Spatially localized Kuzbanian required for specific activation of Notch during border cell migration

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Abstract

The transmembrane receptor Notch is used repeatedly during development for a variety of essential functions. During Drosophila oogenesis, Notch activity is required first to specify particular follicle cell fates, then to promote the differentiation of all follicle cell types, to promote border cell migration, and then to form dorsal appendages, raising the question as to how Notch activity is spatially and temporally regulated. Here we show the Notch activity pattern during oogenesis. Notch activation was found in many follicle cells at stage 6 but then at stage 9 was restricted to migrating border cells, despite uniform expression of Delta. Expression of Kuzbanian (KUZ), a metalloproteinase that can activate Notch as well as cleave other substrates, is enriched in border cells at stage 9; and dominant-negative KUZ caused a strong border cell migration defect, without affecting expression of markers of border cell fate or follicle cell differentiation. Constitutively active Notch rescued the migration defect due to dominant-negative KUZ, and conditional alleles of Delta and Notch also exhibited border cell migration defects. Expression of two different reporters of Notch activity was lost upon expression of dominant-negative KUZ. Taken together these results show that Notch activation and KUZ expression are restricted to border cells at stage 9 of oogenesis and are required for migration, but not differentiation, of these cells. This represents a previously unrecognized mechanism for achieving spatial restriction of Notch signaling.

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Introduction

The Notch family of transmembrane receptors play critical roles in the development of a multitude of cell types and tissues in organisms ranging from worms and fruitflies to mice and humans (Artavanis-Tsakonas et al., 1999). In addition to this extensive repertoire, Notch signaling can stimulate epithelial to mesenchymal transitions (Grego-Bessa et al., 2004; Timmerman et al., 2004), cause T cell leukemia (Aster and Pear, 2001; Ellisen et al., 1991), or act as a tumor suppressor (Lefort and Dotto, 2004).

First discovered in Drosophila, the Notch gene is used and reused throughout fly development. Restriction of Notch signaling through lateral inhibition is required to establish the proper ratio of neuronal precursors to epidermal cells in the early embryo (Knust and Campos-Ortega, 1989). During the formation of sense organs in pupae, asymmetric Notch signaling acts at each cell division to affect binary cell fate choices. In imaginal discs, Notch functions at multiple steps in the formation of the eye (Brennan and Moses, 2000) as well as in dorsal ventral patterning of the wing (Irvine and Vogt, 1997).

In the adult female, Notch is required for multiple steps in oogenesis, including specification of polar cell precursors, formation of polar and stalk cells, differentiation of all epithelial follicle cells, migration of border cells, and dorsal appendage patterning (Gonzalez-Reyes and St Johnston, 1998; Grammont and Irvine, 2001; Ruohola et al., 1991; Schober et al., 2005; Ward et al., 2006).

Notch is notable not only for its myriad biological functions but also for the variety of unusual mechanisms that regulate its activity. Notch is synthesized as a transmembrane precursor, which undergoes several proteolytic cleavages. Processing by a
furin-type protease in the endoplasmic reticulum is necessary for proper trafficking of vertebrate Notch to the cell surface (Logeat et al., 1998) but may not be required for Drosophila Notch (Kidd and Lieber, 2002). Binding of one of the DSL (Delta, Serrate, Lag2) family of ligands is a prerequisite for Notch to be cleaved by an ADAM family protease (KUZ in flies, ADAM10 in mammals) (Lieber et al., 2002; Pan and Rubin, 1997), which renders the protein accessible to presenilin-dependent processing within the transmembrane domain (Fortini, 2001). This final cut liberates the intracellular domain, which travels to the nucleus where it acts as a transcriptional co-activator. The Notch intracellular domain binds Suppressor of Hairless [Su(H)] (Fortini and Artavanis-Tsakonas, 1994), converting it from a repressor to an activator of target gene expression.

Several mechanisms are known that can result in higher Notch signaling in one cell as compared to a neighboring cell. For example, asymmetric localization of the Numb protein specifically inhibits Notch activity in one daughter cell during asymmetric divisions of neuronal precursor cells (Guo et al., 1996). In contrast, elevation of Delta expression in the germline at stage 6 of Drosophila oogenesis is also thought to be responsible for activation of Notch in the surrounding follicle cells, causing them to exit mitosis and initiate endoreplication cycles (Lopez-Schier and St Johnston, 2001b).

