Modulation of Morphogenesis by Egfr during Dorsal Closure in Drosophila

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Abstract

During Drosophila embryogenesis the process of dorsal closure (DC) results in continuity of the embryonic epidermis, and DC is well recognized as a model system for the analysis of epithelial morphogenesis as well as wound healing. During DC the flanking lateral epidermal sheets stretch, align, and fuse along the dorsal midline, thereby sealing a hole in the epidermis occupied by an extra-embryonic tissue known as the amnioserosa (AS). Successful DC requires the regulation of cell shape change via actomyosin contractility in both the epidermis and the AS, and this involves bidirectional communication between these two tissues. We previously demonstrated that transcriptional regulation of myosin from the zipper (zip) locus in both the epidermis and the AS involves the expression of Ack family tyrosine kinases in the AS in conjunction with Dpp secreted from the epidermis. A major function of Ack in other species, however, involves the negative regulation of Egfr. We have, therefore, asked what role Egfr might play in the regulation of DC. Our studies demonstrate that Egfr is required to negatively regulate epidermal expression of dpp during DC. Interestingly, we also find that Egfr signaling in the AS is required to repress zip expression in both the AS and the epidermis, and this may be generally restrictive to the progression of morphogenesis in these tissues. Consistent with this theme of restricting morphogenesis, it has previously been shown that programmed cell death of the AS is essential for proper DC, and we show that Egfr signaling also functions to inhibit or delay AS programmed cell death. Finally, we present evidence that Ack regulates zip expression by promoting the endocytosis of Egfr in the AS. We propose that the general role of Egfr signaling during DC is that of a braking mechanism on the overall progression of DC.

Introduction

Dorsal closure (DC) is a developmental event occurring in the Drosophila embryo between stages 11 and 16, commencing immediately after germband retraction (reviewed in [1]). Upon the completion of germband retraction, a large dorsal opening is evident in the epidermis. The amnioserosa (AS), an extra-embryonic tissue composed of a single layer of large flat epithelial cells, spans the opening. The two opposing lateral epidermal flanks elongate in the dorsal-ventral (D-V) axis and move dorsal ward to seal the dorsal hole. The dorsal-most epidermal (DME) cells from one side of the embryo meet their counterpart DME cells at the dorsal midline. The epidermal sealing process occurs in a zipper-like manner, progressing simultaneously from both the anterior and posterior ends of the dorsal opening and finishing at the center of the dorsal midline. During DC, the AS contracts and its cells become more cuboidal in shape; the AS also actively extrudes approximately 10% of its cells with the effect of increasing the rate of DC [2–4]. Upon the completion of DC, the entire AS degenerates by programmed cell death [2].

The DME cells experience a range of morphogenetic events during DC. These include elongation in the D-V axis, formation of actin-based membrane extensions, and adhesion with their partners from the other side of the embryo. DC is a popular model system to study cell shape change in epithelial morphogenesis and multiple signaling proteins have been characterized in this context. In addition to signaling pathways and proteins, there are a number of mechanical forces driving DC [4](reviewed in [5]). These include a supracellular actomyosin cable that is assembled at the leading edge (LE) of the DME cells to form a contractile “purse string”. This contractile apparatus constricts the DME cells in the anterior-posterior axis and thus contributes to their stretching in the D-V axis and movement towards the dorsal midline. Actin-based filopodia and lamellipodia also project from the leading edge of the DME cells, and these are thought to contribute to the alignment and adhesion of opposing DME cells as DC concludes [6,7]. Finally, as was demonstrated by elegant laser microdissection experiments, AS constriction not only removes this tissue as an impediment to movement of the epidermis, but also pulls the DME cells dorsal ward [4].
Among the numerous signaling proteins known to regulate DC is Dpp, a member of the transforming growth factor-β superfamily of cytokines. Dpp expression in DME cells is required for morphogenesis of both the amnioserosa and the epidermis during DC. This requirement for Dpp expression in the DME cells is associated, at least in part, with the regulation of the expression of zip, which encodes non-muscle myosin II heavy chain [9–26]. We previously demonstrated that two members of the Ack family of nonreceptor tyrosine kinases, Ack and PR2, co-operate with Dpp to regulate myosin levels in the AS and epidermis during DC. This work led us to propose the existence of a diffusible signal that is generated by the AS and is regulated by Ack and PR2 in conjunction with Dpp [26]. The zip product is required for cell shape change in both the AS and the epidermis; zip expression, which is regulated by the Ack/PR2/Dpp signaling network, may ultimately coordinate the overall progression of DC [27].

There is considerable evidence that a major function of Ack is the negative regulation of Egfr, and this is thought to occur through the regulation of Egfr by endocytosis and/or ubiquitination [28–32]. Egfr may, therefore, play a key role in the Ack/PR2/Dpp regulatory pathway during DC. The Egfr pathway is used repeatedly throughout Drosophila development and appears to regulate a myriad of processes including cell proliferation, cell differentiation, apoptosis, cell motility and adhesion [reviewed in [33,34]]. While it has long been recognized that Egfr has multiple roles in regulating morphogenesis, including germband retraction, its role in DC has not been specifically addressed [35,36].

