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Small Wing PLCγ Is Required for ER Retention of Cleaved Spitz during Eye Development in *Drosophila*

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Summary

The *Drosophila* EGF receptor ligand Spitz is cleaved by Rhomboid to generate an active secreted molecule. Surprisingly, when a cleaved variant of Spitz (cSpi) was expressed, it accumulated in the ER, both in embryos and in cell culture. A cell-based RNAi screen for loss-of-function phenotypes that alleviate ER accumulation of cSpi identified several genes, including the *small wing* (*sl*) gene encoding a PLCγ. *sl* mutants compromised ER accumulation of cSpi in embryos, yet they exhibit EGFR hyperactivation phenotypes predominantly in the eye. Spi processing in the eye is carried out primarily by Rhomboid-3/Roughoid, which cleaves Spi in the ER, en route to the Golgi. The *sl* mutant phenotype is consistent with decreased cSpi retention in the R8 cells. Retention of cSpi in the ER provides a novel mechanism for restricting active ligand levels and hence the range of EGFR activation in the developing eye.

Introduction

Morphogens, secreted by a restricted population of producing cells, affect the differentiation of adjacent cells. The final response pattern depends on the spatial distribution of the ligand and is influenced by multiple determinants. In the producing cells, the level of secreted ligand is critical, and so are posttranslational modifications that may alter its range of diffusion. In the receiving cells, mechanisms that affect ligand diffusion and degradation, as well as negative-feedback responses, play a central role in shaping the spatial pattern of activation. This study reveals a novel mechanism regulating the level of a secreted *Drosophila* EGF receptor ligand that is released by the producing cells.

The EGFR pathway triggers multiple developmental decisions throughout the life cycle of the fly (Shilo, 2003). The pathway affects mostly short-range interactions, and several inducible negative-feedback responses contribute to the restricted activation profile. These include the production of the secreted inhibitor Argos, expression of Kekkon (which forms nonfunctional heterodimers with EGFR), and induction of Sprouty (which interferes with the Ras pathway) (Casci et al., 1999; Ghiglione et al., 1999; Golembo et al., 1996; Kramer et al., 1999; Reich et al., 1999). The necessity to restrict EGFR activation range prompted us to examine whether control of the levels of active, secreted ligand represents an additional tier of regulation.

Three EGFR ligands, Spitz (Spi), Keren, and Gurken, are produced as inactive, membrane bound precursors (Neuman-Silberberg and Schupbach, 1993; Reich and Shilo, 2002; Rutledge et al., 1992; Schweitzer et al., 1995b). The fourth activating ligand, Vein, is produced as a secreted ligand (Schnepp et al., 1996). Processing has been studied in greatest detail for the cardinal ligand, Spi. The ligand transmembrane precursor mSpitz (mSpi) is ubiquitously expressed, while the processing machinery determines the spatial and temporal regulation of EGFR activation. mSpi is retained in the endoplasmic reticulum (ER), and its trafficking from the ER to Golgi is facilitated by the transmembrane protein Star (Lee et al., 2001; Tsruya et al., 2002). Following trafficking, the interaction between mSpi and the seven-transmembrane domain protein Rhomboid-1 leads to intramembrane cleavage of Spi by Rhomboid-1 to release the secreted form of the ligand (Urban et al., 2001). Expression of Rhomboid-1 is highly dynamic and restricted, providing the spatial and temporal cues for EGFR activation (Bier et al., 1990; Gabay et al., 1997; Sturtevant et al., 1993).

