue). Note that the border-cell cluster moves evenly with the main body follicle cells (empty arrowheads in **E**). BC, border cell; FC, follicle cell; β-gal, β-galactosidase; Fas III, Fasciclin-III; NC, nurse cell; Oo, oocyte; PC, polar cell.

RESULTS

msn is required for border-cell migration and recruitment

To examine the requirements for *msn* in border-cell migration, we first examined *msn* expression during oogenesis using lacZ enhancer trap lines *msn*[06286], *msn*[S138716] and *msn*[j1E2] (Fig 1 and data not shown; Treisman *et al*, 1997). In all cases, from S2 to S8, *msn-lacZ* is detected at low levels in all follicle cells and at high levels in anterior and posterior polar cells. At S9, *msn-lacZ* is upregulated in the border-cell cluster during migration. The low levels present in the follicular epithelium gradually disappear at S9/10 (Fig 1D,F). Next, we analysed mosaic egg chambers containing clones of *msn*-mutant cells. We refer to 'mutant' clusters when all border cells are homozygous for a given mutant allele and to 'mixed' clusters when only a proportion of them are homozygous. Although wild-type border cells reach the oocyte at S10 (Fig 2A; supplementary Movie S1 online), we found that migration was completely blocked in all of the mosaic S10 egg chambers containing *msn*⁻ border-cell clusters and these cells remained at the anterior pole of the egg chamber (n = 58; Fig 2B,D). In addition, migration was blocked in 89% of the S10 egg chambers

with mixed border-cell clusters (n = 36; Fig 2D; supplementary Movie S2 online). The remaining 11% of mixed clusters showed a delayed migration, with cells advancing only half the distance to the oocyte (Fig 2C,D). In the latter cases, wild-type border cells were found at the front of the migrating cluster and the mutant cells were behind (Fig 2C). This phenomenon has also been observed in other mutants involved in border-cell migration, such as *shg* and *tai* (Niewiadomska *et al*, 1999; Bai *et al*, 2000).

In addition to the migration defects, *msn*⁻ clusters on average contained significantly fewer outer border cells compared with wild type (5 ± 0.6 (n = 30), *msn*⁻ clusters: 3 ± 0.9 (n = 25); Fig 2B,E). Together, the above results show that *msn* is required for proper border cell migration and cluster recruitment. Here, we focus on the role of *msn* during this migration.

