The Drosophila Arf GEF Steppke controls MAPK activation in EGFR signaling

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Summary
Guanine nucleotide exchange factors (GEFs) of the cytohesin protein family are regulators of GDP/GTP exchange for members of the ADP ribosylation factor (Arf) of small GTPases. They have been identified as modulators of various receptor tyrosine kinase signaling pathways including the insulin, the vascular epidermal growth factor (VEGF) and the epidermal growth factor (EGF) pathways. These pathways control many cellular functions, including cell proliferation and differentiation, and their misregulation is often associated with cancerogenesis. In vivo studies on cytohesins using genetic loss of function alleles are lacking, however, since knockout mouse models are not available yet. We have recently identified mutants for the single cytohesin Steppke (Step) in Drosophila and we could demonstrate an essential role of Step in the insulin signaling cascade. In the present study, we provide in vivo evidence for a role of Step in EGFR signaling during wing and eye development. By analyzing step mutants, transgenic RNA interference (RNAi) and overexpression lines for tissue specific as well as clonal analysis, we found that Step acts downstream of the EGFR and is required for the activation of mitogen-activated protein kinase (MAPK) and the induction of EGFR target genes. We further demonstrate that step transcription is induced by EGFR signaling whereas it is negatively regulated by insulin signaling. Furthermore, genetic studies and biochemical analysis show that Step interacts with the Connector Enhancer of KSR (CNK). We propose that Step may be part of a larger signaling scaffold coordinating receptor tyrosine kinase dependent MAPK activation.

Key words: Arf GEF Steppke, Drosophila, EGFR signalling, MAP kinase, RAS

Introduction
The proper development of multicellular organisms requires the coordination of proliferation and differentiation, which is a particular challenge during the formation of the tissues and organs of the body. Numerous studies have shown that receptor tyrosine kinases such as the vascular growth factor receptor (VEGFR; Mannell et al., 2012) epidermal growth factor receptor (EGFR) and insulin/insulin-like growth factor receptors (InR/IGF-Rs) play prominent roles in signaling cell proliferation and differentiation (reviewed by Schlessinger, 2004). Misregulation of both pathways is often causative for tumor development and progression through their effects on uncontrolled cell growth, inhibition of apoptosis, angiogenesis, and tumor-associated inflammation (Witte et al., 2009; Alvarez et al., 2010; Antonarakis et al., 2010; Arkenau, 2009). Determining how growth and differentiation are coordinated by these pathways is thus essential to understanding normal development, as well as disease states such as cancer.

We have recently identified Steppke (Step) as a new and essential component of the insulin signaling pathway in Drosophila (Fuss et al., 2006). The insulin signaling cascade is conserved from flies to humans and was shown to regulate cell and organismal growth in response to extrinsic signals such as growth factors and nutrient availability. In Drosophila, activation of a unique insulin-like receptor (InR) stimulates a conserved downstream cascade that includes the Phosphatidylinositol-3-kinase (PI3K) and Protein Kinase B (PKB or AKT). AKT is involved in enhancement of glucose absorption and glycogen synthesis, and regulates the activity of the Forkhead box O (FoxO) transcription factor, a negative regulator of cell growth (Teleman, 2010; Hafner et al., 2006). Step is a member of the cytohesin family of guanine nucleotide exchange factors (GEFs) which regulate small GTPases of the ADP-ribosylation factor (ARF) family (Kolanus, 2007). Small ARF GTPases are involved in the regulation of many cellular processes including vesicle transport, cell adhesion and migration. In mice, the IIS-dependent larval growth control, we now examined its function in the Drosophila wing, which develops from an epithelial sheet during larval and pupal stages (Bier, 2000; De Celis, 2003). The wing is an ectodermal structure formed by a dorsal and ventral epithelium, interspersed with cuticular ectodermal tubes, the so called wing veins. Stereotypical arrangement of wing veins is determined in the imaginal wing disc in late larval and pupal stages by several signaling pathways including the EGFR cascade (Martın-Blanco et al., 1999). EGFR activation by EGF-like ligands Spitz or Vein results in the
activation of the small GTPase RAS by its loading with guanosine triphosphate (GTP), which as a result triggers the activation of a number of downstream effector proteins including the Ser/Thr-kinase RAF [mitogen-activated protein kinase (MAPK) kinase kinase]. Once activated, RAF phosphorylates and activates MEK (MAPK kinase), which in turn phosphorylates and activates MAPK/ERK (Cobb and Goldsmith, 2000). Phosphorylated MAPK exerts its role in the cytoplasm as well as in the nucleus, where it controls expression of EGFR target genes like pointed (ptn), argos (aos), rhomboid (rho) and ventral nervous system defective (vnd) (Klämbt, 1993; Gabay et al., 1996; Wasserman and Freeman, 1998). The scaffolding protein connector enhancer of KSR (CNK) has been described to facilitate RAS/RAF/MAPK signaling by providing a protein scaffold at the plasma membrane that integrates Src and RAS activities to enhance RAF and MAPK activation (Clapérion and Therrien, 2007). EGFR/MAPK signaling is crucial quite early during wing vein differentiation, where phosphorylation of MAPK determines the positioning of proveins and later during development for maintenance of longitudinal veins (Blair, 2007). In addition to patterning, both EGFR/RAS/MAPK signaling and IIS control general cell proliferation and cell growth during wing development. Thus, EGFR/RAS/MAPK signaling controls both cell fate (vein versus intervein) and general cell proliferation along with IIS at similar times within the wing tissue.