In this study, we examined the capacity of the cleaved Spi ligand to be released from the producing cells and searched for cellular components that impinge on Spi secretion. Expression of a cleaved form of Spi (cSpi) resulted in ER accumulation of the protein. Several observations indicated that this retention might be biologically relevant. Accumulation was not observed for EGFR ligands that are produced in a secreted form such as Argos and Vein. cSpi retention relied on the EGF domain of Spi and appeared to depend on a mechanism that is distinct from COPI-mediated retrograde trafficking of the transmembrane Spi precursor. A screen of 1000 dsRNA molecules, to identify cases in which retention of cSpi would be compromised, identified 11 genes. Especially striking was the identification of *small wing* (*sl*), which encodes the sole *Drosophila* PLCγ homolog and was shown to interact genetically with the EGFR pathway (Thackeray et al., 1998). In *sl* mutant embryos, the retention of cSpi was indeed compromised. Mutations in *sl* are viable and give rise to rough eyes, due to hyperactivation of EGFR (Thackeray et al., 1998).

In most tissues, Spitz is cleaved by the intramembrane protease Rhomboid-1. In the eye, Rhomboid-3/Roughoid functions in parallel to Rhomboid-1 (Wasserman et al., 2000). We demonstrate genetic interactions between *sl* and *roughoid* in the eye, suggesting that cSpi is normally generated in the ER, and therefore that compromising its retention will elicit a phenotype. The activity of Sl in the R8 photoreceptor cells producing the cleaved ligand was indeed demonstrated by rescue of the *sl* eye phenotype following expression of the normal gene product only in R8 cells. We conclude that in the eye a significant

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portion of the Spi precursor is cleaved in the ER. A combination of efficient retention and regulated trafficking by Star determines the amount of ligand that will be secreted from the producing cells.

Results

ER Retention of Cleaved Spitz

We wished to follow directly the cleaved form of Spi (cSpi), in order to identify factors that may affect the distribution of the ligand after it has been processed by Star and Rhomboid. To overcome the processing stages, we used a Spi protein construct lacking the transmembrane and intracellular domains. This variant resembles the mature form and was previously shown to be biologically active both in cell culture and in flies (Schweitzer et al., 1995b). eGFP was inserted immediately after the signal peptide to allow for visualization, and the entire construct was placed in the context of a UAS-based expression vector. The resulting molecule was shown to be highly active in flies, e.g., by expression in the wing and generation of the characteristic EGFR hyperactivation phenotype (not shown).

Typically, an inert secreted molecule, e.g., GFP attached to a signal peptide (sGFP), is observed mostly in the extracellular milieu of the cells expressing the molecule, as well as around the adjacent cells (Figure 1D). Functional ligands such as cSpi are expected to appear in endocytic vesicles in the adjacent cells receiving the Spi signal. Surprisingly, when the distribution of cSpi-GFP was monitored in flies, it could be detected only in the cells where it was expressed. This distribution was observed both in embryos and in the wing disc, in both live and fixed tissue (Figures 1A–1F). Moreover, within the producing cells, cSpi was observed in a perinuclear distribution, typical for ER proteins such as PDI (Figure 1G; Bobinnec et al., 2003).

To examine the biological activity and spatial distribution of cSpi, we followed the activation of EGFR and MAPK by antibodies recognizing dpERK (Gabay et al., 1997). cSpi-GFP was expressed in the embryo in a seven-stripe pattern by *prd-Gal4*. In accordance with our previous observations, cSpi-GFP was indeed potent and capable of triggering EGFR activation, leading to the appearance of dpERK and expression of EGFR target genes (not shown). However, activation was primarily restricted to the cells in which cSpi was expressed and extended at most to one row of neighboring cells that did not express cSpi (Figure 1H).

cSpi Is ER Retained in Cell Culture

When expressed in Schneider cells, cSpi is secreted to the medium (Schweitzer et al., 1995b). However, examination of the intracellular distribution of cSpi in the S_2R^+ producing cells demonstrated that cSpi accumulated in the ER, similar to the observed distribution in flies (Figure 2A). The distribution of cSpi showed a significant overlap with the ER-resident protein BiP (Figures 2G–2I) and only a minor overlap with Golgi markers (Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/7/4/535/DC1/). In view of the eventual secretion of the molecule, it is possible that in Schneider

