

Current Biology

A mismatch in the expression of cell surface molecules induces tissue-intrinsic defense against aberrant cells

Highlights

- Robos, Teneurins, and LRR proteins confer detection of abnormal neighboring cells
- An expression level mismatch between adjacent cells triggers “interface surveillance”
- Robo2 and Robo3 activate interface surveillance independent of the ligand Slit
- Fate-patterning pathways establish a cell surface profile unique to each cell fate

Authors

Friedericke Fischer, Laurin Ernst, Anna Frey, ..., Vanessa Weichselberger, Ramya Balaji, Anne-Kathrin Classen

Correspondence

anne.classen@biologie.uni-freiburg.de

In brief

Fischer et al. find that *Drosophila* tissues rely on a cell surface code of Robo receptors, Teneurins, and other LRR proteins to identify abnormal neighboring cells with deregulated developmental fate, including activation of oncogenic Ras. Cell identification depends on expression differences, leading to cell elimination by “interface surveillance.”



Article

A mismatch in the expression of cell surface molecules induces tissue-intrinsic defense against aberrant cells

Friedericke Fischer,^{1,2,3} Laurin Ernst,^{1,2,3} Anna Frey,^{1,2,4} Katrin Holstein,⁵ Deepti Prasad,^{1,2,4} Vanessa Weichselberger,^{4,6} Ramya Balaji,^{1,2} and Anne-Kathrin Classen^{1,2,7,8,9,10,*}

¹Hilde-Mangold-Haus, University of Freiburg, 79104 Freiburg, Germany

²Faculty of Biology, University of Freiburg, 79104 Freiburg, Germany

³International Max Planck Research School for Immunobiology, Epigenetics, and Metabolism, 79108 Freiburg, Germany

⁴Spemann Graduate School of Biology and Medicine (SGBM), University of Freiburg, 79104 Freiburg, Germany

⁵Department of Vascular Cell Biology, Max Planck Institute for Molecular Biomedicine, 48149 Münster, Germany

⁶Aix Marseille University, CNRS, UMR 7288, IBDM, 13288 Marseille, France

⁷CIBSS Centre for Integrative Biological Signalling Studies, University of Freiburg, 79104 Freiburg, Germany

⁸BIOSS Centre for Biological Signalling Studies, University of Freiburg, 79104 Freiburg, Germany

⁹X (formerly Twitter): @classenlab

¹⁰Lead contact

*Correspondence: anne.classen@biologie.uni-freiburg.de

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SUMMARY

Tissue-intrinsic error correction enables epithelial cells to detect abnormal neighboring cells and facilitate their removal from the tissue. One of these pathways, “interface surveillance,” is triggered by cells with aberrant developmental and cell-fate-patterning pathways. It remains unknown which molecular mechanisms provide cells with the ability to compare fate between neighboring cells. We demonstrate that *Drosophila* imaginal discs express an array of cell surface molecules previously implicated in neuronal axon guidance processes. They include members of the Robo, Teneurin, Ephrin, Toll-like, or atypical cadherin families. Importantly, a mismatch in expression levels of these cell surface molecules between adjacent cells is sufficient to induce interface surveillance, indicating that differences in expression levels between neighboring cells, rather than their absolute expression levels, are crucial. Specifically, a mismatch in Robo2 and Robo3, but not Robo1, induces enrichment of actin, myosin II, and Ena/Vasp, as well as activation of JNK and apoptosis at clonal interfaces. Moreover, Robo2 can induce interface surveillance independently of its cytosolic domain and without the need for the Robo-ligand Slit. The expression of Robo2 and other cell surface molecules, such as Teneurins or the Ephrin receptor is regulated by fate-patterning pathways intrinsic and extrinsic to the wing disc, as well as by expression of oncogenic *Ras*^{V12}. Combined, we demonstrate that neighboring cells respond to a mismatch in surface code patterns mediated by specific transmembrane proteins and reveal a novel function for these cell surface proteins in cell fate recognition and removal of aberrant cells during development and homeostasis of epithelial tissues.

INTRODUCTION

Genetically altered cells are constantly appearing in epithelial tissues, either because of developmental errors or mutagenesis.^{1,2} Surveillance and removal of these cells is necessary to maintain tissue homeostasis and organismal health. “Interface surveillance”—a distinct branch of tissue-intrinsic error correction mechanisms, such as cell-cell competition—mediates the removal of aberrant cells from epithelial tissues.^{3,4} Interface surveillance is specifically activated by aberrant cells that carry mutations in cellular signaling pathways or transcriptional networks, which control cell fate and differentiation programs. For example, mutations in patterning pathways (Decapentaplegic [Dpp]/TGF- β , Wingless [Wg]/WNT, Hedgehog (Hh)/Shh, JAK/STAT, and Notch) or in cell-fate-specifying transcription

factors and transcriptional regulators (Arm, Iro-C, Omb, Yki, En/Inv, Ap, Ci, and Hox genes) induce phenotypes related to interface surveillance.^{5–23} Mosaic clones that deregulate these pathways produce a pronounced difference in cell fate between “aberrant” and surrounding “normal” cells, and we have previously shown that such steep fate differences between neighboring cells cause enrichment of myosin II and filamentous actin at shared junctional and lateral interfaces in *Drosophila* imaginal epithelia. This drives cell segregation between the two cell populations via smoothing of the contractile interface, a characteristic feature of interface surveillance.³ Importantly, this response is induced in a strictly position-dependent manner according to the cell fate of the surrounding cells. For example, Cubitus interruptus (Ci)-expressing clones have normal shapes in anterior wing compartments, where Ci-activation by Hh



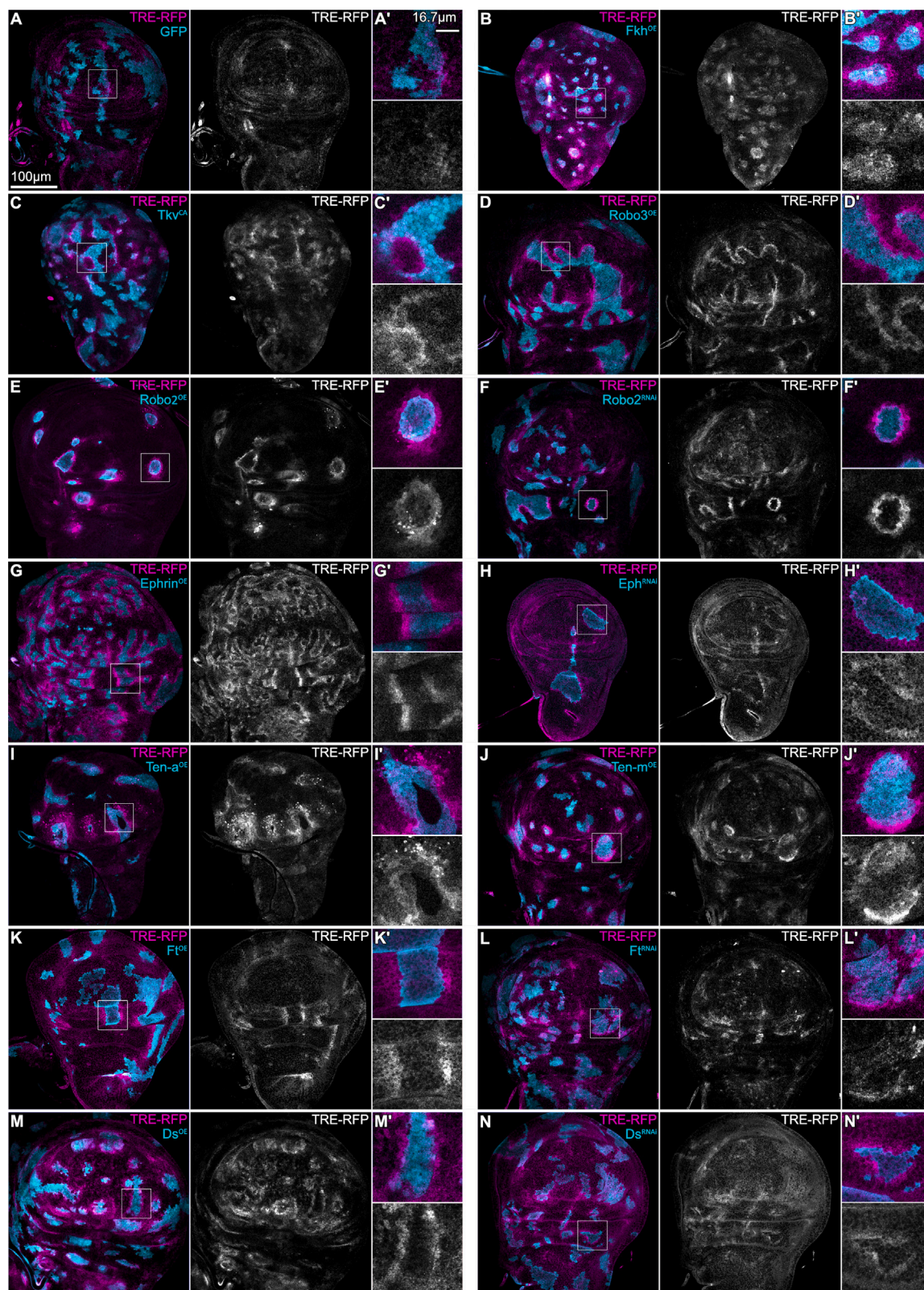


Figure 1. Cell surface molecules with described roles in axon guidance mediate cell-cell recognition in wing discs

TRE-RFP expression is reporting JNK pathway activity (gray or magenta in all panels).

(A) A mosaic wing disc with wild-type clones expressing *UAS-GFP* (GFP, cyan). Please note that JNK activity is physiologically elevated at the A/P compartment boundary in wing discs at this developmental stage. White frame marks region shown in (A').

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signaling is high. However, Ci-expressing clones undergo smoothing and die in posterior compartments, where Ci-signaling is normally low.³ This emphasizes that it is the difference in fate at the clone interface, or in other words, the fate mismatch, rather than the fate per se, which is important for inducing interface surveillance. Curiously, actomyosin recruitment and interface smoothing bear a striking resemblance to the formation of compartment boundaries in developing tissues, where two cell populations of different fate are mechanically separated by the formation of a contractile actomyosin interface between them.^{24–26} However, actomyosin enrichment in interface surveillance is also accompanied by activation of pro-apoptotic c-Jun N-terminal kinase (JNK) signaling in cells on either side of the clonal interface.^{3,4} This drives apoptotic elimination of aberrant cells and is thus an essential hallmark of interface surveillance and its ability to maintain tissue health.

Despite this work, it is still unclear how cells recognize when a neighboring cell acquires a distinct and potentially aberrant fate. Recruitment of actomyosin to the interface and JNK activation in cells on both sides of the interface suggests that surveillance of neighbors is mediated by a cell-contact-dependent mechanism. The molecular machinery responsible for interface surveillance must therefore be composed of cell surface molecules, (1) which modulate actomyosin function and (2) which are competent to transduce contact-dependent signals into signaling pathways, such as JNK. Additionally, (3) expression of these molecules must be regulated by cell-fate-specifying transcription factors and pathways, thereby encoding cell identity. Classical cadherins are prime examples of transmembrane molecules that may fulfill these criteria.²⁷ Yet, the striking pattern-specific activation of interface surveillance, as for Ci-expressing clones, is observed for multiple fate-patterning pathways. This implies that more than one molecule is necessary to distinguish any two of many possible cell fates from each other.^{3,4} Recently, novel and unexpected roles for members of the neuronal axon guidance and neuronal adhesion families emerged in physiology and pathology of epithelial tissues.^{28,29} Axon guidance molecules typically mediate signaling through the interaction of a transmembrane receptor with a cognate ligand. Roundabout (Robo), Plexin, Frazzled (Fra), or Ephrin (Eph) receptor families interact with respective ligands of the Slit, Semaphorin (Sema), Netrin (Net), and Ephrin families.^{30–33} Recent work suggests that Teneurins interact with leucine-rich repeat (LRR)-domain proteins on neighboring cell surfaces.^{33,34} Extensive research on axon guidance effectors has revealed this general principle: when the ligand binds to the receptor, it elicits signals to recruit actomyosin modulators of the small GTPase family (Rho and Rac) and members of the Ena/Vasp, Abl, or Src pathways.³²

This leads to either formation or retraction of actin-based cellular protrusions, thereby driving attractive or repulsive behaviors during axon pathfinding and mediating appropriate spatial positioning of cells within neuronal systems. It is conceivable that expression of these molecules may also have a strong impact on actomyosin and adhesion dynamics in an epithelial tissue thereby regulating epithelial morphogenesis and homeostasis.^{29,30,35} Indeed, Ephrin, Teneurins, and LRR-domain proteins including Toll-like receptors are strongly implicated in mechanical separation of cell populations at compartment boundaries via regulation of a contractile actomyosin interface in epithelial tissues of vertebrate and invertebrate species.^{24–26,36,37}

The phenomenological similarities lead us to explore whether these molecules played a role in interface surveillance against aberrant cells. Importantly, the diverse fates created by developmental patterning in the imaginal disc made this tissue an ideal system to experimentally address the mechanisms of cell fate recognition. We find that neuronal cell surface molecules are widely expressed in epithelia, that misexpression of a single surface molecule is sufficient to induce all hallmarks of interface surveillance, and that these cell surface molecules are co-regulated by cell fate specification pathways.

RESULTS

Cell surface molecules with described roles in axon guidance mediate cell-cell recognition in wing discs

To understand whether epithelial tissues may use cell surface molecules to distinguish fates and detect aberrant cells, we first asked whether misexpression of proteins functioning in axon guidance, neuronal targeting, and cell adhesion is sufficient to induce interface surveillance hallmarks. Mosaic clones expressing aberrant fates generally induce actomyosin recruitment, apoptosis, and, importantly, bilateral JNK signaling at the interface between clonal and wild-type cells (Figures S1A–S1E).⁴ We thus used bilateral activation of the JNK-activity reporter TRE-RFP as a readout for a targeted genetic screen.³⁸ Where possible, we analyzed overexpression, as well as RNAi-mediated downregulation of cell surface molecules using the mosaic GAL4/UAS flip-out system.³⁹ Strikingly, we found that deregulation of single members of 6 cell surface protein families was sufficient to induce TRE-RFP activation at clone boundaries in wing imaginal discs. Specifically, ectopic up- and downregulation of members of the Robo family (Robo 2 and 3), the Teneurin-family (Ten-a and Ten-m), atypical cadherins (Fat and Ds), the Eph/Ephrin-system, Netrins (Netrin-B), and the LRR proteins Toll-2, Toll-8, or Tartan was sufficient to induce bilateral JNK interface signaling (Figures 1 and S1F–S1J). Importantly, several protein

(B and C) Mosaic wing discs with clonal alterations in fate-specifying pathways by the ectopic expression of Fkh (Fkh^{OE}) and the expression of constitutively active Tkiv (Tkv^{CA}) using the “GAL4/UAS flip-out” system. Please note the induction of bilateral JNK signaling at mosaic interfaces. White frame marks regions shown in (B') and (C').

(D–N) Mosaic wing discs with aberrant expression of cell surface molecules. Clones express GFP (cyan) and were exposed to either ectopic expression (OE) of UAS constructs or downregulation of expression by UAS-RNAi (RNAi) constructs for individual cell surface molecules, as indicated in each panel. White frame marks regions shown in (D')–(N').