The results presented here demonstrate that Egfr is required in both the AS and epidermis for proper DC. In addition, we demonstrate that the function of Egfr in the AS involves the transcriptional repression of zip in both the AS and the DME cells, and we suggest that this repression involves the regulation of the same diffusible signal previously proposed to be regulated through Ack and PR2. Consistent with this, we present results suggesting that Ack negatively regulates Egfr in the AS by targeting it for endocytosis. Similar to Ack, we suggest that Egfr regulates zip expression in parallel to Dpp signaling, but we find that Egfr also has a strong negative effect on epidermal Dpp transcription. Finally, we confirm that Egfr signaling has an additional role in the AS as an inhibitor of apoptosis. The various roles identified for Egfr signaling during DC are consistent with negative regulation of morphogenesis, and we propose that Egfr acts as a brake to adjust the rate of closure in response to endocytic regulation.

Results

Egfr is Required for Normal DC

To address the potential function of Egfr in DC we chose three alleles previously addressed to disrupt embryogenesis: Egfr, a severe loss-of-function allele, Egfr, a moderate loss-of-function allele, and Egfr, a conditional allele [35–37]. Two previously uncharacterized embryonic lethal alleles, Egfr and Egfr, were also used in the course of this study [38]. Phenotypic analysis of the latter two alleles indicated that Egfr is a severe loss-of-function allele (equivalent to Egfr) and that Egfr is also a strong loss-of-function allele, but is slightly less severe than either Egfr or Egfr (data not shown). Cuticle preparations of embryos heteroallelic for Egfr and Egfr showed severe defects in morphogenesis. Greater than 95% of embryos exhibited the previously described ‘‘faint little ball’’ phenotype [36,37], being ‘‘curled’’ up, with the posterior end of the embryo in close proximity to the head, indicating a defect in germband retraction (Fig. 1B). In less severely curled embryos it was possible to observe holes in the dorsal surface that typically extended anteriorly into the head (Fig. 1C). In general, severe Egfr mutants were associated with a terminal phenotype that included severe defects in head development as well as a complete failure in germ band retraction – both of which effectively precluded any analysis of DC. We, therefore, sought approaches that would allow us to observe DC defects in individuals with impaired Egfr function. The first of the two approaches involved temperature shift experiments using the conditional allele Egfr [55]. Embryos collected at the permissive temperature of 18°C were aged for various periods of time before being shifted to the restrictive temperature of 29°C. Embryos transferred to 29°C at any stage prior to stage 10 showed severe cuticle defects similar to those seen with other strong loss-of-function Egfr alleles (data not shown). Shifting slightly older (approximately stage 11) Egfr mutant embryos to 29°C, however, resulted in a less severe mutant phenotype comprising a smaller head hole, distinct from other defects in the dorsal surface, and a less severe defect in germband retraction (Fig. 1D). In addition, these embryos consistently displayed creases or ‘‘puckers’’ in the dorsal surface, which together with the mild germband retraction defect, gave them a bowed appearance. Approximately 52% of Egfr embryos were scored as having this bowed phenotype in a temperature shift experiment where a 2.5-hour collection of embryos from the Egfr stock was aged at 18°C for 12 hours and then shifted to 29°C (corresponding to late stage 10/stage 11 at the time of the temperature shift). Of these bowed embryos, half exhibited an additional phenotype of a hole or scab in the dorsal surface (Fig. 1D). Few defects were seen in the dorsal surface of Egfr embryos when they were shifted to 29°C at stage 12 or later (data not shown).

A second approach for facilitating the analysis of DC defects in Egfr-deficient embryos, which also permitted characterization of the tissue specificity of Egfr function, involved the inducible expression of a dominant negative version of Egfr, Egfr. Egfr competes with endogenous Egfr for ligand binding, but lacks the cytoplasmic domain, which contains the tyrosine kinase domain necessary for trans-phosphorylation and receptor activation [39]. As a result, Egfr attenuates activation of the Egfr cascade in a cell autonomous manner. We expressed a UAS-Egfr transgene in various spatial patterns, starting with general epidermal expression and epidermal stripes using the 69B-Gal4 and ptc-Gal4 drivers, respectively [40,41]. Both patterns of Egfr expression resulted in bowed embryos with occasional dorsal holes or scabs, similar to the temperature shift phenotype (Fig. 1E, F). 100% of embryos in which Egfr was expressed with ptc-Gal4 were bowed and 10% had a dorsal hole or scab. The dorsal epidermis plays an important role in DC and we impaired Egfr function in this tissue by expressing Egfr using the LE-Gal4 driver, which is active only during DC primarily within a subset of cells in the first two rows of dorsal epidermal cells flanking the amnioserosa (Fig. 1G, H, and Movie S1) [13]. This resulted in a bowed embryo phenotype in about a quarter of Egfr-expressing embryos (Fig. 1I). Bowed embryos and dorsal holes were also seen when Egfr activity was blocked in the AS using the AS-specific driver Gal4 [42] (Fig. 1J). We conclude that the normal progression of DC requires Egfr signaling in both the epidermis and the AS.