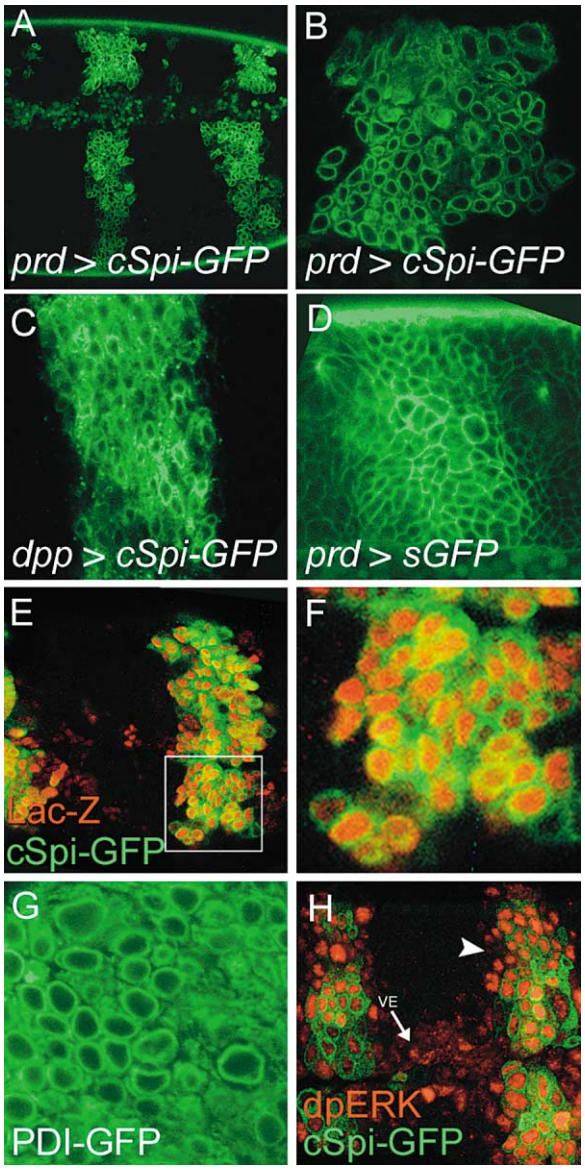


Figure 1. Cleaved Spi Is Retained in the ER in Flies

Cleaved Spi-GFP is retained in a perinuclear distribution in cells in which it was expressed.

(A and B) Expression in the ectoderm of live embryos by *prd-Gal4*.

(C) Expression in live wing-imaginal discs by *dpp-Gal4*.

(D) cSpi distribution is distinct from that of an inert secreted protein such as GFP attached to a signal peptide. In this case, the protein was found in the extracellular region and also diffused beyond the *prd* stripes where it was expressed.

(E and F) In *prd-Gal4* embryos carrying *UAS-cSpi-GFP* and *UAS-LacZ*, cSpi-GFP was detected only in the cells where it was expressed (red, anti-Lac-Z). The nuclear localization of LacZ highlights the perinuclear distribution of cSpi-GFP.

(G) The perinuclear distribution of cSpi-GFP is indicative of ER retention and is similar to proteins that are ER markers such as PDI-GFP.

(H) The activity of cSpi-GFP induced by *prd-Gal4* was monitored by dpERK antibodies (red). Activation within the cells expressing cSpi, as well as in one adjacent cell row, was observed (arrowhead). Arrow indicates the perpendicular stripe of endogenous dpERK in the ventral-most ectodermal cell rows.

