RESEARCH ARTICLE

Novel interplay between JNK and Egfr signaling in Drosophila dorsal closure

Tatyana Kushnir, Sharon Mezuman, Shaked Bar-Cohen, Rotem Lange, Ze’ev Paroush*, Aharon Helman

Department of Developmental Biology and Cancer Research, IMRIC, Faculty of Medicine, The Hebrew University, Jerusalem, Israel

* Current address: Department of Stem Cell and Regenerative Biology, Harvard Stem Cell Institute, Harvard University, 7 Divinity Avenue, Cambridge, MA, United States of America

zparoush@cc.huji.ac.il

Abstract

Dorsal closure (DC) is a developmental process in which two contralateral epithelial sheets migrate to seal a large hole in the dorsal ectoderm of the Drosophila embryo. Two signaling pathways act sequentially to orchestrate this dynamic morphogenetic process. First, c-Jun N-terminal kinase (JNK) signaling activity in the dorsal-most leading edge (LE) cells of the epidermis induces expression of decapentaplegic (dpp). Second, Dpp, a secreted TGF-β homolog, triggers cell shape changes in the adjacent, ventrally located lateral epidermis, that guide the morphogenetic movements and cell migration mandatory for DC. Here we uncover a cell non-autonomous requirement for the Epidermal growth factor receptor (Egfr) pathway in the lateral epidermis for sustained dpp expression in the LE. Specifically, we demonstrate that Egfr pathway activity in the lateral epidermis prevents expression of the gene scarface (scaf), encoding a secreted antagonist of JNK signaling. In embryos with compromised Egfr signaling, upregulated Scaf causes reduction of JNK activity in LE cells, thereby impeding completion of DC. Our results identify a new developmental role for Egfr signaling in regulating epithelial plasticity via crosstalk with the JNK pathway.

Author summary

The developmental process of dorsal closure (DC) in Drosophila embryogenesis has provided many fundamental insights into conserved mechanisms controlling epithelial dynamics and cell migration, and therefore serves as a model for studying tissue morphogenesis and wound repair. DC is known to require the coordinated action of two signaling pathways: (i) the c-Jun N-terminal kinase (JNK) pathway, which is activated in the dorsal-most leading edge (LE) cells of the migrating epidermis; and (ii) the Decapentaplegic (Dpp) pathway, induced by the JNK pathway, which signals to neighboring lateral epidermis cells to drive the cellular changes required for DC. Here we uncover a new tier of regulation essential for DC, mediated by the Epidermal growth factor receptor (Egfr) pathway. Specifically, we demonstrate that Egfr signalling in the lateral epidermis suppresses the expression of the gene scarface, which encodes a secreted JNK antagonist. Through this
regulatory switch the Egfr pathway facilitates JNK signalling in LE cells, ensuring full induction and activity of Dpp signaling that is pivotal for orchestrating the synchronized morphogenetic movements characteristic of DC. Our study thus identifies a novel mechanism of signal integration between the Egfr and JNK pathways, linking Egfr signalling to the core regulatory network controlling DC.

Introduction

Epithelial sheet fusion and collective cell migration are key processes in normal development, wound healing and pathogenesis [1,2]. One system that has offered fundamental insights into the mechanisms controlling epithelial dynamics and cell migration is the embryonic process of dorsal closure (DC) in Drosophila melanogaster. In this developmental setting, two contralateral epithelial sheets from opposing sides of the embryo migrate and converge at the dorsal midline above the amnioserosa (AS), an extraembryonic epithelium tissue, thereby generating a continuous epidermis that seals a large dorsal hole (Fig 1A) [3]. Two cell signaling pathways, which act sequentially, drive and coordinate this process. Initially, c-Jun N-terminal kinase (JNK) pathway activity in the dorsal-most leading edge (LE) cells induces expression of the gene decapentaplegic (dpp), encoding the TGF-β/BMP family member Dpp [4–6]. Secreted Dpp subsequently triggers signal transduction in adjacent, ventrally located lateral epidermis cells, leading to the cell shape changes that are at the basis of the morphogenetic movements of the migrating epithelia [7,8]. Accordingly, embryos mutant for various components of the JNK signaling cascade or for constituents of the Dpp pathway fail to complete DC morphogenesis and consequently display dorsal-open phenotypes [9–11]. Thus, DC is an excellent experimental model system with which to identify and characterize the signaling events regulating complex movements of epithelial layers.

Spatiotemporal refinement of the expression of dpp and other JNK pathway target genes requires input by two negative feedback regulators, which are expressed in response to JNK signaling in LE cells [9]. One is Puckered (Puc), a dual specificity phosphatase that acts as an intracellular inhibitor of pathway activity by dephosphorylating JNK [12]. The other proposed JNK pathway antagonist is Scarface (Scaf), a secreted serine protease homologue that possibly acts by modifying the receptor mediating JNK signaling or an unknown extracellular signal [13,14]. Input from these two feedback inhibitors restricts JNK pathway activity in LE cells. It is not known, however, whether the expression of these negative regulators, or of other JNK pathway target genes in LE cells, is controlled only by JNK signaling, or whether other signaling pathways originating from adjacent epithelial cells might also contribute to this regulation.

Herein, we demonstrate an activity mediated by the Epidermal growth factor receptor (Egfr) pathway in lateral epidermis cells, that is pivotal for JNK function in the adjacent LE cells. Specifically, we identify a positive, cell non-autonomous input by the Egfr pathway, upstream of JNK signaling, into JNK pathway activity. We find that the mechanism underlying this effect involves the repression of scaf expression in lateral epidermis cells. Correspondingly, derepression of scaf in embryos defective in Egfr signaling causes a reduction in JNK activity in nearby LE cells. This leads to impaired expression of the JNK target gene dpp, reduced levels of Dpp effector responses, failure of cell elongation and, consequently, aborted DC resulting in a dorsal-open phenotype. Our results thus identify a novel intercellular crosstalk between the Egfr and JNK signaling pathways, shedding new light on DC regulation and potentially on other related processes involving synchronized cell movements and epithelial fusions [15].
Results

Egfr pathway activity is detectable in the lateral epidermis during dorsal closure

Intercellular signaling mediated by Receptor tyrosine kinases (RTKs) is essential for multiple patterning events during \textit{Drosophila} oogenesis, embryogenesis and adult development [16–]
We therefore reasoned that RTK-dependent signaling might also play unknown roles during the process of DC. Hence, we immunostained embryos for active mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/Erk) as readout for pathway activity [20–22]. Doubly phosphorylated MAPK/Erk (dpErk) was detectable at the time of DC initiation in the lateral epidermis, a region where RTK activity has not been explored before (Fig 1A and 1B).