A major route for signaling by Egfr is the Raf-MAPK pathway, the activation of which can be detected using anti-phospho-MAPK antibodies [43,44]. We observed strong phospho-MAPK immunoreactivity in the central AS cells of wild-type embryos, but little staining in cells of the AS periphery or in the dorsal epidermis (Fig. 1K, K). This result suggests that either Egfr is not using the MAPK pathway in these cells or that the pathway is under tight negative control. Consistent with Egfr signaling generating phospho-MAPK in the AS, the anti-phospho-MAPK immunore-
Figure 1. Embryos with either global or local loss of Egfr signaling have defects in epithelial morphogenesis. (A–F, I, J) Cuticle preparations. Black arrows, posterior spiracles; black arrowheads, puckers in cuticle; white arrowheads, dorsal hole or scab. (G, H, K, K) Confocal micrographs of embryos during dorsal closure (DC). (A) Dorsolateral view of wild-type embryo showing intact dorsal surface. (B) Egfr\textsuperscript{f2}/Egfr\textsuperscript{f2C82} embryo, selected by absence of GFP balancer chromosome, showing ''curled up'' phenotype. (C) Egfr\textsuperscript{f2}/Egfr\textsuperscript{f2C82} embryo showing large dorsal hole. (D) Embryo from temperature-sensitive Egfr\textsuperscript{F26} stock that had been allowed to develop at 18°C before shifting to restrictive temperature of 29°C at about stage 10/11. Embryo has a bowed appearance characterized by pulling in of tail (marked by posterior spiracles) and puckering of cuticle. There is a small dorsal hole in the cuticle. (E) Embryo in which Egfr\textsuperscript{DN} had been expressed in the epidermis using the 69B\textendash Gal4 driver showing dorsal scab and mild bowing. (F) Embryo in which Egfr\textsuperscript{DN} had been expressed in the epidermis using the ptc\textendash Gal4 driver showing bowed appearance and dorsal hole, similar to the embryo in panel D. (G, H) Still images from Movie S1 showing restricted expression pattern of LE\textendash Gal4 driver, revealed using a UAS\textendash GFP-NLS reporter. Cell outlines were revealed through expression of a Ubi\textendash DEcadherin-GFP transgene. (G) LE\textendash Gal4 is not expressed at beginning of DC. (H) Midway through DC, GFP-NLS is expressed in the first two rows of cells flanking the amnioserosa (AS), visualized as GFP signal in nuclei. (I) Embryo
activity in the AS was absent in embryos in which Egfr\textsuperscript{DN} was expressed with Gal\textsuperscript{F-231} (data not shown).

We extended our analysis of Egfr function in DC by live imaging embryos homozygous or heteroallelic for the alleles Egfr\textsuperscript{DD} [37], Egfr\textsuperscript{1154} and Egfr\textsuperscript{1155} [38] and carrying a Ubi-DEcadherin-GFP transgene to visualize cell outlines [45]. At least four movies were taken for each genotype and very consistent phenotypes were observed. Prior to the initiation of germband retraction, degradation of the AS commenced in Egfr mutant embryos, which in the most severe cases led to a complete and dramatic loss of the tissue (Compare Movie S2 to Movies S3 and S4, and Fig. 2A, B to Fig. 2C, D and Fig. 2 E, F). In some embryos the AS persisted throughout germband retraction and DC, but had noticeably fewer cells than wild-type (Movie S3 and Fig. 2G, H, M–P). In such embryos germband retraction proceeded to a point and then appeared to reverse, with the posterior end of the embryo moving anteriorly. This was a bunched-up of the epidermis characterized by inappropriate adhesion between the dorsal end of non-adjacent segments on the same side of the embryo producing (arrowhead in Fig. 2H). In addition, AS morphogenesis was abnormal, with the tissue constricting perpendicularly to the normal anterior-posterior direction (Compare Movie S5 to Movie S6 and Fig. 2I–L to Fig. 2M–P). Finally, heads of Egfr mutant embryos exhibited a precipitous loss of epidermal integrity during embryogenesis with the brain becoming exposed during DC and pushing toward the posterior end of the embryo (Fig. 2G, H, Movie S5).

We also assessed morphogenesis in fixed embryos by staining with an antibody against phosphotyrosine to reveal cell outlines. Heteroallelic Egfr mutant embryos showed highly variable cell shape change of the DME cells compared to wild-type, suggestive of misregulated actomyosin contractility (Fig. 2Q, R). We suspect that this uneven contractility in the dorsal epidermis underlies the bunched-up of the segments in Egfr mutant embryos, and consistent with this expression of Egfr\textsuperscript{DN} with LE-Gal\textsuperscript{4} led to segmental bunching (Fig. 2S).

Egfr Negatively Regulates dpp Expression in the Epidermis During DC

The bowed embryo phenotype associated with reductions in Egfr function is similar to the cuticle phenotype of embryos in which Dpp is ectopically expressed in the dorsal epidermis [17,46–48]. Furthermore, crosstalk between Egfr and Dpp/TGF\textbeta\textsubscript{B} signaling in the form of antagonistic or cooperative interactions has been reported for a number of developmental events [49–82]. We, therefore, next examined dpp expression in embryos having altered Egfr function. We confirmed a previously published observation that ectopic dpp expression extends ventrally along the segmental grooves of Egfr mutant embryos [35], and saw a similar pattern of dpp expression in embryos in which Egfr signaling was attenuated in the epidermis via UAS-Egfr\textsuperscript{DN} expression using 69B-Gal\textsuperscript{4} or LE-Gal\textsuperscript{4} drivers (Fig. 3C–F, arrowheads). The phenocopy of Egfr loss-of-function mutants by UAS-Egfr\textsuperscript{DN} expression was underscored by the fact that widespread epidermal expression of this transgene resulted in a reduction in the separation, from one side of the embryo to the other, between the ventral stripes of dpp expression as previously reported for Egfr and D\textsuperscript{af} mutant embryos [35,83]. This decrease in separation allowed the ventral stripe on the other side of the embryo to be seen in a lateral view (arrows in Fig. 3C, E). Given the many studies indicating communication between the AS and epidermis during DC [10,13,25,26,84–89] and the requirement for Egfr in the AS during DC, we looked at dpp expression in embryos in which Egfr\textsuperscript{DN} had been expressed in the AS using Gal\textsuperscript{F-231}, but found no effect (data not shown).