Recent studies in human lung and breast adenocarcinoma cancer cell lines indicated a function of cytohesins in ErbB (EGFR) signaling, where they facilitate signaling by stabilizing an asymmetric ErbB receptor dimer (Bill et al., 2010). Here we provide the first in vivo model that the cytohesin Step, in addition to its previously characterized function as component of IIS, regulates EGFR signaling dependent wing growth and vein differentiation. Our genetic, immunohistochemical and biochemical experiments indicate that Step acts downstream of the EGF receptor in the EGFR signaling cascade and is necessary and sufficient for MAPK activation and the induction of EGFR target genes. Whereas step transcription is negatively regulated by IIS (Fuss et al., 2006), it is induced by EGFR signaling. We further provide evidences that Step might directly interact with the Connector Enhancer of KSR (CNK) protein that is part of a protein scaffold known to coordinate RAS-dependent RAF and MAPK signaling from tyrosine kinase receptors.

**Results**

The Step protein contains three characteristic motifs, a Sec7 domain responsible for the GEF activity, a coiled-coil domain (CC) mediating protein-protein interactions and a pleckstrin homology (PH) domain (Fig. 1A). The PH domain is required for plasma membrane recruitment via specific binding to phosphatidylinositol-3,4,5-trisphosphate, the second messenger generated by class I PI3Ks (Carnero, 2010). We previously studied the function of Step mainly during larval development where it is acting as a component of the IIS cascade controlling cell and organismal size (Fuss et al., 2006; Becker et al., 2010). Expression studies using an anti-Step antibody (see Materials and Methods) indicate, however, that step is also expressed in imaginal discs and during pupal and adult stages. In wing imaginal discs we found ubiquitous expression of *step* in a vesicle-like pattern with an accumulation of the protein at the cell cortex (Fig. 1B) consistent with previously described localization of the PH domain (Britton et al., 2002).

**Step is required for wing vein differentiation**

Homozygous *step* mutants are lethal at pupal stages (Fuss et al., 2006). Expression of *UAS-step* rescues hypomorphic *step* mutants to adulthood. Whereas this complete rescue requires a strong induction of *step* (Fig. 2C) a weak expression of *step* in homozygous *step* mutants leads to only a partial rescue including defects in wing vein differentiation (Fig. 2B).

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**Fig. 1. Step is expressed ubiquitously in imaginal wing discs.** (A) The cytohesin Steppke (Step) harbours three major domains: a catalytically active Sec7 domain (Sec7), a C-terminal pleckstrin homology (PH) domain and an N-terminal coiled-coiled (CC) domain. Protein fragment used for immunization (dashed line) and target sequence of *step* are indicated. Three UAS constructs were used in this study: a full length *step* version, a full length (FL) *step* construct with an N-terminal GFP tag and a variant lacking the PH domain. (B–B0) Third instar imaginal wing discs stained for E-Cadherin (B) and Step (B0). Step is expressed ubiquitously in imaginal wing discs and localizes in a vesicular pattern (see inset in B0) in close proximity to the cell cortex (marked by E-Cadherin). (C,D) Third instar imaginal wing discs stained for E-Cadherin (B) and Step (B0). Step antibody detects ectopic expression in the posterior imaginal wing disc. Please note that detection channel intensities are adjusted to avoid overexposure. (D–D0) Expression of *step* via *enGal4* leads to reduced Step protein levels in the posterior wing disc.
To complement the genetic analysis of step gene function (see Materials and Methods) based on hypomorphic step alleles and a step deficiency described previously (Fuss et al., 2006; Becker et al., 2010) we generated step RNA interference (RNAi) and overexpression lines enabling tissue specific as well as clonal step knockdown and gain of function experiments. As a driver line, we used engrailed-Gal4 (enGal4) which mediates expression in the posterior compartment of the wing, the anterior compartment serving as internal control (Tabata et al., 1995). UAS-stepRNAi-mediated knockdown of step in the posterior wing compartment results in a strong reduction of Step protein levels (Fig. 1D–D') and causes a reduction of the wing size.

