Collective border cell migration requires the zinc transporter Catsup to limit endoplasmic reticulum stress

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Abstract

Collective cell migration is critical for normal development, wound healing, and in tumor progression and metastasis. Border cells in the Drosophila ovary provide a genetically tractable model to identify molecular mechanisms that drive this important cell behavior. In an unbiased screen for defects in border cell migration in mosaic clones, we identified a mutation in the catsup gene. Catsup, the Drosophila ortholog of Zip7, is a large, multifunctional, transmembrane protein of the endoplasmic reticulum (ER), which has been reported to negatively regulate catecholamine biosynthesis, to regulate Notch signaling, to function as a zinc transporter, and to limit ER stress. Here we report that catsup knockdown caused ER stress in border cells and that ectopic induction of ER stress was sufficient to block migration. Notch and EGFR trafficking were also disrupted. Wild type Catsup rescued the migration defect but point mutations known to disrupt the zinc ion transport of Zip7 did not. We conclude that migrating cells are particularly susceptible to defects in zinc transport and ER homeostasis.

Keywords: Cell migration, Catsup, ER stress, Notch, Zinc transporter

Introduction

Collective cell migration has emerged as a key driver of normal organ development, wound repair, and tumor metastasis. Border cell migration in the Drosophila ovary provides a powerful in vivo model of collective cell migration that is amenable to unbiased genetic screening. Drosophila ovaries are composed of ovariols, which are strings of egg chambers progressing through 14 stages of development to mature eggs (Fig.1A). Each egg chamber is composed of 16 germ cells including 15 nurse cells and one oocyte, which are surrounded by epithelial follicle cells. During stage 9 (Fig.1B and C), 4-8 border cells are specified at the anterior end of the egg chamber, delaminate from the follicular epithelium, and migrate posteriorly, reaching the anterior border of the oocyte by stage 10.

Genetic screens have yielded insights into the molecular mechanisms that specify which of the ~850 follicle cells acquire the ability to migrate, the developmental timing of the migration, collective direction sensing, and cytoskeletal dynamics. While much is understood, insights continue to emerge from border cell studies. The gene catsup was identified both in a large-scale, ethyl methanesulfonate-induced mutagenesis screen for border cell migration defects in mosaic clones and in a whole-genome gene expression profile.

Fig. 1. Catsup expression pattern in ovariols. (A) An ovariol from germarium to developmental stage 10, where border cells (white arrowhead) arrive at the anterior border of the oocyte. (A’) An endogenously tagged Catsup::GFP shows the expression pattern of Catsup. (B) An early stage 9 egg chamber as border cells (arrowhead) initiate migration. (C) A mid stage 9 egg chamber with border cells en route to the oocyte. (D-H) High magnification of a border cell cluster showing the localization of overexpressed CatsupV5 (yellow), anti-PDI staining for ER (green), phalloidin staining for F-actin (magenta), Hoechst staining for DNA (blue). (I-K) 2-dimensional intensity histograms for two selected channels showing colocalization of CatsupV5 relative to ER, F-actin and nuclei. The colocalization regression Pearson’s coefficient displayed in the upper right corner. (L) CatsupV5 is highly colocalized with ER rather than F-actin or nuclei, shown by quantification of the Pearson’s coefficients from 4 border cells. ** P value < 0.01. (M-Q) High magnification of a border cell cluster showing the localization of endogenously tagged Catsup::GFP, ER (PDI), F-actin (Phalloidin) and nuclei (DNA). (R-U) 2-dimensional intensities histograms for two selected channels showing the colocalization and the Pearson’s coefficient of CatsupV5 relative to ER, nuclei, F-actin, and of ER relative to F-actin.
The name catsup is an abbreviation of “catecholamines up”, loss of which increases synthesis of aromatic amines including neurotransmitters such as epinephrine and dopamine\textsuperscript{37}. Catsup is required for Drosophila tracheal morphogenesis, and in this context, it inhibits the Drosophila homolog of tyrosine hydroxylase (Ple) to limit dopamine synthesis\textsuperscript{38}. In contrast, in wing imaginal disc cells, Catsup facilitates proper trafficking of Notch and EGFR\textsuperscript{39}. Its mechanism of action in border cells is unknown.