Several lines of evidence indicate that MAPK/Erk is activated in this region specifically in response to Egfr signaling. First, dpErk staining borders on the striped domains of rhomboid expression (determined using a rhomboid-lacZ enhancer trap line; Fig 1B–1D); this gene encodes the serine protease that is both necessary and sufficient to trigger Egfr pathway activity, and whose expression pattern forecasts Egfr pathway activation [22]. Second, activation of MAPK/Erk is significantly reduced in embryos in which pannier (pnr)-Gal4 [23] drives the expression of a dominant-negative form of Egfr (EgfrDN), thereby blocking Egfr function selectively in the lateral epidermis and the LE cells (Fig 1E and S1 Fig). Third, a reduction in dpErk staining is also evident in rhomboid mutants, as well as in embryos mutant for spitz (spi), which encodes a member of the TGF-α family of Egfr ligands [24], and for Egfr (Fig 1F–1H), though not in mutants for pvr, the gene encoding Drosophila Platelet-derived growth factor/Vascular endothelial growth factor receptor (PVR) (Fig 1I). Taken together, these results show that signaling via the Egfr pathway takes place in the lateral epidermis during the time of DC.

Egfr pathway activity is required for proper dorsal closure

To establish the functionality of Egfr signaling in the lateral epidermis, we examined the effects caused by the loss of Egfr-mediated signal transduction on different aspects of the closure process. Analyses of cuticle preparations revealed that embryos expressing dominant-negative forms of Egfr or Ras (RasDN) via the ectodermal pnr-Gal4 driver (Fig 2C and 2D, respectively), as well as embryos mutant for rhomboid, spi or various alleles of Egfr (Fig 2E–2H), fail to complete DC and exhibit dorsal-open phenotypes (white arrows; cf. wild-type in Fig 2A), characteristic of mutants in genes encoding components of the JNK pathway [e.g., basket (bsk), encoding Drosophila JNK; Fig 2B] [7,25].

To quantify the proportion of embryos that fail to complete closure due to defective Egfr signaling, we scored the percentage of RasDN- and EgfrDN-expressing embryos that completed DC by stage 16 (st16; 13–16 hours after egg lay), when the process normally ends (S2B Fig). In order to unambiguously segregate incomplete DC phenotypes from other secondary effects seen in Egfr-deficient embryos, such as head involution defects, we demarcated the LE cells using the puc-lacZ enhancer trap line, which concurrently also enabled the unequivocal identification of the embryonic genotypes scored (S2A and S2B Fig) (see Materials and Methods). We find that by st16, 92% of control embryos completed the course of DC, whereas only 47% and 39% of embryos expressing RasDN or EgfrDN finalized closure, respectively, confirming that Egfr signaling plays a significant role in DC regulation (S2C Fig).

Consistent with the cuticular defects, the consequences of loss of Egfr-mediated signal transduction are also apparent at the cellular level. Normally, LE cells of the advancing epidermis elongate along the dorsoventral (D/V) axis upon DC initiation. Subsequently, a similar elongation of lateral epidermis cells is observed in more ventral locations, as reflected by DE-Cadherin immunostaining that outlines cell membranes (Fig 3A and 3A’) [26,27]. In contrast, mutations in various constituents of the Egfr pathway, as well as ectodermal EgfrDN or RasDN expression, prevent epithelial cell elongation to a large extent (Fig 3C–3F’, arrowheads; quantification in S3 Fig). Instead, many cells remain polygonal in shape, as do analogous cells in bsk mutant embryos (Fig 3B and 3B’; arrowheads) [28,29].
Collectively, our findings identify a requirement for functional Egfr signaling in the process of DC, which is already apparent at the level of the cell shape changes that normally occur during early DC.

Egfr signaling is required for full induction and activity of Dpp signaling

Dpp signaling is known to coordinate the morphogenetic movements during DC [8]. Considering the impaired cell shape changes observed in embryos defective in Egfr signaling (Fig 3), we next assessed the expression of the JNK pathway target dpp in LE cells of rhomboid mutants, as well as in embryos expressing Egfr\textsuperscript{DN} in the ectoderm, finding that it is reduced in both genotypes (black arrowheads in Fig 4D and 4E, respectively; cf. wild-type dpp expression in Fig 4A; S4 Fig) [6,7]. Noteworthy, in both genetic backgrounds JNK-independent dpp expression is unaffected, for example in the visceral mesoderm and lateral ectoderm (white asterisks and arrowheads, respectively, in Fig 4A, 4D and 4E) [5,25]. Similar outcomes were observed when Egfr\textsuperscript{DN} was expressed in stripes, using paired (prd)-Gal4 (S5 Fig). Reciprocally, the dpp
expression domain expands in embryos expressing \textit{Ras}^{\text{V12}}, a constitutively active form of Ras (Fig 4F; S4 Fig). These effects closely resemble those observed when JNK signaling is blocked or constitutively active, respectively (cf. \textit{bsk} mutants or embryos expressing an active form of the JNK kinase, \textit{Hep}^{\text{Act}}, in Fig 4B and 4C respectively).

In Dpp-responding cells, activation of the Dpp receptor complex brings about the phosphorylation of the SMAD family member Mothers against dpp (Mad) \cite{30}. We find that levels of phosphorylated Mad (pMad) fully mirror the changes in \textit{dpp} expression levels in the different genetic backgrounds (Fig 4B"–4F"; cf. wild-type in Fig 4A"). To determine which cells are most affected by the loss of Egfr signaling, we utilized the \textit{puc-lacZ} enhancer trap line to delineate LE cells. We find that pMad is detectable in LacZ-expressing LE cells in embryos expressing \textit{pnr>Egfr}^{DN} (E) or \textit{pnr>Ras}^{DN} (F), leading to failure of epidermal cells to elongate. Instead, they remain polygonal, thus phenocopying \textit{bsk} mutants (B).