Another mechanism by which Notch signaling can be spatially regulated in development is via localized expression of the glycosyltransferase enzyme encoded by the fringe gene (Bruckner et al., 2000). Fringe acts cell autonomously to modify the Notch protein and this modification increases the affinity of Notch for Delta while decreasing its affinity for Serrate (Panin et al., 2001). Fringe is important in establishing dorsal/ventral patterning in the wing. In addition, Fringe is expressed specifically in polar cells in developing egg chambers where it is required for their Notch-mediated cell fate specification (Grammont and Irvine, 2001). Thus in the fly ovary, at least two different mechanisms are thought to regulate when and where Notch is activated: spatially restricted expression of Fringe in polar cells and uniform upregulation of Delta expression in germ cells at stage 6.

After specifying polar and stalk cells and then inducing differentiation of all follicle cells, Notch activity is required a third time during Drosophila oogenesis, at stage 9 for the normal migration of border cells (Schober et al., 2005). Border cells are a group of 6 to 10 cells that develop from the anterior tip of stage 9 egg chambers (Montell, 2003). Border cells delaminate from the monolayer of approximately 650 epithelial follicle cells, extend protrusions in between the nurse cells, and migrate approximately 100 μm until they reach the border between the nurse cells and the oocyte (Figs. 1A–C). Border cell migration is abnormal in egg chambers from females homozygous for a temperature-sensitive allele of Notch (Nts) (Schober et al., 2005), although it is unclear whether Notch is activated specifically in border cells at stage 9 or whether it is more generally activated and required for follicle cell behavior at this stage.

If Notch activity were restricted at stage 9, it is unclear how such a spatially restricted activation pattern would be achieved. Moreover, it is unclear whether the Nts migration defects are a secondary consequence of the general requirement that all follicle cells have for Notch activity in the process of differentiation.

Here we show that KUZ expression and Notch activity are specifically elevated in border cells during migration. Conditional and/or dominant-negative alleles of KUZ, Notch, and Delta all demonstrate border cell migration defects. Expressing dominant-negative KUZ specifically in border cells does not affect their differentiation detectably but specifically perturbs their ability to migrate and inhibits activation of Notch. Moreover constitutively active Notch rescues border cell migration defects that are due to reduction in KUZ activity. These results provide evidence for a previously undescribed mechanism for the spatial regulation of Notch activation during development through the regulation of the pattern of KUZ gene expression.

Results

Activated Notch rescues border cell migration defects associated with expression of KUZ dominant-negative

We recently reported a microarray analysis, which identified those mRNAs that are more highly expressed in border cells and/or centripetal cells of the Drosophila ovary, which are both migratory cell types, compared to the non-migratory cell population (Wang et al., 2006). KUZ was one of the genes and in situ hybridization confirmed the enrichment of KUZ mRNA in border cells (Wang et al., 2006). KUZ expression was detected at a higher level in border cells than in other follicle cells throughout migration (Figs. 1D, E, and data not shown). In addition, expression of dominant-negative KUZ (KUZ-DN) was previously shown to inhibit border cell migration (Wang et al., 2006); however, the relevant substrate of KUZ in the border cells had not been identified.

In addition to regulating Notch, the mammalian homolog of KUZ has been shown to cleave a ligand that activates the EGF receptor (Sahin et al., 2004) and E-cadherin (Maretzky et al., 2005). Since the EGF receptor and E-cadherin are known to function in border cell migration, we investigated which of the KUZ substrates was most important in this context. To distinguish whether the requirement for KUZ activity was primarily due to a requirement for activation of Notch, or possibly another substrate, we tested the ability of constitutively active Notch to rescue the KUZ-DN phenotype.