Catsup shares a 62\% similarity and 53\% identity with its mammalian homolog ZIP7 (also known as SLC39A7 or HKE4)\textsuperscript{39}, a member of one of the two major families of zinc transporters\textsuperscript{40}. ZIP7 is located within intracellular membranes including ER where ZIP7 transports Zn\textsuperscript{2+} to the cytosol\textsuperscript{41}. Zinc is a necessary trace element vital for many proteins to function, and zinc homeostasis requires 24 zinc transporters in humans, 14 of which are ZIPs\textsuperscript{42}. Zip7 is a conserved protein found in the ER and Golgi in organisms as diverse as yeast, plants and animals\textsuperscript{43–48}. In animal cells, loss of ZIP7 can lead to ER stress and in some cases cell death. Furthermore, increased ZIP7 expression is positively correlated with cancer cell proliferation, growth, invasion, and metastasis\textsuperscript{39} of breast\textsuperscript{50,51}, cervical\textsuperscript{52}, and colorectal cancer\textsuperscript{53}.

In the mammalian intestine, loss of ZIP7 causes an increase in ER stress and loss of stem cells\textsuperscript{54}. Similarly, catsup loss-of-function causes ER stress in fly wing imaginal discs\textsuperscript{39}. Thus, Catsup and ZIP7 are multifunctional proteins. However, the relationships between ER stress, zinc transport, and cell motility remain to be clarified.

Results

Using an endogenously-tagged Catsup::GFP fusion, we found that Catsup is expressed throughout oogenesis, including in all follicle cells (Fig. 1A–C). Mammalian ZIP7 localizes predominantly to the ER\textsuperscript{41}, so we investigated the subcellular localization of Catsup. Both overexpressed, tagged Catsup\textsuperscript{V5} (Fig. 1D–L) and endogenously tagged Catsup::GFP (Fig. 1M–U) significantly co-localized with the ER resident enzyme protein disulfide isomerase (PDI), but not with DNA or F-actin.

Expressing a Catsup RNAi line in border cells using c306Gal4 (Fig. 2A) and FruitlessGal4\textsuperscript{41} led to incomplete migration in 80\% of stage 10 egg chambers examined (Fig.1B and C), a defect that was rescued by UAS-Catsup\textsuperscript{V5} (Fig. 1C). Using the FLP-FRT system, we generated clones of catsup mutant cells in genetically mosaic egg chambers. Compared to control clones (Fig. 2D–E\textsuperscript{\textdagger}), border cell clusters containing cells homozygous mutant for catsup exhibited migration defects (Fig. 2E–E\textsuperscript{\textdagger})\textsuperscript{56}, the severity of which was proportional to the percentage of mutant cells per cluster (Fig. 2F). In addition, in clusters containing both heterozygous and homozygous mutant cells, homozygous mutant cells tended to occupy rear positions (Fig. 2G–G\textsuperscript{\textdagger} and E), which is typical of mutations in genes required for motility\textsuperscript{55}.

One known function of Catsup is direct binding to and inhibition of the tyrosine hydroxylase Ple, which is the rate-limiting enzyme in catecholamine synthesis\textsuperscript{36}. Ple and Catsup are both expressed in embryonic tracheal cells, where they contribute to achieving proper dopamine levels, which regulate Breathless (fibroblast growth factor receptor) endocytosis and signaling\textsuperscript{15}. To test whether inhibition of Ple by Catsup was critical for border cells to migrate, we used an antibody to assess Ple expression in wild-type egg chambers. In contrast to tracheal cells, we detected no Ple protein in wild-type egg chambers (Fig. 3A). The antibody was effective because we could detect Ple ectopically expressed using c306Gal4 (Fig. 3B), as well as endogenous expression of Ple in neurons in the adult brain\textsuperscript{57} (Fig. 3C). Furthermore, Ple overexpression in border cells caused no migration defect (Fig. 3B). Therefore, it is unlikely that negative regulation of Ple activity is the key function of Catsup in border cells, suggesting that the function of Catsup in border cell migration is likely distinct from its role in tracheal development.

