Drosophila USP22/non-stop regulates the Hippo pathway to polarise the actin cytoskeleton during collective border cell migration

Hammed Badmos1,2, Neville Cobbe1, Amy Campbell1, Daimark Bennett1,2

1 Department of Biochemistry, Institute of Integrative Biology, University of Liverpool, Crown Street, Liverpool, L69 7ZB, UK
2 Department of Molecular Physiology & Cell Signalling, Institute of Systems, Molecular and Integrative Biology, University of Liverpool, Crown Street, Liverpool, L69 7ZB, UK

Polarisation of the actin cytoskeleton is vital for the collective migration of cells in vivo. During invasive border cell migration in Drosophila, actin polarisation is directly controlled by Hippo pathway components, which reside at contacts between border cells in the cluster. Here we identify, in a genetic screen for deubiquitinating enzymes involved in border cell migration, an essential role for non-stop/USP22 in the expression of Hippo pathway components expanded and merlin; loss of non-stop function consequently leads to a redistribution of F-actin and the polarity determinant Crumbs, loss of polarised actin protrusions and premature tumbling of the border cell cluster. Non-stop is a component of the Spt-Ada-Gcn5-acetyltransferase (SAGA) transcriptional coactivator complex, but SAGA’s histone acetyltransferase module, which does not bind to expanded or merlin, is dispensable for migration. Taken together, our results uncover novel roles for SAGA-independent non-stop/USP22 in Hippo-mediated collective cell migration, which may help guide studies in other systems where USP22 is necessary for cell motility and invasion.

Introduction

Tightly regulated cell migration is vital for normal development and aberrant migration is involved in a number of human diseases, including tumour invasion and cancer metastasis, inflammatory diseases, and various birth abnormalities (Schumacher, 2019; Stuelten et al., 2018). In many instances, cells move by the process of collective migration in vivo, whereby migratory cells remain connected by cell-cell junctions, show group polarisation and coordinated cytoskeletal dynamics (Haeger et al., 2015; Mishra et al., 2019; Norden and Lecauduy, 2019). This mode of migration is exemplified by the movement of border cells in Drosophila (video S1). In this process, a cluster of five to eight cells are recruited from the follicular epithelium in the ovary by a pair of non-motile polar cells. Both cell types migrate as a cluster from the anterior basal lamina of the egg chamber, invading the underlying germ line, to the anterior border of the oocyte where they are involved in patterning prior to egg fertilization (Montell et al., 2012).

Studies of this process over the past 20 years have identified key features of the genetic programme required for border cell migration, which control the specification of the migratory cluster (Bai et al., 2000; Montell et al., 1992; Silver and Montell, 2001), organisation of cluster polarity and detachment from the epithelium (Abdelilah-Seyfried et al., 2003; McDonald et al., 2008; Pinheiro and Montell, 2004), timing of migration (Godt and Tepass, 2009; Jang et al., 2009), adhesion of the cluster (Cai et al., 2014; Newiadowska et al., 1999) and guidance to the oocyte (Bianco et al., 2007; Duchek and Rorth, 2001; Duchek et al., 2001; McDonald et al., 2003). Details have also emerged regarding the dynamic organisation of the actin cytoskeleton which is an essential driver of this process (Plutoni et al., 2019), with recent studies identifying an important role for the Hippo pathway in linking determinants of cell polarity with polarisation of the actin cytoskeleton in migrating clusters (Lucas et al., 2013). Our understanding of the interplay between polarity determinants and the actin cytoskeleton however remain incomplete, as does knowledge of the regulatory networks responsible for first establishing this polarity.

Ubiquitination of proteins by ubiquitin E3 ligases and removal by deubiquitinating enzymes (DUBs) plays important roles in regulating a raft of intracellular functions from protein stability and enzyme activity to receptor internalization and protein-protein interactions (Clague et al., 2013; Swatek and Komander, 2016). There is a growing body of evidence that ubiquitination plays roles in regulating the motility of single cells in culture (Cai et al., 2018), but little is known about its contribution to collective migration in vivo. Here we report our identification of non-stop (not) from a screen of DUBs involved in border cell migration. not encodes the USP22 orthologue in Drosophila (Martin et al., 1995), and is best known as the enzymatic component of the histone H2B DUB module of the SAGA transcriptional coactivator complex (Koutelou et al., 2010; Lee et al., 2011; Zhang et al., 2008). Histone modifications such as acetylation and ubiquitination are known to modulate the accessibility of genomic loci to transcriptional machinery, with ubiquitination being associated with both activation and repression (Weake and Workman, 2008). Correspondingly, SAGA is associated with the enhancers, promoters and sites of paused RNA polymerase II at genes in multiple tissues during Drosophila embryogenesis, and the Non-stop activity within SAGA is required for full expression of tissue-specific
genes (Weake et al., 2011).

Previous work has revealed essential roles for non-stop/USP22 during embryogenesis in Drosophila and mammals (Li et al., 2017; Lin et al., 2012), as well as in neural development (Weake et al., 2008) and lineage specification (Kosinsky et al., 2015). In the Drosophila nervous system, loss-of-function mutations in non-stop are associated with defects in the migration of a subset of glial cells to their appropriate position in the developing optic lobe and subsequent targeting of photoreceptor axons in the lamina (Martin et al., 1995; Poeck et al., 2001). The underlying mechanisms are not fully understood, but it has recently been suggested that this role may be mediated in part by a SAGA-independent role of Not in deubiquitinating and stabilising the actin regulator Scar (Cloud et al., 2019). Here we find that, in collective border cell migrating and stabilising the actin regulator Scar (Cloud et al., 2019). Here we find that, in collective border cell migration, not functions independently of both Scar and SAGA to regulate the expression of two upstream components of the hippo pathway, resulting in the loss of F-actin polarity, the mislocalisation of polarity determinants, a change in the size and orientation of cellular protrusions and the loss of polarised migration, placing non-stop at the top of a regulatory network underlying collective migration.