Images in (A)–(N) are shown at the same scale. JNK responses in (A)–(C) have been observed in $n \geq 3$ experimental replicates for this study and previous studies.⁴ JNK responses in (F), (H), and (J)–(L) have been observed in $n \geq 3$ experimental replicates for this study. JNK response in (G), (I), (M), and (N) has been observed in $n \geq 3$ wing discs in one experimental replicate, which corresponds to the initial candidate screen (see Figure S1F).

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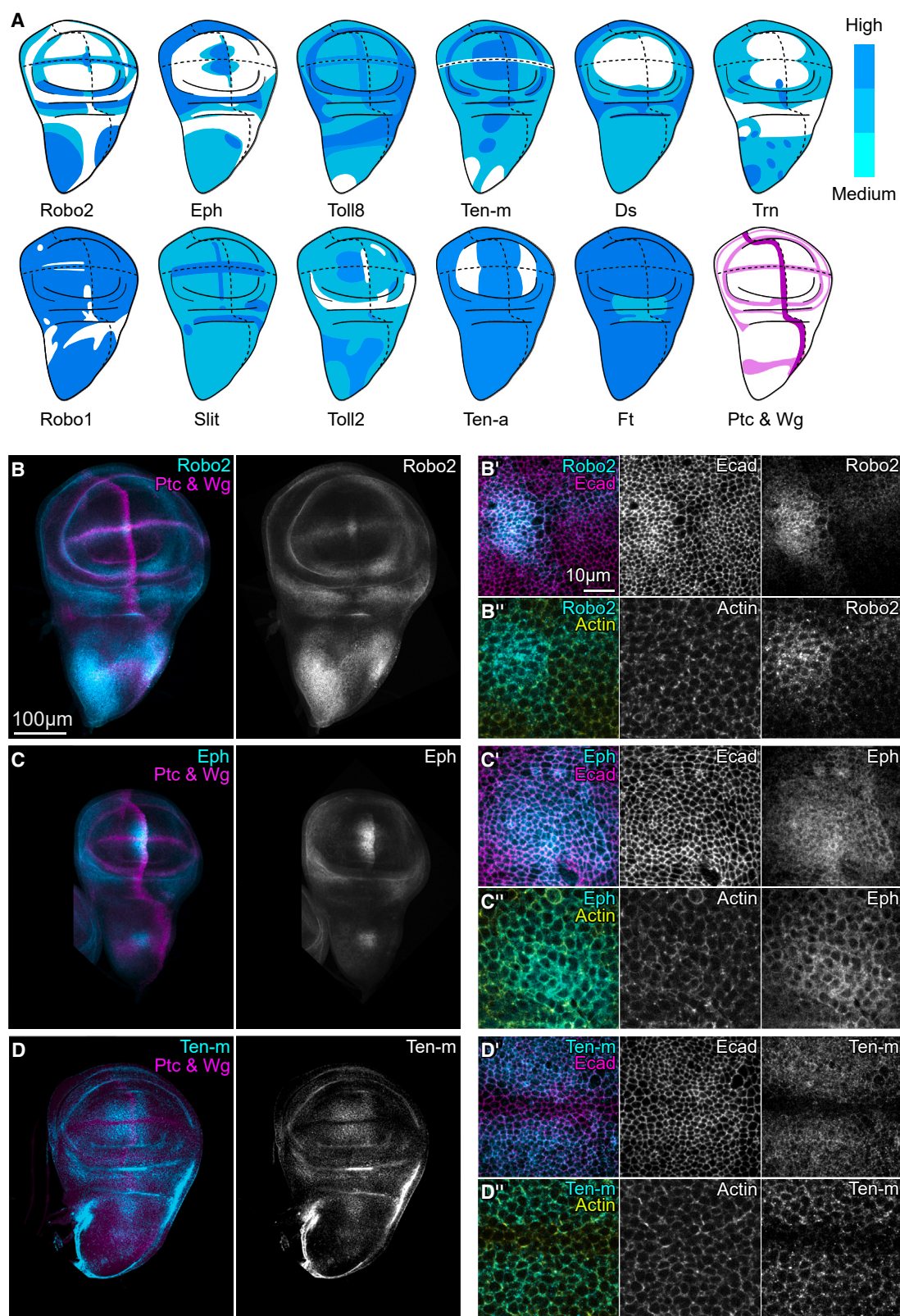


Figure 2. Cell surface molecules with described roles in axon guidance are expressed in wing disc epithelia

(A) Illustrations of expression patterns of cell surface molecules in third instar wing discs at 102 h after egg lay (AEL). Relative fluorescence intensity is depicted by color intensity from low (white), medium (cyan) to high (blue), see Figure S2 and experimental procedures. Expression domains of compartment boundary

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families, such as members of the Plexin/Sema, Irre cell recognition module (IRM), and Dscam-families failed to score positively for JNK interface signaling in our screen, suggesting that not all cell surface molecules with roles in neuronal morphogenesis are involved in interface surveillance responses (Figure S1F). We conclude that a distinct set of cell surface molecules can mediate bilateral JNK signaling, otherwise a characteristic hallmark of interface surveillance toward cells with aberrant fate and specification programs. We thus wanted to examine whether these cell surface molecules may act as cell fate sensors required for the induction of interface surveillance.

Cell surface molecules with described roles in axon guidance are widely expressed in wing disc epithelia

To understand whether these cell surface molecules indeed play a role in aberrant cell recognition, we asked whether these proteins are physiologically expressed in wing discs. We analyzed their expression in early and late wing disc development using tools, such as antibodies, enhancer traps, or GFP-tagged proteins.^{40,41} To provide a spatial reference, we included a staining for Patched (Ptc, anterior-posterior boundary) and Wg (dorsal-ventral boundary). We were surprised to find that most examined molecules were, indeed, expressed in wing discs, with increasingly complex expression patterns toward late 3rd instar stages (Figures 2A–2D and S2). Moreover, certain attributes of those patterns, such as an elevated expression in the pouch, hinge or notum suggest a possible regulation by well-described specification pathways in the wing disc (Figure S2B).⁴² Consequently, each spatial position within the disc is endowed with a unique cell surface code, which, importantly, is composed of multiple different proteins (Figure 2A). The widespread expression of neuronal cell surface molecules suggests that these proteins may be linked to uncharacterized functions during development of imaginal discs.

To confirm that these proteins also locate to the cell surface in wing epithelia, we analyzed their subcellular distribution in more detail. Roundabout 2 (Robo2), Ephrin receptor (Eph), or Teneurin-m (Ten-m) localized to adherens junctions and lateral domains of wing disc cells (Figures 2B'–2D'). Both adherens junctions and lateral surfaces establish contact between neighboring cells, and it is these surfaces that were previously shown to respond to the presence of aberrant cells by recruiting actomyosin.^{3,4} Thus, Robo2, Eph, and Ten-m localize to cell surfaces that are implicated in activation of interface surveillance. Combined, these observations are consistent with a model where wing imaginal discs may utilize the combinatorial expression of cell surface molecules at adherens junctions and lateral surfaces to detect the fate and identity of neighboring cells.

A mismatch of Robo2 receptor expression levels between neighboring cells is detected by interface surveillance

Although atypical cadherins, Teneurins, Ephrin, and LRR proteins have emerging or even established roles in epithelial tissues, almost nothing is known about the epithelial function of Robo receptors.^{36,37,43–45} We thus focused our further analysis on the three *Drosophila* Robo receptors Robo1, Robo2, and Robo3.

Because Robo2 is expressed throughout wing disc development (Figure 3G), we first analyzed the effects of mosaic manipulation of Robo2. We find that Robo2-expressing clones have smooth shapes, recruit actin to the interface and generally induce apoptosis (Figures 3A, 3B, 3D, and 3E). Moreover, the characteristic pattern of bilateral JNK signaling—where both wild-type cells and aberrant cells at the clonal interface activate JNK—can be detected around Robo2-expressing clones (Figures 3B, 3F, 3H, and S3A–S3C). We previously reported that JNK signaling induced by aberrant cells drives apoptosis on either side of the clone interface, whereas a less-well-characterized pathway drives apoptosis in the clone interior.⁴ This characteristic apoptotic pattern could be reproduced by Robo2-expressing clones (Figure 3I). Combined, we conclude that the expression of a single cell surface molecule, such as Robo2, is completely sufficient to induce all hallmarks of interface surveillance.

An important characteristic of interface surveillance is the strict position-dependent recognition of ectopic or missing fate-specifying pathways, which occurs in response to a pronounced mismatch in cell fate between clonal and neighboring cells. Accordingly, the endogenous Robo2-expression pattern should dictate where differences between clonal and neighboring cells are largest. Looking at the Robo2-expression pattern in third instar discs, we noticed that Robo2 is most highly expressed in the anterior notum and the hinge-hinge fold region, whereas medium levels of expression can be found along the dorsal-ventral boundary. In the dorsal and ventral pouch, Robo2 expression is low (Figure 3G). Therefore, we predicted that Robo2-expressing clones should elicit interface surveillance responses in the pouch but not the anterior notum, whereas Robo2-RNAi-expressing clones should elicit responses in the notum and not the pouch. Indeed, Robo2-expressing clones induced actin enrichment and JNK interface signaling in the pouch but not the notum, whereas Robo2-RNAi-expressing clones induced these responses in the notum but not the pouch (Figures 3B and 3C). The Robo2-GFP construct helped to visualize these spatially restricted JNK responses for Robo2-expressing and Robo2-RNAi-expressing clones with respect to the endogenous Robo2 expression pattern (Figures S3D and

markers Patched (Ptc, expressed in anterior cells at the anterior/posterior compartment boundary) and Wingless (Wg, expressed in cells on both sides of the dorsal/ventral compartment boundary) are shown in magenta and light magenta, respectively. All illustrated expression patterns were observed in $n \geq 4$ wing discs in $n \geq 2$ experimental replicates.

(B–D) Wing discs expressing fusion proteins from *robo2-GFP*, *eph-GFP*, and *ten-m-GFP* constructs under native regulatory control (gray or magenta). Ptc and Wg demarcate A/P and D/V compartment boundaries. Images are shown at same scale.

(B'–D') Pouch domains of *robo2-GFP*-, *eph-GFP*-, and *ten-m-GFP*-expressing wing discs (gray or cyan), also stained for the adherens junction marker E-cadherin (Ecad, gray or magenta) and cortical F-actin (by Phalloidin, gray or yellow). Apical (') and lateral (") local-z-projections, displaying receptor localization relative to junctional Ecad and relative to more lateral domains. Images are shown at same scale. Please note that wing discs are not the same as in (B)–(D). Related to Figure S2.

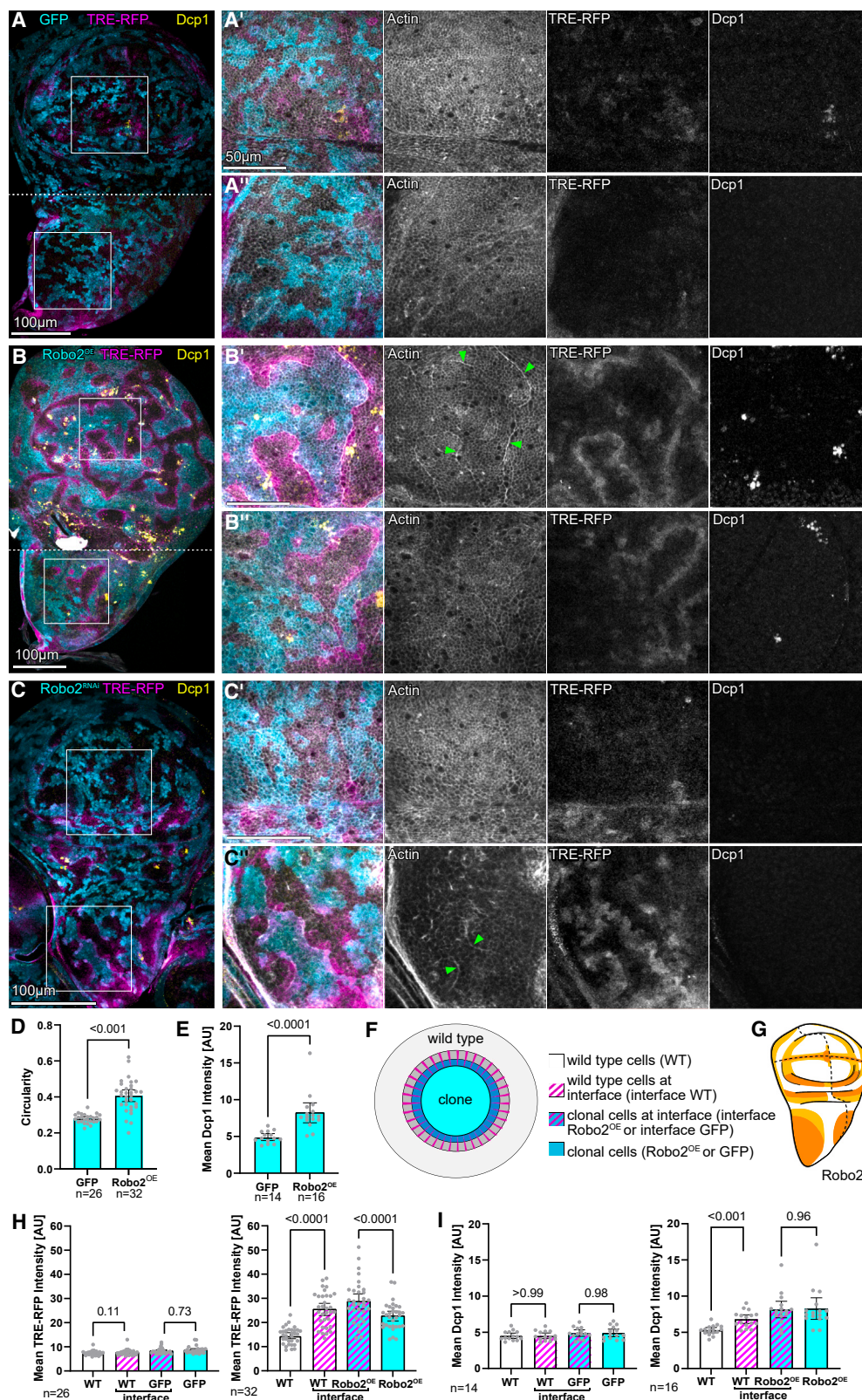


Figure 3. A mismatch of Robo2 receptor expression levels between neighboring cells is detected by interface surveillance

(A–C) Mosaic wing disc with wild-type clones expressing *UAS-GFP* (GFP, cyan) (A), and in addition, either *UAS-rob2* (Robo2^{OE}) (B) or *UAS-rob2-RNAi* (Robo2^{RNAi}) to reduce Robo2 function (C). TRE-RFP expression is reporting JNK pathway activity (gray or magenta). Antibody staining against the cleaved

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S3E). These results demonstrate that cells, which create pronounced differences and thus a mismatch in Robo2 expression levels, are robustly detected by interface surveillance within the endogenous Robo2 expression pattern, suggesting that Robo2 may contribute to detection and removal of aberrant cells in a context-specific manner.

Robo2 and Robo3 mediate interface surveillance in a Slit-independent manner

Intrigued by the ability of Robo2 to induce interface surveillance, we next investigated the functional properties of the Robo2 receptor and the other Robo family members Robo1 and Robo3, as well as the known Robo-ligand Slit.