Cumulative effects due to dysfunction of the Egfr signaling pathway at earlier stages of development could contribute to the dorsal open phenotypes observed in embryos defective in Egfr signaling. To specifically assess the impact of this pathway on dorsal closure, independently from earlier contributions, we used a temperature sensitive \textit{Egfr} allele (\textit{Egfr}^{SH2}) \cite{31}.

https://doi.org/10.1371/journal.pgen.1006860.g003
We find that Egfr embryos shifted from the permissive to the restrictive temperature (i.e., from 18˚C to 29˚C) at the onset of dorsal closure (st12) develop dorsal open phenotypes and other characteristic dorsal closure defects (S7 Fig).

We conclude that, Egfr signaling is required for the full induction of dpp and for the phosphorylation of the downstream Dpp pathway effector molecule, Mad.
The JNK pathway is epistatic to Egfr signaling

Given the phenotypic similarities between embryos defective in Egfr and JNK signaling, we next set out to determine the epistatic relationship between the two pathways using pMad staining. First, we assessed the ability of constitutive JNK pathway activation to suppress loss of Egfr signaling in the double combination of Hep<sup>Act</sup> and Ras<sup>DN</sup>. When individually expressed, pMad staining is reduced in pnr<sup>-&gt;</sup>Ras<sup>DN</sup> embryos compared to controls (Fig 5A; cf. Fig 4A’’), and broader in those expressing pnr<sup>-&gt;</sup>Hep<sup>Act</sup> alone (Fig 5B). Strikingly, the Ras<sup>DN</sup>-mediated reduction in pMad staining is fully suppressed by Hep<sup>Act</sup> co-expression, with the pattern indistinguishable from that observed in embryos singly expressing Hep<sup>Act</sup> (Fig 5C; cf. Fig 5B). This result indicates that the JNK pathway acts downstream of the Egfr pathway.

To further examine this issue we also undertook a reciprocal approach, by testing whether loss of JNK signaling suppresses constitutive activation of the Egfr pathway. Specifically, the pMad pattern expands in embryos expressing Ras<sup>V12</sup> alone (Fig 5D; cf. Fig 4A’’) and narrows in embryos expressing a dominant-negative form of Bsk (Bsk<sup>DN</sup>) by itself (Fig 5E). Markedly, pMad staining in combined pnr<sup>-&gt;</sup>Bsk<sup>DN</sup>; Ras<sup>V12</sup> embryos resembles that seen in embryos expressing Bsk<sup>DN</sup> alone (Fig 5F; cf. Fig 5E; S8 Fig). Taken together, these results demonstrate that the JNK cascade is epistatic to the Egfr pathway. Consistent with this conclusion, we find that MAPK/Erk activation in response to Egfr signaling is unaffected in bsk mutants as well as in embryos expressing Hep<sup>Act</sup> (S9 Fig). Thus, our data identify a positive input by the Egfr pathway, acting upstream of JNK signaling, into the expression of a central JNK pathway target gene, <i>dpp</i>, and consequently into the phosphorylation of the key Dpp pathway effector, Mad.

EGFR signaling is required for repression of <i>scarface</i>, a secreted inhibitor of the JNK pathway, in the lateral epidermis

The rhomboid and dpErk patterns (Fig 1D) indicate that the Egfr pathway is active throughout the dorsal ectoderm. To better understand the hierarchal link between the Egfr and JNK pathways, we co-stained embryos expressing puc-<i>lacZ</i>, a target of JNK signaling in the LE cells [12], for dpErk and LacZ. Surprisingly, dpErk staining does not overlap with the <i>puc-LacZ</i> pattern, and is evident only in the adjacent lateral epidermis (Fig 6A–6B’). The finding that dpErk is excluded from the LE demonstrates that the JNK and Egfr pathways are active in distinct cell types within the dorsal ectoderm, and therefore implies that the Egfr pathway influences JNK signaling cell non-autonomously.

We hypothesized that the indirect input by Egfr signaling in lateral epidermis cells into JNK activity in neighboring LE cells occurs either via induction of a secreted JNK agonist or by the suppression of a secreted JNK antagonist. Among known JNK pathway elements, the secreted JNK antagonist Scarface (Scaf) appeared to be an attractive candidate to perform this intermediate role. <i>scaf</i> is normally expressed in LE cells under JNK regulation (Fig 6C) [13,14]. However, reduced Egfr activity, in <i>rhomboid</i> and <i>spi</i> mutants as well as in embryos expressing Egfr<sup>DN</sup> or Ras<sup>DN</sup>, results in ventral expansion of <i>scaf</i> expression in a striped configuration (Fig 6D–6G; cf. Fig 6C). By contrast, embryos expressing Ras<sup>V12</sup> under pnr-Gal4 regulation show a notable decrease in <i>scaf</i> expression even in LE cells (Fig 6H).

These data strongly suggest that Egfr signaling normally suppresses <i>scaf</i> expression, and provide a plausible explanation for the positive, non-autonomous effect of the Egfr pathway on JNK signaling in the LE. According to this scenario, in wild-type embryos Egfr signaling inhibits <i>scaf</i> expression in lateral epidermis cells, thereby blocking its secretion and antagonistic effect on JNK signaling in neighboring LE cells. Under Egfr loss-of-function conditions, <i>scaf</i> is up-regulated in lateral epidermis cells, leading to reduced JNK activity in LE cells. Accordingly, <i>scaf</i> expression in the LE itself is reduced relative to normal embryos (Fig 6D–
6G'; cf. Fig 6C), and thus responds to the loss of Egfr signaling like other JNK pathway targets, such as dpp.

To establish more directly how scaf deregulation influences JNK pathway output, we assessed dpp transcription and Mad phosphorylation in embryos over-expressing scaf or in scaf mutants. We find that scaf over-expression in the ectoderm obstructs dpp expression (Fig 7A; cf. wild-type in Fig 4A) and reduces Dpp pathway activity as reflected by immunostaining for pMad (Fig 7C; cf. wild-type in Fig 4A''), in a manner resembling Egfr loss-of-function backgrounds in which scaf is derepressed (Fig 4D–4E''). Conversely, both dpp expression and the pMad domain expand in scaf mutants (Fig 7B and 7D). These observations support a role for Scaf as a suppressor of Dpp signaling in the dorsal ectoderm.