msn defines a new, slbo-independent pathway

As *slbo* is expressed and is required in border cells during their migration, we tested whether *slbo* expression was affected in *msn*⁻ mixed clusters and observed that Slbo was expressed
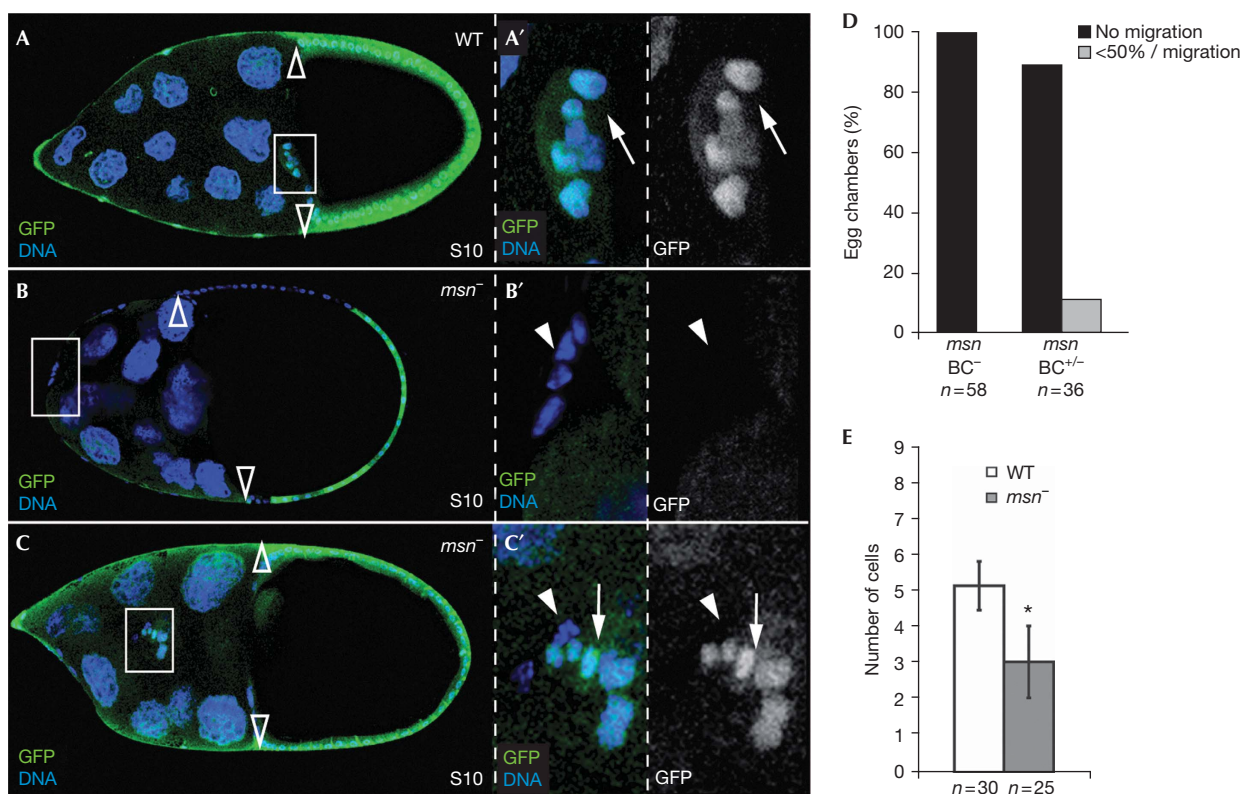
Fig 2 | *msn* is required for border-cell migration. (**A**) WT and (**B,C**) *msn* mosaic stage 10 (S10) egg chambers. In all panels, homozygous mutant cells are labelled by the absence of GFP (green); primed letters (′) are magnifications of the white boxes. (**A**) At S10, posterior migration of border cells is complete. (**B**) An S10 *msn*[102]-mutant border-cell cluster stuck at the anterior pole of the egg chamber. The number of border cells in this *msn*⁻ cluster is reduced compared with the wild type. (**C**) A delayed, mixed border-cell cluster containing wild-type cells at the front and *msn*⁻ cells behind. (**D**) Quantification of the S10 migration phenotype of mutant (BC⁻) and mixed (BC^{+/−}) clusters. (**E**) Quantification of the recruitment phenotype. Only mutant clusters were scored. An asterisk indicates a statistically significant difference (Student's test: $P < 0.01$). Empty arrowheads, rear limit of main body follicle cells; arrowheads, mutant cells; arrow, wild-type cells. BC, border cell; GFP, green fluorescent protein; *msn*, *misshapen*; WT, wild type.

at similar levels in both wild-type and mutant border cells (supplementary Fig S1A online). In addition, expression of the polar-cell-specific marker Fasciclin-III was unaffected (supplementary Fig S1B online), indicating that *msn* is not required to specify border cells or to discriminate between polar cells and outer border cells. Furthermore, as *msn-lacZ* expression was not affected in *slbo*-mutant cells (supplementary Fig S1C online), we concluded that *msn* functions independently of *slbo* in the control of border-cell migration.

We then tested whether *msn* is a component of the other pathways known to regulate border-cell migration, such as the ecdysone, Jak/Stat, and EGFR and PVR pathways, and found that *msn* acts independently of them all (supplementary Fig S2 online).

msn is required for proper distribution of *D*E-Cad

Delamination and migration of the border-cell cluster requires changes in the distribution of the actin cytoskeleton and various cell adhesion molecules, including *D*E-Cad and Dlg. The behaviour of F-actin and Dlg in wild-type and *msn*-mutant border cells is indistinguishable. Thus, F-actin accumulates at the front facing the direction of movement (Verkhusha *et al*, 1999; supplementary Fig S3 online), and Dlg—which initially localizes to the anterior margin of polar cells (Fig 3A,B)—re-localizes to

contacts between border cells in both genotypes during migration (Goode & Perrimon, 1997; Fig 3C,D). *msn*⁻ border cells thereby retain cell polarity cues that allow the redistribution of polarity markers during their migration.