First, we wanted to test, whether in addition to actin, other cytoskeletal effectors were also recruited by Robo2-dependent interface surveillance. We therefore analyzed the localization of a GFP-tagged non-muscle myosin II regulatory light chain (Sqh-GFP) to visualize the actin motor myosin II, which enriches at the interface between wild-type and aberrant cells.³ Moreover, we visualized Enabled (Ena), the *Drosophila* Ena/Vasp actin polymerase and an effector of Robo receptors in neuronal systems.⁴⁶ We also stained for phosphorylated tyrosine (pTyr), a marker of cellular interfaces between normal and oncogenic cells in mammalian epithelial tissue culture models.⁴⁷ We found that all markers enriched at interfaces of Robo2-expressing clones in the pouch (Figures 4A and 4B). However, as expected from the position-dependent recognition of clones, enrichment of Sqh-GFP, Ena, or pTyr was absent from interfaces of clones in the anterior notum (Figures 4C and 4D). These observations strongly indicate that Robo2 targets actomyosin regulation in epithelial tissues to mediate interface surveillance responses.

We next wanted to test if the downstream signal transduction by the Robo2 cytosolic domain is required for the induction of interface surveillance. To this end, we expressed a Robo2-construct that lacks the cytosolic domain (Robo2^{Δcyto}), preventing downstream signaling from the expressed Robo2 receptors. We find that Robo2^{Δcyto}-expressing clones also induced interface surveillance hallmarks, such as clone smoothening, actin enrichment, and JNK signaling at the interface (Figure 4E). Importantly, the presence of endogenous Robo2 in this genetic background only allows for the conclusion that cytosolic signaling by the overexpressed Robo2 is not required for

induction of interface surveillance. We thus wanted to test the requirement of the well-characterized Robo-ligand Slit, which activates canonical receptor signaling. We expressed a Robo2-construct that lacks the extracellular immunoglobulin (Ig)-domains 1 and 2 (Robo2^{ΔIg1+2}) required for Slit ligand binding.^{26,48–50} We find that Robo2^{ΔIg1+2}-expressing clones phenocopy the interface surveillance hallmarks of full-length Robo2-misexpression by displaying smoothening, actin enrichment, and JNK signaling at the interface (Figure 4F). This experiment demonstrates that the Slit-binding domain of Robo2 is not required for interface surveillance. To further provide support for this conclusion, we analyzed the function of Slit in wing imaginal disc. As reported before,⁵¹ Slit is broadly expressed in imaginal discs (Figure S4A), but neither clonal overexpression of Slit or its downregulation using a validated RNAi construct induced JNK signaling at clonal interfaces (Figures 4G, 4H, and S4C). Similarly, Robo1, the Robo family member best described for Slit interaction, is broadly expressed in wing discs (Figure S4B). Yet, neither clonal overexpression nor downregulation of Robo1 using a validated RNAi induced JNK interface surveillance responses (Figures 4I, 4J, S4D, and S4E). Combined, these results suggest that Robo2 misexpression induces interface surveillance via a non-canonical Robo receptor activity and that classical Robo-Slit-mediated signaling may not be required.

Robo3 represents the third and last member of the Robo receptor family in flies. Interestingly, Robo3 shares a higher sequence similarity in extracellular and intracellular signaling domains with Robo2 than with Robo1.⁵² We thus asked if Robo3-misexpression may also induce interface surveillance. In developing wing discs, Robo3 is expressed at barely detectable levels (Figure S5A). Hence, we reasoned that overexpression of Robo3 should strongly induce interface surveillance because Robo3-overexpressing clones would create pronounced differences in Robo3 levels to surrounding wild-type cells (Figure S5B). Indeed, Robo3-expressing clones induced clone smoothening, actin enrichment, JNK signaling, and apoptosis at clonal interfaces (Figures 5A and S5C). Moreover, Sqh-GFP, Ena, and pTyr were enriched at interfaces of Robo3-expressing clones (Figures S5D and S5E). In contrast, we did not observe any response when we expressed a validated Robo3-RNAi construct in mosaic clones (Figures 5B, S5F, and S5G). This result is consistent with a context-dependent model

effector caspase (Dcp1) visualizes apoptosis (gray or yellow). Phalloidin visualizes cortical F-actin (gray). Please note that JNK activity is physiologically elevated at the A/P compartment boundary in wing discs at this developmental stage. White frame marks region shown to the right in (A'), (B'), (C' pouch), (A''), (B''), and (C'' notum). Please note the actin enrichment at the clone interface (green arrows in B' and C'). The dotted line indicates stitching of confocal images with different z positions. Image sets are shown at the same scale.

(D and E) Graphs depicting circularity and apoptosis observed for individual *UAS-GFP*- (GFP) and *UAS-Robo2*- (Robo2^{OE}) expressing clones. Sample number (n) for individual wing discs and p values of a two-tailed, unpaired t test are displayed in graphs. Error bars represent mean and 95% confidence interval.

(F) Illustration of regions of interest (ROIs) relative to the clonal interface defined for quantitative image analysis (see also Figures S3A–S3C). There are non-labeled wild-type cells (white) or *UAS-GFP*-labeled clonal cells (cyan) in the wing disc. Clonal cells which additionally express *UAS-Robo2* are abbreviated as Robo2^{OE}. Both GFP-labeled (cyan) and non-labeled (white) cells are separated into cells, which are in contact with the other cell type at the interface (magenta stripes), and the remaining cells away from the interface.

(G) Illustration of the *robo2-GFP* expression pattern. Relative fluorescence intensity is depicted by the color intensity from low (white), medium (light orange) to high (dark orange).

(H and I) Graphs depicting mean fluorescence intensity of TRE-RFP reporter and mean Dcp1 intensity in the zones of measurement around clones, as depicted in Figure 3F. Graphs display results for mosaic discs with *UAS-GFP*-labeled clonal cells (left) or containing *UAS-Robo2* (Robo2^{OE})-expressing clones (right).

Sample number (n) for individual wing discs and p values of a repeated measured one-way ANOVA with Tukey's multiple comparisons test are displayed in graphs. Error bars represent mean and 95% confidence interval.

Related to Figure S3 and Data S1.

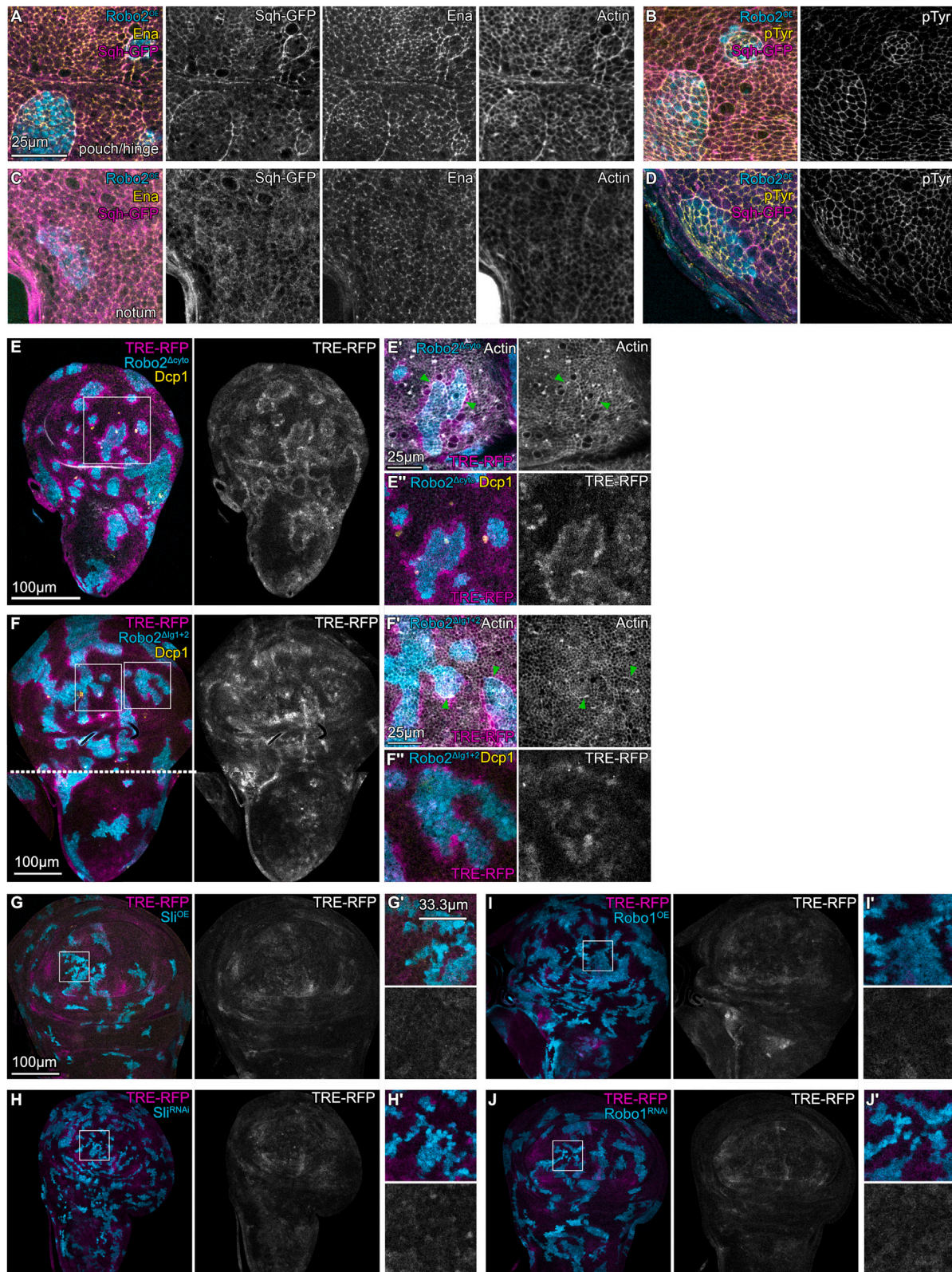


Figure 4. Robo2 induces interfaces surveillance independent of Slit and the receptor's cytosolic domain

(A–D) Mosaic wing disc with clones expressing *UAS-rob2* (*Robo2^{OE}*) (cyan) in either the pouch and hinge (A and B) or the notum region (C and D) of a wing disc. In (A) and (C) A GFP-tagged non-muscle myosin II regulatory light chain (*Sqh-GFP*) tracks the actin motor myosin II. antibody staining against Enabled (*Ena*)

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of Robo3-dependent interface surveillance. Combined, we conclude that misexpression of either Robo2 or Robo3—with respect to levels in the surrounding wild-type cells—are completely sufficient to induce all hallmarks of interface surveillance.

One observation allowed us to further unravel the close functional relationship of Robo2 and Robo3 already suggested by previous studies in the developing nervous system.^{53,54} Because Robo2 elicits interface surveillance depending on its endogenous pattern (Figures 5C–5E), we predicted that Robo3-expressing clones induce interfaces surveillance in the entire disc due to its ubiquitously low expression during development (Figure 5H). Contrary to those predictions, Robo3-misexpressing clones did not induce JNK signaling in the anterior notum, similar to Robo2-expressing clones (Figures 5F and 5G). We thus asked if Robo2 and Robo3 may act redundantly at clonal interfaces. To test this, we expressed Robo3 in Robo2-RNAi-expressing clones. We reasoned that if redundant, Robo3 expression will rescue the mismatch of Robo2 levels induced by Robo2-RNAi clones in the anterior notum. Strikingly, Robo3 expression robustly suppressed induction of JNK signaling and clone smoothening by Robo2-RNAi clones in the anterior notum (Figures 5I and 5J). Combined, our experiments demonstrate a pattern-specific function for Robo2 and Robo3 receptors in inducing interface surveillance and reveal that their high sequence similarity may cause functional redundancy, suggesting that both act through similar mechanisms in interface surveillance.

Expression of Robo receptors and interface surveillance molecules is regulated by fate-patterning pathways

If Robo2, Robo3, and other cell surface molecules identified in our genetic screen are really required to mediate the detection of aberrant and misspecified cells, then deregulation and genetic aberrations of fate-specifying pathways should alter expression levels of these proteins. Such upregulated or downregulated levels in aberrant cells would create pronounced expression level differences in cell surface molecules between neighboring cells, providing the basis for interface recognition.

To test this, we created clones misexpressing known cell-fate-specifying regulators: Thickveins (tkv^{RNAi} , tkv^{CA}), a Dpp/TGF- β -receptor important for wing disc patterning. Eyeless (*ey*), a Pax6 homolog and master regulator of the eye-specification network. Forkhead (*fkh*), a conserved transcription factor required for salivary gland specification. *ci*, the transcription

factor activated by Hh signaling in the developing wing disc. Previous studies demonstrated that misexpression of these factors can drive dramatic respecification of cell fates and alterations of entire gene expression programs in imaginal discs.^{55,56} We first tested if ectopic misspecification by these pathways affects Robo2 expression. Strikingly, expression levels of Robo2 were significantly altered, showing both upregulation and downregulation upon clonal misexpression of *Tkv*-RNAi, *Fkh*, or *Ey* in wing imaginal discs (Figures 6A–6D). Importantly, ectopic misspecification did not regulate Robo2 equally in every wing disc position. For instance, *Ey* increased expression levels of Robo2 in the pouch but not the notum, whereas *Fkh* downregulated Robo2 in the notum but not the pouch. The developing disc undergoes extensive patterning and different cell-type-specific signaling networks are active in the pouch, hinge, and notum cells. Therefore, our findings emphasize that the misexpression of one fate-specifying pathway is integrated into the intrinsic transcriptional network of different cell types, consistent with models of transcriptional networks in tissue patterning.^{57,58} We thus suggest that—in response to ectopic deregulation of cell-fate-patterning pathways—the highly cell-type-specific transcriptional context determines whether to express a specific cell surface molecule or not.

Such reasoning predicts that changes to a single cell specification program may alter expression of several cell surface molecules simultaneously, including positive hits identified in our genetic screen. We thus wanted to understand how aberrant cell fate specification may reflect more complex changes to the composition of a cell surface code. We analyzed which cell surface molecules competent for interface surveillance are affected by deregulated *Tkv* signaling. Importantly, when we analyzed *Tkv*-misexpressing clones in the pouch, we found that they altered expression not just of Robo2 but also of Eph, Ten-m, Ten-a, and Tartan (Figures 6E–6N). Similarly, *Fkh* or *Ci*-expressing clones induced changes in expression of at least three cell surface molecules simultaneously (Figures S6A–S6I). These experiments strongly demonstrate that the alteration in activity of just one cell fate specification pathway causes changes in expression levels of multiple cell surface molecules. This alteration dramatically modifies the cell surface code of misspecified cells in comparison with surrounding wild-type cells.

To better understand how regulation of cell surface molecules may also be relevant for oncogenic processes driven by cell fate changes, we turned to the analysis of oncogenic mutations

visualizes localization of the *Drosophila* Ena/Vasp actin polymerase. Phalloidin visualizes cortical F-actin. In (B) and (D), antibody staining against phosphorylated tyrosine (pTyr) visualizes enhanced signaling activity at cellular junctions. Image sets are shown at the same scale. Projections of the apical junctional network were generated using the LocalZ-projector.

(E) Mosaic wing discs with clones (cyan) expressing *UAS-Robo2.DeltaC* (Robo2^{Δcyto}), a Robo2 construct lacking the cytosolic domains of the receptor. White frame marks the region shown in (E') and (E''). Please note the actin enrichment at the clone interface (green arrows in E').

(F) Mosaic wing discs with clones (cyan) expressing *UAS-Robo2.DeltaIg1+2* (Robo2^{ΔIg1+2}), a Robo2 construct lacking the Ig1 and Ig2 domains of the extracellular part of the receptor. Please note that the Ig1 receptor domain has been shown to convey the binding of the Slit ligand. White frame marks region shown in (F') and (F''). Please note the actin enrichment at the clone interface (green arrows in F').