**Fig. 2. Step is required for wing vein differentiation.** (A) Wild-type adult Drosophila wings contain five lateral wing veins (L1–L5) and two cross veins (anterior cross vein, acv; posterior cross vein, pcv). Yellow pseudocolouring indicates the Engrailed expression domain (Simpson, 2007). Anterior is up, distal is right. (B, C) Expression of step via hsFLP rescues wing vein defects of step+/step+ mutants. Depending on the duration of the heat shock, rescue is either partial (B) or complete (C). (D–K) enGal4 was used to express gene specific RNAi constructs or gain of function transgenes respectively in the posterior wing compartment (see yellow area in A) and to score for wing vein differentiation and growth in mutant clones (posterior compartment) versus unaffected control clones (anterior compartment). stepRNAi expression (D) in a wild-type background results in loss of the cross veins (arrows); in a haploinsufficient step deficiency background parts of L4 (asterisks) are lost as well (F). Full length step (E) but not stepDPH overexpression (G) leads to excess wing vein tissue (see arrows). Reduction of IIS by InR RNAi (H) leads to a decreased posterior wing area (see M for quantification); but wing vein tissue is not lost. Enhanced IIS by InR overexpression (I) results in increased wing area posterior from lateral vein L4 (for quantification see M); wing vein formation is not affected. A reduction of EGFR signaling via expression of EGF RNAi results in loss of wing veins (see arrows in J). acv and L4 are lost almost entirely. EGFR signaling gain of function by EGFR overexpression leads to additional wing vein tissue in the posterior wing (see asterisks in K). (L, M) The effect of step modulations on wing size was assessed by measuring the area of the posterior part of the wing (see yellow area in inset) relative to the total wing area. Yellow bar indicates size range of control wings. (L) step loss (stepRNAi) and gain of function (UAS-step) leads to an decrease in wing size; ectopic expression of a step variant lacking the pleckstrin homology (PH) domain (stepΔPH) does not affect wing size. (M) Activation of IIS by InR or dp110 expression results in significantly larger wings, whereas inhibition of IIS by InR RNAi decreases wing size significantly. Activation and inhibition of Egfr signaling (Egfr and Egfr RNAi expression) resulted in significantly smaller wings. n>20. Error bars indicate s.e.m. Statistical significance was analyzed by Student’s t-test. P-values are indicated.
(Fig. 2L) due to a reduction in cell number but not size (supplementary material Fig. S1). Furthermore, loss of wing vein tissue can be observed and both cross veins are not formed properly (Fig. 2D, compare with wild-type wing in Fig. 2A). Reducing step levels further by RNAi mediated knockdown in the background of a step heterozygous deficiency (step 0/+, Materials and Methods), the phenotype is enhanced and lateral vein L4 displays gaps in addition (Fig. 2F). In contrast, overexpression of step in the posterior wing compartment induces the formation of ectopic longitudinal and cross vein tissue and excess veins at the distal end of lateral vein L4 (Fig. 2E). Of note, overexpression of a step variant lacking the PH domain (stepPH) does not result in excess wing vein tissue (Fig. 2G), indicating that membrane recruitment of Step is essential for its function on vein differentiation.

The wing growth phenotypes observed upon modulation of step levels are similar to the effects observed when InsR is reduced (Fig. 2F,H,L,M) (Böhn et al., 1999; Brogliolo et al., 2001), consistent with an expected role of Step in IIS. However, wing vein defects or ectopic veins which are found upon step reduction or overexpression, respectively, cannot be explained by a role in IIS, since vein formation is not affected in wings expressing UAS-InsR or UAS-InsR(RNAi) (Fig. 2H,I). Indeed, they rather resemble EGFR-dependent phenotypes in the wing. Reducing EGFR levels by RNAi-mediated knockdown in the posterior compartment leads to loss of lateral vein L4 and the apical cross vein (Fig. 2J) (Martin-Blanco et al., 1999) and growth defects (Fig. 2M). Since expression of several transgenes activating EGFR signaling (like UAS-RasV12, UAS-Rafgof or the activated form of the EGFR) via eGal4 lead to early larval lethality, we made use of an UAS-EGFR constructs to mildly induce the EGFR pathway. Overexpression of the wild-type receptor results in mild EGFR signaling gain of function effects: excess wing vein tissue is formed between L4 and L5 (Fig. 2K). This effect is more severe but similar to phenotypes observed for Step modulation.