The scaf over-expression and loss-of-function phenotypes closely parallel those observed for Egfr loss-of-function (Fig 4D–4E'') and RTK constitutive activation (Fig 4F and 4F''), respectively. To establish a regulatory link between Egfr signaling and scaf, we stained embryos, singly or doubly mutant for rhomboid and scaf, for pMad. The pMad domain broadens in scaf mutants (Fig 7D) and narrows considerably in rhomboid mutant embryos (Fig 7E). Importantly, embryos doubly mutant for rhomboid and scaf show an expanded pMad pattern (Fig 7F), similarly to embryos mutant for scaf alone (Fig 7D). These results indicate that Egfr signaling acts as an upstream negative regulator of scaf in the lateral epidermis cells. Thus, in the absence of scaf, JNK activity and consequent Dpp pathway activity is robust whether or not the Egfr pathway is functional. Collectively, our data indicate that Egfr-mediated suppression of scaf in lateral epidermis cells is required for full JNK signaling activity in LE cells, accounting for how Egfr activity affects JNK signaling in nearby cells and explaining why the Egfr pathway is required for the completion of the DC process (Fig 7G and 7H).

We next addressed the mechanism by which Egfr signaling impacts on scaf expression. scaf could represent an exceptional example of a gene that is directly repressed by the Egfr pathway. Still, previous studies indicated that, at least in flies, RTK signaling pathways predominantly
activate gene expression, often by downregulating negative transcriptional regulators such as the ETS transcription factor Yan (also known as Anterior open) [32]. Therefore, Egfr signaling could be affecting sco expression indirectly by inducing an intermediary repressor of this gene. To begin testing this idea, we expressed a non-phosphorylatable derivative of Yan, Yan\(^\text{Act}\), that is insensitive to attenuation by Egfr-mediated signaling [33] and, hence, should dominantly block the induction of such a repressor. We find that pnr>Yan\(^\text{Act}\) embryos display dorsal open phenotypes (Fig 8A and 8A'; cf. Fig 2A), loss of dpp expression and reduced pMad staining (Fig 8B and 8C; cf. Fig 4A–4A'). Remarkably, sco is derepressed in these embryos (Fig 8D; cf. Fig 6C), consistent with the idea that Yan normally represses a sco repressor.

S. Noselli and colleagues recently demonstrated that sco is under complex transcriptional control, involving several activators as well as at least two repressors: Engrailed (En) and Abdominal-A (Abd-A) [34]. They showed that in embryos mutant for en, sco is derepressed only in LE cells, perhaps due to the combined repressor activity of Abd-A. Nevertheless, when they expressed a form of En that was converted into an activator, sco was ectopically expressed.
Fig 7. Over-expression of *scarface* mimics the loss of Egfr pathway activity. (A-B) Embryos hybridized using a digoxigenin-labeled RNA probe for *dpp* (blue). Over-expression of *scarface* brings about a reduction in *dpp* expression (A) whereas *dpp* expression expands in *scarface* mutant embryos (B), similarly to loss- and gain-of-function mutations in the Egfr pathway, respectively. (C-F) Embryos stained for pMad (red). (C, D) In keeping with *dpp* expression levels, pMad staining decreases upon *scarface* over-expression (C), and is augmented in a *scarface* mutant (D). (E, F) Although pMad staining is reduced in *rhomboid* single mutant embryo (E), it expands in embryo doubly mutant for *scarface* and *rhomboid* (F), as in *scarface* single mutant (D), indicating that *scarface* is epistatic to *Egfr* signaling. (G-H) Model showing how Egfr signaling in the lateral epidermis positively and non-autonomously contributes to JNK pathway activity in LE cells and to DC. (G) The Egfr pathway normally acts...
in the lateral epidermis [34]. This raised the question whether the Egfr-Yan axis induces En, which in turn directly represses scaf. Indeed, we find that En is dominantly repressed in the lateral epidermis to prevent expression of the JNK antagonist, scaf, thus supporting maximal JNK activity in LE cells. (H) When Egfr signaling is defective, deregulated Scaf subsequently attenuates functional JNK signaling in LE cells, thus hindering the process of DC. Bold text and arrows/bars indicate normal levels of gene expression and regulation, whereas gray fonts designate abnormally lower levels of expression and regulation, respectively.

https://doi.org/10.1371/journal.pgen.1006860.g007

in the lateral epidermis [34]. This raised the question whether the Egfr-Yan axis induces En, which in turn directly represses scaf. Indeed, we find that En is dominantly repressed in the

Fig 8. The Egfr pathway induces expression of Engrailed, a scarface repressor, in the lateral epidermis. (A, A') Cuticle preparation. Dark (A) and bright field (A') images of an embryo expressing pnr>Yan^{act}. Note the dorsal open hole. (B, C) Embryos expressing pnr>Yan^{act}, hybridized using a digoxigenin-labeled RNA probe for dpp (blue; B) or stained for pMad (red; C). (B') Magnified view of the region marked by a rectangle in (B). Note that Yan^{act} brings about a reduction in dpp expression and, as a consequence, a reduction in the pMad domain, similarly to other Egfr pathway mutants. (D) Embryo expressing pnr>Yan^{act} hybridized using a digoxigenin-labeled RNA probe for scaf (blue). (D') Magnified view of the region marked by a rectangle in (D). Note that scaf expression expands into the lateral epidermis. (E, F) Yan^{act} dominantly represses En. Control embryo expressing pnr-GFP (E) and embryo expressing pnr>Yan^{act} (F) stained for En (green), as well as for LacZ (magenta; puc-lacZ) to mark the LE. Yan^{act} activity reduces En expression in the LacZ-positive LE cells, as well as in the adjacent lateral epidermis (F). (G) Model explaining how Egfr signaling prevents expression of scaf in the lateral epidermis.

https://doi.org/10.1371/journal.pgen.1006860.g008
lateral epidermis of embryos expressing pnr\textsuperscript{>Yan\textsuperscript{ACT}}, specifically in the pnr domain (Fig 8F; cf. 8E). Thus, a transcriptional regulatory cascade could explain how the Egfr pathway represses scaf (Fig 8G): by downregulating Yan, Egfr signaling induces expression of En (and perhaps other repressors) which, in turn, silences scaf expression. In embryos in which Egfr pathway activity is blocked, En is not induced and scaf is derepressed in the lateral epidermis, thereby hindering JNK signaling in LE cells.