**Results**

**non-stop is required for invasive border cell migration.** We identified the Drosophila USP22 homologue non-stop in an RNA interference (RNAi) screen for deubiquitinases (DUBs) required for border cell migration. Wild-type border cell clusters normally reach the oocyte by stage 10 of oogenesis, whereas expression of transgenic inverted repeat constructs for non-stop (notIR) in the outer border cells severely delayed border cell migration (mean percentage migration of the distance to the oocyte ±SEM was 2.5 ±2.5%, n=40, Student’s t-test, P<0.0001) (Fig.1A-E). These migration defects could be significantly rescued by a full-length synthetic RNAi-resistant transgene (notIR, see Methods) confirming the requirement for non-stop in migration (Fig.1D,E). Incomplete rescue is most likely an indication of some off-target effects of notIR. Expression of notIR alone in both polar and outer border

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**Fig.1 non-stop is required for invasive border cell migration.** A-D, Confocal micrographs of egg chambers at stage 10 labelled with GFP (green) under the control of slbo-GAL4 to mark border cells (arrows) and TOPRO-3 (blue) to stain all nuclei. Anterior is left, posterior is right. Some GFP expression is also evident in centripetal follicle cells (arrowhead). Bars, 25µm. A, Image of slbo-GAL4 control (slbo>) showing complete migration of the border cell cluster. B, RNAi knockdown of not under the control of slbo-GAL4 (slbo>notIR) abrogates border cell migration. C, In contrast, overexpression of notIR in the whole border cell cluster using c306-GAL4 (c306>notIR) did not affect migration, indicating non-stop is not limiting for migration. Clusters expressing notIR with Slbo-GAL4 also migrated normally (not shown). D, Migration index for quantification of border cell migration at stage 10, see Methods. E, Stacked bar chart summarising migration defects in the indicated genotypes (n= number of egg chambers). The effect of not RNAi knockdown can be partially rescued by transgenic overexpression of RNAi-resistant not (slbo>notIRIR). F-G, Confocal micrographs of egg chambers labelled with GFP (green) to mark clones of cells induced with the MARCM technique and TOPRO-3 (blue) to stain all nuclei. Bars, 25µm. Compared to control clones, which routinely complete migration at stage 10 (F), notIR mutant border cell clusters display defective migration, with clusters lagging behind overlying centripetal cells (position marked with dotted line) at stage 9 (G). H, Quantitation of migration defects at stage 10, reveal that the clusters containing >50% mutant cells are more severely affected than those with <50% mutant cells in the cluster; migration is largely restored by notIR overexpression (tub>notIRIR; notIR). n= number of egg chambers. I, Stage 10 egg chamber showing splitting of notIR mutant border cell clusters; 18% of clusters displayed splitting into two groups of cells, 10% of clusters split into >2 groups of cells (J, frequency of cluster splitting, n= number of egg chambers).
cells had no effect on migration (Fig.1C,E). To further confirm the requirement for non-stop in border cell migration, we generated homozygous clones for an amorphic non-stop mutant allele (not1). Notably, border cell clusters genetically mosaic for not1 showed greatly retarded migration, with the severity of the effect being dependent on the proportion of mutant cells in the cluster (Fig.1F-H).

Mean migration was reduced by 61.3 ±2.9% (P<0.0001, n=101) in clusters containing >50% non-stop mutant cells compared to clusters with control clones, where cells migrated normally; these defects were almost fully rescued by transgenic expression of not+r (Fig.1H). Unlike in controls, splitting of border cell clusters was also observed in 28% of stage 9 or 10 non-stop mutant egg chambers (n=138) (Fig.1I-J), indicative of a defect in maintaining the integrity of border cell-border cell contact. Taken together, these data identify non-stop as a novel regulator of border cell migration.

**non-stop regulates polar cell number.** At stage 8 of oogenesis, a pair of anterior polar cells secrete Unpaired (Upd) ligand, which activates the JAK-STAT (Janus kinase-signal transducer and activation of transcription) signalling pathway in surrounding follicle cells, leading to the recruitment of 5-8 follicle cells into a migratory cluster (Beccari et al., 2002; Silver and Montell, 2001). To explore the requirement for non-stop in border cell signalling we looked at the expression of slbo, a downstream target of Upd-JAK/STAT signalling in the migratory outer border cells, which induces the expression of genes required for migration. The level of a transcriptional reporter, slbo-lacZ, was not significantly different between not1 mutant cells and their wild type siblings within mosaic border cell clusters (Fig.2A-C; arbitrary units, mean intensity ±SEM was 47 ±5.3 for not1 n=21, compared to 56 ±5.5 for controls n=23, P=0.22). non-stop was also not required for the expression pattern of Eyes absent (Fig.2D,E), which is expressed in outer border cells to repress polar cell fate in these cells (Bai and Montell, 2003).

**Fig.2 non-stop regulates polar cell number.** A, Control egg chamber at mid stage 9 showing slow border cells expression with the slbo-lacZ reporter (red) in migrating border cells. Nuclei are labelled with TOPRO-3 (blue). Inset shows magnified image of slbo-lacZ alone (greyscale). B, Stage 10 egg chamber with not1 mutant border cells labelled by MARCM with GFP (green). The normal pattern of slbo-lacZ is detected. C, Quantitation of relative slbo-lacZ signal intensity (GFP, internal control: GFP+ homozygous sibling cell, see Methods for genotypes), showing no significant difference in slbo expression between wild type and not1 mutant cells. D, Control egg chamber at stage 10 showing anti-Eyes absent antibody staining (Eya, red). Nuclei are labelled with TOPRO-3 (blue). Inset shows magnified image, showing upd-lacZ expression (greyscale) is restricted to the two polar cells (red arrows). G-J, 32% of not1 mutant clusters possessed more than two upd-lacZ positive polar cells, which is associated with an increase in border cell numbers. G, GFP-labelled not1 mutant border cell cluster possessing two upd-lacZ+ nuclei, which represents the most abundant category, but some clusters contain up to 6 upd-lacZ+ nuclei (H). I, Quantitation of polar and border cell numbers, reveals a significant increase in numbers of both upd-lacZ+ polar cells and border cells in not1 mutant clusters compared to controls. J, Graph showing the relationship between number of upd-lacZ+ polar cells and border cells in individual not1 border cell clusters, colour coded according to numbers of polar cells: 2, purple (n=36); 3, red (n=12); 4, green (n=3); 5, blue (n=1), 6, white (n=1). Bars in confocal images are 25 µm (10 µm for insets).
Therefore, we conclude that non-stop does not affect the expression levels of genes in migratory outer border cells that specify their fate. When we looked at upstream signalling using a upd-lacZ reporter (Fig.2F-H), we observed that 32% of not1 mutant clusters possessed more than two upd-lacZ positive polar cells (Fig.2I; mean ±SEM was 2.47 ±0.12, n=53), suggesting that some not1 polar cells continue proliferating after stage 2 of egg chamber development when divisions would normally cease (Margolis and Spradling, 1995). not1 clusters also contained on average a 1.7-fold higher number of border cells than controls (Fig.2I; mean ±SEM was 11.1 ±0.2 for not1 n=138, compared to 6.4 ±0.12 for controls n=47, \( P<0.0001 \)) and this was correlated with the number of upd-lacZ positive polar cells (Fig.2I; multiple regression \( R^2=0.54, n=53, P<0.0001 \)), suggesting the presence of additional polar cells led to the recruitment of additional border cells into the cluster. Clusters with more than two polar cells had a significantly reduced degree of migration compared to those with just two polar cells, (mean migration was 3.8% (n=17) compared to 22.2% (n=36), respectively, t-test \( P=0.003 \)), suggesting that larger not1 clusters had particular difficulty in making their way successfully to the oocyte. 