We quantified the migration defects by dividing the migration path into four quadrants and observing the extent of migration in >100 stage 10 egg chambers for each of the genotypes examined. By stage 10, border cells reach the fourth quadrant, and are at or near the oocyte/nurse cell border, in >95% of control egg chambers, whereas this is true for <25% of border cell clusters expressing dominant-negative KUZ (Figs. 1G–J). This phenotype was even
stronger at 29°C (Wang et al., 2006). In contrast, the full-length form of KUZ (KUZ-F) alone, expressed in wild-type egg chambers had no detectable effect (Wang et al., 2006). Conditional expression of KUZ-DN was employed rather than loss of function mutant alleles, because of the requirement for KUZ and Notch signaling earlier in oogenesis. As a consequence, follicle cell clones lacking the function of either gene fail to form border cells at all (not shown).

The Notch intracellular domain (N\textsuperscript{intra}) expressed together with KUZ-DN, ameliorated the migration defect compared to KUZ-DN alone (Fig. 1J). In fact, N\textsuperscript{intra} rescued border cell migration to the same extent as KUZ-F. This result suggested that Notch was the most important substrate for KUZ in border cells, although it was not possible to rule out some contribution from another substrate. Expression of the activated EGF receptor failed to rescue the KUZ-DN phenotype (not shown); however, the interpretation of this
result is confounded by the strong dominant inhibitory effect that the activated EGF receptor has on border cell migration (Duchek and Rorth, 2001).

Notch has previously been shown to be required for proper differentiation of all follicle cells at stage 6 and clones of follicle cells lacking Notch expression fail to differentiate (Lopez-Schier and St Johnston, 2001a). Notch is widely expressed in ovaries, including in border cells (Xu et al., 1992). To investigate the possibility that in addition to the general requirement for Notch in follicle cell differentiation, there is a specific requirement for Notch activity during border cell migration, we examined egg chambers from females homozygous for a temperature-sensitive allele of Notch (Nts2), shifted to the non-permissive temperature after follicle cell differentiation was complete. Stage 9 lasts approximately 6 h. Therefore, we evaluated border cell migration in stage 10 Nts2 egg chambers that were shifted to the non-permissive temperature for less than 6 h (Fig. 1K). Migration defects were apparent even though these egg chambers had already developed to stage 9, and follicle cell differentiation was allowed to occur, prior to the temperature shift. In the same experiment, wild-type egg chambers incubated at the same temperature showed no migration defect (not shown).

**KUZ-DN border cells exhibit altered morphology but normal differentiation**

To address the possibility that the KUZ-DN border cell migration defects were due to problems with border cell differentiation, we examined the expression of a number of markers. In Notch mutant follicle cells that fail to differentiate properly, expression of Fasciclin 3 (FAS3) is observed (Lopez-Schier and St Johnston, 2001a). FAS3 is a marker of undifferentiated follicle cells as well as of differentiated polar cells. In all egg chambers examined in which KUZ-DN was...
expressed specifically in border cell clusters, FAS3 expression appeared normal and was restricted to polar cells, as it normally is at these stages (Figs. 2A–C). We tested several other markers of border cell differentiation as well. slbo<sup>PZ1310</sup>, Singed, and Eyes Absent were all expressed normally in all border cells examined that were expressing KUZ-DN, indicating that a general problem with cell differentiation was not the reason for the observed migration defect (Figs. 2D–L). Although gene expression appeared normal, the morphology of the border cell clusters was frequently different from wild-type. The clusters appeared less round and the cells less tightly clustered (Figs. 2B, H, K) than wild-type (Figs. 2C, I, L).

Migration defects in Delta mutants

Delta and Serrate are the best-characterized ligands for Notch. Delta and Serrate are widely expressed in oogenesis (Bender et al., 1993; Dobens et al., 2005) (Figs. 3A–C). Delta mutant germline clones cause early defects in polar and stalk cell specification and as a consequence border cells fail to form (Lopez-Schier and St Johnston, 2001a). Therefore we examined the contribution of DI to border cell migration using dominant-negative and temperature-sensitive alleles. In Dl<sup>6B</sup>/Dl<sup>RF</sup> flies at the permissive temperature of 18°C, border cells migrate to reach the oocyte at stage 10 (Figs. 3D, E). Following incubation of Dl<sup>6B</sup>/Dl<sup>RF</sup> flies at the non-permissive temperature of 32°C overnight, border cells failed to migrate in 12 out of 72 stage 10 egg chambers examined (Fig. 3F), even though the clusters formed (Fig. 3G). Border cell migration was severely impaired following expression of a dominant-negative form of Delta specifically in the border cells using slbo-Gal4 (Fig. 3H). In contrast, border cells migrated normally in egg chambers bearing Serrate mutant germline or somatic cell clones (not shown).