**Discussion**

Although extensively investigated, it is not fully understood how complex morphogenetic processes such as DC are controlled, and by which signaling pathways. In this manuscript we report that, in addition to the well-established JNK and Dpp pathways, signaling mediated by the Egfr is also instrumental to DC. We uncover a novel interplay between the Egfr and JNK pathways, specifically by demonstrating that Egfr signaling in the lateral epidermis suppresses the expression of the gene scaf, which encodes a proposed secreted JNK antagonist. Through this regulatory switch the Egfr pathway facilitates JNK signaling in LE cells (Fig 7G and 7H). Egfr signaling thus contributes cell non-autonomously to the expression of the JNK target dpp, to the phosphorylation of the downstream Dpp effector Mad and, consequently, to the synchronized morphogenetic movements orchestrated by Dpp signaling that are essential to successful DC.

It is currently unknown whether Scaf, the protein product of an established JNK pathway target gene [14,34–36], impinges on JNK pathway activity directly or indirectly. Scaf could be negatively regulating JNK signaling directly, by acting on an extracellular signal or on a putative receptor of the pathway [13]. However, it could also be playing a more general role, for example by degrading the extracellular matrix [35] or by establishing correct basement membrane protein localization [14], thus influencing JNK pathway outcomes indirectly. Although our study does not distinguish between these mechanisms, our results showing that Scaf impacts on the expression and activity of Dpp are consistent with the notion that Scaf is an antagonist of the JNK pathway. Rousset et al. reached similar conclusions, based on their findings that scaf loss-of-function mimics JNK over-activity as well as on other data [13]. Further research, however, will be required to conclusively elucidate the molecular mechanism(s) underlying Scaf function in the context of dorsal closure.

Our findings that the Egfr and JNK pathways are linked at the level of a JNK feedback inhibitor exemplify an important emerging theme in cell signaling: that Egfr signaling frequently impacts on the activity of other developmental pathways or master regulators via the induction of genes, whose protein products subsequently modulate the activity of these secondary pathways and/or factors. For instance, in the fly eye imaginal disc, Egfr signaling induces expression of the Delta ligand in photoreceptor cells, and thus positively stimulates Notch signaling in neighbouring cone cells [37,38]. In other cases, Egfr-regulated targets act as negative feedback regulators. For example, the Egfr pathway induces expression of the gene wntD, which encodes an antagonist of the Rel transcription factor, Dorsal. Through this negative feedback regulation, Egfr signaling opposes the nuclear localization of Dorsal, thereby affecting the expression of multiple Dorsal targets along the D/V axis of the embryo [39].

In our analyses, we have focused on the input to DC by Egfr signaling taking place in the lateral epidermis. It is conceivable that Egfr-mediated signal transduction also plays additional regulatory roles during DC. For example, this pathway has been previously implicated in the suppression of zipper, the gene encoding Drosophila non-muscle myosin II heavy chain, in the AS and in a cell non-autonomous manner also in LE cells [40]. Furthermore, our results do not preclude the involvement of additional RTK pathways in this developmental process. As a
case in point, signaling by PVR in DC supports proper midline zippering in addition to AS internalization and removal, via the PI3K pathway and independently of JNK signaling [41]. Future studies will uncover the full impact of RTK-mediated signal transduction in DC.

In conclusion, our work illuminates a novel mechanism of signal integration between the Egfr and JNK pathways, linking Egfr signaling to the core regulatory network controlling DC. Our results thus reinforce the idea that different signaling pathways that regulate morphogenesis are interlinked, acting in a coordinated manner. A deeper understanding of the cross-regulation between these pathways, and the elucidation of further roles for Egfr signaling in DC, should facilitate our understanding of how diverse signal transduction pathways intersect to synchronize collective cell behavior, and how this circuitry ultimately leads to precise and coordinated morphogenetic processes.

Materials and methods

Fly culture and stocks
Flies were cultured and crossed on standard yeast-cornmeal-molasses-malt extract-agar medium at 25°C. The following mutant stocks and Gal4 drivers were used: EgfrEA [31], scf11;5, UAS-Scaf [14], rhomboidTM, spiST14, UAS-EgfrDN, UAS-RasDN, UAS-RasV12, bsk1, UAS-BskDN, UAS-HepAc1, UAS-YanAct and pnr-Gal4, puc-lacZ (pucE69). In general, mutant chromosomes were maintained over wg-LacZ- or dfd-YFP-marked balancer chromosomes, allowing the unambiguous identification of embryos of the correct genotype. Yellow white flies served as wild-type controls.

To quantify the proportion of embryos compromised in Egfr signaling that fail to complete closure at st16, the following genotypes were used: 1) white; pnr-Gal4, puc-lacZ/UAS-GFP (control); 2) white; UAS-EgfrDN; pnr-Gal4, puc-lacZ; 3) and UAS-RasDN; pnr-Gal4, puc-lacZ.

Cuticle preparation
Unhatched larvae (>24 hours old) were dechorionated in bleach, devitellinised in 1:1 methanol/heptane, rehydrated in PBS/methanol and mounted in 1:1 Hoyer’s medium/double-distilled water and cleared overnight at 70°C.

In situ hybridization and antibody staining
Embryos were dechorionated in bleach and fixed in 8% formaldehyde/PBS/heptane for 20 minutes. Expression patterns of dpp and scf were visualized by whole-mount in situ hybridization using digoxigenin-UTP labeled antisense RNA probes and anti-digoxigenin antibodies conjugated to alkaline phosphatase (Roche).

Fluorescent immunodetection of dpErk, in freshly fixed embryos (10% formaldehyde/PBS/Heptane buffer), was attained using rabbit αdpErk (1:100; Cell Signaling) [20]. Other antibodies used were: mouse αLacZ (1:1000; Promega), rabbit αLacZ (1:2000; Cappel), rabbit αpMad (1:100; Epitomics), mouse αEn (1:20; Developmental Studies Hybridoma Bank) and rat αDE-Cadherin (1:50; Developmental Studies Hybridoma Bank). Secondary antibodies were Alexa Fluor 488-, 546-, and 633-conjugated (1:400; Jackson Laboratories). Embryos were mounted using Vectashield medium (Vector Laboratories).