**non-stop is required for normal actin polarity in migratory border cells.** Following their specification, border cells undergo two phases of cell migration, an initial polarised phase, and a second phase that utilises collective migration (Bianco et al., 2007). In the initial phase, leader cells exhibit long, highly polarised F-actin protrusions that are required for adhesion to and migration through the substratum (Fulga and Rorth, 2002). Later, F-actin accumulates around the cortex of the cluster, as cells alternate their position in the cluster as they move collectively (Bianco et al., 2007). In not1 mutant clones, we observed a loss of initial F-actin polarity, and F-actin accumulation was subsequently not restrained to the cluster cortex but it also accumulated along border cell-border cell junctions (Fig. 3A,B). Quantification of F-actin staining confirmed a 2.6-fold shift in relative distribution towards the interior border cell junctions in not1 mutant clusters compared to controls (two-way Anova \( P<0.0001 \), Fig.3C). This change in distribution was rescued by transgenic not+r overexpression (Fig.3C). When we examined egg chambers by live imaging, we found that progressive migration was reduced by 80%, from 0.45 \( \mu \text{m/min} \) in controls to 0.09 \( \mu \text{m/min} \) in not1 mutant border cell clusters (\( P<0.01 \)). This was accompanied by loss of initial F-actin polarity (Fig.4A,B, video S2 and S3) and a premature

![Fig.3](https://example.com/fig3.png)

**Fig.3** **non-stop is required for normal actin polarity in migratory border cells.** A, Confocal micrographs of egg chambers harbouring wild type, not1 or rescued not1 GFP-labelled clones (not1; tub>not+r) labelled with Phalloidin to visualise F-actin (red), TO-PRO-3 to label nuclei (blue). Egg chambers are stage 10 except the wt control, which is shown at mid-migration at stage 9 (dotted line indicates expected position of the cluster at this stage of migration). Border cell clusters are indicated with arrows. In wt, F-actin is normally polarised, with high levels around the cortex of the cluster, at border cell-nurse cell junctions. In contrast, in not1 clusters, F-actin predominantly accumulates at internal border cell-border cell junctions; this is rescued by transgenic not+r overexpression (Fig.3C). B, Representative line scans of the same genotypes showing signal intensities of F-actin from anterior (left) to posterior (right), showing the change in F-actin profile in not1 mutant clusters. C, Mean ratios of area under curve for front, middle and back of the cluster derived from lines scans taken from several egg chambers (wt control clusters, n=7; not1 clusters, n=9; tub>not+r, not1 clusters, n=16) showing a consistent defect in F-actin polarisation in not1 clusters.
tumbling motion (Fig.4A-C). Further analysis revealed that while there was not a global reduction in the number of protrusions in not1 mutant clusters (Fig.4D), there was a significant change in the distribution of the number (Fig.4E) and size (Fig.4F,G) of protrusions, from a front bias in controls (54% of protrusions) to the sides (63% of protrusions) in not1 mutants (Fig.4E, P<0.01), consistent with a failure of these clusters to move in a polarised fashion.

**non-stop acts independently of Scar during border cell migration.** Recent data suggest Non-stop is capable of interacting with Arp2/3 and the WAVE regulatory complexes (WRC) in the cytoplasm to prevent polyubiquitination and subsequent proteasomal degradation of the WRC subunit Scar (Cloud et al., 2019). Scar/WAVE-Arp2/3 interactions result in nucleation of branched actin filament networks and in that way regulate migration (Buracco et al., 2019; Krause and Gautreau, 2014). This prompted us to test whether loss of **non-stop** function resulted in destabilisation of Scar levels in border cells. Endogenous Scar staining was very faint (Fig.5A,B) compared to ectopically overexpressed Scar (Fig.5C), but we did not observe any difference in Scar protein staining between not1 mutant border cells and their heterozygous siblings (Fig.5A,B). To test whether Scar loss-of-function phenocopied not1 clusters, we generated homozygous clones for an amorphic Scar mutant allele, ScarΔ37 (Zallen et al., 2002). Notably, we found F-actin polarity was unaffected with F-actin being predominantly distributed at the cortex of ScarΔ37 clusters. Migration of ScarΔ37 clusters was retarded. However, previous live imaging analysis of clusters in which Scar had been knocked down by RNAi, revealed that Scar loss of function resulted in a reduction in the number of cellular protrusions, with a higher proportion of protrusions at the rear of the cluster, and fewer in the front and middle compared to controls (Law et al., 2013). These phenotypes are consistent with a reduction in migration, but not with the not1 phenotypes described above (Fig.3,4). Polarisation of the polarity determinant Crb was also normal in Scar mutant clones suggesting the architecture of the clusters was unaffected. Taken together, we conclude that, in border cells, **non-stop** acts independently of Scar to drive collective migration.
**non-stop** is required for the normal level and/or distribution of Hippo signalling components in border cells. The loss of normal actin polarity, early tumbling of the border cell cluster, increased polar cell number are all features of Hippo signalling loss-of-function (Lin et al., 2014; Lucas et al., 2013). In outer border cells, the key upstream components of the Hippo pathway (Crumbs, Kibra, Expanded, Merlin) are found at sites of border-cell border-cell contact (Lucas et al., 2013; Niewiadomska et al., 1999), where the pathway acts independently of the canonical downstream effectors Yorkie to limit the activity, but not the recruitment, of the actin polymerisation protein Enabled (Lucas et al., 2013). This prompted us to test whether non-stop may be required for the normal level or distribution of Hippo signalling components in outer border cells. Using a transcriptional reporter of expanded expression (ex-lacZ), we found a 2.46 fold reduction in expanded levels in not1 mutant cells compared to heterozygous sister cells in mosaic border cell clusters (Fig.6A,B; P=0.003, n=26). Similarly, we saw a reduction in Merlin protein levels at border cell-border cell junctions in not1 mutant cells (Fig.6C,D). The distribution of Enabled appeared largely unaffected in not1 mutant clusters (Fig.6E,F). In follicle cells, Expanded and Merlin are redundantly required for normal localisation of the apical transmembrane protein Crumbs (Crb) (Aguilar-Aragon et al., 2020; Fletcher et al., 2012). Strikingly, when we examined the distribution of Crb, we found that rather than being distributed in the junctions between neighbouring border cells (Niewiadomska et al., 1999), it was localised around the cortex of the cluster, at the interface between border cells and nurse cells (Fig.6G,H). Crb is required for polarisation of other polarity determinants, including aPKC in border cells (Wang et al., 2018). Correspondingly, the distribution of aPKC was somewhat disrupted in not1 mutant cells (Fig.6J). We also observed a modest effect on the distribution of the adherens junction protein Armadillo/β-catenin (Arm; Fig.6K,L). Taken together, these data show that non-stop is required for expression of hippo signalling components and correct recruitment of polarity determinants in outer border cells.