To examine the effects of excessive Egfr signaling on dpp expression we used two transgenes, UAS-sp\textit{p} and UAS-Egfr-EGFP. UAS-sp\textit{p} encodes a secreted, active version of the Egfr ligand, Spitz, which can directly bind to Egfr to activate the Egfr pathway, whereas UAS-Egfr-EGFP encodes a biologically active Egfr tagged with enhanced green fluorescent protein (EGFP) [90,91]. These transgenes were expressed in stripes in the embryo using ptc-Gal\textsuperscript{4} and effects on dpp expression assessed by in situ hybridization. For both transgenes, the dpp expression stripes in the dorsal and ventrolateral epidermis became fragmented (Fig. 3G, H). Staining with anti-GFP antibodies revealed that remaining patches of dpp expression were in areas where Egfr-EGFP had not been expressed (Fig. 3H, H). We conclude from our loss- and gain-of-function studies that Egfr signaling negatively regulates dpp expression in the epidermis during DC.

Egfr Negatively Regulates zip Expression in the Epidermis and AS During DC

An important target of Dpp regulation during DC is zip, and we evaluated zip expression in Egfr mutant embryos and embryos with tissue-specific attenuation of Egfr signaling. zip shows two major events of transcriptional upregulation that are relevant to the morphogenetic events during DC: first, a burst of expression occurs in the AS during germband retraction and terminates around the beginning of DC; and, second, upregulation occurs in the DME cells beginning during the germband retraction stage and persists throughout DC [12,24,26] (Fig. 4A). Egfr mutant embryos showed excessive accumulation of zip transcripts in the DME cells, in addition to some ectopic zip transcription in the epidermis (Fig. 4B). We occasionally found less severely disrupted Egfr mutant embryos where the AS was intact; in these zip transcripts persisted in the AS during DC, in contrast to wild-type embryos where the AS was devoid of transcripts by this stage (compare Fig. 4C with Fig. 4A). Reduction of Egfr function in the epidermis through expression of UAS-Egfr\textsuperscript{DN} using the LE- and 69B-Gal\textsuperscript{4} drivers also caused excessive epidermal zip expression (data not shown). We previously demonstrated that the tyrosine kinase Ack, a putative negative regulator of Egfr, controls zip levels in the AS [26]; we, therefore, also tested the effect of disrupting Egfr specifically in this tissue by expressing UAS-Egfr\textsuperscript{DN} using the AS-specific Gal\textsuperscript{F-231} driver. In Gal\textsuperscript{F-231} > UAS-Egfr\textsuperscript{DN} embryos we observed ectopic zip expression throughout the AS during DC as well as elevated zip levels in the head, the latter indicating some degree of cell non-autonomous control of zip by Egfr (Fig. 4D).

A major downstream effector for Egfr is Ras and expression of a dominant negative Ras transgene, Ras\textit{N17} [92], in the AS was found to be associated with a similar increase in zip transcript levels (Fig. 4E). Given the robust levels of zip transcripts normally seen in the DME cells, we found it difficult to ascertain if knockdown of Egfr in the AS affected zip expression in the DME cells, but we suspect that it caused a modest elevation (Fig. 4D). To
determine if excessive Egfr signaling in the AS would have the opposite effect on zip expression, we expressed the Egfr-EGFP transgene with Gal4c381 (Fig. 4F). zip levels in the DME cells were decreased in association with excessive Egfr signaling in the AS and we conclude that Egfr represses zip transcription in a cell non-autonomous manner during DC.