**Step induces EGFR target genes and is required for MAPK activation**

To further investigate a potential role of step in EGFR signaling, we analyzed the effect of step on the EGFR target genes argos (aos), pointed2 (pnt), and rhomboid (rho). All of these target genes were significantly reduced in hypomorphic step mutants (Fig. 3A) as well as wing discs expressing stepRNAi (Fig. 3B). EGFR target genes are induced in imaginal eye discs overexpressing step (Fig. 3C). To further study the impact of step on EGFR target gene expression, we analyzed Aos protein levels in imaginal wing discs and found enhanced Aos levels in clones expressing step (Fig. 3D–D'). Similarly, overexpression of step in the posterior compartment of an imaginal wing disc carrying the reporter aos-lacZ induces ectopic Argos expression in the posterior wing disc (Fig. 3E–E').

To test where Step may act in the EGFR cascade we analyzed MAPK activity in imaginal wing discs, in which step expression was reduced or elevated in the posterior wing compartment.

It is known that the EGFRs signals are transduced by a cascade that includes RAS, RAF and MEK, which dually phosphorylates MAPK directly to generate phosphory-MAPK (pMAPK) (reviewed by Wasserman and Freeman, 1997). pMAPK then disassociates from MEK and forms active homodimers that can phosphorylate targets in the cytoplasm, and/or translocate to the nucleus to activate nuclear targets, and thus controls gene expression (Brunet et al., 1999; Chen et al., 1992; Lenormand et al., 1998). To monitor MAPK activity, we made use of an antibody specific for the doubly phosphorylated form of MAPK (pMAPK).

Reduction of step levels causes an inhibition of MAPK phosphorylation (Fig. 3F–F') whereas overexpression of step in the posterior wing compartment results in elevated pMAPK levels (Fig. 3G–G'). Induction of EGFR signaling by EGFR overexpression in imaginal wing discs results in similarly increased pMAPK levels (supplementary material Fig. S2D–D'). Of note, expression of the InsR does not lead to increased pMAPK levels (supplementary material Fig. S2F–F'), indicating that MAPK activation does not occur in response to a Step dependent IIS function.

**Step acts downstream of the EGF receptor in the EGFR pathway**

To identify the level at which step is involved in with the EGFR pathway we tested whether step and several EGFR pathway components interact genetically, similar to approaches published recently (Gaengel and Mlodzik, 2003; Yoshida et al., 2004). Knocking down step transcript and protein levels in an EGFR(RNAi) background using eGall4 increases the effect of EGFR(RNAi) in the posterior compartment of the developing wing (compare Fig. 4A,A'). Lateral vein L4 was entirely lost, both cross veins were not differentiated and the distal end of lateral vein L5 was missing (see arrows in Fig. 4A). Similarly, stepRNAi enhances wing vein defects caused by reduction of RAS, RAF and MAPK transcript levels (compare Fig. 4B,B', Fig. 4C,C' and Fig. 4D,D'). Furthermore, UAS-step expression could rescue wing vein defects caused by EGFR(RNAi) (Fig. 4A') as well as it improves wing vein phenotypes due to RAS(RNAi) expression (Fig. 4B'). In contrast, wing vein differentiation defects caused by RAF(RNAi) or MAPK(RNAi) were not rescued upon UAS-step expression (Fig. 4C',D'). There results point to a function of Step between RAS and RAF in the EGFR pathway.

Similar to the genetic interaction of step and EGFR pathway components observed during wing vein development, we found step to genetically interact with the EGFR component Starr in another EGFR dependent process. An analysis of eye development in late step (0/+) pupae clearly shows a loss of photoreceptors in the step hypomorph (supplementary material Fig. S3A,A') which is also found in EGFR mutants (but not in mutants for the insulin receptor; McNeill et al., 2008). Flies heterozygote for the Starr allele display a very mild loss of photoreceptors differentiation phenotype (supplementary material Fig. S3A') which can be enhanced by removal of one copy of essential EGFR/ MAPK signaling components (Gaengel and Mlodzik, 2003). A similar phenotype enhancement occurs in flies transheterozygous for the step (0/+) and the Starr (0/+) allele: the mild defects of photoreceptor differentiation observed in heterozygous Starr (0/+) mutants (supplementary material Fig. S3B) are significantly increased in transheterozygosity with step (0/+) mutants (Starr(0/+) step(0/+) A; supplementary material Fig. S3C). The allelic combination Starr(0/+) InsR(05545/+); InsR(05545/+); step(0/+) does not lead to an enhanced phenotype, indicating an IIS independent function of step during photoreceptor development. Furthermore, Step function also mediates MAPK activation in context of eye development. A clonal reduction of step activity in imaginal eye discs via UAS-stepRNAi results in lowered pMAPK levels (supplementary material Fig. S3C,C'), whereas clonal overexpression of step leads to increased pMAPK levels (supplementary material Fig. S3D,D').
The observations on the role of Step in EGFR signaling during wing and eye development are consistent with reports obtained in mammalian cell culture models where cytohesins have been implicated with ErbB (EGFR) in human lung and breast adenosarcoma cancer cell lines. Bill et al. could show that cytohesin 2 is a cytoplasmic ErbB receptor activator by stabilizing active asymmetric ErbB dimers (Bill et al., 2010). Interestingly, our data point towards a more downstream role of Step in the signaling cascade, since step overexpression is able to rescue EGFR\(\text{RNAi}\) and Ras\(\text{RNAi}\) wings. Moreover, our data point to a function of Step upstream of MAPK in the EGFR pathway.