In wing imaginal discs, Notch and EGFR signaling are disrupted by Catsup loss of function, and both of these pathways are required for border cell migration. Notch signaling facilitates initiation of border cell migration, specifically detachment from the anterior\textsuperscript{16,23}, while EGFR is required for border cells to take a dorsal turn near the end of their migration to the oocyte.\textsuperscript{16,23} We found abnormal intracellular accumulation of Notch in epithelial cells generally (Fig. 3D, D\textsuperscript{\textdagger}) and border cells specifically (Fig. 3E, E\textsuperscript{\textdagger}) upon Catsup knockdown (Fig. 3D and E). Cells lacking Catsup also exhibited defective Notch signaling, detected by the Notch responsive element reporter\textsuperscript{19} (Fig. 3E, E\textsuperscript{\textdagger}). As in imaginal discs, EGFR also accumulated abnormally (Fig. 3G–I). Since Notch signaling is essential for border cell migration and expression of
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constitutively active Notch (the Notch intracellular domain, NICD), which does not require intracellular trafficking or processing, rescues impaired Notch signaling in border cells58, we asked whether NICD expression might rescue Catsup knockdown. However, neither NICD expression nor overexpression of the Notch specific chaperone O-fucosyltransferase-160 was sufficient to rescue Catsup RNAi (Fig. S1). This suggests that multiple Catsup functions are essential for border cell migration.

Since suppression of ER stress is a conserved function of Catsup and ZIP7, we used the ER stress marker XBP1-EGFP65 to compare heterozygous and homozygous catsup mutant cells in mosaic clusters (Fig. 4A). We found high levels of Xbp1 protein in homozygous cells compared to heterozygous border cells. Cells with elevated Xbp1 also exhibited reduced expression of Eyes Absent (Eya) (Fig. 4A), a nuclear protein that is required to repress polar cell fate and maintain border cell identity6. By contrast, the nuclear protein STAT, a transcription factor required for border cell fate specification6, was not decreased. Nuclear size was reduced by about half in mutant cells, possibly due to defective Notch signaling62,63 (Fig. 4B).

To test whether ER stress impairs migration, we genetically induced ER stress by overexpressing a misfolded rhodopsin protein RH1G69D64. As expected, RH1G69D induced Xbp1 expression in border cells (Fig. 4C). It also blocked migration (Fig. 4C), showing that high levels of ER stress are sufficient to inhibit border cell migration. In Xbp1-positive, RH1G69D-expressing cells, Eya expression was again reduced (Fig. 4C, insets), suggesting that ER stress, rather than another function of Catsup, was likely responsible for reduced Eya protein levels.