Microscopy
Light microscope images were acquired using a Zeiss Axioplan2 microscope and confocal images were taken using a Zeiss LSM710 confocal microscope. Images were processed using.
Adobe Photoshop software, and the ZEN 2012 blue edition was used to measure LE cell length in embryos compromised in Egfr signaling.

**Supporting information**

**S1 Fig.** *pannier*-Gal4 drives ectodermal GFP expression throughout the lateral epidermis and LE cells. (A-C) *pnr*-Gal4>GFP, *puc*-lacZ enhancer-trap embryo, stained for LacZ (red; A) and for GFP (green; B). (C) Merge. Note that ectodermal expression of GFP, driven by *pnr*-Gal4, is restricted to the lateral epidermis and LE cells.

**S2 Fig.** Quantifying incomplete dorsal closure in embryos defective in Egfr signaling.

(A-B) Confocal images of st16 *puc*-lacZ enhancer-trap line embryos, in which *pnr*-Gal4 drives the expression either of GFP (control), Ras\(^{DN}\) or Egfr\(^{DN}\), stained for LacZ to demarcate LE cells. The numbers of st16 embryos, displaying a dorsal-open hole and therefore incomplete closure (A; red), or those that have completed closure (B; blue), were scored. (C) Percentage of st16 embryos, expressing GFP, Ras\(^{DN}\) or Egfr\(^{DN}\) via *pnr*-Gal4, that have completed closure (blue) or not (red). n = number of embryos from each definitive genotype that were scored.

**S3 Fig.** Egfr pathway activity is required for proper LE cell elongation. Quantification of LE cell length in wild-type or in *bsk*, *spi* and *rhomboid* mutant embryos, as well as in embryos expressing *pnr>*Egfr\(^{DN}\) or *pnr>*Ras\(^{DN}\). The data represent the mean ± SD derived from 8–10 different embryos. *** P<0.0001 compared to wild-type embryos (Mann-Whitney U-test). n = number of LE cells from each definitive genotype that were scored.

**S4 Fig.** Egfr signaling is required for the full expression of *dpp*. (A-D) High magnification (x40) lateral views of embryos hybridized using a digoxigenin-labeled RNA probe for *dpp* (blue). Levels of *dpp* are reduced in a *rhomboid* mutant (B) as well as in embryo expressing *pnr>*Egfr\(^{DN}\) (C). Conversely, the *dpp* domain expands ventrally in embryo expressing *pnr>*Ras\(^{V12}\) (D).

**S5 Fig.** Expression of Egfr\(^{DN}\) in stripes leads to defective dorsal closure. (A) Cuticle preparation of embryo expressing *prd>*Egfr\(^{DN}\) showing an open dorsal phenotype (white arrowhead). (B) St13 embryo expressing *prd>*Egfr\(^{DN}\) hybridized using a digoxigenin-labeled RNA probe for *dpp* (blue). Loss of *dpp* expression (black arrowheads) in both stripe and inter-stripe regions of *prd>*Egfr\(^{DN}\) embryos indicates that the resulting ectopic Scaf acts on LE cells non-autonomously (see below). White asterisks and arrowheads mark JNK-independent *dpp* expression in the visceral mesoderm and lateral ectoderm, respectively.

**S6 Fig.** Egfr pathway perturbations primarily affect the lateral epidermis. Control *puc*-lacZ, *pnr*-Gal4 embryo expressing GFP (A), or *puc*-lacZ, *pnr*-Gal4 embryos expressing Egfr\(^{DN}\) (B) and Ras\(^{DN}\) (C), stained for pMad (red) and LacZ (blue). Note that LE cells, distinguishable by LacZ staining, co-stain for pMad, whereas pMad staining is markedly reduced in the lateral epidermis.
S7 Fig. Egfr loss-of-function from stage 12 onwards leads to dorsal closure defects. (A, B) Cuticle preparations of embryos carrying the temperature sensitive Egfr<sup>SH2</sup> allele, maintained at permissive (18˚C) (A) or restrictive (29˚C) (B) temperatures. The embryo in (B) was shifted from the permissive to the restrictive temperature at the onset of dorsal closure (st12). Note the dorsal open phenotype (arrowhead). Wild-type embryos subjected to the same regime hatched normally. The embryo in (A) has mild segmental defects. (C, D) The domain of pMad staining (red) decreases in Egfr<sup>SH2</sup> embryo shifted to 29˚C at st12 (D) but not in embryo of the same genotype raised at 18˚C (C). (E, F) Embryos stained for DE-cadherin (green) to outline cell membranes. Corresponding primed panels (E’ and F’) show magnified views of the regions marked with arrowheads. Note the occurrence of cell elongation defects in F’.

(TIF)

S8 Fig. The loss of pMad staining in pnr<sup>+</sup>Bst<sup>DN;Ras</sup><sup>V12</sup> embryos is not due to Gal4 dilution. (A-F) Lateral views of st13 embryos stained for GFP (green) and pMad (red). (A-C) Control embryo, expressing pnr<sup>+</sup>GFP, stained for (A) GFP and (B) pMad. (C) Merge. (D-F) Embryo co-expressing pnr<sup>+</sup>Ras<sup>V12;GFP</sup>, stained for (D) GFP and (E) pMad. (F) Merge. Note the strong pMad staining in pnr<sup>+</sup>Ras<sup>V12;GFP</sup> embryo.

(TIF)

S9 Fig. The dpErk pattern is unaltered in embryos with loss- or gain-of-function JNK signaling. (A-C) Embryos stained for dpErk (red). No significant change in the dpErk pattern is observed in bsk mutant embryo (B) or upon pnr<sup>+</sup>Hep<sup>Act</sup> expression (C), compared to control (A). The signal in the AS is an artifact caused by auto-florescence.