S8 wild-type follicle cells adjacent to polar cells upregulate *D*E-Cad levels, which mainly concentrate at the zonula adherens (Niewiadomska *et al*, 1999). At early S9, *D*E-Cad becomes uniformly distributed in a depolarized manner over the surface of border cells (Fig 3E; supplementary Movie S3 online). On border-cell migration, the highest levels of *D*E-Cad are seen at border-cell, polar-cell and mixed contacts, whereas contacts between border cells and nurse cells show lower levels of *D*E-Cad (Fig 3G,I; supplementary Movie S4 online). By contrast, although the initial distribution of *D*E-Cad in *msn*⁻ cells is normal (Fig 3F; supplementary Movie S5 online), *D*E-Cad levels remain distributed in a non-polarized manner, with high levels in contacts between border cells and nurse cells (Fig 3H,J; supplementary Movie S6 online). Armadillo (Arm, β-catenin) distribution in *msn*⁻ cells is indistinguishable from that of *D*E-Cad (supplementary Fig S3C online). Together with the normal accumulation of actin and Dlg in mutant cells, the aberrant localization of *D*E-Cad and Arm in *msn*⁻ border cells demonstrates that *msn*⁻ cells do not exhibit a general defect in cell polarity, but instead have a specific
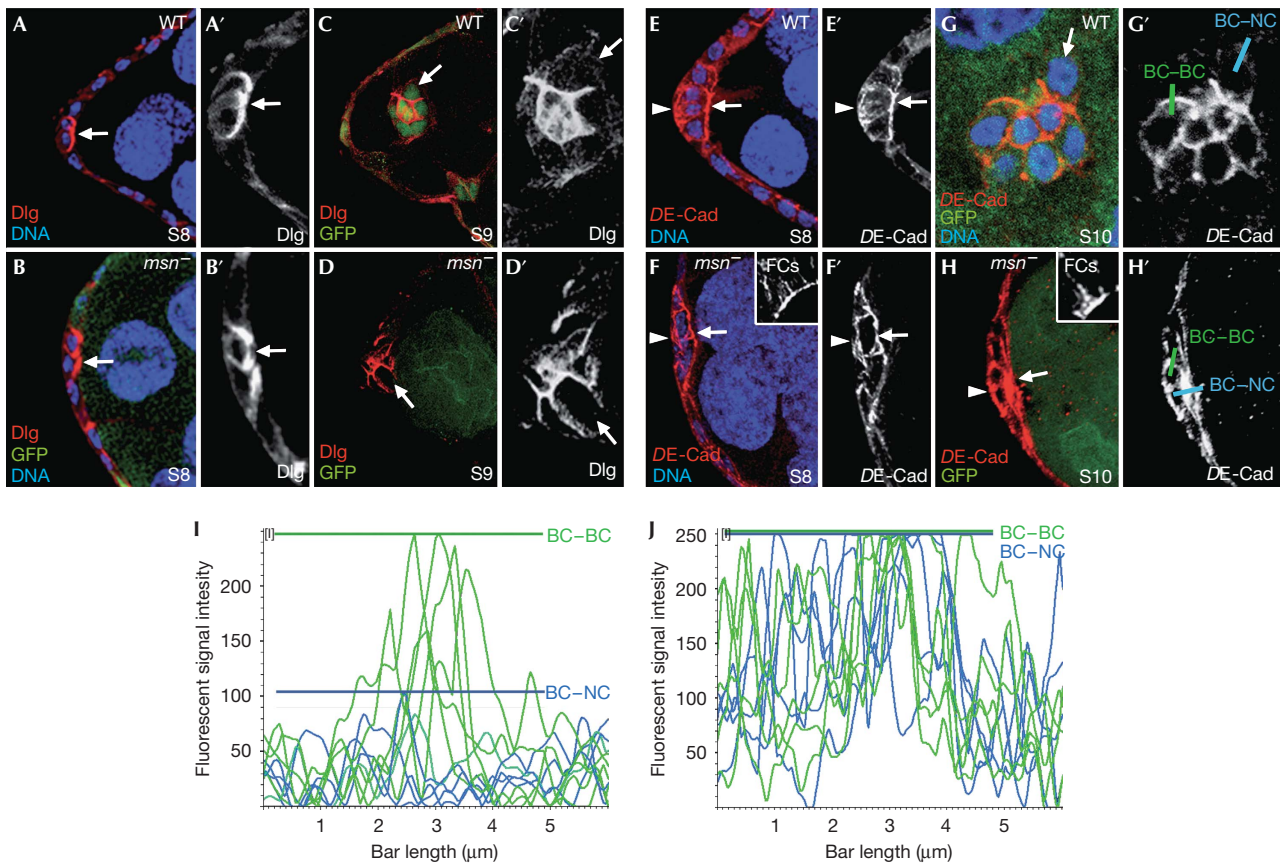
Fig 3 | *msn* regulates *D*E-Cad levels in border cells. At stage 8 (S8), (**A**) WT and (**B**) *msn⁻* border cells localize Dlg (red) at the front, facing the germ line. (**C,D**) At S9, Dlg is found at high levels at PC–BC and BC–BC interfaces and low levels at the BC–NC interface in both wild-type and *msn⁻* border cells. (**E,F**) *D*E-Cad distribution in S8 egg chambers is uniform around the surface of both (**E**) wild-type and (**F**) *msn⁻* border cells. (**G,H**) At S10, while in wild-type S10 clusters *D*E-Cad is found at high levels at the PC–BC and BC–BC boundaries and at low levels at BC–NC boundaries, *msn⁻* border cells also accumulate high *D*E-Cad levels at BC–NC interfaces. Insets in **F** and **H** are internal controls for *D*E-Cad staining in follicle cells. (**I,J**) Histogram of relative fluorescent intensities of *D*E-Cad along BC–BC (green) and BC–NC (blue) boundaries, as indicated in **G′** and **H′**. Coloured bars indicate the maximum *D*E-Cad fluorescence intensity found at BC–BC and BC–NC boundaries. Arrows, BC–NC contacts; arrowheads, basal side of border cells. Primed letters are single channels of corresponding merged panels. BC, border cell; *D*E-Cad, *D*E-cadherin; Dlg, Discs large; FC, follicle cell; GFP, green fluorescent protein; *msn*, misshapen; NC, nurse cell; PC, polar cell; WT, wild type.