ZIP7 transports zinc from the ER into the cytosol65. To test whether the zinc transporter activity of Catsup was likely required for border cell migration, we designed point mutations that alter amino acids that are conserved between Catsup, ZIP7 and a more distant family member from Arabidopsis IRT1, and that are required for zinc transport(Fig. 5B). Histidine is an amino acid that coordinates zinc66, and it appears in the highly conserved HELP domain and the CHEXPHEXGD motif that are important for Zinc transport60. The mutation CatsupH315A replaces a
conserved histidine within the HELP domain with alanine. CatsupH344A changes a histidine within the CHEXPHEXGD motif to alanine. We made transgenic flies expressing the mutants under Ga4 control and included a V5 tag so that we could monitor protein expression and localization. As a control, we made UAS-CatsupG178D, the point mutation present in the mutant line (CatsupH88) isolated from the screen for mutations that cause border cell migration defects in mosaic clones. We then co-expressed each of the mutant lines with CatsupRNAi and quantified border cell migration (Fig. 5C). UAS-CatsupG178D provided no significant rescue compared to UAS-GFP-nls, though the mutation is likely a hypomorph and may have provided slight rescue that did not reach statistical significance (P=0.1). CatsupH344A failed to rescue, as did expression of UAS-CatsupH315A, which though not statistically significantly different, might have caused an even more severe migration defect, possibly due to a dominant-negative effect (Fig. 5C). The point mutations did not destabilize the proteins or alter their localization (Fig. 5D, E), therefore the lack of rescue was likely a consequence of impaired activity rather than impaired expression.

**Discussion**

In this study we explored the roles of the multifunctional protein Catsup in border cell migration. Catsup is a conserved protein that goes by names including ZRT1 in yeast, IRT1 in plants, SLC39a7/ZIP7/Kc4 in mammals. Despite the name Catsup (Catecholamines up), the most conserved features include subcellular localization to intracellular membranes including ER and Golgi, and bivalent cation transport. In Drosophila, Catsup has been implicated in direct binding and inhibition of tyrosine hydroxylase (TH/Ple), and thus in limiting dopamine production. This is important in the nervous system where as a neurotransmitter, dopamine levels must be tightly regulated. Somewhat surprisingly, the negative regulation of dopamine by Catsup is also important in tracheal development. Hsouna et al showed that Catsup and Ple are both required to achieve the appropriate level of dopamine, which regulates internalization of the Breathless (Btl) receptor tyrosine kinase. Excessive dopamine in catsup mutants leads to excessive endocytosis and thus downregulation of Btl, which inhibits tracheal cell migration. Ple mutations on the other hand result in reduced dopamine and Btl endocytosis, excess Btl signaling and therefore ectopic branching. Border cells also rely on chemotaxis via receptor tyrosine kinase signaling, so our first hypothesis was that the mechanism would be similar in tracheal and border cells. However, Ple is not detectable in border cells, so it is unlikely that negative regulation of dopamine synthesis is the primary function of Catsup in border cells.

In Drosophila wing imaginal discs, Catsup was found to regulate trafficking of Notch and EGFR. Although the biological effects of Notch and EGFR are different in imaginal disc cells compared to border cells, both pathways are required in both cell types, and our results support a general role for Catsup in Notch and EGFR trafficking. Perhaps as a consequence of defective trafficking, Catsup knockdown also induced ER stress in border cells, supporting that this is a general and conserved function. Moreover, we show for the first time that ER stress is sufficient to block migration and thereby regulate the abundance of the nuclear protein Eya, whereas STAT is unaffected. It is not immediately obvious how ER stress affects Eya abundance. Eya is an unusual protein in that it possesses protein phosphatase activity, at least in vitro, and serves as a transcriptional activator protein. Together the results demonstrate multiple essential functions for Catsup in border cells.

The point mutations designed to disrupt zinc transport based on mutations in ZIP7 indicated that border cell migration requires not only expression and ER localization of Catsup but also its ability to transport zinc. Given the requirement for ZIP7 in cancer cell motility, its over-expression in numerous cancers, and its correlation with disease progression, invasion, and metastasis, the border cell system offers an excellent model for deciphering the key effects of this multifunctional protein on collective cell motility in vivo.

