A non-cell-autonomous role for Ras signaling in *C. elegans* neuroblast delamination

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ABSTRACT
Receptor tyrosine kinase (RTK) signaling through Ras influences many aspects of normal cell behavior, including epithelial-to-mesenchymal transition, and aberrant signaling promotes both tumorigenesis and metastasis. Although many such effects are cell-autonomous, here we show a non-cell-autonomous role for RTK-Ras signaling in the delamination of a neuroblast from an epithelial organ. The *C. elegans* renal-like excretory organ is initially composed of three unicellular epithelial tubes, namely the canal, duct and G1 pore cells; however, the G1 cell later delaminates from the excretory system to become a neuroblast and is replaced by the G2 cell. G1 delamination and G2 intercalation involve cytoskeletal remodeling, interconversion of autacellular and intercellular junctions and migration over a luminal extracellular matrix, followed by G1 junction loss. LET-23(EGFR) and SOS-1, an exchange factor for Ras, are required for G1 junction loss but not for initial cytoskeletal or junction remodeling. Surprisingly, expression of activated LET-60/Ras, are required for G1 junction loss but not for initial cytoskeletal or junction remodeling. This suggests that Ras acts non-cell-autonomously to permit G1 delamination. We suggest that, similarly, oncogenic mutations in cells within a tumor might help create a microenvironment that is permissive for other cells to detach and ultimately metastasize.

KEY WORDS: Caenorhabditis elegans, EMT, Delamination, Epithelia, Junction, Ras

INTRODUCTION
Whereas some epithelial cells maintain their identities throughout their lifetime, others will undergo a transition to form another cell type. This is a common occurrence during normal developmental programs such as epithelial-to-mesenchymal transition (EMT) or neuroblast delamination and in disease states such as cancer cell metastasis (Acloque et al., 2009; Lim and Thiery, 2012). When epithelial cells transition to form another cell type, they require many cell-autonomous changes including altered gene expression, gaining motility, remodeling junctions, and detaching from or invading through the extracellular matrix (ECM). Furthermore, neighboring cells that remain within the epithelium must loosen their connection to the departing cell and rescale the tissue after its departure. Most studies of epithelial transitions have focused on the cell-autonomous changes, while less is known about the role of neighboring epithelial cells in facilitating such transitions.

Many signaling pathways, including receptor tyrosine kinase (RTK) signaling through Ras, promote EMT, neuronal delamination and/or cancer cell metastasis (Yang and Weinberg, 2008). One clear role for RTK-Ras signaling during cell transitions in vivo is as a cell-autonomous trigger for transcription factors such as Snail and Twist that repress epithelial identity (Lim and Thiery, 2012). On the other hand, work in cell culture has shown that RTK signaling can alter the adhesive dynamics of cells in various other ways (Janda et al., 2006; Lu et al., 2003; Orlichenko et al., 2009; Palacios et al., 2001, 2002). Here we show that RTK-Ras signaling in a cell that remains in the epithelium is crucial to allow delamination of a neighboring cell.

RESULTS AND DISCUSSION
Background
The *C. elegans* excretory system is composed of three unicellular epithelial tubes, namely the canal, duct and pore cells, which connect in tandem to provide a conduit for fluid waste excretion (Buechner, 2002; Nelson et al., 1983; Nelson and Riddle, 1984) (Fig. 1A). In the first (L1) larval stage, the initial pore cell, named G1, delaminates from the excretory system and divides to form a pair of neurons (Stone et al., 2009; Sulston and Horvitz, 1977; Sulston et al., 1983) (Fig. 1B,C). Simultaneous with its departure, the G1 cell is replaced as excretory pore by the neighboring G2 epidermal cell. As G1 departs the excretory system, it must remodel its junctions to the duct cell and to G2 and the epidermis, as well as eliminate an autacellular junction (AJ) that maintains it in the shape of a tube. Correspondingly, the duct cell must remodel its intercellular junction (IJ) to detach from G1 and connect to the entering G2 cell.