(G and H) Mosaic wing discs with clones (cyan) expressing either *UAS-sli.D* (Sli^{OE}) (G) or *UAS-sli-RNAi* (Sli^{RNAi}) (H). White frame marks the region shown in (G') and (H'). Image sets are shown at the same scale.

(I and J) Mosaic wing discs with clones (cyan) expressing either *UAS-robo1* (Robo1^{OE}) (I) or *UAS-robo1-RNAi* (Robo1^{RNAi}) (J). White frame marks the region shown in (I') and (J'). Image sets are shown at the same scale as (G) and (H).

TRE-RFP expression is reporting JNK pathway activity (gray or magenta). Antibody staining against the cleaved effector caspase (Dcp1) visualizes apoptosis (yellow). Phalloidin visualizes cortical F-actin (gray).

Related to Figure S4.

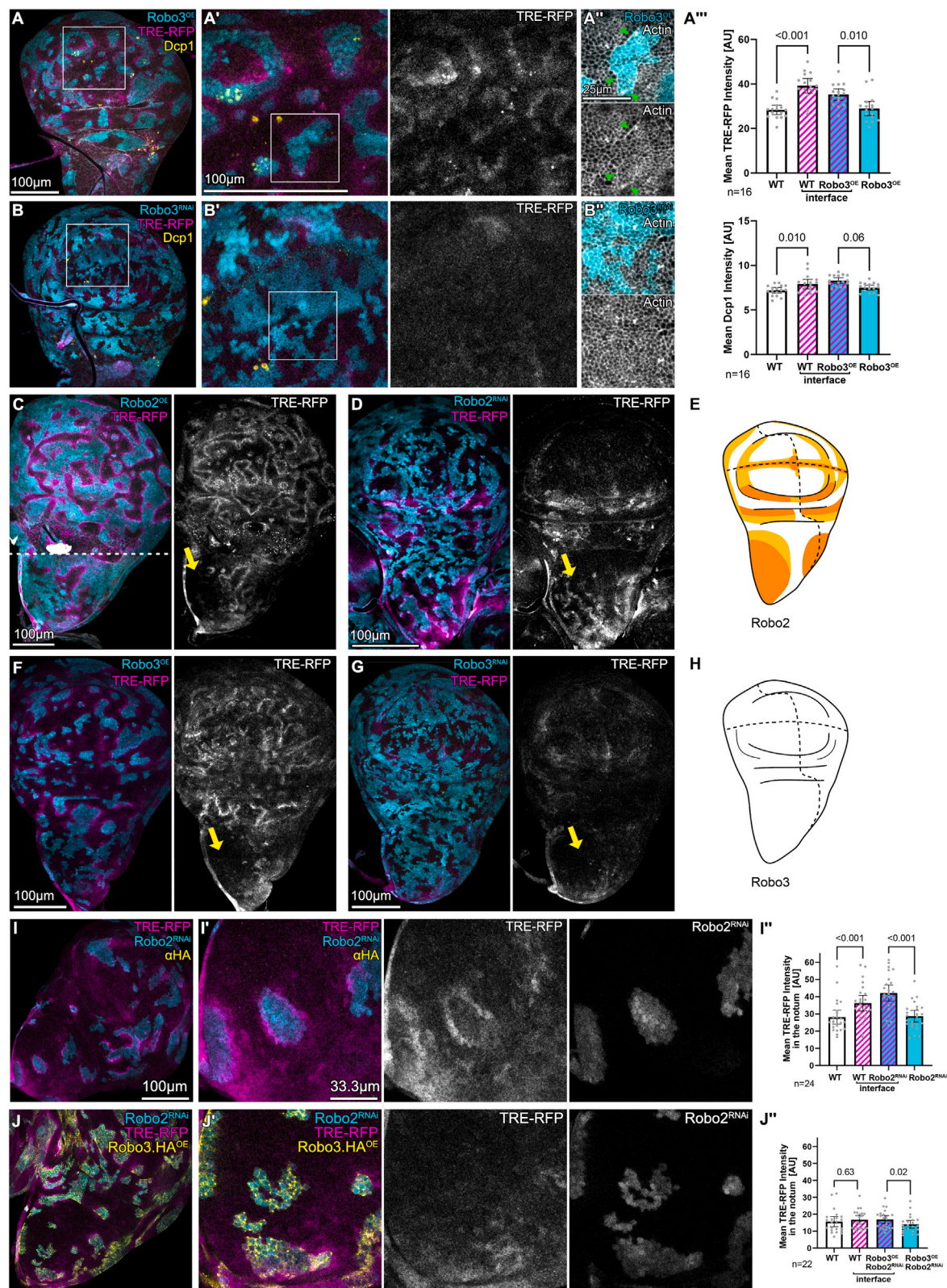


Figure 5. Robo3 induces interface surveillance and functions redundantly with Robo2

(A and B) Mosaic wing discs with clones (cyan) expressing either *UAS-robo3.HA* (*Robo3^{OE}*) (A) or *UAS-robo3-RNAi* (*Robo3^{RNAi}*) (B). TRE-RFP expression is reporting JNK pathway activity (gray or magenta in all panels). Antibody staining against the cleaved effector caspase (Dcp1) visualizes apoptosis (yellow).

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incurred by *Ras*^{V12}. Ras signals through the EGF/ERK pathway and regulates both proliferation and wing fate patterning.⁵⁹ We previously reported that *Ras*^{V12}-expressing cells induce clone smoothening, actin enrichment, and JNK signaling at clonal interfaces (Figure S6J).^{3,60} Yet, *Ras*^{V12} cells evade apoptosis, thereby allowing them to form slow-growing tumors.⁴ To understand if *Ras*^{V12}-expressing cells may be initially detected by altered expression of cell surface markers, we monitored expression of cell surface molecules in *Ras*^{V12} mosaic discs. Indeed, *Ras*^{V12}-expressing clones deregulated expression of Robo2, Eph, Ten-m, and others (Figures 6O–6T and S6K–S6R). These observations provide further support for our conclusion that just a single fate-specifying aberration will alter the expression of multiple cell surface molecules and thereby drive complex changes in the cell surface code composition.

To understand whether expression of these cell surface molecules may also be utilized by other tissue-intrinsic error correction mechanisms, we analyzed genetic models of classical cell competition. In cell competition, the comparison of proteostatic or metabolic fitness between neighboring cells drives cell elimination, such that less fit “loser” cells are eliminated by fit “winner” cells.^{61–68} Previous studies indicate that loser cells start to express a specific isoform of the cell surface molecule Flower, which marks them for elimination by winner cells in a cell-contact-dependent manner.^{69,70} To test whether also a Robo2, Eph, or Ten-m-dependent cell surface code is associated with winner or loser cell state, we analyzed expression of these cell surface molecules in imaginal discs mosaic for a Myc-expressing winner genotype. In agreement with the model that cell competition and interface surveillance are mechanistically distinct error correction mechanisms, we found that Myc-expressing clones did not display pronounced alterations of cell surface receptor expression patterns that were predictive of either winner or loser fate (Figures 6U–6Z). Similarly, other genotypes linked to cell competition, tissue growth or cell survival, such as moderate activation or inhibition of Hippo/Warts

signaling, did not alter cell surface molecule expression patterns (Figures S6U–S6AB). These observations demonstrate that a change in expression of the analyzed cell surface molecules is specific to cell-fate-specifying pathways and consequently highly correlates with activation of interface surveillance. This conclusion is strongly supported by our previous studies, in which cell competition and interface surveillance form distinct branches of tissue-intrinsic error correction and tumor suppression mechanisms that maintain the health of epithelial tissues.⁴

All our data presented so far provide strong genetic evidence that individual cell surface molecules are regulated by cell-fate-patterning pathways and that each is individually fully sufficient to induce interface surveillance. We now wanted to develop an approach to also provide evidence that deregulation of individual cell surface molecules is indeed genetically necessary for the detection of aberrant cells, even though we expected that genetically targeting a single molecule may not be sufficient to interfere with interface surveillance in response to changes in multiple cell surface molecules. Yet, we designed rescue experiments where we co-expressed a RNAi construct or overexpression construct for either Robo2, Eph, or Ten-m in clones with aberrant cell fates. We find that cells that aberrantly express *ey* and *fkh* still induced clone smoothening, enrichment of actin and even apoptosis, despite very targeted experiments to restore expression levels of Eph, Robo2, or Ten-m to that of surrounding cells (Figures S7A–S7C). Similarly, *Ras*^{V12} clones that ectopically induce expression of Eph or Robo2 in the peripheral pouch still activated interface surveillance responses despite targeted knockdown of Robo2 or Eph (Figures S7D and S7E). Yet, to really test whether equalizing expression levels of just one cell surface molecule between aberrant cells and surrounding cells can suppress interface surveillance, we expressed UAS-RNAi constructs for molecules that are upregulated in the peripheral pouch and hinge domain by *tkv*^{CA} (where *Tkv* is not normally active) under the control of *en*-GAL4. Thereby, all cells in the posterior compartment lacked either Eph or Ten-m at the time

Phalloidin visualizes cortical F-actin (gray). White frames in (A) and (B) mark the region shown in (A') and (B'). White frames in (A') and (B') mark the regions shown in (A'') and (B''). Image sets are shown at the same scale. Please note the actin enrichment at clone interfaces in (A'') (green arrows). (A''') Graphs depicting mean fluorescence intensity of TRE-RFP reporter and mean Dcp1 intensity in the zones of measurement around clones, as depicted in Figure 3F. Graphs display results for mosaic discs containing *UAS-Robo3.HA* (*Robo3*^{OE})-expressing clones. See Figure S5C''' for *UAS-GFP*-labeled clonal cells as wild-type control. Sample number (n) for individual wing discs and p values of a repeated measured one-way ANOVA with Tukey's multiple comparisons test are displayed in graphs. Error bars represent mean and 95% confidence interval.

(C and D) Mosaic wing discs where clones (cyan) express either *UAS-rob2* (*Robo2*^{OE}) (C) or express *UAS-rob2-RNAi* (*Robo2*^{RNAi}) (D). Yellow arrows indicate anterior notum region. TRE-RFP expression is reporting JNK pathway activity (gray or magenta). The dotted line indicates stitching of confocal images, with different z positions.

(E) Illustration of the *rob2-GFP* expression pattern. Relative fluorescence intensity is depicted by the color intensity from low (white), medium (light orange) to high (dark orange).

(F and G) Mosaic wing discs where clones (cyan) express either *UAS-rob3* (*Robo3*^{OE}) (F) or express *UAS-rob3-RNAi* (*Robo3*^{RNAi}) (G). Yellow arrows indicate anterior notum region. TRE-RFP expression is reporting JNK pathway activity (gray or magenta). Please note the absence of JNK activation in the notum region in (F), reminiscent of JNK activation by *Robo2*^{OE} in (C).

(H) Illustration of the Robo3 expression in the wing disc as visualized by Robo3 antibodies. Please note that Robo3 is hardly expressed in the wing disc. See Figure S5A.

(I and J) Mosaic wing discs with clones expressing either *UAS-rob2-RNAi* (*Robo2*^{RNAi}) (I) or both *UAS-rob2-RNAi* (*Robo2*^{RNAi}) and *UAS-rob3.HA* (*Robo3.HA*^{OE}) (J). TRE-RFP expression is reporting JNK pathway activity (gray or magenta). Antibody staining against HA tag (αHA) visualizes the presence of the *UAS-rob3.HA* construct.

(I'' and J'') Graphs depicting the mean fluorescence intensity of TRE-RFP reporter in the zones of measurement around clones, as depicted in Figure 3F. Graphs display the results for mosaic discs containing *UAS-rob2-RNAi* (*Robo2*^{RNAi}) (I''), or both *UAS-rob2-RNAi* (*Robo2*^{RNAi})- and *UAS-Robo3.HA* (*Robo3*^{OE})-expressing clones. Sample number (n) for individual wing discs and p values of a repeated measured one-way ANOVA with Tukey's multiple comparisons test are displayed in graphs. Error bars represent mean and 95% confidence interval.

Related to Figure S5 and Data S2.

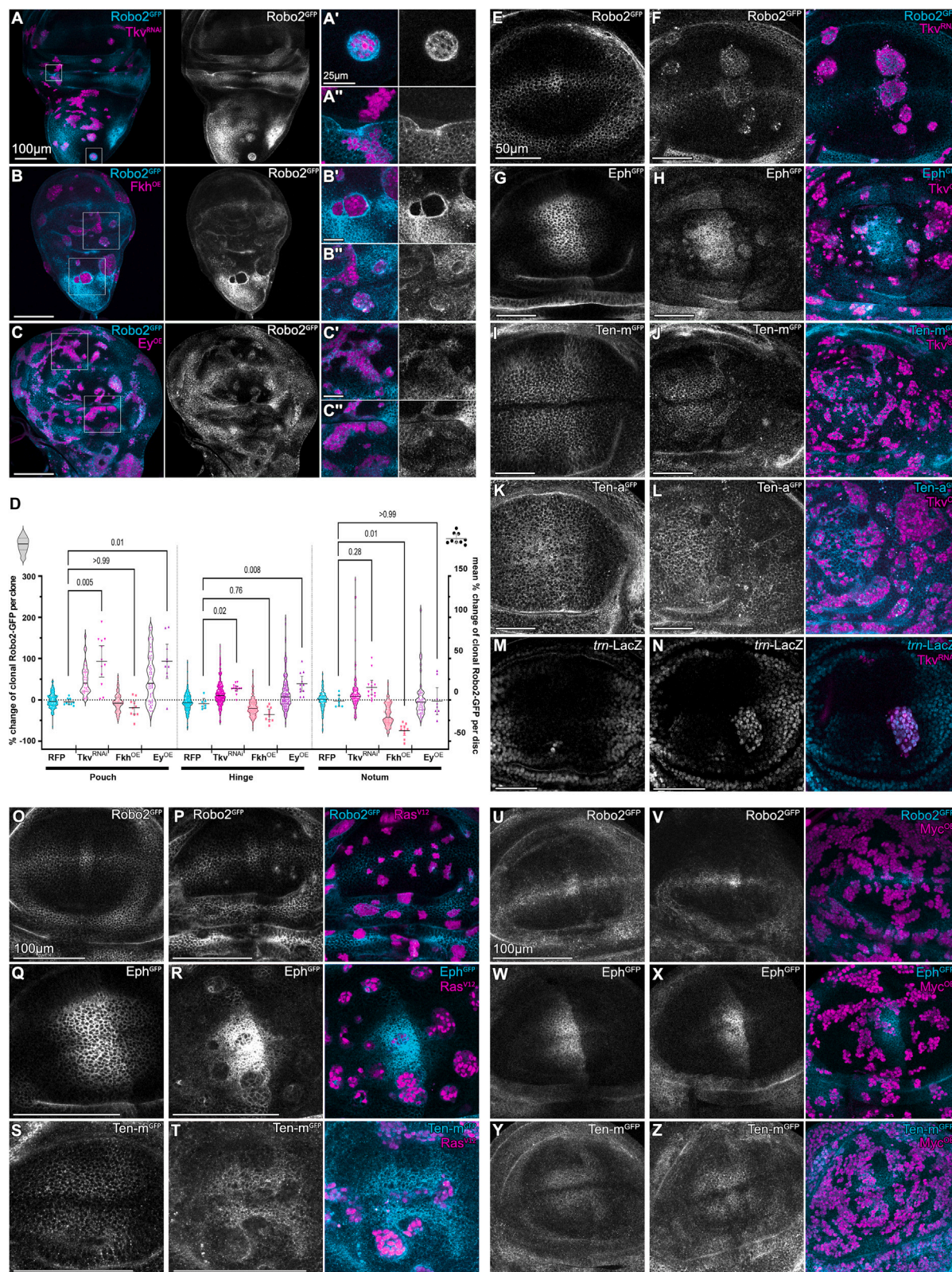


Figure 6. Expression of Robo receptors and other cell surface molecules is regulated by fate-patterning pathways

(A–C) Wing discs expressing *Robo2-GFP* (Robo2^{GFP}) (gray or cyan). Mosaic clones (magenta) deregulate fate-specifying pathways by the expression of *UAS-tkv-RNAi* (Tkvr^{RNAi}) (A), *UAS-fkh* (Fkh^{OE}) (B), or *UAS-ey* (Ey^{OE}) (C). White frames mark the regions shown in (A')–(C'').