defect in the distribution of adherent junction components. This abnormal accumulation of *D*E-Cad complexes in *msn⁻* border cells is not a consequence of failed cell migration, as no defects in *D*E-Cad distribution are observed in other genes with similar phenotypes, such as *jing* or *Hsp70* (Bai *et al*, 2000; Cobreros *et al*, 2008). The overexpression of UAS-*msn* using two different drivers—*slbo*-Gal4 and *c306*-Gal4—did not produce any visible phenotypes (data not shown).

To test whether *msn* regulates *D*E-Cad at the transcriptional level, we examined both *D*E-Cad levels and β-galactosidase activity from the enhancer trap line *shg^{k03401}* in mixed clusters, with wild-type cells of the cluster as an internal control. *msn⁻* border cells with elevated levels of *D*E-Cad did not exhibit increased β-galactosidase activity compared with wild-type siblings (Fig 4A,B), indicating that *msn* does not affect *shg* transcription. Finally, we found that the abnormal levels of *D*E-Cad observed in *msn⁻* border cells were linked to inhibition of migration, as removing one copy of *shg* diminished the migration defects of *msn* mixed clusters (Fig 4C).

Endocytosis has been identified as a key step in regulating E-Cad turnover. In fact, *D*E-Cad is a known cargo of the Rab11 recycling endosomes in several contexts (Lock & Stow, 2005), and Rab11-mediated transport regulates cadherin-based adhesion in *Drosophila* wing and trachea development (Classen *et al*, 2005; Shaye *et al*, 2008). We found that Rab11-dependent transport was required for border-cell migration, as expression of Rab11^DN (Marois *et al*, 2006) reduced *D*E-Cad levels substantially and delayed migration (Fig 5B,D). However, expression of Rab11^DN was not able to rescue the abnormal levels of *D*E-Cad or the migration phenotype of *msn⁻* border cells (Fig 5C), suggesting that *msn* does not regulate *D*E-Cad through Rab11-mediated transport.