**ex and mer are targets of Non-stop but not the HAT module of SAGA, which is dispensable for border cell migration.** A key and highly conserved role of Non-stop/USP22 is to regulate gene expression, acting as a central component in the DUB module of the SAGA complex (Lee et al., 2011). By exploiting genome-wide ChiPSeq data from a recent study of the Drosophila SAGA complex (Li et al., 2017), we asked whether any of the canonical hippo signalling components are transcriptional targets of Non-stop. To do this we looked for binding sites at the gene promoters, -1000 to +200bp of the transcription start sites. We found that the expanded promoter is bound by Non-stop (n=2, Fig.7A); furthermore, depletion of non-stop leads to a 2.5 fold reduction expanded expression in embryos (Li et al., 2017), comparable to the effect we observed in border cells (see above). Similarly, we also found evidence that Not binds the merlin promoter (n=1, Fig.7B). Interestingly, Ada2b, a SAGA-specific HAT module subunit, that anchors the HAT module to
Fig. 6. non-stop is required for the normal level and/or distribution of Hippo signalling components and polarity determinants in border cells A-L. Confocal micrographs showing egg chambers with either wt or not1 GFP-labelled MARCM clones (green) stained with antibodies against β-gal (to detect ex-lacZ expression, A,B); Merlin (C,D), Ena (E,F), Crb (G,H), aPKC (I,J), or Arm (K,L) in red. Nuclei are stained with TO-PRO-3 (blue). Bars 25 μm (10 μm for insets). Arrows, border cells. The stage of egg chamber development is as indicated. A, Mosaic order cell clusters, showing the normal expression of ex-lacZ in both GFP-labelled control clones (green outline), and control sibling cells (white outline). B, Notably, there is a reduction in ex-lacZ expression in not1 clones (green outline) compared to control sibling cells (white outline). C, Merlin staining is weak but clearly detectable at the inner-border cell junctions in control clones (green outline), but D, is lost in GFP-labelled not1 cells (green outline) and not adjacent control cells of the same cluster. E-F, Ena is predominantly located at cell junctions around the polar cells, and at inner and outer border cell membranes, in both control (E) and not1 (F) clones. G, Crb is normally distributed at inner border cell junctions in control border cell clusters, but H, is strikingly redistributed to the cortex of not1 border cell clusters. I, aPKC is normally distributed at inner border cell junctions in control border cell clusters, but J, this distribution is disrupted in not1 clones, with some loss of aPKC at the inner membranes and a more cytoplasmic distribution in the border cells. K, the adherens junction protein Arm is apically localises at inner junctions in controls. L, in not1 border cell clusters Arm appears more spread out, although remains restricted to inner junctions.

SAGA and is required for its HAT activity (Kusch et al., 2003; Lee et al., 2011; Muratoglu et al., 2003; Pankotai et al., 2005; Zsindely et al., 2009), did not bind either of these loci (n=4, Fig. 7A,B), suggesting that expanded and merlin promoters are DUB specific targets. Correspondingly, we did not see a reduction in ex-lacZ levels in ada2b mutant clones (Fig. 7C). Furthermore, when we tested the requirement for ada2b in F-actin polarity and border cell migration, we found that ada2b mutant border cells migrated normally with cortically-localised F-actin (Fig. 7D, mean migration 82.3% ± 3.6%, n=45). Taken together, these data indicate that the DUB module can regulate transcription of expanded and merlin independently of the HAT module in border cells.
Overexpression of expanded partially rescues cell migration and polarity defects. To further explore the functional significance of reduced expanded levels, we examined the effect of expanded loss-of-function on border cell polarity and migration (Fig.8). We found that border cells mutant for an expanded loss-of-function allele (ex+) phenocopied the effect of not1, albeit more weakly (Fig.8A-F), with some loss of cortical F-actin staining and a significant disruption of Crumbs distribution (Fig.8K-L), accompanied by abrogated migration (Fig.8M). Strikingly, expanded overexpression (ex+) substantially restored more normal Crumbs and F-actin distributions in not1 mutants (Fig.8G-H and K-L) and significantly suppressed the effect of not1 on migration (Fig.8M; the mean percentage migration of ex+ not1 border cell clusters was 55.2 ±3.0%, n=75 compared to 38.7 ±2.9%, n=101 for not1 alone, P<0.0001). Taken together with the data above, we conclude that expanded is a critical transcriptional target of non-stop required for its function in border cells. Previous studies have shown that overexpression of Capping protein B (cpb+), which antagonises Enabled by competing for binding F-actin barbed ends and preventing actin polymerisation, is capable of complementing impaired hippo signalling (loss of warts) in border cells. Correspondingly, we find that cpb+ has a similar ability as ex+ to rescue not1-associated defects in F-actin polarity and collective cell migration (Fig.8I-M). Interestingly, we also saw a partial recovery in the Crb distribution in cpb+ not1 mutant cells compared to sibling control cells. D, Confocal micrograph of a stage 10 egg chamber (arrow) with ada2b1+ GFP-labelled MARCM clone (green) stained with Phalloidin to label F-actin (red), showing F-actin is localised to outer border cell junctions, as wild type, compare Fig2A.

**Discussion**

A non-stop-mediated transcriptional programme establishes F-actin polarity during collective migration

Here we report that Drosophila USP22, encoded by non-stop, is necessary for F-actin polarity and collective cell migration of invasive border cells. Collective border cell migration requires actomyosin polymerisation and contraction at the cortex around the cluster as it moves over the nurse cell substrate; F-actin is effectively excluded from the center of the cluster where polarity determinants acting via the Hippo complex block the activity of the F-actin regulator Enabled. Mechanistically, our experiments suggest non-stop regulates inside-out F-actin polarity by regulating the expression of hippo signalling components, ex and mer, which are direct Not targets. Not has been reported to regulate the actin cytoskeleton directly by promoting the stability of Scar/WAVE. However, we did not observe a reduction in Scar levels in not mutant clones and Scar loss-of-function did not disrupt F-actin polarity.