Egfr Inhibits Apoptosis in the AS

The similarity of Egfr mutant embryonic phenotypes to those of the U-shaped group of genes, which are required for maintenance of the AS, has been noted and studies on fixed preparations suggest premature apoptosis in Egfr mutant embryos [35,93,94]. Apoptosis of the AS cells contributes to the forces driving DC, and negative regulation of cell death in the AS could be an important component of the participation of Egfr in DC [2,3]. As described above, AS cells are lost prematurely in Egfr mutant embryos (Fig. 2D, F) suggesting an early onset of apoptosis. To visualize the effects of losses or gains of Egfr signaling on AS apoptosis in live embryos, we used the caspase sensor Apoliner [97,98]. Apoliner consists of a monomeric red fluorescent protein (RFP) tethered to EGFP by a caspase-sensitive linker [97]. Furthermore, the design of the Apoliner construct includes a transmembrane domain that precedes the RFP component while the EGFP component includes a nuclear localization signal (NLS). As a consequence, the two fluorophores co-localize to membranes in live cells lacking caspase activity, but caspase activation in live cells results in separation of the fluorophores, with Apoliner-RFP remaining at membranes while Apoliner-EGFP is translocated to the nucleus due to its NLS. At the beginning of germband retraction in wild-type embryos there was little nuclear EGFP in the AS, indicating minimal caspase activity [95]. Furthermore, Ras appears to negatively regulate apoptosis throughout the embryo, as revealed by acridine orange staining of embryos with global gains or losses of Ras signaling [96]. As described above, AS cells are lost prematurely in Egfr mutant embryos (Fig. 2D, F) suggesting an early onset of apoptosis. To visualize the effects of losses or gains of Egfr signaling on AS apoptosis in live embryos, we used the caspase sensor Apoliner [97,98]. Apoliner consists of a monomeric red fluorescent protein (RFP) tethered to EGFP by a caspase-sensitive linker [97]. Furthermore, the design of the Apoliner construct includes a transmembrane domain that precedes the RFP component while the EGFP component includes a nuclear localization signal (NLS). As a consequence, the two fluorophores co-localize to membranes in live cells lacking caspase activity, but caspase activation in live cells results in separation of the fluorophores, with Apoliner-RFP remaining at membranes while Apoliner-EGFP is translocated to the nucleus due to its NLS. At the beginning of germband retraction in wild-type embryos there was little nuclear EGFP in the AS, indicating minimal caspase activity.
Figure 3. dpp transcription is repressed by Egfr signaling during DC. Panels A, C, E and F are digoxigenin in situ hybridizations and panels B, D and H–H are FISH, with all embryos at beginning of DC. (A, B) Wild-type embryos showing horizontal dorsal and ventrolateral stripes of dpp expression. The dorsal stripe is dpp expression in the DME cells. (C, D) Egfr\textsuperscript{f2} embryo (C) and Egfr\textsuperscript{f2}/Egfr\textsuperscript{CR2} embryo (D) showing ectopic dpp expression ventral to the DME cells (arrowheads). Arrow in (C) shows ventrolateral stripe visible on other side of embryo due to decreased distance between stripes compared to wild-type. (E) Embryo in which Egfr\textsuperscript{DN} had been expressed in the epidermis using the 69B-Gal4 driver showing ectopic dpp expression (arrowhead). Arrow shows ventrolateral stripe visible on other side of embryo. (F) Embryo in which Egfr\textsuperscript{DN} had been expressed using the LE-Gal4 driver showing elevated dpp expression in the dorsal epidermis (arrowhead). (G, H–H) Increasing EGFR signaling by expression of ssPi (G) or Egfr-EGFP (H–H) in vertical stripes using the ptc-Gal4 driver causes breaks in the dorsal and ventrolateral dpp stripes. Anti-GFP staining (H, H) reveals the expression pattern of Egfr-EGFP. Note that remnants of dpp expression (arrowheads in H–H) are seen where Egfr-EGFP was not expressed. Scale bar: 50 μm.
doi:10.1371/journal.pone.0060180.g003
Figure 5. A–A), but there was strong nuclear EGFP in the AS of Egfr
mutant embryos at the same stage (Fig. 5 B–B). As DC proceeded
nuclear EGFP accumulated in the AS of wild-type embryos (Fig. 5C–C) and this accumulation could be blocked by expression
of the baculovirus caspase inhibitor p35 [99] (Fig. 5D–D). To
promote Egfr signaling in the AS, we expressed either sSpi or
RasV12 and found that, in both cases, cells showed little nuclear
EGFP even late in DC, similar to what was seen with p35
expression (Fig. 5E–F). We conclude that Egfr signaling inhibits
caspase activation in the AS. If Egfr impedes apoptosis in the AS,
then excessive Egfr signaling might be expected to affect AS
morphogenesis. A robust increase in Egfr levels in the AS through
expression of Egfr-EGFP using the double driver combination
Gal4NP3312 + GAL4NP5328 resulted in a failure of the AS to properly
complete morphogenesis (compare Fig. 5G to Fig. 5H, I and
Movie S7 to Movies S8 and S9). In addition, the AS of these
embryos persisted beyond the normal time of AS programmed cell
death.

Evidence that Ack and Endocytosis Negatively Regulate
Egfr Levels in the AS
An important route through which Egfr signaling is down
regulated is by clathrin-mediated endocytosis (reviewed in [100]).
When imaging Egfr-EGFP in the AS for the apoptosis study, we
noticed that in addition to localizing cortically in AS cells, much of
the protein appeared to be accumulating in vesicles (Fig. 6F).
Given the literature demonstrating that Ack family tyrosine kinases
promote down regulation of Egfr by endocytosis and subsequent
degradation [29–32], we looked for evidence that AS Ack was
controlling zip expression through down regulation of Egfr in this
tissue. Over-expression of Ack in the AS during germband
retraction causes a dramatic increase in zip levels in this tissue
[26](Compare Fig. 6A to Fig. 6B), but co-expression with Egfr-
EGFP (but not a control lacZ transgene) restored wild-type
zip levels, suggesting that Ack controls zip through down regulation of
Egfr (Fig. 6C, D). We subsequently over-expressed Ack in prd
stripes in the AS and examined the effect on Egfr distribution by
comparison with adjacent amnioserosa cells not over-expressing
Ack. AS cells with endogenous levels of Ack showed strong cortical
Egfr immunostaining as well as staining in cytoplasmic puncta,
some of which were Rab5 positive and therefore early endosomes
(Fig. 6E–E). In Ack-over-expressing cells (identified by increased
levels of phosphotyrosine [101]) there was a decrease in cortical
Egfr staining and an increase in Egfr-positive cytoplasmic puncta,
with some of these being Rab5-positive; these cells also showed
a general increase in the levels of early endosomes (Fig. 6E–E).
Many of the Egfr-positive puncta in these cells were Rab5-negative
and we suspect that they may be multivesicular bodies, where