**Step interacts with the scaffolding protein dCNK to control EGFR/MAPK signaling**

The Connector Enhancer of KSR (CNK) protein family has been proposed to function as protein scaffolds (Therrien et al., 1998; Douziech et al., 2003). Data obtained in HepG2 cell experiments indicate that CNK1 binds the coiled-coil domain of cytohesin 2 via a C-terminal region (CBD, cytohesin binding domain) and mediates plasma membrane association via its PH domain. At the plasma membrane, cytohesin 2 activates ARF6 and thereby enhances PIP5K activation. This leads to PIP2 enrichment at the plasma membrane, which in turn promotes IRS membrane recruitment and thereby facilitates IIS (Lim et al., 2010). In contrast, the Drosophila CNK homologue, dCNK, has been associated with EGFR/MAPK signaling: dCNK directly associates with the kinase domain of RAF via a short amino-acid sequence, called the RAF-interacting motif (RIM), and modulates RAF activity according to the RTK signaling status (Douziech et al., 2006; Laberge et al., 2005). Without RTK signals, CNK-bound RAF is inhibited by a second motif adjacent to the RIM, called the inhibitory sequence (IS). Upon RTK activation, CNK integrates Src and RAS activities, which then leads to RAF activation.
To test whether Step function in EGFR signaling during wing differentiation is at the level of dCNK we tested genetic and biochemical interaction of the two factors. We monitored CNK expression using a CNK antibody (Douziech et al., 2003). dCNK is expressed ubiquitously in the wing imaginal disc in a vesicle-like pattern (supplementary material Fig. S4A).

$dCNK$ knockdown in the posterior compartment of the imaginal wing disc using $enGal4$ in combination with $UAS-dCNK^{RNAi}$ results in loss of wing vein tissue (Fig. 5A; supplementary material Fig. S4A), consistent with its described role in EGFR/MAPK signaling. This reduction of vein tissue can be further increased when $step$ is downregulated in addition (Fig. 5A'). In contrast, $step$ overexpression in a $dCNK^{RNAi}$ background rescues the wing vein differentiation defects (Fig. 5A'').

![Fig. 4. Step acts downstream of the EGF receptor in the EGFR pathway. (A–D) Knockdown of the EGFR pathway components EGFR, RAS, RAF and MAPK in the posterior wing compartment via RNAi leads to reduction of wing vein differentiation (see arrows in A’–D’). For each EGFR pathway component knockdown, coexpression of $step^{RNAi}$ enhances the loss of wing vein differentiation (compare A and A’, B and B’, C and C’, D and D’). $UAS-step^{RNAi}$ expression can rescue wing differentiation defects in $UAS-EGFR^{RNAi}$ wings (A’') as well as enhance wing vein differentiation defects caused by $RAS^{RNAi}$ (B’'). In contrast, wing vein defects in $RAS^{RNAi}$ and $MAPK^{RNAi}$ wings were not rescued by $UAS-step$ (compare C’ and C'', D’ and D'’).