msn acts independently of JNK

The *msn* gene activates mitogen-activated protein kinase cascades in various organisms (Dan *et al*, 2001). In *Drosophila*, *msn* acts upstream from *basket* (*Drosophila* Jun amino-terminal kinase (JNK)) in dorsal closure (Su *et al*, 1998), but not during photoreceptor axon targeting (Ruan *et al*, 1999). In border cells, mutations in
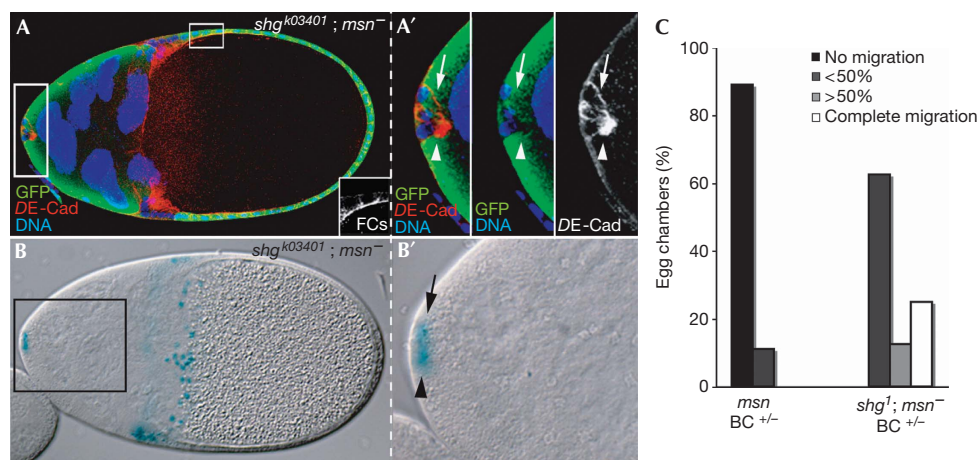
Fig 4 | *msn* does not regulate *D*E-cadherin transcription. S11 egg chamber carrying a mixed border-cell cluster stained with anti-GFP (green) and anti-*D*E-Cad (red), and assayed for β-galactosidase activity. (**A**) *D*E-Cad staining in *msn⁻* cells (white arrowheads) is abnormally elevated compared with sibling wild-type controls (white arrows). The inset shows an internal control for *D*E-Cad staining in follicle cells. (**B**) *msn⁻* cells (black arrowhead) show normal β-galactosidase activity (black arrow: sibling controls). (**C**) Reducing *D*E-Cad alleviates the border-cell migration defects found in *msn* mixed clusters. Migration is blocked in 89% of the mixed S10 border-cell clusters and delayed in 11% ($n = 36$). In an $shg^{-/+}$ background, migration is completed in 25% of the cases and delayed in the remaining 75% ($n = 15$). Primed letters are magnifications of the corresponding white boxes. BC, border cell; *D*E-Cad, *Drosophila* E-cadherin; FC, follicle cell; GFP, green fluorescent protein; *msn*, misshapen; S11, stage 11; *shg*, shotgun.

basket or overexpression of the JNK phosphatase *puckered* result in cluster dissociation and slightly delayed migration (Llense & Martin-Blanco, 2008). Similarly, we found that removal of either the *Drosophila* JNK kinase *hep* or the transcription factor *Djun* caused a delay in migration (supplementary Fig S4A,D online and data not shown). This suggests either that additional pathways cooperate in this process or that JNK is not downstream from *msn* in border-cell migration. We used a phospho-Jun antibody as a readout of JNK activation and found that this pathway was not altered in *msn⁻* border cells (supplementary Fig S4B online). To test for redundancy between the p38 kinase and JNK downstream from *msn*—as is the case in planar polarity generation (Paricio *et al*, 1999)—we analysed border cells lacking both *hep* and *Dp38K/lic* ($n = 36$) and found that they behaved similarly to *hep⁻* border cells (supplementary Fig S4C online). Our observations indicate that *msn* acts independently of the JNK pathway to regulate border cells. Finally, a known target of the Ste20-like Nck-interacting kinase are the Ezrin–Radixin–Moesin proteins (Baumgatner *et al*, 2006). However, removal of the sole member of the Ezrin–Radixin–Moesin family in *Drosophila*, *moesin*, in border cells did not affect migration (supplementary Fig S4E online).

DISCUSSION
A defining feature in stationary-to-migratory transitions of epithelial cells is the regulation of E-Cad distribution. Modulation of E-cad levels is also crucial during tumour progression (Peinado *et al*, 2007) and therefore understanding the molecular mechanisms behind this regulation is important to cancer biology.