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**Figure 7** Ex and Mer are targets of Non-stop but not the HAT module of SAGA, which is dispensable for border cell migration. A-B, Non-stop, but not Ada2b bind to the expanded and merlin promoters. At the top are the ChIP binding profiles for all replicates of Not (green, n=2) and Ada2b (blue, n=4) at expanded and merlin promoters in Drosophila embryos as determined from data reported in (Li et al., 2017). Position of the transcription start site (TSS) is shown with a dotted line. Below is a schematic of the gene structure at the respective genomic loci with exons (thick lines) and introns (thin lines). Scale bar, 1 kb intervals. C, Confocal micrograph of a stage 10 egg chamber (arrow) with ada2b1+ GFP-labelled MARCM clone (green) stained with antibodies against β-gal (red) to detect ex-lacZ expression. Nuclei labelled with TO-PRO-3 (blue). Inset, ex-lacZ staining in grayscale, with mutant cells outlined (green dotted line). There is no reduction in ex-lacZ staining in ada2b1 mutant cells compared to sibling control cells. D, Confocal micrograph of a stage 10 egg chamber (arrow) with ada2b1+ GFP-labelled MARCM clone (green) stained with Phalloidin to label F-actin (red), showing F-actin is localised to outer border cell junctions, as wild type, compare Fig2A.
Fig. 8 Overexpression of expanded or the actin capping protein cpB partially rescues cell migration and polarity defects A-J. Confocal micrographs of Crb or F-actin (red) staining in egg chambers harbouring GFP-labelled MARCM clones (green) of different genotypes. TOPRO-3 (blue) labels all nuclei. Bars, 25µm (insets 10 µm). The stage of egg chamber development is as indicated, with dotted line showing position of overlying centripetal follicle cells in stage 9 chambers. Border cells are indicated with arrows. A, Control showing normal distribution of Crb at contacts between the border cells inside the cluster. B, Control showing normal cortical distribution of F-actin around the outer membrane of the cluster. C, exe1 clones showing partial disruption of Crb. D, F-actin polarisation is also partially impaired in exe1 border cells, with some F-actin visible at inner junctions of the migrating clusters. E, Crb is redistributed away from inner junctions to the cortex of the cluster in not1 clones. F, F-actin is found distributed on inner junctions of not1 clusters between border cells. G, The disruption of Crb localisation in not1 clones is partially rescued by overexpression of ex (not1 ex+). H, F-actin also is more normally polarised in not1 ex+ border cells, although some weak staining is also evident between border cell-border cell junctions. I, Overexpression of cpB weakly restores some Crb distribution in not1 mutant cells (not1 cpB+) and J, F-actin is displaced from border cell junctions inside the not1 cpB+ clusters. K, Quantification of mean percentage of Crb staining at the front, middle and back of the cluster (area under curve measurements) derived from lines scans taken from several egg chambers (n=number of clusters). * P<0.05; ** P<0.001; ns, not significant, 2-way Anova comparisons of mean ratio of Crb staining at the front of the cluster; comparable results were obtained for comparisons of staining in the middle of the cluster (not shown). L, Quantification of mean percentage of F-actin staining at the front, middle and back of the cluster (area under curve measurements) derived from lines scans taken from several egg chambers (n=number of clusters). ** P<0.001; ns, not significant, 2-way Anova comparisons of mean ratio of F-actin staining at the front of the cluster; comparable results were obtained for comparisons of staining in the middle of the cluster (not shown). M, Histograms summarising border cell migration defects at stage 10 in the indicated genotypes (n= number of egg chambers), alongside the migration index for quantification of migration.
Furthermore, we did not observe a significant change in the number of actin protrusions following not loss of function, which might be expected if Scar were a target in border cells. Notably, we find that overexpression of expanded suppressed not\textsuperscript{1} induced F-actin accumulation at inner border cell junctions, consistent with partial restoration of Hippo function and inhibition of Enabled function. We also observed that cpb overexpression rescued loss of non\textsuperscript{-stop}, again consistent with disruption of Enabled function due to competitive binding of Cpb to F-actin barbed ends and the inhibition of F-actin polymerisation at inner border cell junctions. Interestingly, studies of maternally-provided not in the early embryo have identified a requirement for not in membrane invagination and nuclear anchoring during cellularisation (Li et al., 2017). Invagination is driven by actin, which is highly polarised at the base of invaginating membranes, and transiently in apical microvilli. Enabled plays an important role in establishing actin dynamics during invagination (Grevengoed et al., 2003), raising the question of whether the regulatory network between Not and the Hippo complex we have uncovered also has a role to play in this context.

non-stop regulates the distribution of polarity determinants. A striking effect of not loss of function in border cells is the redistribution of Crb from inner to outer border cell junctions. When we looked at possible effects of this on other polarity determinants, we found localisation of aPKC to the inside apical junction between border cells was disrupted, consistent with previous studies showing that Crb, acting together with the Par complex and endo-cytic recycling machinery, is necessary for ensuring its correct distribution (Wang et al., 2018). Mislocalised aPKC generates protrusions at the side and back of border cells (Wang et al., 2018), just as we have seen in not\textsuperscript{1} clusters. Hence, whilst loss of Hippo components leads to loss of inside-out actin polarity, disruption of Crb and aPKC might account for the change in orientation of protrusions. Why is Crumbs mis-localised to the cortex of the border cell complex? Our complementation experiments (Fig. 8) suggest that this might be at least partially accounted for by loss of expression of the FERM domain proteins Expanded and Merlin, which in follicle cells act together with Moesin (Moe) to recruit Crb to the apical surface (Aguilar-Aragon et al., 2020). Moe stabilises Crb at the apical membrane of epithelia by linking Crb to cortical actin (Medina et al., 2002). Although the physical interaction between Moe and Crb may be weak (Sherrard and Fehon, 2015), Moe is an important regulator of dynamic Crb localisation in follicle cells, as it acts to antagonise interactions between Crb and aPKC at the marginal zone of the apical membrane domain, while stabilising interactions between Crb and the apical surface (Sherrard and Fehon, 2015). Importantly, in border cells, Moe is cortically localised where it organises a supercellular actin cytoskeleton network and promotes cortical stiffness (Ramel et al., 2013). An attractive hypothesis therefore is that Moe, perhaps along with other proteins, is a sink for Crb at the cortex of the border cell cluster following loss of Ex and Mer at inner border cell junctions in non-stop mutants. When we overexpressed expanded the normal pattern of Crb localisation was partially restored, in support of a competitive binding model. Interestingly, we also observed weak rescue of Crb localisation following Cpb overexpression. This might be because Moe, or other proteins that tether Crb on the outer membrane are only accessible in the absence of a strong supercellular F-actin cortex and that restoration of cortical F-actin in not\textsuperscript{1} cpb\textsuperscript{+} cells displaces Crb. In wild type border cells, Crb needs to be constantly moved from the outside membrane in a dynamin- and Rab5-dependent manner (Wang et al., 2018). Another possibility therefore, which is not mutually exclusive with the first, is that polarisation of the F-actin cytoskeleton is important for correct trafficking of Crb in border cells, as it is in follicle cells (Aguilar-Aragon et al., 2020).