![Fig. 5. Step interacts with CNK. Knockdown of CNK levels in the posterior wing leads to loss of wing veins (A). The $CNK^{RNAi}$ phenotype can be increased by coexpression of $step^{RNAi}$ (compare A and A’). $step$ overexpression is able to rescue wing vein differentiation defects caused by $CNK$ knockdown (A’). (B) Direct interaction of Step and CNK was tested in a yeast two-hybrid approach. The CNK C-terminus (aa 818–1557) was fused to the Gal4 activation domain of plasmid pGAD. Binding of three Step deletion variants fused to the Gal4 DNA-binding domain of plasmid pGB was analysed: Versions lacking the coiled-coiled (CC; step-CC), the Sec7 (step-Sec7) and the PH domain (PH; step-PH) were cloned. Growth in the two-hybrid assay is indicated by ‘+’; ‘−’ indicates the inability of corresponding transformants to grow on selection medium. Step mutants lacking the PH and the Sec7 domain were tested positive in this binary test system.]
Fig. 6. **Step levels are increased upon EGFR overexpression.** Clonal expression of EGFR and GFP (A,B) or GFP only (C) in third instar imaginal wing discs stained for EGFR, Step and Schlank (as a negative control). Clonal EGFR overexpression via hsFLP leads to increased Step levels (A'), whereas Schlank levels are not affected (B'). Clonal GFP expression alone does not influence Step levels (C–C'). **Step expression is induced in L3 larvae overexpressing UAS-EGFR (D; induction of EGFR signaling validated by aos induction) and downregulated in specimens expressing UAS-EGFRDN (E; inhibition of EGFR signaling validated by downregulation of aos).** Identically treated L3 larvae of the genotype hsGal4;+ served as control. n=5; error bars indicate s.e.m. Statistical significance was analyzed by Mann–Whitney U-test. P values are as indicated. aos, argos; step, steppke. (F) Model of potential function of Step in wing vein and eye development. Step acts in the EGFR pathway downstream of CNK. Step expression is regulated by EGFR signaling.
Step acts downstream of dCNK in the EGFR/MAPK cascade. Both Step and dCNK are localized in a vesicle-like pattern throughout the cells. Subcellular localization studies reveal a partial co-localization of Step and dCNK in the cortical region of cells (supplementary material Fig. S4C–C'); for quantification see supplementary material Fig. S4'C,'C'). To address whether Step has the potential to directly interact with dCNK we used the binary yeast-two-hybrid system (see Materials and Methods). We tested interactions of Step protein variants that were lacking either one of the three functional domains of Step, the coiled-coil, the Sec7 or the PH domain, respectively, with the C-terminus of dCNK (see Fig. S5B; supplementary material Fig. S4B). The results suggest that Step, similar to data obtained in mammalian cell culture models, might interact with the C-terminus of CNK via a stretch containing the coiled-coil domain and thereby might act as part of the CNK scaffolding complex.

EGFR signaling induces Step expression

The importance of Step for EGFR signaling may further be indicated by the following observation: upon induction of the EGFR pathway, Step levels are enhanced. Clones in imaginal wing discs overexpressing UAS-EGFR show increased levels of Step protein (Fig. 6A–A'). This increase cannot be seen when only GFP is clonally expressed (Fig. 6C–C'). The modulation of Step levels upon EGFR pathway activity can further be seen on transcript levels. Overexpression of UAS-EGFR leads to a strong induction of Step transcript levels (Fig. 6D), whereas overexpression of a dominant negative version of the EGFR inhibiting the signaling pathway leads to a downregulation of step (Fig. 6E). This indicates that step transcriptional levels are positively regulated by the EGFR pathway as a part of a positive feedback loop. This is opposite to effects seen on step levels upon IIS modulation, where step transcription is negatively regulated by the pathway and activated by FOXO (Fuss et al., 2006).

Discussion

Our study demonstrates an in vivo function of the Arf GEF Step as an essential component of the EGFR signaling pathway which acts downstream of the EGFR (Fig. 6F). Step is necessary and sufficient for activation of MAPK and the induction of EGFR target genes in Drosophila. Based on our biochemical, immunohistochemical and the genetic data we propose a mechanistic model in which Step and dCNK interaction is important for EGFR signaling. dCNK is the single member of the CNK protein family in Drosophila. CNK proteins are scaffolding proteins that have been linked with Ras, Rho, Rac, Ras and Arf GTPases and are proposed to act as general regulators of GTPase-mediated events downstream of receptor tyrosine kinases, including EGFR and InR-insulin-like growth factor receptors (Clapéron and Therrien, 2007). Together with the kinase suppressor of Ras (KSR), CNK was shown to assemble a signaling complex including RAF and MEK which promotes Ras-dependent RAF activation and the subsequent phosphorylation of MAPK (Roy et al., 2002; Therrien et al., 1998; Douziech et al., 2006; Rajakulendran et al., 2008). We suggest that Step is a functional part of this scaffolding complex via its direct interaction with CNK. This is also consistent with recent data in HeLa and 393T cells showing that human CNK1 directly interacts with cytohesin-2 to coordinate PI3K/AKT signaling downstream of Inr/IGF-R (Lim et al., 2010). It was proposed that CNK1 recruits cytohesin-2 to the plasma membrane, where activity of plasma membrane bound GTPases leads to a PI3K rich microenvironment, which enhances IRS1 recruitment and hence facilitates PI3K/AKT signaling (Lim et al., 2010). Similarly, Drosophila cytohesin Step was shown to be required for PI3K activation (Fuss et al., 2006). Together, several lines of evidence support a role of cytohesins and CNK in similar signaling contexts (RAS/RAF/ MAPK and PI3K/AKT signaling), where a direct interaction of both proteins as part of a signaling platform might promote downstream signaling events like MAPK phosphorylation and PI3K activation. This does not exclude other functions of cytohesins, e.g. the stabilization of asymmetric ErbB (EGFR) dimers, as shown recently in human lung and breast adenosarcoma cancer cell lines (Bill et al., 2010). Our data indicate, however, that a major function of the Drosophila cytohesin Step in EGFR signaling resides downstream of the EGFR and upstream of MAPK.