Figure 4. zip transcription is repressed by Egfr signaling during DC. zip FISH on embryos at beginning of DC. (A) Wild-type embryo showing
high levels of zip transcription in DME cells and absence of zip expression in the AS. Prior to completion of germband retraction there are high levels
of zip in the AS of wild-type embryos (see Fig. 6A). (B) Egfrf2 embryo showing intense zip signal in DME cells and ectopic zip expression (arrowheads).
(C) Mildly affected Egfrf2 embryo showing modest retention of zip in AS. (D, E) Embryos in which Egfr signaling had been impaired in the AS by
expression of either EgfrDN (D) or RasN17 (E) showing significant retention of zip in AS, modest elevation of zip expression in the DME cells and ectopic
zip transcripts in the head. (F) Elevation of Egfr signaling in the AS through expression of Egfr-EGFP causes down-regulation of zip expression in DME
cells. Scale bar: 50 µm.
doi:10.1371/journal.pone.0060180.g004
endocytosed Egfr is known to accumulate (reviewed in [100]). The increase in early endosomes in Ack-over-expressing cells indicates that Ack over-expression leads to a general increase in endocytosis in AS cells. In support of this is an observation we made when trying to observe the effects of Ack on apoptosis using the Apoliner reporter. Here, with the expression of kinase-dead Ack, which is more effective than wild-type Ack at inducing zip expression [26], a highly punctate distribution of membrane-localized Apoliner-RFP signal was observed while control embryos at the same stage of DC displayed a typical homogeneous distribution (Fig. 6G, H). We interpret this difference as reflecting a general increase in intracellular vesicular traffic, consistent with the effect of Ack over-expression in promoting Egfr endocytosis.

Discussion

We have determined that Egfr is required in both the AS and epidermis for DC to proceed normally and our results suggest that Egfr signaling has a least three distinct roles in DC, all of which act to repress morphogenesis (see model in Fig. 7). Egfr is a negative regulator of dpp expression in the epidermis as loss of Egfr function in either Egfr mutant embryos or as a result of EgfrDf expression leads to ectopic dpp expression. dpp is expressed in two stripes during DC, one composed of the DME cells and the other running along the ventrolateral epidermis, where dpp expression in the DME cells, but not the ventrolateral stripe, is dependent on a JNK MAPK cascade [13–18]. Consistent with the notion that Egfr functions as a negative regulator of dpp expression, activation of the Egfr pathway can repress dpp expression in either stripe. The down regulation of dpp expression in both stripes, however, supports the view that Egfr does not reduce dpp transcription by impacting the JNK pathway, in which case we would expect to observe down regulation of dpp expression only in the DME stripe.

How might Egfr signaling be regulating dpp expression? Wingless (Wg) is a diffusible signal required for proper dpp expression in both the dorsal and ventrolateral stripes during DC [102,103]. Egfr negatively regulates Wg levels in the eye imaginal disc by transcriptionally regulating phyllopod [104]; we looked for evidence that Egfr might be controlling dpp expression through Wg, but altering Egfr signaling had no discernable effect on phyllopod transcription or Wg distribution in the embryo (X. C., unpublished results).

Figure 5. Egfr inhibits apoptosis and morphogenesis in the AS. (A–F) Apoliner signals in the AS. Apoliner reporter had been expressed either globally with the tub-Gal4 driver or in the AS using the LP1-Gal4 driver. For each embryo RFP, EGFP signals and merge are shown. On the right side of each panel is a higher power view of AS cells. In the absence of caspase activity, RFP and EGFP co-localize at various membranes and there is little EGFP signal in the nucleus. In the presence of caspase activity, EGFP is cleaved away from RFP and moves into the nucleus. (A–A) AS of wild-type embryo prior to germband retraction showing co-localization of RFP and EGFP signals and weak EGFP signals in the nucleus. (B–B) AS of Egfr mutant embryo prior to germband retraction showing strong EGFP signals in the nucleus. (C–C) AS of wild-type embryo during DC showing strong EGFP signals in the nucleus. (D–D) AS of p35-expressing embryo during DC showing weak EGFP signals in the nucleus. (E–E) AS of sspi-expressing-expressing embryo during DC showing weak EGFP signals in the nucleus. (F–F) AS of RasV12-expressing embryo during DC showing weak EGFP signals in the nucleus. (G) Still from Movie S7 showing AS of stage 15 wild-type embryo in which GFP had been expressed with the Gal4NP3312 AS driver, showing narrow, tube-like AS. (H, I) Stills from Movies S8 (H) and S9 (I) showing AS of stage 15 embryos in which Egfr-EGFP and GFP-NLS had been expressed with the double driver combination Gal4NP3312+Gal4NP5328 showing failure of AS morphogenesis. The AS in panel H has failed to narrow throughout while that in panel I has failed to narrow at the anterior end. Scale bars: 50 μm (A–B); 10 μm (C–I).

doi:10.1371/journal.pone.0060180.g005
The defects in morphogenesis seen in embryos with impaired Egfr signaling are likely at least in part due to misregulated actomyosin contractility. A recurring theme associated with various circumstances of Egfr impairment is the bowed embryo phenotype, where segments are bunched together at the leading edge of the epidermis during DC. We suggest this is due to uneven actomyosin contractility in the dorsal epidermis associated with excessive zip expression. The loss of epithelial integrity or “pulling apart” of the head seen in live imaging of Egfr mutant embryos may similarly be due to misregulated actomyosin contractility and/or excessive apoptosis. Previous work has indicated that a major function for Egfr in the head is inhibition of apoptosis, similar to its role in the AS [74,105,106]. Parallels between the regulation of head involution and DC have been noted and Egfr may function to modulate these two morphogenetic events through similar mechanisms [107].