The transition from stationary epithelial follicle cells to migratory border cells also requires tight regulation of *D*E-Cad levels and distribution (Niewiadomska *et al*, 1999; Bai *et al*, 2000). What are the signals that regulate *D*E-Cad levels in border cells? Several genes have been identified that, when mutated, result in increased or reduced *D*E-Cad levels and severe defects in

border-cell migration, including *slbo, tai, hnt, yan* and *Stat92E* (Niewiadomska *et al*, 1999; Bai *et al*, 2000; Silver & Montell, 2001; Schober *et al*, 2005; Melani *et al*, 2008). However, a causal link between alterations in *D*E-Cad surface levels and migration defects has not been demonstrated. Here, we show that *msn⁻* border cells display abnormal accumulation of *D*E-Cad and Arm, and fail to delaminate from the epithelium. Importantly, a reduction in *D*E-Cad function ameliorates the *msn⁻* migration defects. Although *msn* might also control other aspects of the initiation of their migration, we propose that Msn acts to regulate proper redistribution of *D*E-Cad before migration by controlling the reorganization of *D*E-Cad-containing adhesion complexes, either by affecting its turnover in a Rab11-independent manner or by modifying *D*E-Cad directly or indirectly so that it can be efficiently recycled.

E-Cad activity during organogenesis and tumorigenesis is often regulated at the level of transcription (Peinado *et al*, 2007). For processes in which a fast and transient regulation of E-Cad is required—such as during *Drosophila* tracheal intercalation—transport of E-Cad has been identified as an alternative mechanism (Shaye *et al*, 2008). Our results and those of others suggest that endocytosis might also have a role in modulating E-Cad turnover during border-cell migration. Expression of dominant-negative forms of Rab11—as we have demonstrated here—or Rab5 in border cells (Schober *et al*, 2005) leads to a reduction or an increase, respectively, in *D*E-Cad levels at the cell cortex and in defective migration. As interference with Rab5 function causes a phenotype similar to that of blocking *msn* function, and as the activity of mitogen-activated protein kinase p38, downstream from *msn*, accelerates endocytosis by stimulating formation of the GDI–Rab5 complex (Cavalli *et al*, 2001), *msn* could activate Rab5-mediated endocytosis. However, the overexpression of an activated form of Rab5 in wild-type border cells was not sufficient to cause a consistent reduction of *D*E-Cad