Materials and Methods

Fly stocks

Flies were raised on standard fly food at 25°C if not mentioned otherwise. The following fly stocks were used: wild-type (Oregon R); stepK08110 (Bloomington stock #10770); ML2JT/65 (referred as stepK', Bloomington stock #1602); enGal4, UAS-Dcr2, UAS-EGFPRNAi (Bloomington stock #25752); hsFLP;act >CD2 >Gal4, UAS-EGFP (Bloomington stock #9431); UAS-EGFR (a gift of C. Klambt); UAS-dp110E,A14 (Bloomington stock #25908); UAS-step (Fuss et al., 2006); UAS-InRA1325D (Bloomington stock #8263).

The following stocks were obtained from the Vienna Drosophila RNAi Centre (Dietzl et al., 2007): UAS-EGFR/EK215 (Bloomington stock #107130); UAS-InR/EK410 (Bloomington stock #992); UAS-CNKR64K (Bloomington stock #11960); UAS-RAS/EK215 (Bloomington stock #106642); UAS-RAP/R65H (Bloomington stock #20099); UAS-MAPK/R4124 (Bloomington stock #43124). To generate a transgenic steppe RNAi line, a 443 bp step cDNA fragment (bp 589–1031 of step-RA cDNA) was cloned in reverse orientation into pWiz via XbaI (using primers: 5'-aaccacagcagcactgca-3' and 5'gtgcagcaatgcaacaga-3'). Transgenic flies were generated via transposase-mediated P-element insertion. step knockdown of RNAi construct was tested using real-time RT-PCR and immunohistochemistry (see Fig. 1C–C').

Rescue experiments were of the following genotype: hsFLP;stepK'/stepK'; act >CD2 >Gal4, UAS-EGFP/UAS-step.

To generate a steppe null allele, several gene targeting approaches were performed according to the modified ‘Rong and Golic’ protocol (Rong and Golic, 2001; Huang et al., 2008). In contrast to parallel gene targeting approaches for other genes in the lab, several steppe specific targeting attempts failed, most probably due to the low recombination rate in the vicinity of the centromere region. Moreover, Flp/Frt-based clonal analysis of steppe gene function using the hypomorphic alleles is prevented by the close localization of the steppe gene locus with respect to the FRT40a insertion.

Generation of a Steppe antibody

The coding sequence corresponding to amino acids 5 to 266 was cloned into the pTRex-4 Neo vector via NcoI and XhoI to generate an expression vector containing the coding sequence for a 6×His tagged Steppe fusion protein. Steppe5–266-HIS was expressed in E. coli BL21 and purified via the 6×His tag using NiNTA Agarose (Machery-Nagel). Guinea pigs were immunized with the recombinant Steppe-5–266-HIS protein. 2 mg of purified Steppe5–266-HIS was immobilized to CNBr activated Sepharose 4B in a column according to manufacturer’s instructions (GE healthcare) to generate affinity purification columns. 2 ml serum of immunized animals was loaded to the column for 2 hours at room temperature. The column was further washed with PBT, and the purified antibody was eluted via 0.1 M glycine (pH 2.3). Fractions were neutralized with Tris-HCl (pH 8.1) and amount and quantity of purified antibody was assessed via Coomassie SDS page. Specificity of antibody was tested in step gain and loss of function (Fig. 1B,C).