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when we introduced *tkv^{CA}*-expressing clones using the LexA/LexO system.^{71,72} However, removing either Eph or Ten-m from the surface code of posterior cells failed to affect interface surveillance responses of aberrant *tkv^{CA}*-expressing clones in the posterior pouch and hinge (Figures S7H–S7J).

These experiments, while challenging, support the multifactorial nature of a cell surface code, where multiple cell surface molecules are simultaneously deregulated upon cell fate mis-specification, and deregulation of any one of them is sufficient to robustly induce interface surveillance. As a consequence, genetic experiments to analyze the necessity of individual molecules in the detection of aberrant cells are technically not possible, as our current genetic tools are incompatible with the possibly highly redundant nature of these molecules within the surface code. While we demonstrate that 12 proteins from our genetic screen are each sufficient to activate all hallmarks of interface surveillance, our study is thus limited by the lack of genetic evidence for the necessity of these molecules in mediating detection and recognition of aberrant cells in tissues.

DISCUSSION

Cell fate, cell surface code, and interface surveillance

In summary, we demonstrate that wing disc cells are equipped with various cell surface molecules, such as Robo2, Teneurin, or Eph. Almost all of them are expressed during development and deregulation of expression levels and the resulting expression level mismatch created at clonal interfaces is sufficient to induce all hallmarks of interface surveillance (Figure 7). Furthermore, aberrant cell fate programs deregulate expression of multiple cell surface molecules simultaneously. We propose that this reflects a physiological process of cell fate specification during development, in which cell fates establish a cell surface profile unique to each fate. Importantly, a single transcription factor does not always target the same cell surface molecule. Instead, the molecular composition is shaped by the cumulative activity of cell-type-specific transcriptional networks unique to each cell type. Finally, an altered surface code is a unique feature of cells experiencing cell fate deregulation and interface surveillance but not classical cell competition.

Among the protein families that we identified in our genetic screen, Eph/Ephrin, specifically, have previously been described to promote interface smoothening via actomyosin regulation in mosaic tissues and at compartment boundaries, both in *Drosophila* and mammalian systems.^{36,43} Indeed, deregulation of Eph/Ephrin signaling induces actomyosin and Ena enrichment at clonal interfaces, clone smoothening and bilateral JNK

signaling in our assays (Figures S7K–S7R). Similarly, Teneurins, or LRR proteins, such as Tartan, have been implicated in modulating actomyosin contractility or compartment boundary smoothening in different tissues. Moreover, regulation of their expression has been previously linked to fate specification in *Drosophila*.^{36,37,44,73–77} Yet, we also describe a novel function of these molecules by implicating them in the induction of bilateral JNK activation. This suggests that these very different cell surface molecules may act not only through a shared set of cytoskeleton effectors but also through similar signaling effectors. The cell surface molecules identified in our screen are composed of very different structural motifs, including leucine-rich, Ig, YD, or cadherin repeats.^{29,30,35,78} How these extracellular motifs or intracellular domains contribute to these shared effector modalities of actomyosin contractility and JNK signaling will be an important future avenue of our research.

Robo receptors in epithelia

Robo receptors are increasingly recognized to play a role in epithelia.²⁹ For example, recent molecular data from the mouse pancreas show that epithelial islet cells express Robo receptors. Disrupting Slit-Robo signaling disrupts embryonic islet formation and adult islet architecture.^{79,80} In *Drosophila*, a JNK-Robo2-Ena axis is required for the elimination of *scrib* mutant cells in eye discs.⁴⁵ Importantly, we propose a distinct model for Robo2 and Robo3 receptors, where their mismatch at clonal boundaries is crucial rather than the mere presence of Robo2 receptors in aberrant cells. Moreover, JNK serves as a downstream effector of this mismatch, rather than an upstream regulator of Robo2 expression levels. In addition, neither the ligand Slit nor the cytosolic domain in Robo2 expression constructs are required for the induction of interface surveillance in our assays. In fact, ectopic levels of solely the extracellular domain of Robo2 are sufficient to activate interface signaling, suggesting that the extracellular domain of Robo2 may act as interaction partner for cell surface molecules. Curiously, hRobo2 can interact in *trans* on neighboring cell surfaces, and the binding of LRR domains present in Slit result in conformation changes favoring the formation of an activating Robo2 dimer in *cis*.^{50,81} Similarly, recent studies have focused on heterophilic receptor interactions involving Toll-like receptors containing LRR domains with Trm and Ten-m in *Drosophila* embryo patterning, receptors that we also find to induce interface surveillance.^{44,82} Whether heterophilic interactions through LRR-domain proteins other than Slit play a role in Robo2-mediated interface surveillance remains to be investigated. Of note, we also find that Robo3 is capable of compensating for the loss of Robo2. Robo2 and Robo3 are

(D) Graph depicting the change of *robo2-GFP* in clonal cells at the interface expressing *UAS-RFP* (RFP), *UAS-*tkv*-RNAi* (*Tkv^{RNAi}*), *UAS-*fkh** (*Fkh^{OE}*), or *UAS-*ey** (*Ey^{OE}*) relative to the surrounding wild-type cells in the pouch, hinge, or notum region of the wing disc. Left axis shows the relative change for individual clones as depicted in the violin plots. Right axis shows the mean change of all the relative changes of individual clones per disc. $n \geq 7$ wing discs per genotype are plotted, and p values of a Kruskal-Wallis test with Dunn's multiple comparison test are displayed in the graph. Error bars represent mean and 95% confidence interval. (E, G, I, K, M, O, Q, S, U, W, and Y) Wing discs presenting the specific expression patterns of *robo2-GFP* (*Robo2^{GFP}*) (E, O, and U), *eph-GFP* (*Eph^{GFP}*) (G, Q, and W), *ten-m-GFP* (*ten-m^{GFP}*) (I, S, and Y), *ten-a-GFP* (*Ten-a^{GFP}*) (K), or *trn-LacZ* (*trn-LacZ*) (M) serving as a reference for the experimentally induced changes in (F), (H), (J), (L), (N), (P), (R), (T), (V), (X), and (Z).

(F, H, J, L, N, P, R, T, V, X, and Z) Wing discs expressing *robo2-GFP* (*Robo2^{GFP}*) (F, P, V), *eph-GFP* (*Eph^{GFP}*) (H, R, and X), *ten-m-GFP* (*ten-m^{GFP}*) (J, T, and Z), *ten-a-GFP* (*Ten-a^{GFP}*) (L), or *trn-LacZ* (*trn-LacZ*) (N) (gray or cyan) and carrying mosaic clones (magenta) with the knockdown of *Tkv* (*Tkv^{RNAi}*) (F and N), expression of a constitutively active *Tkv* (*Tkv^{CA}*) (H, J, and L), expression of *UAS-RasV12* (*Ras^{V12}*) (P, R, and T) or expression of *Myc* (*Myc^{OE}*) (V, X, and Z). Increasing *Myc*-levels creates super competitor cells, which will outcompete surrounding wild-type cells. Related to Figures S6 and S7 and Data S3 and S4.

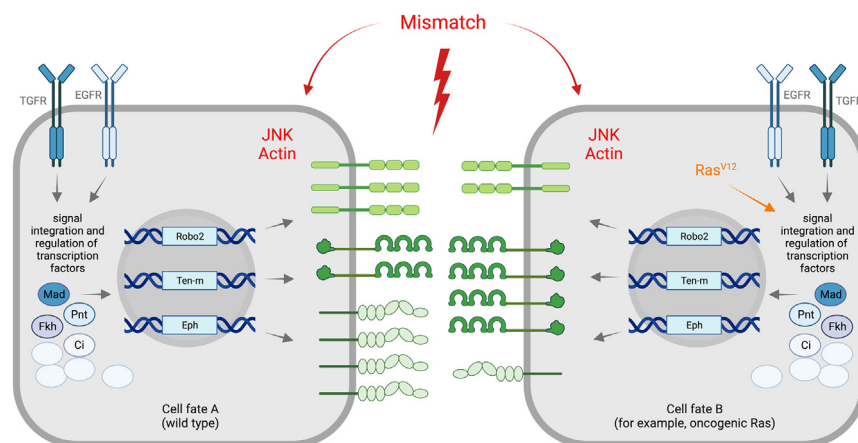


Figure 7. A mismatch in cell surface molecules induces interface surveillance

Signaling by different developmental patterning pathways (blue) is integrated in a cell-type-specific manner and a cell-type-specific set of transcription factors (blue) to drive the expression of a set of genes (blue) encoding for cell surface molecules (green), which are known to play a role in neuronal guidance and targeting. Thus, the combination of molecules displayed at the cell surface depends on the signal integration and fate specification at any position within the imaginal disc. Aberrant fate signals, for example, by the expression of oncogenic Ras^{V12} (orange), disrupt this cell-type-specific regulation of gene expression leading to a changed combination of cell surface molecules relative to that of the neighboring wild-type cell. The mismatch in cell surface molecules between neighboring cells induces all hallmarks of interface surveillance (red). Related to Figure S7.

closely related molecules with previously described functions in neuronal cell-cell recognition and differ from Robo1.^{53,54} While all Robo receptors share similar extracellular domains, Robo2 and 3 retain only two of the four conserved cytoplasmic (CC) motifs found in Robo1 and lack the strongest Ena-recruiting CC-motif.⁴⁶ Interestingly, all Robo receptors retain a WAVE regulatory-complex-interacting receptor sequence (WIRS) through which actin regulation may be facilitated independent of Slit ligand binding.^{83,84}

Regulation of neuronal cell surface molecules in cancer

Multiple recent studies revealed significant changes to the expression patterns of neuronal cell surface molecules in tumors, for example.^{85,86} We demonstrate that Ras^{V12}-expressing cells disrupt the endogenous pattern of surface molecules, causing pronounced differences in the surface code and activation of interface surveillance responses. In mammalian epithelia, cells with oncogenic Ras signaling are detected and eliminated by a process termed epithelial defense against cancer (EDAC) akin to interface surveillance.^{4,87,88} Eph/Ephrin signaling was shown to regulate the interaction of oncogenic Ras^{V12}-expressing cells with neighboring wild-type cells in cultured MDCK monolayers and pancreas *in vivo*.^{89,90} Here, Ras^{V12}-expression induces EphA2 which underlies actin enrichment and cell shape changes upon interaction of wild-type and Ras^{V12}-expressing cells. This supports a model where interface surveillance mediated by altered expression of cell surface molecules may have important roles in tumor suppression by regulating the interaction between wild-type and aberrant cells. However, this very machinery can also promote tumor progression by triggering signaling pathways and actomyosin dynamics at the interface with wild-type cells. Aberrant cells may exhibit resistance to apoptosis, as exemplified by the case of Ras^{V12}-expressing cells, which would channel activation of JNK signaling and actomyosin dynamics toward migratory behaviors.⁴

Interface surveillance in development

Interface surveillance shares striking similarities with compartment boundary formation in developing tissues, where two cell populations of distinct fate mechanically segregate via the formation of a

contractile actomyosin interface between them. Indeed, many of the molecules identified in our genetic screen facilitate actomyosin regulation during compartment boundary formation in vertebrate and invertebrate species.^{24–26,36,37} The similarities between developmental morphogenesis and tissue-intrinsic error correction, relying on the same principle of receptor-mediated recognition and actomyosin driven segregation of cell populations, point toward a common evolutionary origin of the surface code subsequently adapted for developmental and homeostatic processes. In fact, cellular function of neuronal axon guidance in neuronal tissues may have originally evolved in evolutionarily more ancient epithelia. Ultimately, the cell surface code formed by so-called neuronal axon guidance molecules and related factors may represent an ancient system to distinguish self from non-self in physiological and pathological contexts.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
 - Lead contact
 - Materials availability
 - Data and code availability
- EXPERIMENTAL MODEL
- METHOD DETAILS
 - Drosophila genetics
 - Immunohistochemistry
 - Image acquisition and image display
 - Illustration of expression patterns
- IMAGE QUANTIFICATION AND STATISTICAL ANALYSIS
 - Region of interest (ROI) segmentation and quantification workflow

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.cub.2024.01.053>.

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AUTHOR CONTRIBUTIONS

Conceptualization, F.F., A.F., and A.-K.C.; data curation, F.F., L.E., A.F., and K.H.; formal analysis, F.F., A.F., L.E., and V.W.; funding acquisition, A.-K.C.; investigation, F.F., A.F., L.E., K.H., D.P., V.W., and R.B.; methodology, F.F., A.F., L.E., K.H., D.P., V.W., and R.B.; project administration, A.-K.C.; supervision, A.-K.C.; validation, F.F., A.F., L.E., K.H., D.P., V.W., and R.B.; visualization, F.F., L.E., and A.F.; writing – original draft, F.F. and A.-K.C.; writing – review & editing, L.E., A.F., F.F., and A.-K.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES

- Starostik, M.R., Sosina, O.A., and McCoy, R.C. (2020). Single-cell analysis of human embryos reveals diverse patterns of aneuploidy and mosaicism. *Genome Res.* 30, 814–825.
- Martincorena, I. (2019). Somatic mutation and clonal expansions in human tissues. *Genome Med.* 11, 35.
- Bielmeier, C., Alt, S., Weichselberger, V., La Fortezza, M., Harz, H., Jülicher, F., Salbreux, G., and Classen, A.K. (2016). Interface Contractility between Differently Fated Cells Drives Cell Elimination and Cyst Formation. *Curr. Biol.* 26, 563–574.
- Prasad, D., Illek, K., Fischer, F., Holstein, K., and Classen, A.K. (2023). Bilateral JNK activation is a hallmark of interface surveillance and promotes elimination of aberrant cells. *eLife* 12.
- Gibson, M.C., and Perrimon, N. (2005). Extrusion and death of DPP/BMP-compromised epithelial cells in the developing Drosophila wing. *Science* 307, 1785–1789. 307/5716/1785.
- Shen, J., and Dahmann, C. (2005). Extrusion of cells with inappropriate Dpp signaling from Drosophila wing disc epithelia. *Science* 307, 1789–1790.
- Widmann, T.J., and Dahmann, C. (2009). Wingless signaling and the control of cell shape in Drosophila wing imaginal discs. *Dev. Biol.* 334, 161–173. S0012-1606(09)01063-X.
- Widmann, T.J., and Dahmann, C. (2009). Dpp signaling promotes the cuboidal-to-columnar shape transition of Drosophila wing disc epithelia by regulating Rho1. *J. Cell Sci.* 122, 1362–1373.
- Pallavi, S.K., Ho, D.M., Hicks, C., Miele, L., and Artavanis-Tsakonas, S. (2012). Notch and Mef2 synergize to promote proliferation and metastasis through JNK signal activation in Drosophila. *EMBO J.* 31, 2895–2907.
- Gandille, P., Narbonne-Reveau, K., Boissonneau, E., Randsholt, N., Busson, D., and Pret, A.M. (2010). Mutations in the polycomb group gene polyhomeotic lead to epithelial instability in both the ovary and wing imaginal disc in Drosophila. *PLoS One* 5, e13946.
- Bessa, J., Carmona, L., and Casares, F. (2009). Zinc-finger paralogues tsh and tio are functionally equivalent during imaginal development in Drosophila and maintain their expression levels through auto- and cross-negative feedback loops. *Dev. Dyn.* 238, 19–28.
- Aldaz, S., Morata, G., and Azpiazu, N. (2005). Patterning function of homothorax/extradenticle in the thorax of Drosophila. *Development* 132, 439–446.
- Beuchle, D., Struhl, G., and Müller, J. (2001). Polycomb group proteins and heritable silencing of Drosophila Hox genes. *Development* 128, 993–1004.
- Prober, D.A., and Edgar, B.A. (2000). Ras1 promotes cellular growth in the Drosophila wing. *Cell* 100, 435–446.
- Liu, X., Grammont, M., and Irvine, K.D. (2000). Roles for scalloped and vestigial in regulating cell affinity and interactions between the wing blade and the wing hinge. *Dev. Biol.* 228, 287–303.
- Worley, M.I., Setiawan, L., and Hariharan, I.K. (2013). TIE-DYE: a combinatorial marking system to visualize and genetically manipulate clones during development in Drosophila melanogaster. *Development* 140, 3275–3284.
- Perea, D., Molohon, K., Edwards, K., and Díaz-Benjumea, F.J. (2013). Multiple roles of the gene zinc finger homeodomain-2 in the development of the Drosophila wing. *Mech. Dev.* 130, 467–481.
- Gold, K.S., and Brand, A.H. (2014). Optix defines a neuroepithelial compartment in the optic lobe of the Drosophila brain. *Neural Dev.* 9, 18.
- Classen, A.K., Bunker, B.D., Harvey, K.F., Vaccari, T., and Bilder, D. (2009). A tumor suppressor activity of Drosophila Polycomb genes mediated by JAK-STAT signaling. *Nat. Genet.* 41, 1150–1155.
- Bell, G.P., and Thompson, B.J. (2014). Colorectal cancer progression: lessons from Drosophila? *Semin. Cell Dev. Biol.* 28, 70–77.
- Organista, M.F., and De Celis, J.F. (2013). The Spalt transcription factors regulate cell proliferation, survival and epithelial integrity downstream of the Decapentaplegic signalling pathway. *Biol. Open* 2, 37–48.
- Villa-Cuesta, E., González-Pérez, E., and Modolell, J. (2007). Apposition of iroquois expressing and non-expressing cells leads to cell sorting and fold formation in the Drosophila imaginal wing disc. *BMC Dev. Biol.* 7, 106.
- Shen, J., Dahmann, C., and Pflugfelder, G.O. (2010). Spatial discontinuity of optomotor-blind expression in the Drosophila wing imaginal disc disrupts epithelial architecture and promotes cell sorting. *BMC Dev. Biol.* 10, 23.
- Sharrock, T.E., and Sanson, B. (2020). Cell sorting and morphogenesis in early Drosophila embryos. *Semin. Cell Dev. Biol.* 107, 147–160.
- Wang, J., and Dahmann, C. (2020). Establishing compartment boundaries in Drosophila wing imaginal discs: An interplay between selector genes, signaling pathways and cell mechanics. *Semin. Cell Dev. Biol.* 107, 161–169.
- Addison, M., and Wilkinson, D.G. (2016). Segment Identity and Cell Segregation in the Vertebrate Hindbrain. *Curr. Top. Dev. Biol.* 117, 581–596.
- Tsai, T.Y., Garner, R.M., and Megason, S.G. (2022). Adhesion-Based Self-Organization in Tissue Patterning. *Annu. Rev. Cell Dev. Biol.* 38, 349–374.
- Kania, A., and Klein, R. (2016). Mechanisms of ephrin-Eph signalling in development, physiology and disease. *Nat. Rev. Mol. Cell Biol.* 17, 240–256.

29. Beamish, I.V., Hinck, L., and Kennedy, T.E. (2018). Making Connections: Guidance Cues and Receptors at Nonneural Cell-Cell Junctions. *Cold Spring Harb. Perspect. Biol.* **10**, a029165.
30. Moreland, T., and Poulain, F.E. (2022). To Stick or Not to Stick: The Multiple Roles of Cell Adhesion Molecules in Neural Circuit Assembly. *Front. Neurosci.* **16**, 889155.
31. Cortés, E., Pak, J.S., and Özkan, E. (2023). Structure and evolution of neuronal wiring receptors and ligands. *Dev. Dyn.* **252**, 27–60.
32. Zang, Y., Chaudhari, K., and Bashaw, G.J. (2021). New insights into the molecular mechanisms of axon guidance receptor regulation and signaling. *Curr. Top. Dev. Biol.* **142**, 147–196.
33. Klein, R., and Pasterkamp, R.J. (2021). Recent advances in inter-cellular interactions during neural circuit assembly. *Curr. Opin. Neurobiol.* **69**, 25–32.
34. Del Toro, D., Carrasquero-Ordaz, M.A., Chu, A., Ruff, T., Shahin, M., Jackson, V.A., Chavent, M., Berbeira-Santana, M., Seyit-Bremer, G., Brignani, S., et al. (2020). Structural Basis of Teneurin-Latrophilin Interaction in Repulsive Guidance of Migrating Neurons. *Cell* **180**, 323–339.e19.
35. Honig, B., and Shapiro, L. (2020). Adhesion Protein Structure, Molecular Affinities, and Principles of Cell-Cell Recognition. *Cell* **181**, 520–535.
36. Umetsu, D., Dunst, S., and Dahmann, C. (2014). An RNA interference screen for genes required to shape the anteroposterior compartment boundary in *Drosophila* identifies the Eph receptor. *PLoS One* **9**, e114340.
37. Paré, A.C., Naik, P., Shi, J., Mirman, Z., Palmquist, K.H., and Zallen, J.A. (2019). An LRR Receptor-Teneurin System Directs Planar Polarity at Compartment Boundaries. *Dev. Cell* **51**, 208–221.e6.
38. Chatterjee, N., and Bohmann, D. (2012). A versatile PhiC31 based reporter system for measuring AP-1 and Nrf2 signaling in *Drosophila* and in tissue culture. *PLoS One* **7**, e34063.
39. del Valle Rodríguez, A., Didiano, D., and Desplan, C. (2011). Power tools for gene expression and clonal analysis in *Drosophila*. *Nat. Methods* **9**, 47–55.
40. Sarov, M., Barz, C., Jambor, H., Hein, M.Y., Schmied, C., Suchold, D., Stender, B., Janosch, S., K J, V.V., Krishnan, R.T., et al. (2016). A genome-wide resource for the analysis of protein localisation in *Drosophila*. *eLife* **5**, e12068.
41. Venken, K.J.T., Schulze, K.L., Haelterman, N.A., Pan, H., He, Y., Evans-Holm, M., Carlson, J.W., Levis, R.W., Spradling, A.C., Hoskins, R.A., et al. (2011). MiMIC: a highly versatile transposon insertion resource for engineering *Drosophila* melanogaster genes. *Nat. Methods* **8**, 737–743.
42. Held, L.I., Jr. (2002). *Imaginal Discs: the Genetic and Cellular Logic of Pattern Formation* (Cambridge University Press).
43. Calzolari, S., Terriente, J., and Pujades, C. (2014). Cell segregation in the vertebrate hindbrain relies on actomyosin cables located at the interhomomeric boundaries. *EMBO J.* **33**, 686–701.
44. Lavalou, J., Mao, Q., Harmansa, S., Kerridge, S., Lellouch, A.C., Philippe, J.M., Audebert, S., Camoin, L., and Lecuit, T. (2021). Formation of polarized contractile interfaces by self-organized Toll-8/Cir1 GPCR asymmetry. *Dev. Cell* **56**, 1574–1588.e7.
45. Vaughn, J., and Igaki, T. (2016). Slit-Robo Repulsive Signaling Extrudes Tumorigenic Cells from Epithelia. *Dev. Cell* **39**, 683–695.
46. Bashaw, G.J., Kidd, T., Murray, D., Pawson, T., and Goodman, C.S. (2000). Repulsive axon guidance: Abelson and Enabled play opposing roles downstream of the roundabout receptor. *Cell* **101**, 703–715.
47. Kajita, M., Sugimura, K., Ohoka, A., Burden, J., Suganuma, H., Ikegawa, M., Shimada, T., Kitamura, T., Shindoh, M., Ishikawa, S., et al. (2014). Filamin acts as a key regulator in epithelial defence against transformed cells. *Nat. Commun.* **5**, 4428.
48. Howard, L.J., Reichert, M.C., and Evans, T.A. (2021). The Slit-binding Ig1 domain is required for multiple axon guidance activities of *Drosophila* Robo2. *Genesis* **59**, e23443.
49. Fukuhara, N., Howitt, J.A., Hussain, S.A., and Hohenester, E. (2008). Structural and functional analysis of slit and heparin binding to immunoglobulin-like domains 1 and 2 of *Drosophila* Robo. *J. Biol. Chem.* **283**, 16226–16234.
50. Barak, R., Yom-Tov, G., Guez-Haddad, J., Gasri-Plotnitsky, L., Maimon, R., Cohen-Berkman, M., McCarthy, A.A., Perlson, E., Henis-Korenblit, S., Isupov, M.N., et al. (2019). Structural Principles in Robo Activation and Auto-inhibition. *Cell* **177**, 272–285.e16.
51. Sasse, S., and Klämbt, C. (2016). Repulsive Epithelial Cues Direct Glial Migration along the Nerve. *Dev. Cell* **39**, 696–707.
52. Simpson, J.H., Kidd, T., Bland, K.S., and Goodman, C.S. (2000). Short-Range and Long-Range Guidance by Slit and Its Robo Receptors: Robo and Robo2 Play Distinct Roles in Midline Guidance. *Neuron* **28**, 753–766.
53. Pappu, K.S., Morey, M., Nern, A., Spitzweck, B., Dickson, B.J., and Zipursky, S.L. (2011). Robo-3-mediated repulsive interactions guide R8 axons during *Drosophila* visual system development. *Proc. Natl. Acad. Sci. USA* **108**, 7571–7576.
54. Rajagopalan, S., Vivancos, V., Nicolas, E., and Dickson, B.J. (2000). Selecting a longitudinal pathway: Robo receptors specify the lateral position of axons in the *Drosophila* CNS. *Cell* **103**, 1033–1045.
55. Halder, G., Callaerts, P., and Gehring, W.J. (1995). Induction of ectopic eyes by targeted expression of the eyeless gene in *Drosophila*. *Science* **267**, 1788–1792.
56. Zecca, M., Basler, K., and Struhl, G. (1995). Sequential organizing activities of engrailed, hedgehog and decapentaplegic in the *Drosophila* wing. *Development* **121**, 2265–2278.
57. Beira, J.V., and Paro, R. (2016). The legacy of *Drosophila* imaginal discs. *Chromosoma* **125**, 573–592.
58. Ruiz-Losada, M., Blom-Dahl, D., Córdoba, S., and Estella, C. (2018). Specification and Patterning of *Drosophila* Appendages. *J. Dev. Biol.* **6**, 17.
59. De Celis, J.F. (2003). Pattern formation in the *Drosophila* wing: The development of the veins. *BioEssays* **25**, 443–451.
60. Prasad, D., Illek, K., Fischer, F., Holstein, K., and Classen, A.-K. (2022). Bilateral JNK activation is a hallmark of interface contractility and promotes elimination of aberrant cells. <https://doi.org/10.1101/2022.06.20.496791>.
61. Baumgartner, M.E., Dinan, M.P., Langton, P.F., Kucinski, I., and Piddini, E. (2021). Proteotoxic stress is a driver of the loser status and cell competition. *Nat. Cell Biol.* **23**, 136–146.
62. Blanco, J., Cooper, J.C., and Baker, N.E. (2020). Roles of C/EBP class bZip proteins in the growth and cell competition of Rp ('Minute') mutants in *Drosophila*. *eLife* **9**, e50535.
63. Ji, Z., Kiparaki, M., Folgado, V., Kumar, A., Blanco, J., Rimesso, G., Chuen, J., Liu, Y., Zheng, D., and Baker, N.E. (2019). *Drosophila* RpS12 controls translation, growth, and cell competition through Xrp1. *PLoS Genet.* **15**, e1008513.
64. Baker, N.E., Kiparaki, M., and Khan, C. (2019). A potential link between p53, cell competition and ribosomopathy in mammals and in *Drosophila*. *Dev. Biol.* **446**, 17–19.
65. Lee, C.H., Kiparaki, M., Blanco, J., Folgado, V., Ji, Z., Kumar, A., Rimesso, G., and Baker, N.E. (2018). A Regulatory Response to Ribosomal Protein Mutations Controls Translation, Growth, and Cell Competition. *Dev. Cell* **46**, 456–469.e4.
66. Baillon, L., Germani, F., Rockel, C., Hilchenbach, J., and Basler, K. (2018). Xrp1 is a transcription factor required for cell competition-driven elimination of loser cells. *Sci. Rep.* **8**, 17712.
67. Kale, A., Ji, Z., Kiparaki, M., Blanco, J., Rimesso, G., Flibotte, S., and Baker, N.E. (2018). Ribosomal Protein S12e Has a Distinct Function in Cell Competition. *Dev. Cell* **44**, 42–55.e4.
68. Ochi, N., Nakamura, M., Nagata, R., Wakasa, N., Nakano, R., and Igaki, T. (2021). Cell competition is driven by Xrp1-mediated phosphorylation of eukaryotic initiation factor 2alpha. *PLoS Genet.* **17**, e1009958.
69. Madan, E., Pelham, C.J., Nagane, M., Parker, T.M., Canas-Marques, R., Fazio, K., Shaik, K., Yuan, Y., Henriques, V., Galzerano, A., et al. (2019).