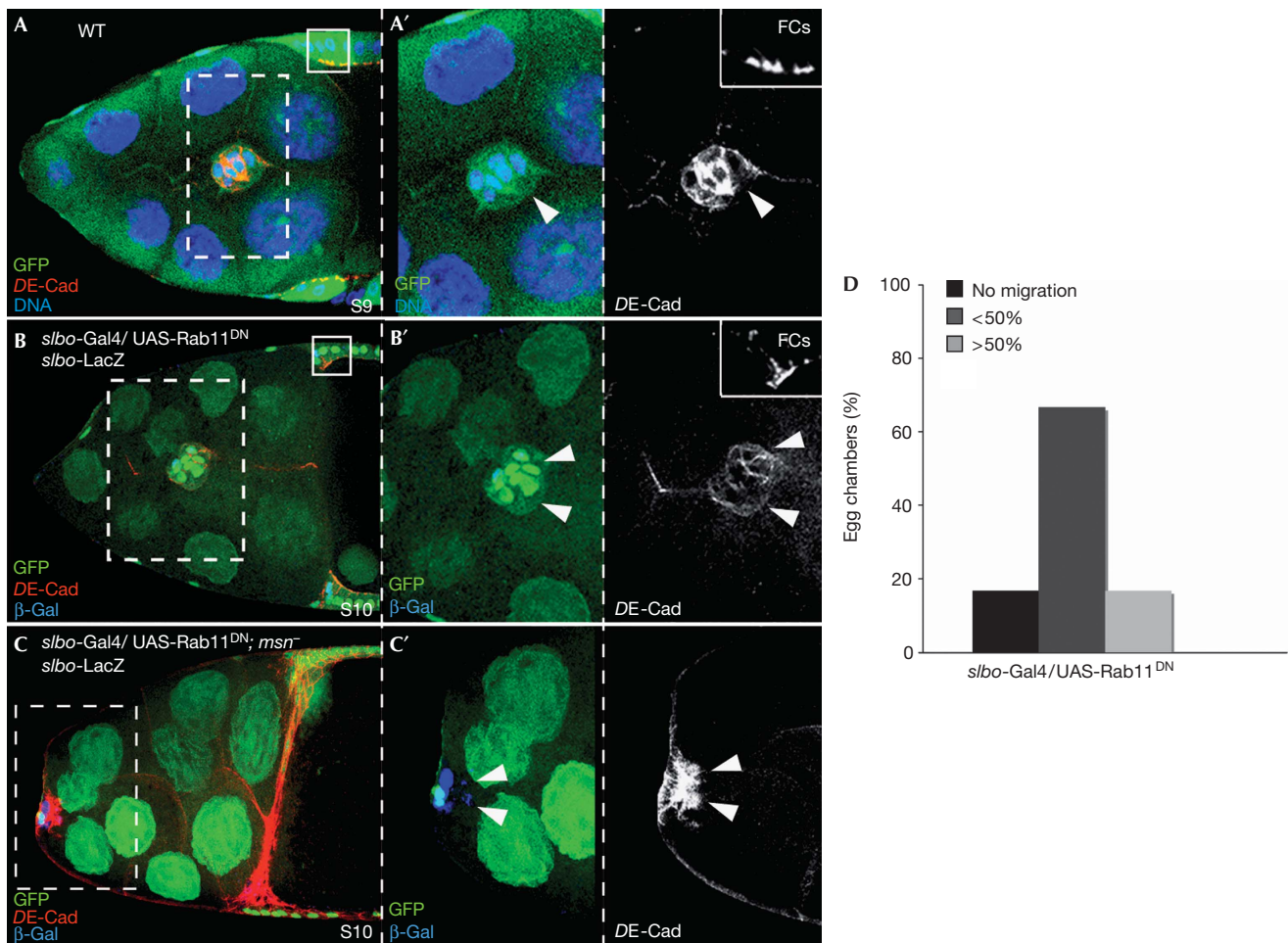
Fig 5 | Interfering with Rab11 function affects *D*E-Cad levels and border-cell migration. (**A**) S9 controls the border-cell cluster. (**B,C**) S9 border cells labelled with *slbo*-lacZ. (**B**) Overexpression of UAS-Rab11^DN reduces *D*E-Cad levels and impairs migration. (**C**) Overexpression of UAS-Rab11^DN does not rescue the migration defects or the elevated *D*E-Cad levels of *msn*⁻ border cells. (**D**) Quantification of the border-cell migration phenotype of S10 egg chambers overexpressing UAS-Rab11^DN. Small insets in **A** and **B** are internal controls of *D*E-Cad staining. Arrowheads, BCs. Primed letters are magnifications of white boxes. BC, border cell; *D*E-Cad, *Drosophila* E-cadherin; *msn*, misshapen; S9, stage 9; *slbo*, *slow border cells*; WT, wild type.

levels and we were therefore unable to test this hypothesis (supplementary Fig S5 online).

The role of Ste20 group kinases in cell migration seems to be pleiotropic and, in many instances, remains poorly understood. They modulate a variety of intracellular pathways through the extensive structural diversity of their non-catalytic domains. *Caenorhabditis elegans* NIK MIG-15 regulates guidance rather than migration itself (Su *et al*, 2000; Poinat *et al*, 2002), and mammalian *Nik* has been proposed to regulate the production of factors required for proper cell migration and not migration itself (Xue *et al*, 2001). Similarly to our results here, expression of dominant-negative forms of human Misshapen/NIKs-related kinase-β (hMINK-β) in MCF7 epithelial cells results in an upregulation of membrane-associated β-catenin, promoting cell–cell adhesion and inhibiting cell motility (Hu *et al*, 2004). The fact that *msn* is essential for the invasive behaviour of border cells supports a role for these kinases during invasion and/or tumorigenesis. In fact, human homologues of this family of kinases are broadly expressed in tumour cells and modulate cellular

transformation, invasion and adhesion (Wright *et al*, 2003). The identification of downstream targets of Msn during border-cell migration and its mechanisms of action might result in the discovery of new proteins that contribute to tumour invasion.

METHODS

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.

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