(TIF)

Acknowledgments

We thank members of our laboratory, especially Einat Cinnamon and Rona Grossman, for continued help and encouragement; Ksenia Yurkovsky for her help with the graphics; and Martin Blum, Einat Cinnamon, Nicole Gorfinkel, António Jacinto, Gerardo Jiménez, Amir Orian, Susan Parkhurst, Eyal Schejter and Benny Shilo for their insightful comments on the manuscript. We are grateful to Dirk Bohmann, Einat Cinnamon, Abraham Fainsod, Nicole Gorfinkel, Tom Jessell, Gerardo Jiménez, Ed Laufer, Marco Milan, Susan Morton, Benny Shilo, Beth Stronach, Dan Vasiliauskas, Tonia von Ohlen, Trudi Schüpbach, the Developmental Studies Hybridoma Bank, and the Bloomington Stock Centre for DNA constructs, antibodies, reagents and fly stocks.

Author Contributions

Conceptualization: Tatyana Kushnir, Sharon Mezuman, Shaked Bar-Cohen, Ze’ev Paroush, Aharon Helman.

Formal analysis: Tatyana Kushnir, Sharon Mezuman, Shaked Bar-Cohen, Ze’ev Paroush, Aharon Helman.

Funding acquisition: Ze’ev Paroush.

Investigation: Tatyana Kushnir, Sharon Mezuman, Shaked Bar-Cohen, Rotem Lange, Aharon Helman.

Methodology: Tatyana Kushnir, Sharon Mezuman, Shaked Bar-Cohen, Ze’ev Paroush, Aharon Helman.

Project administration: Ze’ev Paroush.
Supervision: Ze’ev Paroush, Aharon Helman.

Validation: Tatyana Kushnir, Sharon Mezuman, Shaked Bar-Cohen, Rotem Lange, Aharon Helman.

Writing – original draft: Tatyana Kushnir, Sharon Mezuman, Shaked Bar-Cohen, Ze’ev Paroush, Aharon Helman.

Writing – review & editing: Tatyana Kushnir, Sharon Mezuman, Shaked Bar-Cohen, Ze’ev Paroush, Aharon Helman.

References

1. Schock F, Perrimon N (2002) Molecular mechanisms of epithelial morphogenesis. Annu Rev Cell Dev Biol 18: 463–493. https://doi.org/10.1146/annurev.cellbio.18.022602.131838 PMID: 12142280

2. Friedl P, Hegerfeldt Y, Tusch M (2004) Collective cell migration in morphogenesis and cancer. Int J Dev Biol 48: 441–449. https://doi.org/10.1387/ijdb.041821 PMID: 15349818

3. Jacinto A, Woolner S, Martin P (2002) Dynamic analysis of dorsal closure in Drosophila: from genetics to cell biology. Dev Cell 3: 9–19. PMID: 12110163

4. Riesgo-Escovar JR, Hafen E (1997) Drosophila Jun kinase regulates expression of decapentaplegic via the ETS-domain protein Aop and the AP-1 transcription factor DJun during dorsal closure. Genes Dev 11: 1717–1727. PMID: 9224720

5. Hou XS, Goldstein ES, Perrimon N (1997) Drosophila Jun relays the Jun amino-terminal kinase signal transduction pathway to the Decapentaplegic signal transduction pathway in regulating epithelial cell sheet movement. Genes Dev 11: 1728–1737. PMID: 9224721

6. Glise B, Noselli S (1997) Coupling of Jun amino-terminal kinase and Decapentaplegic signaling pathways in Drosophila morphogenesis. Genes Dev 11: 1738–1747. PMID: 9224722

7. Riesgo-Escovar JR, Hafen E (1997) Common and distinct roles of Dfos and DJun during Drosophila development. Science 278: 669–672. PMID: 9381174

8. Fernandez BG, Arias AM, Jacinto A (2007) Dpp signalling orchestrates dorsal closure by regulating cell shape changes both in the amnioserosa and in the epidermis. Mech Dev 124: 884–897. https://doi.org/10.1016/j.mod.2007.09.002 PMID: 17950580

9. Stronach BE, Perrimon N (1999) Stress signaling in Drosophila. Oncogene 18: 6172–6182. https://doi.org/10.1038/sj.onc.1203125 PMID: 10557109

10. Zeitlinger J, Kockel L, Peverali FA, Jackson DB, Modzik M, et al. (1997) Defective dorsal closure and loss of epidermal decapentaplegic expression in Drosophila fos mutants. EMBO J 16: 7393–7401. https://doi.org/10.1093/emboj/16.24.7393 PMID: 9405368

11. Sluss HK, Han Z, Barrett T, Goberdhan DC, Wilson C, et al. (1996) A JNK signal transduction pathway that mediates morphogenesis and an immune response in Drosophila. Genes Dev 10: 2745–2758. PMID: 8946915

12. Martin-Blanco E, Gampel A, Ring J, Virdee K, Kirov N, et al. (1998) puckered encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in Drosophila. Genes Dev 12: 557–570. PMID: 9472024

13. Roussel R, Bono-Lauriol S, Gettings M, Suzannet M, Speder P, et al. (2010) The Drosophila serine protease homologue Scarface regulates JNK signalling in a negative-feedback loop during epithelial morphogenesis. Development 137: 2177–2186. https://doi.org/10.1242/dev.050781 PMID: 20530545

14. Sorrosal G, Perez L, Herranz H, Milan M (2010) Scarface, a secreted serine protease-like protein, regulates polarized localization of laminin A at the basement membrane of the Drosophila embryo. EMBO Rep 11: 373–379. https://doi.org/10.1038/embor.2010.43 PMID: 20379222

15. Martin P, Parkhurst SM (2004) Parallels between tissue repair and embryo morphogenesis. Development 131: 3021–3034. https://doi.org/10.1242/dev.01253 PMID: 15197160

16. Schweitzer R, Shilo BZ (1997) A thousand and one roles for the Drosophila EGF receptor. Trends Genet 13: 191–196. PMID: 9154002

17. Nilson LA, Schubbach T (1999) EGF receptor signaling in Drosophila oogenesis. Curr Top Dev Biol 44: 203–243. PMID: 9891881