G1 and G2 migrate over a luminal matrix during delamination and intercalation
To visualize G1 delamination, we marked the duct and G1 cell bodies with *det-Spro::mCherry* and apical junctions with *AJM-1::GFP* or *HMR-1::GFP* (Fig. 1; supplementary material Fig. S1). We imaged carefully staged L1 larvae hourly at 25°C (see Materials and Methods) to construct a timeline of events (Fig. 1B,C). Beginning 4 h after hatch, the G1 cell began to extend cytoplasm dorsally and then migrated in an anterior/dorsal direction as its AJ gradually shortened. Concomitantly, the G2 cell invaded at the base of the G1 cell and began to wrap into the shape of a tube and form a lengthening AJ. At 7 h post hatch, the G1 cell retained only a remnant of junction at its base, where it contacted the duct and now fully incorporated G2 pore. Around this time, G1 divided in the right/left plane to produce daughter cells G1.l and G1.r (Sulston and Horvitz, 1977) (supplementary material Fig. S2).

As G2 invaded the excretory system, we noted a thickening of the AJ at the moving G1-G2 cell boundary (Fig. 1B,C). Archival transmission electron microscope (TEM) images reveal that this thickening corresponds to adjacent junctions at a short region of overlap where the G1 and G2 cells transiently share the pore lumen.


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as G2 intercalates dorsally (Fig. 1D). Thus, the departing G1 cell appears to unzip, first converting its AJ to IJ before losing junctions entirely, while the entering G2 cell zips up beneath it, with a short region of two-celled tube at their intersection.

The excretory duct and pore cell lumen is lined with a thick collagenous cuticle that is contiguous with the epidermal cuticle and will be shed during molting, ∼6 h after G1 departs the excretory system (Mancuso et al., 2012; Nelson et al., 1983). The cuticular lining of the duct/pore channel remains clearly visible by TEM during delamination and intercalation (Fig. 1D), suggesting that the G1 and G2 cells migrate over this lumenal cuticle as they change positions, although we cannot exclude the possibility of some new matrix synthesis.

Re-organization of the actin cytoskeleton precedes G1 delamination

Epithelial cells in transition often gain motility by stimulating F-actin protrusions (Ridley, 2011). We visualized actin dynamics in the G1 and duct cells using a GFP-tagged VAB-10 (spectraplakin) actin-binding domain (ABD) (Liegeois et al., 2007) under control of the dct-5 promoter (Fig. 2). From hatching to just before 3 h post hatching, actin was very strongly enriched along the G1 AJ and, to a lesser degree, along the duct lumen and at the IJ between the duct and canal cell. However, at 3 h post hatch, the actin relocalized away from the AJ to become generally distributed throughout the cytoplasm; this occurred ∼1 h before G1 began to migrate. As delamination completed, actin accumulated at a narrow, anteriorly protruding tip of the G1 cell.

sos-1 is required for G1 delamination

RTK signaling promotes many epithelial transitions. In C. elegans, the RTK LET-23/EGFR signals through a well-described pathway that includes the guanine nucleotide exchange factor (GEF) SOS-1 and LET-60/Ras (Sundaram, 2013). During embryogenesis, LIN-3/EGF is expressed in the excretory canal cell and stimulates the EGFR-Ras pathway in the duct cell to specify the duct versus G1 cell fate (Abdus-Saboor et al., 2011; Yochem et al., 1997). A temperature sensitive (ts) allele of sos-1 that is specifically defective in the Ras GEF domain (Rocheleau et al., 2002) revealed a continued requirement for signaling during the L1 stage in order to maintain excretory system integrity (Abdus-Saboor et al., 2011). These observations suggested a possible role for Ras signaling in the process of G1 delamination.