non-stop is necessary for the expression of ex and mer independently of the requirement for yki. Abnormal accumulation of F-actin in epithelial tissues, e.g. resulting from loss of CpB, has been shown to lead to Yki-induced expression of ex, mer, and other target genes to reinforce Hippo activity at the cortex (Fernandez et al., 2011; Ko et al., 2016; Sanso-ses-Garcia et al., 2011). It is known that the Hippo pathway integrates multiple inputs at the level of Yki and that Yki interacts with a number of chromatin-modifying factors for transcriptional activation of target genes (Hillmer and Link, 2019). Is it possible that non-stop acts to support Yki-mediated expression of ex and mer? In border cells, ectopic overexpression of Yki has been reported to accelerate border cell migration, resulting in clusters prematurely reaching the oocyte during stage 9, suggesting that there may be a Yorkie-mediated negative feedback loop to maintain F-actin homeostasis (Lucas et al., 2013). However, yki mutant border cells or clusters in which yki has been knocked down in the outer border cells migrate normally, suggesting that yki is normally dispensable in outer border cells for invasive migration (Lin et al., 2014). We therefore favour a model whereby non-stop provides independent transcriptional control of ex and mer in this context. The situation is different in polar cells, where the Hippo pathway is proposed to act by suppressing Yki activity and cell proliferation to maintain normal polar cell numbers. However, similar to Hippo loss-of-function or yki gain-of-function, we find that loss of non-stop leads to increased numbers of polar cells, which, again, argues against a role for non-stop in supporting yki-mediated gene expression. Nevertheless, what this does suggest is that the requirement for non-stop in Hippo complex formation is not limited to situations where the Hippo complex acts in a yki-independent fashion.

SAGA-independent roles for non-stop during development and disease. The growth, specification and migration of cells during tissue development requires precisely regulated patterns of gene expression, that depend on numerous cues for temporal and spatial gene activa-
tion, involving crosstalk with multiple signalling pathways. Strikingly, it has emerged that factors once considered to be ubiquitous regulators of transcription, including the SAGA chromatin-modifying complex, can have specific roles in discrete developmental processes. Although it has been suggested that SAGA is required for all transcribed genes in some contexts (Bonnet et al., 2014), numerous studies have shown that loss of SAGA components affects the expression of only a subset of genes (Pahi et al., 2015; Pankotai et al., 2013; Zsindely et al., 2009) and different components modulate distinct and overlapping subsets (Helmlinger et al., 2008; Helmlinger et al., 2011; Lee et al., 2000; Weake et al., 2008). These differences in expression are likely to explain their different physiological roles; for instance, during female germline development in Drosophila, ada2B affects the expression of many genes and is required for oogenesis, whereas non-stop affects relatively few and is dispensable (Li et al., 2017). Elegant genome-wide ChIP studies indicate that even though both DUB and HAT modules bind the same genes, many of the targets do not require the DUB module for expression, explaining the observed dependencies. These experiments also reveal non-overlapping sites of chromatin occupancy for the DUB and HAT modules of SAGA in Drosophila (Li et al., 2017), but the significance of differences in transcriptional targeting for cell function had not been established. Notably, in this respect, we find that the requirement for non-stop in border cell migration is not matched by a requirement for ada2b. Furthermore, Ada2b has not been found to bind the ex and mer promoters, providing a molecular explanation for non-stop’s SAGA-independent role. Importantly, these findings challenge the perceived view that transcriptional roles for non-stop/USP22 are mediated solely by SAGA. This may have broader relevance to situations where USP22, but not other members of SAGA are associated with human disease states, particularly where cell polarity is frequently disrupted, such as cancer (Glinsky et al., 2005). Our current efforts are directed at identifying SAGA-independent factors that facilitate Non-stop’s chromatin binding and function.

Methods
Non-stop transgene. An RNAi-resistant, full-length non-stop expression construct was synthesised by GeneArt (Invitrogen). RNAi-resistance was achieved by incorporating numerous silent polymorphic mutations, such that, in the regions targeted by dsRNAs, homology with the inverted repeat sequences was limited to no more than 8 contiguous base pairs (Jonchere and Bennett, 2013). The non-stop open reading frame was shuttled into pPMW-atB (Chen et al., 2015) by gateway cloning, placing the non-stop open-reading frame downstream of a Myc epitope tag. Stable transgenic flies were made by phiC31 integrase-mediated transgenesis at a landing site on the second (attP40, at 25C6) and third (attP2, at 68A4) chromosomes by the Cambridge fly facility (University of Cambridge).

Drosophila stocks and genetics. Flies were raised and crossed at 25°C according to standard procedures. w1118 or FRT80B flies were used as the wild-type control strains. 138 RNAi lines, corresponding to 45 Drosophila DUBs (details available on request), were screened for border cell defects at 25°C. UAS-notR (Vienna Drosophila Resource Center #45776) was identified as having the most severe effect on migration. The FLP/FRT site-specific recombination system was used to generate mutant clones with a heat-shock promoter (Xu and Rubin, 1993). The following fly lines were obtained from the Bloomington Drosophila Stock Center: FRT80B (BL1988), w1118 (BL6409), slbo-Gal4, UAS-GFP (BL6458, Montell Lab), slbo-lacZ enhancer trap line (BL12227), slbo-Lifeact-GFP (BL58364), c306-Gal4, UAS-GFP (BL3743). For clonal analysis we used the following strains: hsFLP, tub-Gal4, UAS-GFP; +/-; tubGAL80 FRT80B/TM6B (generated from BL42732, BL5191), hsFLP, tub-Gal4, UAS-GFP; tubGAL80 FRT40A/+; +/- TM6B (generated from BL42732, BL5192), hsFLP, tub-Gal4, UAS-GFP; +/-; FRT82B tubGAL80/TM6B (generated from BL42732, BL4408). The amorphic not allele, notf, was obtained from Margarete Heck and recombined with FRT80B. FRT82B Ada2B was a gift from Jerry Workman (Li et al., 2017). UAS-Scar and FRT40A ScarΔ37 were gifts from Eyal Scheter. UAS-cpB, UAS-ex (Lucas et al., 2013), upd-lacZ (Jiang et al., 2009) and ex-lacZ (Fletcher et al., 2012), were gifts from Nic Tapon. Information on these strains is also available at http://www.flybase.org.