Fig. 3. Expression and function of DI. (A–C) Delta protein expression pattern (green) and DAPI staining (blue) in egg chambers of the indicated stages. (D–G) Border cells (arrows) in egg chambers dissected from Dl<sup>6B</sup>/Dl<sup>RF</sup> females incubated at the permissive temperature 18°C (D, E) or non-permissive temperature 32°C (F, G). In panel F, border cells and centripetal cells are labeled with anti-armadillo antibody staining (red) and all nuclei are stained with DAPI (blue). Panels E and G show high magnification of border cell cluster stained with DAPI. (H) Bar graph demonstrating the migration defect associated with expression of dominant-negative Delta (DI–DN).
Notch activity restricted to migrating border cells at stage 9

As mentioned above, Notch is known to be required early in oogenesis for polar cell fate specification and then at stage 6 for differentiation of all the follicle cells. Our results implicated KUZ and Notch at stage 9 in border cell migration, independent of its earlier functions. We therefore investigated the spatial and temporal pattern of Notch activation in egg chambers. To do so, we employed the reporter system, Notch-GV, developed by Struhl and Adachi (2000). The basis of this reporter is that the intracellular domain of Notch can only enter the nucleus following ligand binding and ligand-dependent proteolytic cleavage. Struhl and Adachi fused the Gal4VP16 transcriptional activator coding sequence to the C-terminus of Notch and expressed this as a heat-inducible transgene. Heat shock induces relatively uniform expression of the transgene; however, only in those cells in which Notch is cleaved can the NintraGal4VP16 moiety translocate to the nucleus and Activate UAS-lacZ. Therefore beta-galactosidase can only be expressed in cells in which Notch is activated.

Flies of the genotype hs-Notch-GV;UAS-lacZ were heat shocked for 1 h to induce ubiquitous expression of the Notch-GV fusion protein. Three hours later, we dissected the ovaries and stained for the beta-galactosidase expression pattern. Beta-gal expression was observed in the majority of follicle cells at stage 6, consistent with the previously described requirement for Notch activity at this stage (Fig. 4D). Strikingly, throughout the period of border cell migration, beta-gal expression was specific to the migratory population of border cells surrounding the polar cells (Figs. 4A–C and F). Although Notch activity is required for polar cell fate specification earlier in oogenesis, there was a conspicuous absence of reporter gene expression in polar cells at stage 9.

To test whether KUZ contributed to this specific Notch activation pattern, we examined Notch-GV driven beta-gal expression in heat shock KUZ-DN flies. Expression of dominant-negative KUZ significantly reduced Notch reporter gene expression in border cells (Figs. 4E and G).

In addition to Notch-GV, Su(H)lacZ serves as a reporter of Notch activity (Furriols and Bray, 2001). Su(H)lacZ is a beta-galactosidase reporter construct that contains Su(H) and Grainy Head binding sites and shows highly enriched expression in border cells at stages 9–10 (Figs. 4H and J and Schober et al., 2005). To test whether the border cell enrichment of KUZ expression contributed to the spatially localized pattern of Notch activity, we inhibited KUZ specifically in border cells and assayed the effect on Su(H)lacZ. Su(H)lacZ expression was dramatically reduced (Figs. 4I and K). Thus border cell expression of KUZ is required to achieve the spatially localized pattern of Notch activity in stage 9.