18. Shilo BZ (2003) Signaling by the Drosophila epidermal growth factor receptor pathway during development. Exp Cell Res 284: 140–149. PMID: 12648473
20. Helman A, Paroush Z (2010) Detection of RTK pathway activation in Drosophila using anti-dpERK immunofluorescence staining. Methods Mol Biol 661: 401–408. https://doi.org/10.1007/978-1-60761-795-2_24 PMID: 20811997
21. Gabay L, Seger R, Shilo BZ (1997) MAP kinase in situ activation atlas during Drosophila embryogenesis. Development 124: 3535–3541. PMID: 9342046
22. Gabay L, Seger R, Shilo BZ (1997) In situ activation pattern of Drosophila EGF receptor pathway during development. Science 277: 1103–1106. PMID: 9262480
23. Calleja M, Herranz H, Estella C, Casal J, Lawrence P, et al. (2000) Generation of medial and lateral dorsal body domains by the pannier gene of Drosophila. Development 127: 3971–3980. PMID: 10952895
24. Rutledge BJ, Zhang K, Bier E, Jan YN, Perrimon N (1992) The Drosophila spitz gene encodes a putative EGF-like growth factor involved in dorsal-ventral axis formation and neurogenesis. Genes Dev 6: 1503–1517. PMID: 1644644
25. Glise B, Bourbon H, Noselli S (1995) hemipterous encodes a novel Drosophila MAP kinase kinase, required for epithelial cell sheet movement. Cell 83: 451–461. PMID: 8521475
26. Kiehart DP, Galbraith CG, Edwards KA, Rickoll WL, Montague RA (2000) Multiple forces contribute to required for epithelial cell sheet movement. Cell 83: 451–461. PMID: 8521475
27. Noselli S (1998) JNK signaling and morphogenesis in Drosophila. Development 124: 3535–3541. PMID: 9342046
28. Rios-Barrera LD, Riesgo-Escovar JR (2013) Regulating cell morphogenesis: the Drosophila Jun-N-terminal kinase pathway. Genesis 51: 147–162. https://doi.org/10.1002/dvg.23103 PMID: 23103
29. Noselli S (1998) JNK signaling and morphogenesis in Drosophila. Trends Genet 14: 33–38. https://doi.org/10.1016/S0168-9525(97)01320-6 PMID: 9448464
30. Affolter M, Marty T, Vigano MA, Jazwinska A (2001) Nuclear interpretation of Dpp signaling in Drosophila. EMBO J 20: 3298–3305. https://doi.org/10.1093/emboj/20.13.3298 PMID: 11432617
31. Clifford R, Schupbach T (1994) Molecular analysis of the Drosophila EGF receptor homolog reveals that several genetically defined classes of alleles cluster in subdomains of the receptor protein. Genetics 137: 531–550. PMID: 8070664
32. Gabay L, Scholz H, Golembo M, Klaes A, Shilo BZ, et al. (1996) EGF receptor signaling induces pointed EGFR signaling is required for dorsal closure in Drosophila. J Cell Biol 149: 471–490. PMID: 10769037
33. Rios-Barrera LD, Riesgo-Escovar JR (2013) Regulating cell morphogenesis: the Drosophila Jun-N-terminal kinase pathway. Genesis 51: 147–162. https://doi.org/10.1002/dvg.23103 PMID: 23103
34. Affolter M, Marty T, Vigano MA, Jazwinska A (2001) Nuclear interpretation of Dpp signaling in Drosophila. EMBO J 20: 3298–3305. https://doi.org/10.1093/emboj/20.13.3298 PMID: 11432617
35. Clifford R, Schupbach T (1994) Molecular analysis of the Drosophila EGF receptor homolog reveals that several genetically defined classes of alleles cluster in subdomains of the receptor protein. Genetics 137: 531–550. PMID: 8070664
36. Gabay L, Scholz H, Golembo M, Klaes A, Shilo BZ, et al. (1996) EGF receptor signaling induces pointed P1 transcription and inactivates Yan protein in the Drosophila embryonic ventral ectoderm. Development 122: 3535–3542. PMID: 8951052
37. Rebay I, Rubin GM (1995) Yan functions as a general inhibitor of differentiation and is negatively regulated by activation of the Ras1/MAPK pathway. Cell 81: 857–866. PMID: 7781063
38. Rousset R, Carballes F, Parassol N, Schaub S, Cerezo D, et al. (2017) Signalling crosstalk at the leading edge controls tissue closure dynamics in the Drosophila embryo. PLoS Genet 13: e1006640. https://doi.org/10.1371/journal.pgen.1006640 PMID: 28321245
39. Lopez-Otin C, Matrisian LM (2007) Emerging roles of proteases in tumour suppression. Nat Rev Cancer 7: 800–808. https://doi.org/10.1038/nrc2228 PMID: 17851543
40. Srivastava A, Dong Q (2015) Regulation of a serine protease homolog by the JNK pathway during thoracic development of Drosophila melanogaster. FEBS Open Bio 5: 117–123. https://doi.org/10.1002/fob.2015.01.008 PMID: 25737837
41. Tsuda L, Nagaraj R, Zipsrusk SL, Banerjee U (2002) An EGF/Erk/Sno pathway promotes Delta expression by inactivating Su(H)/SMRTER repression during inductive notch signaling. Cell 110: 625–637. PMID: 12203979
42. Nagaraj R, Banerjee U (2007) Combinatorial signaling in the specification of primary pigment cells in the Drosophila eye. Development 134: 825–831. https://doi.org/10.1242/dev.02788 PMID: 17251265
43. Helman A, Lim B, Andreu MJ, Kim Y, Shestkin T, et al. (2012) RTK signaling modulates the Dorsal gradient. Development 139: 3032–3039. https://doi.org/10.1242/dev.075812 PMID: 22791891
44. Shen W, Chen X, Cormier O, Cheng DC, Reed B, et al. (2013) Modulation of morphogenesis by Egfr during dorsal closure in Drosophila. PLoS One 8: e60180. https://doi.org/10.1371/journal.pone.0060180 PMID: 23579691
45. Garlena RA, Lennox AL, Baker LR, Parsons TE, Weinberg SM, et al. (2015) The receptor tyrosine kinase Pvr promotes tissue closure by coordinating corpse removal and epidermal sealing. Development 142: 3403–3415. https://doi.org/10.1242/dev.122226 PMID: 26293306