Generation of mosaic clones using MARCM. Mosaic Analysis with a Repressible Cell Marker (MARCM) was used to generate positively marked clones labelled with GFP (Lee and Luo, 2001). Expression of genes under GAL4-UAS is inhibited in the presence of GAL80. Heat shocking induces the expression of heat shock (hs) driven FLP, which acts to induce recombination at Flippase Recognition Targets (FRT). Homozygous daughter cells lacking GAL80 are then capable of GAL4-mediated gene expression of GFP and other UAS-transgenes. Mitotic recombination is initiated after heat shock where some daughter cells are GFP+ while others are GFP- due to the presence of GAL80. To obtain border cell mitotic (mosaic) clones, progeny of the right genotypes were heat shocked twice a day for 1 hour each with at least 5 hr intervals between treatments, from pupae to adult at 37°C. Newly enclosed adults (2-3 d old) were fattened for 2 d on yeast paste.

Immunofluorescent staining. Ovaries were dissected in PBS (Phosphate buffer saline) and fixed with 3.7% paraformaldehyde in PBS. The ovaries were washed with PBST (1x PBS, 0.2% Tween 20) 3 times for 15 minutes each time. Ovaries were then blocked with PBTB (1x PBS, 0.2% Tween 20, 5% fetal bovine serum) for 1 hour at room temperature. The ovaries were treated with primary antibodies in PBTB at 4°C overnight. The following
primary antibodies were used for immunostaining. Developmental Studies Hybridoma Bank (DSHB): mouse anti-Armadillo (N27A1, 1:200, concentrate), mouse anti-Enabled (5G2, 1:25, concentrate), mouse anti-B-gal (40-1a, 1:300, concentrate), mouse anti-eyes absent (eya10H6, 1:100, supernatant), mouse anti-SCAR (P1C1, 1:200, concentrate). Mouse anti-aPKC ζ (sc-17781, 1:200) from Santa Cruz. Guinea pig anti-Merlin (1:7500) from R Fehon lab. The primary antibodies were washed with PBST 3 times 15 min and then blocked with PBTB for 1 hr at room temperature. Ovaries were incubated with Alexafluor-conjugated secondary antibodies (1:500, Life technologies) in PBTB at 4°C overnight. Phalloidin 555 (1:50, Molecular Probes) was used to stain F-actin. Ovaries were washed with PBST for 15 minutes before staining nuclei with TO-PRO-3 (Life technologies, 1:1000) in PBST for 15 minutes. Ovaries were mounted in Vectashield (Vector laboratories). For Crumbs staining, Ovaries were dissected in PBS (Phosphate buffer saline) and fixed with boiled 8% paraformaldehyde in PBS and heptane (6:1) for 10 minutes. Samples were treated with heptane and methanol (1:2) for 30 seconds. They were then washed in methanol for 10 minutes. The ovaries were washed with PBST (1x PBS, 0.2% Tween 20) 2 times for 15 minutes each time. Ovaries were then blocked with PBTB (1x PBS, 0.2% Tween 20, 5% fetal bovine serum) for 30 minutes at room temperature. The ovaries were treated with mouse anti-Crumbs (Cq4, 1:100, concentrate, DSHB) in PBTB at 4°C overnight.

Image acquisition and analysis of fixed samples. Images were taken on a confocal microscope (LSM710 or LSM780, Carl Zeiss) using 20x/0.5NA air objectives. Three laser lines were used based on the excitation of wavelength of the staining dyes which includes 488 nm, 561 nm and 633 nm wavelengths. Extent of migration (the migration index) was measured as a percentage of the distance travelled to the oocyte/nurse cell boundary in stage 10 egg chambers. ImageJ was used for quantification of signal intensities in mosaic clusters using z-stack maximum projections. Raw integrated density was used as intensity values. For line scan profiles, maximum intensity images of Actin and Crumbs staining were generated in ImageJ. Background signal were subtracted. The plot profile function in ImageJ was used to measure signal intensities along lines drawn through the centre of border cell clusters and the peak analyser tool in OriginPro (Origin Lab) was used to calculate the area under peaks that were identified. The ratio of intensities at front, middle and back, were compared and normalised in Prism8 (Graphpad). The following statistical tests were performed using Prism 8 (GraphPad): Student’s t-tests; one-way or two-way Anova, with Tukey correction for multiple comparisons; multiple linear regression with least squares. Figures were made using FigureApp in OMERO (Allan et al., 2012; Burel et al., 2015) and final assembly in Adobe Photoshop.

Egg chamber culture and time-lapse imaging of live egg chambers. Live imaging of egg chamber culture were as previously described (Law et al., 2013; Prasad et al., 2007) with slight modification. Briefly, media for both dissection and live-imaging, comprised of Schneider media (Gibco), 15% fetal bovine serum, 0.1 mg/ml acidified insulin (Sigma), 9 µM FM4-64 dye (Molecular Probes) and 0.1 mg/ml Pen-strep (Gibco) was freshly prepared. The pH of the media was adjusted to 6.90-6.95. Individual egg chambers from well fattened progeny of the right genotype were dissected and transferred to borosilicate glass bottom chambered coverglasses (ThermoFisher) for imaging. Imaging was done at 25°C. Time-lapse movies were acquired on an inverted confocal microscope (LSM 710; Carl Zeiss) using 20x/0.5NA air objectives. Two laser lines were used based on the excitation of wavelength of the endogenous GFP and FM4-64 dye, which are 488 nm, and 561 nm wavelengths respectively. 16-20 slices of Z-stacks were taken with 2.5 µm slices every 3 min.

Analysis of time-lapse images. Time-lapse image analyses were performed using a custom macro for ImageJ to analyse the behaviour of border cell migration and extension dynamics (Law et al., 2013; Poukkula et al., 2011) with slight modification. Briefly, time-lapse movies were split into different channels. Maximum projections of the GFP-channel were created. Egg chambers were rotated so that anterior ends were at the left. Border cells were manually thresholded to mask nuclear GFP generated from the MARCM system through the first or early phase of migration. Images of border cells clusters were then segmented into cell body and cellular extensions using signals from sibo-LifeAct-GFP. Extensions were grouped based on their positions in relation to the leading edge of the cluster: front (315-45°), side (45-135° or 225-315°) and back (135-225°). The macro also enabled tracking of the movement of cluster to measure the migration speed. Forward directed speed was calculated on x-axis by taking distance of the centre of cluster at one time point relative to the next time point. The tumbling index was calculated as the mean percentage of frames per time lapse movie that showed rounded clusters, exhibiting changes in the position of individual cells within the cluster for two or more consecutive frames in the first half of migration. Data were collated in Microsoft Excel and independent Student’s t-tests were done with Prism 8 (GraphPad). For visualisation of stills (Fig4A,A’-B,B’), GFP-labelled nuclei were segmented in Imaris (Bitplane) and labelled in white.