- Flower isoforms promote competitive growth in cancer. *Nature* 572, 260–264.
70. Rhiner, C., López-Gay, J.M., Soldini, D., Casas-Tinto, S., Martín, F.A., Lombardía, L., and Moreno, E. (2010). Flower forms an extracellular code that reveals the fitness of a cell to its neighbors in *Drosophila*. *Dev. Cell* 18, 985–998.
71. Bosch, J.A., Tran, N.H., and Hariharan, I.K. (2015). CoinFLP: a system for efficient mosaic screening and for visualizing clonal boundaries in *Drosophila*. *Development* 142, 597–606.
72. Yagi, R., Mayer, F., and Basler, K. (2010). Refined LexA transactivators and their use in combination with the *Drosophila* Gal4 system. *Proc. Natl. Acad. Sci. USA* 107, 16166–16171.
73. Milán, M., Weihe, U., Pérez, L., and Cohen, S.M. (2001). The LRR proteins capricious and Tartan mediate cell interactions during DV boundary formation in the *Drosophila* wing. *Cell* 106, 785–794.
74. Milán, M., Pérez, L., and Cohen, S.M. (2005). Boundary formation in the *Drosophila* wing: functional dissection of Capricious and Tartan. *Dev. Dyn.* 233, 804–810.
75. Willecke, M., Hamaratoglu, F., Sansores-Garcia, L., Tao, C., and Halder, G. (2008). Boundaries of Dachsous Cadherin activity modulate the Hippo signaling pathway to induce cell proliferation. *Proc. Natl. Acad. Sci. USA* 105, 14897–14902.
76. Bosveld, F., Guirao, B., Wang, Z., Rivière, M., Bonnet, I., Graner, F., and Bellaïche, Y. (2016). Modulation of junction tension by tumor suppressors and proto-oncogenes regulates cell-cell contacts. *Development* 143, 623–634.
77. Milán, M., Pérez, L., and Cohen, S.M. (2002). Short-range cell interactions and cell survival in the *Drosophila* wing. *Dev. Cell* 2, 797–805.
78. Fulford, A.D., and McNeill, H. (2020). Fat/Dachsous family cadherins in cell and tissue organisation. *Curr. Opin. Cell Biol.* 62, 96–103.
79. Gilbert, J.M., Adams, M.T., Sharon, N., Jayaraman, H., and Blum, B. (2021). Morphogenesis of the Islets of Langerhans Is Guided by Extraendocrine Slit2 and Slit3 Signals. *Mol. Cell. Biol.* 41, e0045120.
80. Waters, B.J., and Blum, B. (2022). Axon Guidance Molecules in the Islets of Langerhans. *Front. Endocrinol.* 13, 869780.
81. Evans, T.A., Santiago, C., Arbelles, E., and Bashaw, G.J. (2015). Robo2 acts in trans to inhibit Slit-Robo1 repulsion in pre-crossing commissural axons. *eLife* 4, e08407.
82. Paré, A.C., Vichas, A., Fincher, C.T., Mirman, Z., Farrell, D.L., Mainieri, A., and Zallen, J.A. (2014). A positional Toll receptor code directs convergent extension in *Drosophila*. *Nature* 515, 523–527.
83. Chaudhari, K., Gorla, M., Chang, C., Kania, A., and Bashaw, G.J. (2021). Robo recruitment of the Wave regulatory complex plays an essential and conserved role in midline repulsion. *eLife* 10, e64474.
84. Chen, B., Brinkmann, K., Chen, Z., Pak, C.W., Liao, Y., Shi, S., Henry, L., Grishin, N.V., Bogdan, S., and Rosen, M.K. (2014). The WAVE regulatory complex links diverse receptors to the actin cytoskeleton. *Cell* 156, 195–207.
85. Gara, R.K., Kumari, S., Ganju, A., Yallapu, M.M., Jaggi, M., and Chauhan, S.C. (2015). Slit/Robo pathway: a promising therapeutic target for cancer. *Drug Discov. Today* 20, 156–164.
86. Ahammad, I. (2020). A comprehensive review of tumor proliferative and suppressive role of semaphorins and therapeutic approaches. *Biophys. Rev.* 12, 1233–1247.
87. Maruyama, T., and Fujita, Y. (2017). Cell competition in mammals — novel homeostatic machinery for embryonic development and cancer prevention. *Curr. Opin. Cell Biol.* 48, 106–112.
88. Tanimura, N., and Fujita, Y. (2020). Epithelial defense against cancer (EDAC). *Semin. Cancer Biol.* 63, 44–48.
89. Porazinski, S., de Navascués, J., Yako, Y., Hill, W., Jones, M.R., Maddison, R., Fujita, Y., and Hogan, C. (2016). EphA2 Drives the Segregation of Ras-Transformed Epithelial Cells from Normal Neighbors. *Curr. Biol.* 26, 3220–3229.
90. Hill, W., Zaragoulas, A., Salvador-Barbero, B., Parfitt, G.J., Alatsianos, M., Padilha, A., Porazinski, S., Woolley, T.E., Morton, J.P., Sansom, O.J., et al. (2021). EPHA2-dependent outcompetition of KRASG12D mutant cells by wild-type neighbors in the adult pancreas. *Curr. Biol.* 31, 2550–2560.e5.
91. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682.
92. Herbert, S., Valon, L., Mancini, L., Dray, N., Caldarelli, P., Gros, J., Esposito, E., Shorte, S.L., Bally-Cuif, L., Aulner, N., et al. (2021). LocalZProjector and DeProj: a toolbox for local 2D projection and accurate morphometrics of large 3D microscopy images. *BMC Biol.* 19, 136.
93. Sui, L., Alt, S., Weigert, M., Dye, N., Eaton, S., Jug, F., Myers, E.W., Jülicher, F., Salbreux, G., and Dahmann, C. (2018). Differential lateral and basal tension drive folding of *Drosophila* wing discs through two distinct mechanisms. *Nat. Commun.* 9, 4620.
94. Hammer, O., Harper, D., and Ryan, P. (2001). Past: Paleontological Statistics Software Package for Education and Data Analysis. *Palaeontol. Electron.* 4, 9.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
mouse monoclonal anti-Robo1	Developmental Studies Hybridoma Bank	DSHB-13C9 RRID: AB_2181861
mouse monoclonal anti-Robo3 extracellular	Developmental Studies Hybridoma Bank	DSHB-14C9 RRID: AB_528454
mouse monoclonal anti-Robo3 cytoplasmic	Developmental Studies Hybridoma Bank	DSHB-15H2 RRID: AB_531800
mouse anti-Ds	Suzanne Eaton	N/A
mouse monoclonal anti-Nrt	Developmental Studies Hybridoma Bank	DSHB-BP106 RRID: AB_528404
mouse anti-Sema2	Developmental Studies Hybridoma Bank	DSHB-19C2 RRID: AB_528460
mouse anti-Ptc	Developmental Studies Hybridoma Bank	DSHB-Apa1 RRID: AB_528441
mouse anti-Wg	Developmental Studies Hybridoma Bank	DSHB-4D4 RRID: AB_528512
mouse monoclonal anti-β-gal	Promega	Z378B, RRID: AB_2323752
Rabbit polyclonal anti-β-gal	MP Biomedicals	55976, SKU: 085597-CF
rabbit anti-Ephrin	Andrea Brand	N/A
Rabbit polyclonal anti-Dcp1	Cell Signaling Technology	CST-9578S; RRID: AB_2721060
rabbit anti-GFP	Thermo Fisher Scientific	G10362, RRID: AB_2536526
rat anti-E-cadherin	Developmental Studies Hybridoma Bank	DSHB-DCAD2 RRID: AB_528120
rat monoclonal anti-RFP	ChromoTek	5F8 RRID: AB_2336064
Chemicals, peptides, and recombinant proteins		
DAPI	Sigma-Aldrich	D9564
Phalloidin-Alexa Fluor 405	Abcam	ab176752
Phalloidin-Alexa Fluor 488	Invitrogen	A12379
Phalloidin-Alexa Fluor 555	Sigma-Aldrich	P1951
Phalloidin-Alexa Fluor 647	Invitrogen	A22287
Experimental models: Organisms/strains		
<i>w¹¹⁸</i>	David Bilder	FBal0018157
<i>hsflp¹²²</i>	Ishwar Hariharan	FBtp0001101
<i>act>y⁺>GAL4, UAS-GFP</i>	David Bilder	FBti0012290
<i>act>CD2>GAL4, UAS-RFP</i>	David Bilder	FBal0058800
<i>tub>CD2>GAL4, UAS-nLacZ</i>	George Pyrowolakis	FBtp0010169
<i>UAS-LacZ</i>	Bloomington Drosophila Stock Center	BDSC 1776
<i>UAS-flkh-3xHA</i>	Martin Juenger	FBal0249318
<i>UAS-tnv^{CA}</i>	Bloomington Drosophila Stock Center	BDSC 36537
<i>UAS-ey</i>	Bloomington Drosophila Stock Center	BDSC 6294
<i>UAS-arm^{S10}</i>	Suzanne Eaton	FBtp0001723
<i>UAS-ci-HA</i>	Bloomington Drosophila Stock Center	BDSC 32570
<i>dad⁴-LacZ</i>	George Pyrowolakis	FBsf0000872988
<i>TRE-RFP</i>	Dirk Bohmann	FBti0147636
<i>puc^{A251.1F3}>LacZ</i>	Bloomington Drosophila Stock Center	BDSC 11173

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>CoinFLP-LexA::GAD.GAL4</i>	Bloomington Drosophila Stock Center	BDSC 59271
<i>en-GAL4</i>	Mirka Uhlirova	FBal0052377
<i>brk-GAL4; UAS-mCD8::GFP, LexO-mCherry::CAAX/SM5-TM6b; LexO-<i>tkv</i>RD/SM5-TM6b</i>	George Pyrowolakis	FBal0295113
<i>UAS-<i>tkv</i>-RNAi (HMS02185)</i>	Bloomington Drosophila Stock Center	BDSC 40937
<i>UAS-Ras^{V12}</i>	Helena Richardson	FBtp0001705
<i>UAS-Ras^{V12}</i>	Bloomington Drosophila Stock Center	BDSC 4847
<i>UAS-myc.HA</i>	Bloomington Drosophila Stock Center	BDSC 64759
<i>UAS-<i>hpo</i></i>	Ishwar Hariharan	FBal0191267
<i>UAS-wts-RNAi (101055/KK)</i>	Vienna Drosophila Resource Center	VDRC 111002
<i>elav-GAL4, UAS-GFP</i>	Bloomington Drosophila Stock Center	BDSC 6923
<i>toll-2-GFP</i>	Vienna Drosophila Resource Center	VDRC 318319
<i>toll-8-YFP/CyO,hbLacZ</i>	Jennifer Zallen	FBtp0148072
<i>rst-GFP</i>	Bloomington Drosophila Stock Center	BDSC 59410
<i>hbs-GFP</i>	Bloomington Drosophila Stock Center	BDSC 65321
<i>hbs-LacZ</i>	Ruth Johnson	FBal0131086
<i>sns-GFP</i>	Bloomington Drosophila Stock Center	BDSC 59801
<i>sema1a-GFP</i>	Bloomington Drosophila Stock Center	BDSC 50816
<i>netA-GFP</i>	Bloomington Drosophila Stock Center	BDSC 59409
<i>eph-GFP</i>	Frank Schnorrer	FlyFos015198
<i>ten-m-GFP</i>	Bloomington Drosophila Stock Center	BDSC 59798
<i>ten-a-GFP</i>	Bloomington Drosophila Stock Center	BDSC 60541
<i>trn-LacZ</i>	Bloomington Drosophila Stock Center	BDSC 4550
<i>ft-EGFP</i>	David Strutt	FBal0385338
<i>ds-LacZ</i>	Bloomington Drosophila Stock Center	BDSC 11394
<i>sli-GFP</i>	Bloomington Drosophila Stock Center	BDSC 64472
<i>robo2-GFP</i>	Bloomington Drosophila Stock Center	BDSC 61774
<i>netB-GFP</i>	Bloomington Drosophila Stock Center	BDSC 67644
<i>unc5-GFP</i>	Bloomington Drosophila Stock Center	BDSC 60547
<i>UAS-robo3-HA</i>	Bloomington Drosophila Stock Center	BDSC 66890
<i>UAS-robo2-HA</i>	Bloomington Drosophila Stock Center	BDSC 66886
<i>UAS-robo2-RNAi</i>	Bloomington Drosophila Stock Center	BDSC 34589
<i>UAS-DEphrin</i>	Andrea Brand	FBal0269900
<i>UAS-eph-RNAi</i>	Bloomington Drosophila Stock Center	BDSC 35290
<i>UAS-ten-a</i>	Bloomington Drosophila Stock Center	BDSC 42018
<i>UAS-ten-m</i>	Bloomington Drosophila Stock Center	BDSC 41570
<i>UAS-toll-8</i>	Yoshimasa Yagi	N/A
<i>UAS-toll-2</i>	Yoshimasa Yagi	N/A
<i>UAS-netB-RNAi</i>	Vienna Drosophila Resource Center	VDRC 100840
<i>UAS-trn-HA</i>	Jennifer Zallen	N/A
<i>UAS-ft</i>	David Strutt	FBtp0019924
<i>UAS-ft-RNAi</i>	Bloomington Drosophila Stock Center	BDSC 34970
<i>UAS-ds</i>	David Strutt	FBtp0019964
<i>UAS-ds-RNAi (GD14350)</i>	David Strutt	VDRC 36219
<i>UAS-robo1-RNAi</i>	Vienna Drosophila Resource Center	VDRC 42241
<i>UAS-robo3-RNAi</i>	Vienna Drosophila Resource Center	VDRC 44702
<i>UAS-ten-m-RNAi</i>	Bloomington Drosophila Stock Center	BDSC 29390
<i>UAS-rst</i>	Ruth Johnson	RJ459

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>UAS-kirre</i>	Karl-Friedrich Fischbach	FBtp0013068
<i>UAS-hbs</i>	Ruth Johnson	RJ404
<i>UAS-eph</i>	Mel Feany	Probably FBtp0055391, not FBtp0055389, nor FBtp0015743
<i>UAS-plexA-RNAi</i> (HM05221, GD/14483, GL01870, KK/101499)	Bloomington Drosophila Stock Center Vienna Drosophila Resource Center	BDSC 30483, VDRC 27240, BDSC 67845, VDRC 107004
<i>UAS-plexB-RNAi</i> (HM05122, GD/16420)	Bloomington Drosophila Stock Center Vienna Drosophila Resource Center	BDSC 28911, VDRC 46687
<i>UAS-sema2a-RNAi</i> (GL00356)	Bloomington Drosophila Stock Center	BDSC 35432
<i>UAS-plexA</i>	Bloomington Drosophila Stock Center	BDSC 8479
<i>UAS-sli.D</i>	Bloomington Drosophila Stock Center	BDSC 67689
<i>UAS-slit-RNAi</i> (KK/100803)	Vienna Drosophila Resource Center	VDRC 108853
<i>UAS-cir1-RNAi</i> (KK/108383, JF02674, HMS00136)	Vienna Drosophila Resource Center Bloomington Drosophila Stock Center	VDRC100749, BDSC 27524, BDSC 34821
<i>UAS-tl</i>	Bloomington Drosophila Stock Center	BDSC 58987
<i>UAS-tl-RNAi</i> (JF01276, JF01491)	Bloomington Drosophila Stock Center	BDSC 31477, BDSC 31044
<i>UAS-toll-2-RNAi</i>	Bloomington Drosophila Stock Center	BDSC 30498
<i>UAS-toll-3</i>	Yoshimasa Yagi	N/A
<i>UAS-toll-3-RNAi</i> (HM05012)	Bloomington Drosophila Stock Center	BDSC 28526
<i>UAS-toll-4</i>	Yoshimasa Yagi	N/A
<i>UAS-toll-4-RNAi</i> (HM050297)	Bloomington Drosophila Stock Center	BDSC 28543
<i>UAS-toll-5-RNAi</i> (HM05212)	Bloomington Drosophila Stock Center	BDSC 29533
<i>UAS-toll-6</i>	Yoshimasa Yagi	N/A
<i>UAS-toll-6-RNAi</i> (HMC0584, HMS04251)	Bloomington Drosophila Stock Center	BDSC 64968, BDSC 56048
<i>UAS-toll-7</i>	Yoshimasa Yagi	N/A
<i>UAS-toll-7-RNAi</i> (HM05230)	Bloomington Drosophila Stock Center	BDSC 30488
<i>UAS-toll-8-RNAi</i>	Bloomington Drosophila Stock Center	BDSC 28519
<i>UAS-toll-9-RNAi</i> (HM05227, HMS00171)	Bloomington Drosophila Stock Center	BDSC 30535, BDSC 34853
<i>UAS-dachs.V5</i>	Bloomington Drosophila Stock Center	BDSC 28814
<i>UAS-dachs-RNAi</i> (JF02743)	Bloomington Drosophila Stock Center	BDSC 27664
<i>UAS-caps-RNAi</i> (GD/2530, GD/14433, JF02854)	Vienna Drosophila Resource Center Bloomington Drosophila Stock Center	VDRC 3046, VDRC 27097, BDSC 28020
<i>UAS-trn-RNAi</i> (GLC01638, KK/104688, HM05011)	Vienna Drosophila Resource Center Bloomington Drosophila Stock Center	BDSC 50520, VDRC 107883, BDSC 28525
<i>UAS-netB-RNAi</i> (KK/103672)	Vienna Drosophila Resource Center	VDRC 100840
<i>UAS-unc5-RNAi</i> (HMS01099)	Bloomington Drosophila Stock Center	BDSC 33756
<i>UAS-fra</i>	Bloomington Drosophila Stock Center	BDSC 8814
<i>UAS-fra-RNAi</i> (JF01231, JF01457)	Bloomington Drosophila Stock Center	BDSC 31469, BDSC 31664
<i>UAS-kek1-RNAi</i> (HMC04442)	Bloomington Drosophila Stock Center	BDSC 57000
<i>UAS-kek3-RNAi</i> (HMS05948)	Bloomington Drosophila Stock Center	BDSC 77354
<i>UAS-kek4-RNAi</i> (HMC06307)	Bloomington Drosophila Stock Center	BDSC 67206
<i>UAS-kek5-RNAi</i> (HMS01996)	Bloomington Drosophila Stock Center	BDSC 40830
<i>UAS-ret.L</i>	Bloomington Drosophila Stock Center	BDSC 59002