We tested a requirement for SOS-1 by shifting sos-1(ts) animals to non-permissive temperature (25°C) directly after hatching and then examining the dynamics of G1 departure (Fig. 3). In upshifted animals, cell and junction morphology and cytoskeletal organization initially appeared normal. Actin relocalized away from the G1 AJ, G2 began to intercalate and G1 began to extend cytoplasm and migrate dorsally with approximately normal timing.
LET-23, we examined hypomorphic (supplementary material Fig. S4). To confirm the involvement of pathway to control G1 delamination (Abdus-Saboor et al., 2011) consistent with SOS-1 acting in the canonical EGF-Ras-ERK the canal cell and duct/pore, respectively, during remodeling, lin-3 autonomously permit G1 delamination (Fig. 3A,B). The duct lumen began to dilate, suggesting that the pore channel might have closed. G1 rarely formed a protruding tip, and actin remained partly associated with the remaining G1 junction and accumulated at the sites of duct lumen dilation (Fig. 3C). Despite its failure to delamate, G1 still divided with approximately normal timing, with the G1.r cell maintaining a junction (supplementary material Fig. S2). Thus, sos-1 is required for G1 junction loss and detachment from the excretory system, but is not required for many other G1 behaviors, such as initial actin and junction reorganization, gain of motility and cell cycle re-entry. Despite its failure to delaminate, G1 still divided with approximately normal timing, with the G1.r cell maintaining a junction (supplementary material Fig. S2). Thus, sos-1 is required for G1 junction loss and detachment from the excretory system, but is not required for many other G1 behaviors, such as initial actin and junction reorganization, gain of motility and cell cycle re-entry.

**LET-23 and SOS-1 act upstream of Ras in the duct cell to non-autonomously permit G1 delamination**

lin-3/EGF and let-23/EGFR reporters continue to be expressed in the canal cell and duct/pore, respectively, during remodeling, consistent with SOS-1 acting in the canonical EGF-Ras-ERK pathway to control G1 delamination (Abdus-Saboor et al., 2011) (supplementary material Fig. S4). To confirm the involvement of LET-23, we examined hypomorphic let-23(sy97) mutants in which initial duct versus pore cell fate specification was normal. let-23 (sy97) mutants had a G1 delamination defect similar to that of sos-1(ts) mutants (Fig. 4A,B). To confirm that SOS-1 acts through Ras and to determine where it carries out its function, we used a transgenic approach to express a constitutively active version of LET-60/Ras (G13E) in a tissue-specific manner. The lin-48 promoter drives expression in the duct, rectum and neuronal support cells (Abdus-Saboor et al., 2011; Johnson et al., 2001), whereas the dpy-7 promoter drives expression in G1, G2 and the epidermis (Johnstone and Barry, 1996; Stone et al., 2009) (supplementary material Fig. S4). Using these tissue-specific promoters, we found that expression of LET-60/G13E in the duct and G1 cells, or in the duct cell only, but not in the G1 cell only, significantly rescued the junction-retention defects in the G1 cell (Fig. 4C,D). Consistent with these data, removal of let-60 from G1 or G2, but not the duct, is compatible with normal excretory function and animal viability (Yochem et al., 1997). Thus, SOS-1 acts through Ras, and Ras signaling in the duct cell acts non-cell-autonomously to permit G1 delamination.

Notably, LET-60 (G13E) did not significantly affect the timing or execution of G1 delamination in a wild-type background (Fig. 4C), suggesting that Ras acts permissively, rather than instructively, to allow G1 delamination. Furthermore, G1 delamination still initiated in earlier upshifted sos-1(ts) animals that had a two G1, no duct phenotype (Fig. 4E); thus, the duct cell is not required to stimulate G1 delamination. Previous work showed that G1 delamination also occurs in the absence of G2 (Abdus-Saboor et al., 2011). Together, these data suggest that a G1-intrinsic clock or a signal from somewhere other than the duct or G2 stimulates G1 delamination at the appropriate time, and that Ras signaling in the duct permits successful execution of the delamination program.