Analysis of previously reported ChIP datasets. ChIP-seq data were downloaded from GEO (https://www.ncbi.nlm.nih.gov/geo/) using accession GSE98862; the dm3 assembly of the D. melanogaster genome was obtained from UCSC (http://www.genome.ucsc.edu/cgi-bin/hgTables). Peaks from Ada2b and Non-stop ChIP experiments were mapped to the dm3 genome assembly using BEDtools software (Quinlan and Hall, 2010), and
any genes matching to peaks from -1000 to +200 of the transcription start site (TSS) were identified. For visualisation of ChIP-seq peaks on the genome, we utilised the 'karyoplateR' R/Bioconductor package (Gel and Serra, 2017).

Genotypes of strains

Fig 1.

A. w1118/+; Slbo-Gal4, UAS-GFP/+  
B. Slbo-Gal4, UAS-GFP/UAS-notR  
C. c306-Gal4, UAS-GFP; UAS-not/+  
E. (as A-C with) Slbo-Gal4, UAS-GFP/UAS-notR; UAS-not/+  
F. hsFLP, tub-Gal4, UAS-GFP/++; +, FRT80B/tub-Gal80, FRT80B  
G.I. hsFLP, tub-Gal4, UAS-GFP/++; not1, FRT80B/tub-Gal80, FRT80B  
H. (as F,G with) hsFLP, tub-Gal4, UAS-GFP/++; UAS-not/+; not1, FRT80B/tub-Gal80, FRT80B

Fig 2.

A. hsFLP, tub-Gal4, UAS-GFP/++; slbo-lacZ/+; +, FRT80B/tub-Gal80, FRT80B  
B. hsFLP, tub-Gal4, UAS-GFP/++; slbo-lacZ/+; not1, FRT80B/tub-Gal80, FRT80B  
C. Quantification of A,B:  
Wild type GFP+: hsFLP, tub-Gal4, UAS-GFP/++; slbo-lacZ/+; +, FRT80B/tub-Gal80, FRT80B (or homozygous for tub-Gal80, FRT80B)  
Wild type GFP+: hsFLP, tub-Gal4, UAS-GFP/++; slbo-lacZ/+; +, FRT80B/tub-Gal80, FRT80B  
slbo-GFP: hsFLP, tub-Gal4, UAS-GFP/++; slbo-lacZ/+; +, FRT80B/tub-Gal80, FRT80B (or homozygous for tub-Gal80, FRT80B)  
slbo-GFP: hsFLP, tub-Gal4, UAS-GFP/++; slbo-lacZ/+; +, FRT80B/tub-Gal80, FRT80B  
slbo-GFP: hsFLP, tub-Gal4, UAS-GFP/++; slbo-lacZ/+; +, FRT80B/tub-Gal80, FRT80B  
not1: hsFLP, tub-Gal4, UAS-GFP/++; slbo-lacZ/+; +, FRT80B/tub-Gal80, FRT80B  
D. hsFLP, tub-Gal4, UAS-GFP/++; +, FRT80B/tub-Gal80, FRT80B  
E. hsFLP, tub-Gal4, UAS-GFP/+; not1, FRT80B/tub-Gal80, FRT80B  
F. hsFLP, tub-Gal4, UAS-GFP/udp-lacZ/++; +, FRT80B/tub-Gal80, FRT80B  
G.H. hsFLP, tub-Gal4, UAS-GFP/udp-lacZ/++; +, FRT80B/tub-Gal80, FRT80B  
I,J. (quantification of F-H)

Fig 3.

wt control: hsFLP, tub-Gal4, UAS-GFP/++; +, FRT80B/tub-Gal80, FRT80B  
not1: hsFLP, tub-Gal4, UAS-GFP/++; not1, FRT80B/tub-Gal80, FRT80B  
not1: tub>not1: hsFLP, tub-Gal4, UAS-GFP/++; UAS-not1/+; +; not1, FRT80B/tub-Gal80, FRT80B

Fig 4.

Control: hsFLP, tub-Gal4, UAS-GFP/++; slbo-LifeAct-GFP/+; +, FRT80B/tub-Gal80, FRT80B  
not1: hsFLP, tub-Gal4, UAS-GFP/++; slbo-LifeAct-GFP/+; not1, FRT80B/tub-Gal80, FRT80B

Online supplementary material

Video S1: 4 h time-lapse of border cell migration starting from specification of the cluster and the ability of the cluster to acquire forward protrusion, followed by cell-on-cell migration to the anterior border of the oocyte. GFP expression is driven by slbo-Gal4 to label the border cell cluster in green. Nuclei are labelled with Ub-His2A-RFP in magenta.

Video S2: 4 h time-lapse movie of normal border cell migration showing onset of migration including the ability of cluster to acquire forward actin protrusions. MARCM clones are labelled with nuclear GFP, F-actin is labelled with LifeAct-GFP. Egg chamber genotype: hsFLP, tub-
Gal4, UAS-GFP/+; slbo-LifeAct-GFP/+; +, FRT80B/tub-Gal80, FRT80B.

**Video S3:** 4 h time-lapse movie of abnormal border cell migration showing early tumbling of the cluster and multi-directional actin protrusions in not1 mutant cells labelled with nuclear GFP using MARCM. F-actin is labelled with LifeAct-GFP. Egg chamber genotype: hsFLP, tub-Gal4, UAS-GFP/++; slbo-LifeAct-GFP/++; not1, FRT80B/tub-Gal80, FRT80B

**Acknowledgments**

We thank Rick Fehon, Margarete Heck, Timothy Megraw, Eyal Schejter, Nic Tapon, Jerry Workman the Developmental Studies Hybridoma Bank (DSHB), and Bloomington Stock Center for antibodies, vectors and fly stocks. Thanks also to the Liverpool Computational Biology Facility and Chris Seidel (Stowers Institute) for assistance with ChIP data analysis, and to the Liverpool Centre for Cell Imaging (https://cci.liv.ac.uk/) for help with microscopy and image analysis. The work was funded by the MRC (MR/K015931/1), NWCR (CRC847), Liverpool CRUK Centre and the University of Liverpool international PhD fees waiver scheme.

**References**