Fig. 4. Notch activation pattern. (A–G) The Notch-GV reporter was used to monitor the pattern of activation of Notch during oogenesis (see text for details). Beta-galactosidase expression (green) indicates cells in which Notch is activated. FAS3 (red) staining marks the interface between the two polar cells, which are surrounded by the migratory cells. (A–D) Wild-type egg chambers of the indicated stages. (E) Effect of expressing KUZ-DN on Notch-GV reporter expression. (F) High magnification view of Notch-GV reporter expression in a wild-type border cell cluster. (G) High magnification view of Notch-GV reporter expression in a border cell cluster after heat-shock induced expression of KUZ-DN. (H) Pattern of expression of Su(H)-lacZ (green) in a wild-type stage 10 egg chamber. (I) Pattern of expression of Su(H)-lacZ (green) in a stage 10 egg chamber expressing KUZ-DN in border cells. DAPI staining is shown in blue. Panels J and K are high magnification views of the clusters shown in panels H and I, respectively.
Discussion

KUZ, Notch, and Delta are required for normal border cell migration

We previously reported that KUZ expression is enriched in border cells and required for their migration (Wang et al., 2006). Here we demonstrate that Notch, rather than other described substrates for KUZ and its vertebrate homologs, is the major substrate of KUZ during border cell migration. KUZ, Delta, and Notch are all required for normal border cell migration, and Notch is specifically activated in border cells, but not in polar cells, throughout the 6 h period of migration. This high degree of specificity in the Notch activation pattern was surprising, given the pattern of Delta expression. Delta is expressed throughout the germline (Bender et al., 1993), at highest levels between stages 5 and 7 (Lopez-Schier and St Johnston, 2001b). Delta can also be observed in follicle cells. Our mutant analysis suggests that, despite its global expression pattern, Delta is likely to be the functionally relevant ligand for Notch with respect to border cell migration because Dl^{1B}/Dl^{RF} mutants exhibit border cell migration defects at the non-permissive temperature whereas loss of Serrate had no effect.

The question then arises as to how the spatial localization of Notch activity is achieved. We demonstrate that KUZ is expressed at highest levels in the border cells and is required for the localized activation of Notch. This mechanism of localization of Notch signaling has not been reported previously. Notch appears to be enriched in border cells during the period of their migration relative to the expression level in other follicle cells (Xu et al., 1992). However, this enrichment is not as great as the specificity of Notch-GV driven reporter gene expression. Therefore, multiple factors may contribute to the highly specific pattern of Notch activity at stage 9.

As a transmembrane receptor, there are a variety of functions Notch could possibly carry out in cell migration, including transcripational activation of downstream target genes or cell-cell adhesion. The finding that KUZ is required for migration suggests that ligand-induced proteolytic cleavage of Notch is important, implicating the transcriptional activation function. Further evidence for this is that N^{intra} rescued migration in border cells expressing dominant-negative KUZ, at least as well as full-length KUZ. This result also implies that the level of Notch activity need not be carefully regulated. This contrasts with activation of several other receptors and transcription factors in border cells, such as PVR, EGF-R, and STAT, hyperactivation of which is detrimental to border cell migration (Silver and Montell, 2001).

Our analysis of the N^{52} phenotype demonstrates that activation of Notch is required for migration, independent of the earlier requirement for Notch in cell differentiation. This conclusion is based on several observations and is supported by the finding that migration defects begin to appear even after relatively short temperature shifts in the N^{52} mutants. If Notch activity were required only at the earlier stage, then we would expect that egg chambers would have to be shifted to the non-permissive temperature at stage 6, about 18 h prior to migration, for the defect to show up. However, we saw migration defects as early as 2 to 5 h after temperature shift. Further support of a specific effect on migration was that border cell migration was severely impaired following expression of dominant-negative KUZ using slbo-GAL4, which does not drive high levels of expression until stage 9. In addition, markers of border cell fate and differentiation appeared normal in border cells that failed to migrate due to expression of dominant-negative KUZ.