**How does Ras signaling in the duct facilitate G1 remodeling?**

Several (non-mutually exclusive) models could explain the non-cell-autonomous effects of Ras signaling. First, Ras signaling in the duct could directly alter junction components on the duct side of the duct-G1 connection, thereby allowing freer G1 mobility and remodeling. Extensive work in cell culture has shown that Ras signaling can stimulate cadherin endocytosis (Janda et al., 2006; Lu et al., 2003; Orlichenko et al., 2009; Palacios et al., 2001, 2002), although the in vivo significance of this role is unclear. Second, Ras signaling in the duct could act indirectly by stimulating the production of another signaling ligand. Much earlier in development, Ras signaling in the duct appears to trigger an unknown, non-Notch-mediated lateral inhibitory signal that prevents G1 from also adopting a duct fate (Abdus-Saboor et al., 2011; Sulston et al., 1983; Yochem et al., 1997); potentially, continued signaling could facilitate later G1 behaviors such as junction remodeling. Third, Ras signaling might alter components of the luminal matrix that the duct shares with G1, facilitating detachment of G1 from that matrix. Ras signaling in mammalian cells can upregulate the expression of various matrix metalloproteinases (Sanchez-Laorden et al., 2014), and we showed previously that mutations that disrupt apical matrix organization lead to premature separation of the duct and pore cells (Mancuso et al., 2012). Further studies of mutants with G1 delamination...
defects should provide insights into the mechanisms by which Ras signaling non-autonomously promotes this epithelial transition.

Implications for the role of EGFR and Ras in cancer cell metastasis
Activating mutations in EGFR and Ras are among the most common changes in epithelial-derived cancers (Pylayeva-Gupta et al., 2011). The lethal effects of such cancers are predominantly due to tumor cell metastasis (Nguyen and Massagué, 2007). Although cell-autonomous effects of EGFR-Ras signaling clearly contribute to tumorigenesis and metastasis, the results presented here emphasize the possibility that mutant cells that remain in the original tumor could also help create a microenvironment that is permissive for other cells to detach and ultimately metastasize.

MATERIALS AND METHODS
Strains and transgenes
See supplementary material Tables S1-S3 for strains, transgenes and plasmids used. All strains were grown at 25°C under standard conditions (Brenner, 1974) unless specifically noted otherwise.
Staging of larvae

Embryos were hand-picked at 1.5-fold stage, incubated at 15°C until hatch, and then incubated at 25°C to reach the designated stage. Staging was confirmed based on the morphology of seam hypodermal cells, which are actively dividing and migrating during L1 (Austin and Kenyon, 1994; Podbilewicz and White, 1994; Sulston and Horvitz, 1977).

Microscopy

Confocal microscopy utilized a Leica TCS CP. Images were processed using ImageJ (Schneider et al., 2012). TEM data correspond to animal L1Q (available at wormimage.org).

Measurements of junction length

At least 15 animals per stage were analyzed for each genotype (except N2 4 h, n=12).

For manual measurements (Fig. 3), confocal z-stacks were processed as average projections. ImageJ was used to draw a line from the base of the AJ to the base of the G1 cell body (G2 AJ length), or from the base of the G1 cell body to the top of the AJ (G1 AJ length). G1 junction length did not change from hatch to 4 h.

Volocity software (PerkinElmer) was used to quantify the area of overlap between the G1 cell body and AJ (supplementary material Fig. S3; Fig. 4). A single raw section from a confocal z-stack corresponding to approximately the center of the excretory system was imported to Volocity as an image z-stack corresponding to approximately 75%-90%, with outliers as individual points.

Immunostaining

Embryos in supplementary materials Fig. S4A were freeze-cracked and fixed in methanol as described previously (Duer et al., 1999) and incubated with primary antibodies [goat polyclonal anti-GFP (Rockland, 600-101-215; 1:50); rabbit polyclonal anti-DLG-1 (1:50 to 1:100; Segbert et al., 2004)] overnight at 4°C and with secondary antibodies (Jackson ImmunoResearch Laboratories, 705-095-003 and 111-295-003; 1:50 to 1:200) for 2 hours at room temperature.

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Competing interests
The authors declare no competing financial interests.

Author contributions
J.M.P. and M.V.S. jointly conceived the approach and prepared the manuscript. J.M.P. performed the data experiments and data analysis.

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