While it is likely that increased levels of Dpp in Egfr mutant embryos contribute to the elevated zip levels, our results of manipulating Egfr signaling support the interpretation of a separate route for zip regulation that involves signaling from the AS to both the AS and the epidermis. This signaling is not operating through the regulation of dpp expression as impairment of Egfr signaling in the AS does not affect Dpp levels. Thus, we consider this zip regulation a second distinct role for Egfr in DC and we believe this

Figure 6. Evidence that Egfr signaling is negatively regulated by endocytosis in the AS. (A–D) zip FISH on embryos late in germband retraction. (A) Wild-type embryo showing zip expression in AS. (B) Expression of Ack in the AS using the Gal4c381 driver causes an increase in zip levels in this tissue relative to wild-type. (C) Ack fails to elevate zip levels when co-expressed with Egfr–EGFP. (D) zip levels are elevated when Ack is co-expressed with control lacZ gene. (E–E) AS in which Ack had been over-expressed in prd stripes, triple-stained with anti-phosphotyrosine (anti-PY) (E), anti-Egfr (E) and anti-Rab5 (E). (E) Cells over-expressing Ack are marked by high levels of anti-PY (outlined with dotted lines). (E) Egfr shows strong cortical localization in wild-type AS cells but a more cytoplasmic distribution in Ack-over-expressing cells. (E) There is an increase in Rab5-positive early endosomes in Ack-over-expressing cells. (E) Merge of panels E and E. Arrowheads and arrows mark Egfr-positive early endosomes in wild-type cells and Ack-over-expressing cells, respectively. (F) Egfr-EGFP expressed in the AS using the Gal4NP3312 driver shows vesicular accumulation in addition to being at the plasma membrane. (G) AS cells in embryo in which Apoliner has been expressed with LP1-Gal4 driver showing localization of Apoliner-RFP signal to membranes. (H) AS cells in embryo in which Apoliner and kinase-dead Ack have been co-expressed with LP1-Gal4 driver showing punctate localization of Apoliner-RFP signal. Scale bars: 50 μm in A–D; 5 μm in E-H.

doi:10.1371/journal.pone.0060180.g006
signaling is the same as that regulated by Ack in its control of zip expression. Consistent with this, gains or losses of Ack do not affect the Dpp pathway, supporting the view that Ack operates in parallel to Dpp signaling [26,101]. The Ack/Egfr-regulated signal could be a diffusible ligand ("X" in Fig. 7) produced in the AS cells that activates a pathway in the AS and DME cells where it activates a pathway promoting transcription of myosin from the zip locus. Previous work from our group and others, and unpublished results from our group, suggest that Dpp from the DME cells diffuses to the AS where it regulates production of a second diffusible signal "Y" providing a parallel input into zip transcription. Myosin produced through the cooperation of the two pathways then drives morphogenesis of the AS and DME cells. Egfr additionally regulates this signaling network by negatively regulating dpp transcription in the epidermis, including the DME cells. Egfr further regulates AS morphogenesis by inhibiting apoptosis in this tissue.

doi:10.1371/journal.pone.0060180.g007

Figure 7. Model for Egfr acting as a brake on DC. Egfr negatively regulates the production and/or secretion of a diffusible signal "X" in the AS (AS) and is itself negatively regulated by Ack through endocytosis. "X" signals into both the AS and the DME cells where it activates a pathway promoting transcription of myosin from the zip locus. Previous work from our group and others, and unpublished results from our group, suggest that Dpp from the DME cells diffuses to the AS where it regulates production of a second diffusible signal "Y" providing a parallel input into zip transcription. Myosin produced through the cooperation of the two pathways then drives morphogenesis of the AS and DME cells. Egfr additionally regulates this signaling network by negatively regulating dpp transcription in the epidermis, including the DME cells. Egfr further regulates AS morphogenesis by inhibiting apoptosis in this tissue.

A third major role for Egfr in DC is as a negative regulator of apoptosis in the AS. Enhancement of apoptosis accelerates DC whereas suppression of apoptosis slows it, indicating that apoptosis, similar to actomyosin contractility, provides a force for morphogenesis [3]. Thus, down regulation of Egfr in the AS during DC provides two means to accelerate the process: increased myosin expression and increased cell death. The "tweaking" of Egfr function in the AS could constitute an important regulatory mechanism for controlling the rate of closure. We have provided evidence that endocytosis, promoted by Ack, is a route by which Egfr signaling is controlled in the AS cells. Our results suggest that Ack would have a pro-apoptotic role in the AS through promotion of Egfr endocytosis. This in contrast to the Drosophila eye in which Ack has an anti-apoptotic function that is independent of Egfr [108].

A recent study has demonstrated that endocytosis in the AS is required for its correct morphogenesis during DC, but this work focused on the role of endocytosis in removing membrane to promote cell shape change [109]. Our results indicate that another route of action for endocytosis in the AS is in regulation of Egfr signaling. It has been suggested that endocytosis could act as a rheostat in which membrane area is adjusted in response to actomyosin contractility [109]; such a rheostat could also be used to adjust Egfr signaling throughout DC.