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>UAS-ret-RNAi</i> (HMC04143)	Bloomington Drosophila Stock Center	BDSC 55872
<i>UAS-GP150</i> (HMS00395)	Bloomington Drosophila Stock Center	BDSC 32400
<i>UAS-dscam4-RNAi</i> (HMC03277)	Bloomington Drosophila Stock Center	BDSC 51508
<i>UAS-dscam4</i>	Bloomington Drosophila Stock Center	BDSC 17117
<i>UAS-dscam2-RNAi</i> (HMC03411)	Bloomington Drosophila Stock Center	BDSC 51839
<i>UAS-pyd-RNAi</i>	Ruth Johnson	RJ452
<i>UAS-ics.O</i>	Bloomington Drosophila Stock Center	BDSC 66567
<i>UAS-sas-RNAi</i> (KK/106177)	Vienna Drosophila Resource Center	VDRC 100901
<i>UAS-fili-RNAi</i> (HM05054)	Bloomington Drosophila Stock Center	BDSC 28568
<i>UAS-bsg-RNAi</i> (HMC03195)	Bloomington Drosophila Stock Center	BDSC 52110
<i>UAS-con-RNAi</i> (HM05178)	Bloomington Drosophila Stock Center	BDSC 28967
<i>UAS-beat-VII-RNAi</i> (HMC05049)	Bloomington Drosophila Stock Center	BDSC 60056
<i>UAS-beat-IIIc-RNAi</i> (JF03286)	Bloomington Drosophila Stock Center	BDSC 29607
<i>UAS-tnc-RNAi</i> (HMC05051)	Bloomington Drosophila Stock Center	BDSC 60058

Software and algorithms

FIJI (ImageJ 1.48b)	Schindelin et al. ⁹¹	https://fiji.sc/
local-z-projector Fiji plugin v1.5.4	Herbert et al. ⁹²	https://gitlab.pasteur.fr/iah-public/Local_Zprojector
GraphPad Prism	Graph Pad	version 9.5.0
Affinity Designer	Affinity	Up to version 1.10.8
Microsoft Excel	Microsoft	Microsoft Excel 365
Procreate	Procreate	Version 5.2.9
Inkscape (0.92.4)	Inkscape	https://inkscape.org/release/inkscape-0.92.4/
Biorender	Science Suite Inc. DBA BioRender	Biorender

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Anne-Kathrin Classen (anne.classen@biologie.uni-freiburg.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Quantitative data sets are provided as supplemental source files with this publication. Raw imaging data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL

All experiments were performed on *Drosophila melanogaster*. Fly strains (see [key resources table](#)) were maintained on standard fly food (10L water, 74.5g agar, 243g dry yeast, 580g corn flour, 552ml molasses, 20.7g Nipagin, 35ml propionic acid) at 18°C – 22°C. Larvae from experimental crosses were allowed to feed on Bloomington formulation (175.7g Nutry-Fly, 1100ml water 20g dry yeast, 1.45g Nipagin in 15ml Ethanol, 4.8ml Propionic acid) and raised at 25°C. Our experimental design did not consider differences between sexes unless for genetic crossing schemes.

METHOD DETAILS

Drosophila genetics

Mosaic gene expression was induced by Flip-out (act or tub>GAL4/UAS, CoinFLP-LexA/LexO) or mitotic FLP/FRT.^{39,71} Parental adult flies were allowed to lay eggs for 72 h at 25°C before flippase expression was induced by heat shock (HS) at 37°C for

5–15 min. Detailed genotypes and experimental conditions are listed in Table S1. For the JNK interface signaling screen (Figure S1F), larvae were dissected 48 h after a 10 min HS (AHS). Due to genetic limitations, the CoinFLP experiments (Figures S7H–S7J) contained two GAL4-drivers, the Actin5C-FRT/STOP-GAL4 and the *en*-GAL4, resulting in mosaic GAL4 expression in the total disc and GAL4 expression in the posterior compartment. For experiments examining expression patterns (Figures 2 and S2), larvae were dissected 80 h or 102 h after egg lay (AEL).

Immunohistochemistry

Larvae were dissected and the inverted cuticula with attached wing discs were fixed in 4% paraformaldehyde in PBS for 15 min at 22°C. Samples were washed in 0.1% Triton X-100 in PBS (PBT) and incubated with primary and secondary antibodies in 400 µl PBT on a nutator overnight at 4°C and 1–3 h at 22°C, respectively. Following a final wash in PBS, wing discs were mounted with Molecular Probes Antifade Reagents (#S2828). Following antibodies were used: Mouse anti-Robo1 (1:20, DSHB-13C9), mouse anti-Robo3 (1:20, DSHB-14C9&15H2), mouse anti-Ds (1:1000, Suzanne Eaton), mouse anti-Nrt (1:20, DSHB-BP106), mouse anti-Sema2 (1:20, DSHB-19C2), mouse anti-Ptc (1:20, DSHB-apa1), mouse anti-Wg (1:100, DSHB-4D4), rabbit anti-Ephrin (1:1000, Andrea Brand), rabbit anti-Dcp1 (1:400, Cell Signaling-9578S), rat anti-E-cadherin (1:50, DSHB-DCAD2), mouse anti-β-gal (1:1000, Promega Z378B), mouse anti-β-gal (1:1000, MP Biochemicals-55976), rabbit anti-HA (1:200, Invitrogen 715500), rabbit anti-GFP (1:400, Thermo Fisher-G10362), rat anti-RFP (1:20, Heinrich Leonhardt, 5F8) and rat anti-RFP (1:2000, ChromoTek-5F8). Fluorescent dyes DAPI (0.25 ng/µl, Sigma), Phalloidin-Alexa Fluor 405 (1:2000, Abcam-ab176752), Phalloidin-Alexa Fluor 488 (1:500, Invitrogen A12379), Phalloidin-Alexa Fluor 555 (1:500, sigma-P1951) and Phalloidin-Alexa Fluor 647 (1:500, Invitrogen A22287) were added together with secondary antibodies.

Image acquisition and image display

Samples were imaged using Leica TCS SP8 confocal microscopes. Figure panels were assembled in Affinity Designer. In most figure panels, individual channels represent the same z-position from a confocal image stack. However, some panels may assemble channels from different z-positions in the wing disc (as listed in Table S2). Such a portrayal was chosen to better visualize the spatially distinct phenotypes at different positions within the cell, specifically of (1) junctional actin (apical), (2) cytoplasmic/nuclear RFP (lateral) and (3) Dcp1 (basal) in one image panel. This was meant to reduce the (peripheral) data load in the manuscript that would be otherwise required to visualize all channels at all positions.

If mentioned in the figure legends, the local-z-projector Fiji plugin (<https://gitlab.pasteur.fr/iah-public/Local-Zprojector>, v1.5.4)⁹² was used to project the curved apical surface of the wing disc epithelium into the same plane. The local-z-projector generates a surface height-map based on a reference channel (i.e., Ecad or Phalloidin staining) and other channels of interest were displayed in relation to this surface height-map.

Illustration of expression patterns

Procreate (Version 5.2.9) was used for illustration of cell surface receptor expression patterns (Figure 2). Characteristic points of reference, such as hinge folds, A/P and D/V boundary, were used to transfer the expression domains manually into a wing disc template. The grading of fluorescence intensity is based on overall impression of intensity across the wing disc and is presented as a subjective approximation. Bitmap tracing was done in Inkscape (0.92.4) to convert the areas of expression into vector graphics.

IMAGE QUANTIFICATION AND STATISTICAL ANALYSIS

To provide a measure for reproducibility and robustness, number of samples (wing discs) and experimental replicates are mentioned in the figure legends. An experimental replicate refers to an independent event for dissection and sample processing. From each larva, only one wing disc per larva was dissected and considered to be an independent sample for statistical analysis. Please note that several discs were visually analyzed for each experimental replicate. For all quantifications, control and experimental samples were processed together and imaged in parallel, using the same confocal settings. For comparison of TRE-RFP and Dcp1 fluorescence intensities in different bands around the clone, a repeated measured one-way ANOVA with Tukey's multiple comparisons test was used. Kruskal-Wallis test with Dunnett's multiple comparison test was used for comparison of changes in Robo2-GFP intensities in aberrant clones. For other comparisons, a two tailed, unpaired t-test was performed. Images were processed in FIJI.⁹¹ GraphPad Prism (version 9.5.0) was used for statistical analysis and generation of graphs. Source data for our statistical analysis is provided in Data S1, S2, S3, and S4.

Region of interest (ROI) segmentation and quantification workflow

(ROI1) of total disc area: RFP or Phalloidin signal was thresholded and the ROI created by Convert to Mask, Fill Holes, and selection by the wand tool. (ROI2) of clone area: clonal GFP signal was thresholded and ROIs were created by analyze particles=10 µm-infinity. For Dcp1 quantification, the lower cutoff was 2 µm. (ROI3) WT cells at interface: the Make Band, 4 µm command created a band around the ROI2. (ROI4) clonal cells not at the interface: the Enlarge, -4 µm command applied on ROI2 created a ROI of the inner clone area. (ROI5) Clonal cells at the interface: XOR of ROI2 and ROI4. (ROI6) WT area around the ROI3: First, Make Band, 16 µm created a band around ROI2. XOR of this band and ROI3 resulted in ROI6.

Subsequent general procedures: by using the Combine command, several ROIs of a category, such as individual clones in ROI2, were combined to one ROI and used for further analysis. Signal outside the wing disc was excluded by AND of ROI1 and other ROIs. ROIs of WT cells, such as ROI3 and ROI6, might cover clonal area due to insufficient spacing of clones. This was prevented, in example for ROI3, by first selecting OR of ROI3 and ROI2, followed by XOR of that selection and ROI2. After the disc segmentation into ROIs, the Measure command was applied.

Workflow for quantification of TRE-RFP intensity in UAS-eph-RNAi and UAS-GFP clones

7min HS at 37°C, 72 h AEL, reared at 25°C, dissected 72 h AHS. TRE-RFP signal was elevated by anti-RFP antibody staining. One lateral section was imaged and quantified. Area and mean intensity of TRE-RFP was measured in each ROI. For circularity, the shape of individual clones in ROI2 was measured.

Workflow for quantification of TRE-RFP intensity in UAS-robo2-HA and UAS-GFP clones

8min HS at 37°C, 72 h AEL, reared at 25°C, dissected 48 h AHS. TRE-RFP signal with and without rat anti-RFP antibody was compared (Figure S3). One lateral section of native TRE-RFP was imaged and quantified. Area and mean intensity of TRE-RFP was measured in each ROI. For circularity, the shape of individual clones in ROI2 was measured.

Workflow for quantification of Dcp1 intensity in UAS-robo2-HA and UAS-GFP clones

9min HS at 37°C, 72 h AEL, reared at 25°C, dissected 30 h AHS. In total, 4 confocal z-sections were quantified. As apoptotic cells are localized basally, imaging of the stack was started at the most basal position, moving towards the apical surface in 2μm steps, resulting in the quantification of basal and lateral sections. The above-described ROI segmentation and measurement of Dcp1 intensity was applied individually for each z-section in all ROIs. Eventually, the intensities of all four z-sections were averaged before doing the statistics.

Workflow for quantification of TRE-RFP and Dcp1 intensity in UAS-robo3-HA and UAS-GFP clones

19min HS at 37°C, 72h AEL, reared at 25°C, dissected 48 h AHS. The workflow for the quantification of Dcp1 was as described in DCP1 quantification for UAS-robo2-HA. TRE-RFP intensities were quantified for the second most apical slice of the z-stack.

Workflow for quantification of TRE-RFP intensities in the wing disc notum with UAS-robo2-RNAi alone or with expression with UAS-robo3-HA

13min HS at 37°C, 80h AEL, reared at 25°C, dissected 40h AHS. Of a z-stack, the z-position with best aerial coverage of the epithelial cells of the wing disc notum was selected. The anterior-notum area was manually converted into a ROI and TRE-RFP intensities were measured as mentioned before, but only within this ROI.

Workflow for quantification of Robo2-GFP regulation in UAS-GFP, UAS-ey, UAS-tkv-RNAi, UAS-fkh clones

Crosses: 10min HS at 37°C, 72 h AEL, reared at 25°C, dissected 44 h AHS. Confocal z-stack had a step size of 4μm. Of those, 4 contiguous lateral z-sections beneath the peripodium were chosen for quantification. First, the wing disc was manually separated into approximate pouch, hinge and notum areas by using the polygon selections in Fiji. For the pouch, a shape was drawn along the apical hinge-pouch fold. For the notum, the apical hinge-notum fold and wing disc outline was used.⁹³ The area between those two areas and the wing disc outline was used as hinge area. Then, the above-described ROI segmentation with measurement of Robo2-GFP intensity in ROI3 and ROI5 was applied individually for each z-section. The results were then processed as follows. Individual clones were tracked through the z-stack by their centroids and the Similarity And Distance Indices function in Past4,⁹⁴ resulting in up to 4 data points per clone. To avoid non genotype specific changes in Robo2-GFP intensity due to cyst formation and consequent changes of the apical plane in clones, the data points were filtered based on changes in actin levels. Data points from z-sections in which the clone had actin changes of >20% between ROI3 and ROI5, while Robo2-GFP intensity increased, were excluded. Vice versa, clones with actin changes of <-20% between ROI3 and ROI5, while Robo2-GFP intensity decreased, were excluded as well. Furthermore, small clones beneath 30μm² were excluded, as well as clones which were only detected in one of the 4 z-sections. Eventually, the data point of a clone, which was used for further statistics, derived from the most apical z-section that fulfilled all those criteria.