Immunohistochemistry

Larval imaginal discs were fixed in 4% paraformaldehyde/PBS for 45 minutes at room temperature. Specimen were blocked for at least 1 hour in 1% Rotip–Block (Carl Roth) PBT (containing 0.1% Triton X-100) and incubated with primary antibodies in PBT overnight at 4°C. Primary antibodies used were: anti-E-Cadherin (1:10, Santa-Cruz), anti-GFP (1:100, Sigma-Aldrich), anti-dpERK.
performed the Y2H analysis. D.G. generated the Step antibody. M.H. planned and supervised the project. I.H., B.F. and M.H. wrote the manuscript.

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Supplementary material available online at

References


Fig. S1. Step affects cell number but not cell size in adult wings. Expression of \textit{UAS-step}^{RNAi} via \textit{enGal4} leads to reduced size of the posterior wing (A; ratio of posterior wing area versus total wing area) due to a reduction of total cell number (C). Cell size (number of cells/defined square) remains unaffected (B). Overexpression of \textit{UAS-step} via \textit{enGal4} has a dominant negative effect on size of the posterior wing (A). Cell size (number of cells/defined square) is unaffected, however, the number of total cells in the posterior wing is reduced (C).
Fig. S2. Step mediates EGFR target gene expression. EGFR and IIS target gene expression in L3 homozygous stepK larvae (A) and L3 larvae overexpressing UAS-step (B). Step mutant L3 larvae were compared to control (oreR) larvae. step knock down in stepK mutants was validated. 4EBP induction serves as a positive control for step dependent target gene regulation. (B) hsGal4;UAS-step L3 larvae were heat shocked @ 37°C for 1 hour. Identically treated L3 larvae of the genotype hsGal4;+ served as control. hsGal4;UAS-EGFR larvae are positive controls for EGFR target gene regulation. Gene expression was analyzed 2 hours after heat shock. Step overexpression is verified. n≥5, error bars indicate SEM. Statistical significance was analyzed by Mann-Witney-U test. P values are as indicated: *p<0.05; **p<0.01; ***p<0.001; step, stepK; aos, argos; vnd, ventral nerve cord defective; pnt, pointed. (C–F) Imaginal wing of third instar larvae stained for double phosphorylated MAPK (pMAPK). Posterior expression of transgenes in the imaginal wing disc compartment via enGal4 is marked by coexpression of GFP (C’–F’). Knock down of EGFR via RNAi leads to decreased MAPK phosphorylation in the posterior wing disc (see arrow in D). Activation of the EGFR pathway in the posterior wing disc via UAS-EGFR expression results in increased pMAPK levels (see asterisk in D). Neither inhibition of IIS in the posterior wing discs by expression of UAS-InR RNAi nor activation of the pathway via UAS-InR affects MAPK phosphorylation (see arrows in E and F).
Fig. S3. *Step* also acts in *EGFR* signaling in context of photoreceptor differentiation. Figures show tangential sections of adult compound eyes (A – A’’) of heterozygous step^+/+ (A), Star^+/+ (A’) and transheterozygous step^+/Star^mutants (A’’) and homozygous step^/step^ mutants (A’’’). Ommatidia displaying differentiation defects are indicated by red circles. Compound eyes, heterozygous for the Star^ allele display mild rotation and differentiation defects (A’), that are increased in transheterozygosity with step^ (A’’). (B) Note the increase of differentiation defects of Star^ mutants in step^ as well as step^def. This effect cannot be observed in Star^/+; InR^5545/+ mutant compound eyes. n>200 from at least five independent eyes. Error bars indicate SEM. Statistical significance was analyzed by Student’s t-test. P values are indicated. (C – D) Imaginal eye discs of third instar larvae stained for phosphorylated MAPK (pMAPK). Anterior is left. Clonal expression of step (C – C’) and step^RNAi (D – D’) via hsFLP in imaginal eye discs marked by GFP (area inside dashed line in C’ and D’). step gain of function increases MAPK phosphorylation in clonal areas compared to surrounding tissue (C’); step knock down (step^RNAi) leads to less phosphorylation of MAPK in cells expressing step^RNAi (D’).
**Fig. S4. CNKRNAi and anti-CNK antibody verification.** Third instar imaginal discs expressing CNKRNAi in the posterior compartment (left; marked by GFP expression in A and A’’) via enGal4, stained for CNK. Please note reduction in CNK levels in posterior wing compartment (A’). (B) Yeast two-hybrid analysis results: Binding of three Step deletion variants fused to the Gal4 DNA-binding domain of plasmid pGB was analysed: Growth in the two-hybrid assay is indicated by “+,” “−” indicates the inability of corresponding transformants to grow on selective medium (compare to plate). (C-C’) A putative interaction of Step and dCNK is in line with immunohistochemical stainings of imaginal discs stained for CNK and Step, which show partial colocalization at the cell cortex. Co-localization was analyzed using BioImageXD software.
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