![Fig. 5. Point mutations reveal likely requirement for Zinc transport in cell motility.](image)
Materials and Methods

**Drosophila genetics**

Cat5p mutant fly was generated by ethyl methanesulfonate (EMS) mutagen. The mutation results in Glycine(G) to Aspartic acid(D) replacement at the 178th amino acid. FLP/FRT system was used to generate the Cat5pG178D homozygous mutant clones by combining FRT40A-Cat5pG178D with hsFLP12;w;ubi:GFPlns, FRT40A or hsFLP12;w;ubi:RFPlns, FRT40A (Cyo). Cat5p knock down experiment uses border cell specific C306Gal4 and FruitlessGal4 to drive UASCat5pRNAi that is found in VDRC 100095 P[KK103630] VIE-260B. Wild type rescue w[+]; snf(Sco)/CyO; P[w[+mC]=UAS- Cat5p.V5]6 Bloomington 63229. Transgenic drosophila stocks used UASwRNAi/Cyo is a lab stock, UASPlERNAi Bloomington 25796 y[1] v[1]; P{y(7.7)v[1.8]=TRIP.FJ01813}attP2, UASPlE is Bloomington 37539 w[+]; P[w[+mC]=UAS-plE.T]3312, O-fucosyltransferase1 Bloomington 9376 P[UAS-O-fut.I.O]11.1, UAS-Notch.Intracellular.Domain on third chromosome is a gift from Artavants-Tsakonas Lab55, ER stress marker UASXbpl-EGFP.HG Bloomington 60731 w[+]; P[w[+mC]=UAS-Xbpl.EGFP.HG]13, UASHSC70-3 Bloomington 5843 w[126]; P[w[+mC]=UAS-Hsc70-3.WTB. B. Cat5p point mutations were cloned into vector pUAS-attb and injected to attp2 flies y w67c23; P{CaryP}attP2 by BestGene Inc.

**Design of UAS-RNAi-resistant Cat5p point mutations**

When generating UAS-Cat5p-point-mutations, we designed the construct so it can not be targeted by the Cat5pRNAi sequences. The region that is targeted by RNAi changed with the redundant codon for the same amino acids.

**Immunostaining and confocal imaging**

Female flies were fattened with yeast for 2 days at 29°C. Egg chambers are dissected from ovaries of female fly bodies in Schneider’s medium with 10% FBS (pH=6.85-6.95) as described previously51. Freshly dissected egg chambers are fixed in 4% paraformaldehyde and then incubated overnight in 1xPBS with 0.4% triton with primary antibody to stain for ER mouse PDI (1:200) AD1-SPA-891-D Enzo Life Sciences, Inc, chicken GFP (1:200) ab13970 Abcam plc., Ple (anti-TI) antibody is a gift from Craig Montell lab, mouse anti-Notch intracellular domain (1:100) C17.9C6 DSHB, rat Ecadherin antibody DCAD2 (1:50) DSHB, V5 Tag Monoclonal Antibody-Alpha Fluor 555 (2F11F7) Invitrogen, mouse anti-deGFR (1:200) E2960 Sigma Aldrich. O-fut1 antibody was used to confirm O-fut1 overexpression, and it is a gift from Kenneth D. Irvine lab72. Secondary antibodies were incubated for 2 hours, together with Hoechst stains for nuclei, and Phalloidin stains for F-actin. Mouse anti-PDI and mouse anti-V5-555 co-staining was done by first stain with PDI primary and secondary, after through wash out, apply anti-V5-555 for overnight then wash out. Immunostained samples are mounted in VECTASHIELD Mounting Medium from Vector Laboratories. Zeiss LSM780 and LSM800 confocal microscopes were used to acquire images. Images were visualised by FIJI, rotated and cropped for presentation.

**Sequence alignment**

Cat5p and ZIP7 amino acid sequences were acquired from NCBI in a FASTA format. The files were input into T-coffee in the T-coffee.erg.cat/apps/tcoffee/do:regular to generate multiple sequence alignment. The output was fed into Boxshade http://www.ch.embnet.org/software/BOX_form.html to generate the sequence alignment with black and grey shades to show conserved sequence region.

**End Matter**

**Author Contributions and Notes**

X.G., W.D., and D.J.M. designed experiments and prepared the manuscript. X.G., and W.D., performed experiments. The authors declare no conflict of interest.

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