Sequential activation of the Notch pathway during oogenesis occurs via distinct mechanisms

Notch activity is required in multiple cell types and tissues throughout development in many organisms. Even within one tissue, Notch signaling can be employed over and over again. For example, during sensory organ development, differential Notch signaling occurs following each cell division to specify the fates of the daughter cells (Jan, 1993). In the Drosophila ovary, Notch signaling is deployed multiple times for different purposes and each time the regulation of Notch activation is achieved via a distinct mechanism. Polar cells express high levels of Fringe, a protein known to modify Notch so that it is more sensitive to Delta (Panin et al., 1997). Fringe is required for polar cell fate specification (Grammont and Irvine, 2001). However, Fringe is not required for the other functions of Notch in the ovary. On the other hand, elevated Delta expression at stage 6 correlates with increased Notch activity in all the follicle cells at this stage, leading to their differentiation (Lopez-Schier and St Johnston, 2001a). Based on the work presented here, elevated KUZ expression in border cells appears to be critical for achieving specific activation of Notch during their migration. It may be that the great variety of mechanisms available to regulate Notch activation contribute to the extreme versatility of this molecule during development.

A general role for Notch activation in cell migration

While the role of Notch in cell fate specification has been studied for decades, only recently has the importance of Notch signaling in cell migration been appreciated (Grego-Bessa et al., 2004; Timmerman et al., 2004). Notch is required for epithelial to mesenchymal transitions in heart development and in tumor metastasis. One key target of Notch in those contexts is the transcriptional repressor Snail. In border cells, Notch promotes cell motility as well, even though border cells undergo only a partial EMT and retain significant epithelial polarity during their migration (Niewiadomska et al., 1999; Pinheiro and Montell, 2004). It is interesting that one key downstream target of Notch in border cells is Yan which, like snail, is a transcriptional repressor (Schober et al., 2005). In border cells, Yan modulates E-cadherin expression and/or distribution to allow cell migration and this function is similar to one of the key functions of Snail in EMT. It will be of interest in the future to determine if the KUZ–Notch–Yan–E-cadherin pathway is found in other examples of epithelial cell migration and how generally the spatial pattern of KUZ expression contributes to the spatial pattern of Notch activation.
Experimental procedures

Drosophila genetics

US-KUZ-DN, US-KUZ-F, D\(f^{Rh}\), N\(m^{2}\), and US-DI-DN were obtained from the Bloomington stock center. US-\(X^{men}\) (Nint.G.Scr/UAS) (Schultz and Brand, 1999) and Su(Hy)lacZ were from Dr Norbert Perrimon. The Notch GV construct hs-Notch-\(+\)-GV was a gift from Dr. Gary Struhl. slbo-Gal4 was from Dr. Pernille Rorth (Rorth et al., 1998) and recombined with UAS-mCD8-GFP from Dr. Tzumin Lee (Lee and Luo, 1999). D\(f^{Rh}\) was from Dr. Marc Muskavitch. hs-KUZ-DN was from Dr. Duoja Pan. All flies were kept at 25°C except where indicated.

For temperature-sensitive experiments, N\(m^{2}\) was kept at 18°C and was shifted to 32°C for 1 to 5 h. D\(f^{Rh}\) was crossed to D\(f^{Rh}\) at 18°C, the D\(f^{Rh}\)-D\(f^{Rh}\) progeny were shifted to 32°C overnight. For hs-Notch-\(+\)-GV; adult flies 2–7 days old were well fattened and shifted to 37°C for 1 h and returned to 25°C for 3 h before dissection.

For analysis of border cell migration phenotype using Gal4/UAS system, slbo-Gal4/Cyo was crossed to UAS transgenic flies. Non-balancer progeny were fattened at 25°C or 29°C overnight before dissection.

In situ hybridization

In situ hybridization was performed as described (Wang et al., 2006).

Immunohistochemistry

All staining procedures followed the protocol as described (McDonald and Montell, 2005). The antibodies used were: mouse anti-armedillo at 1:25 (N2 7A1, DSHB); mouse anti-singed at 1:50 (eya7c, DSHB); mouse anti-eya at 1:50 (N2 7A1, DSHB); mouse anti-delta at 1:50 (from Dr. Marc Muskavitch) (Bender et al., 1993). Secondary antibodies conjugated to Alexa fluor 488 and 568 were used at 1:200. DAPI was used to stain cell nuclei. Images were acquired using ApoTome system on a Zeiss Axioplan 2 microscope or a Zeiss LSM 510 Meta confocal microscope.

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References