Additional avenues for Egfr regulation during DC could be control of ligands binding to Egfr and feedback inhibition [110,111], but we have yet to address these. In summary, we have identified Egfr signaling as an inhibitor of morphogenesis during DC that acts at several distinct levels. Having a single pathway control multiple aspects of this complex process may simplify feedback regulation, ensuring that morphogenesis occurs in a coordinated fashion. In essence, Egfr signaling acts as a brake that can be applied when required to ensure that closure proceeds smoothly and without loss of epidermal integrity. DC shows striking parallels to the healing of induced wounds in the Drosophila embryo, with the two processes using similar cytoskeletal and signaling machineries [112–114]. Egfr has recently been shown to be required for healing of induced wounds in the embryo and it will be of interest to determine if it uses similar routes of action in this as we have shown in DC [115].

Materials and Methods

Fly STOCKS

Egfr<sup>1C82</sup> and Egfr<sup>1F26</sup> were gifts from T. Schupbach, UAS-Egfr-

EGFP from J. Duffy, UAS-sSp from B. Shilo, UAS-RasN17 from T. Lee, LE-Gal4 from S. Noselli, Ubi-DEcadherin-GFP from H. Oda, LP1-Gal4 from G. Morata, Gal4<sup>NP2528</sup> and Gal4<sup>NP3312</sup> from the Kyoto Drosophila Resource Center and UAS-Apoliner and tub-

Apoliner from P.L. Bardet. Egfr<sup>1C82</sup> and Egfr<sup>1F26</sup> were isolated from a collection of EMS-mutagenized second chromosomes [30]. cn<sup>+</sup> Egfr<sup>bw</sup> sp<sup>1</sup> (CyO) flies and all other stocks were obtained from the Bloomington Drosophila Stock Center. Crosses were performed at 25°C.

Cuticle Preparations

Cuticles were prepared as described but with the fixation step removed [116]. At least 100 embryos were examined in each experiment.

Immunohistochemistry and RNA in Situ Hybridization

Fixing and antibody staining of embryos were done as previously described [117,118]. The following primary antibodies were used: mouse anti-phosphotyrosine (Cell Signaling;1:1000),

PLOS ONE | www.plosone.org 10 April 2013 | Volume 8 | Issue 4 | e60180
rabit anti-GFP (1:500), mouse anti-GFP (1:500), goat anti-Egfr (Santa Cruz 1:5), rabbit anti-phospho-MAPK (Cell Signaling 1:50) and rabbit anti-Rab5 (Abcam 1:1000). All secondary antibodies were from Vector Laboratories and used at a 1:200 dilution. In situ mRNA hybridizations using digoxigenin-labeled RNA probes and FISH were performed as described [119,120]. cDNAs for in situ hybridization probes were obtained from the Canadian Drosophila Microarray Centre. Fluorescently-stained embryos were examined on either a Zeiss LSM 410 laser-scanning confocal microscope or a Quorum spinning disk confocal microscope, and digoxigenin-labeled embryos imaged using a Zeiss Axioplan 2 microscope. Images were processed in Adobe Photoshop. The genotypes of all fluorescently-stained embryos were established by tracking balancer chromosomes bearing GFP reporters.

Live Imaging of Embryos

Chromosomes carrying Ubi-DEcadherin-GFP in combination with the alleles Egfrf2, EgfrH25, and Egfr1a15 were recovered by mictic recombination. Similarly, the tub-Apoliner insertion, which expresses Apoliner under the control of the tubulin 1 α promoter (described in [97]), was recombined with Egfrf2. All recombinant chromosomes were maintained over so-called “GFP-balancer” chromosomes obtained from the Bloomington Drosophila Stock Center. Each CyO, P[w/+mc] = GAL4-kr.C, P[wUAS-GFP,S65T]/DC7, CyO, P[w/+mc] = GAL4-tux.G]2,2, P(UAS-2×EGFP)AH2.2. For live imaging experiments Egfr mutant embryos were unambiguously identified as those lacking GFP expression derived from the GFP-balancer. Since the onset of the Egfr mutant phenotype preceded the timing of GFP expression associated with either GFP-balancer stock, the selection of embryos for live imaging was random and identification of mutant embryos was achieved subsequent to image acquisition. The early onset of the Egfr mutant phenotype also preceded the time at which all available amnioserosa specific GAL4 drivers could induce reporter gene expression, and for this reason our analysis of caspase activity in Egfr mutant embryos required using tub-Apoliner carried by the maternal parent.

Embryos were prepared for live imaging using the hanging drop protocol, which eliminates effects of compression on the mounted embryo [121]. Time-lapse confocal microscopy was performed using a 20X Plan Apo VC objective on a Nikon Eclipse 90 i microscope with a Nikon D-eclipse C1 scan head. Images were saved as animated projections using the Nikon EZ-C1 3.70 software and further processed using ImageJ (NIH).

Supporting Information

Movie S1 Time-lapse showing restricted expression pattern of LE-Gal4 driver, revealed using a UAS-GFP-NLS reporter. Cell outlines were revealed through expression of a Ubi-DEcadherin-GFP transgene.

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Author Contributions

Conceived and designed the experiments: NH BR. Performed the experiments: WS XC DC BR. Analyzed the data: NH WS XC BR. Wrote the paper: NH BR.
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