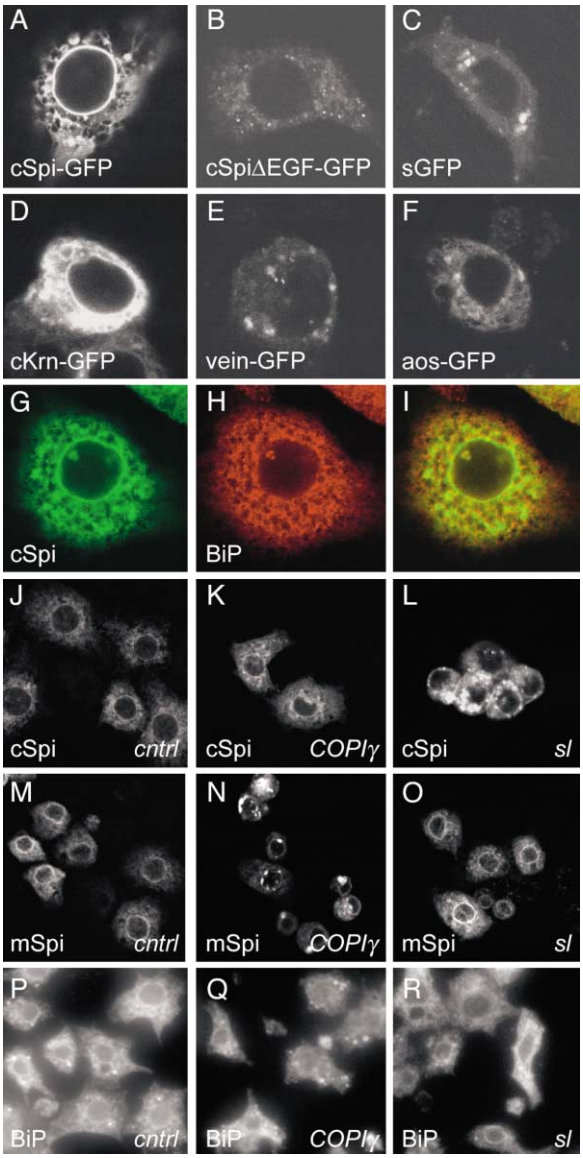


Figure 2. cSpi Is Retained in the ER in Cell Culture by a Novel Mechanism

Images (A)–(O) were derived from transfected S_2R^+ cells expressing different UAS-based constructs that were induced by *actin-Gal4*.

(A) cSpi-GFP showed a perinuclear distribution.

(B and C) Deletion of the EGF domain of cSpi abolished the retention (B) and gave rise to a typical secretory distribution, similar to secreted GFP (C).

(D–F) A cleaved version of Keren also exhibited ER retention similar to cSpi (D), while Vein and Argos, which are naturally secreted ligands, displayed a typical secretory distribution (E and F).

(G–I) The localization of cSpi overlaps with the ER-resident protein BiP.

(J and K) Incubation of cells expressing cSpi with dsRNA for COPIγ, which compromises retrograde trafficking from the Golgi to the ER, did not affect the distribution of cSpi.

(L) dsRNA for *sl* compromised the ER retention of cSpi and gave rise to accumulation in punctate cytoplasmic structures.

(M and N) dsRNA for COPIγ compromised the ER retention of the Spi precursor and gave rise to its accumulation in punctate structures.

(O) *sl* dsRNA had no effect on the distribution of mSpi.

(P–R) The dsRNA for COPIγ but not for *sl* affected the localization of the ER-resident protein BiP. Control RNAi was generated for GFP.

cells this accumulation represents only a transient feature, in contrast to the distribution in flies. Elimination of the EGF domain of cSpi alleviated the ER retention of the molecule in the cells and resulted in a distribution that was similar to secreted molecules such as sGFP (Figures 2B and 2C). A similar alteration in the distribution profile following elimination of the EGF domain was monitored in embryos (not shown).

To examine if ER retention is a general property of EGFR ligands, we tested GFP fusions of cleaved Keren, Vein, and Argos. Keren is a ligand that is structurally highly similar to Spi in the extracellular domain (Reich and Shilo, 2002), and indeed cleaved Keren-GFP was similarly retained in the ER (Figure 2D). Vein and Argos are naturally secreted ligands (Freeman et al., 1992; Schnepp et al., 1996). In both cases, the ligands exhibited a typical secretory distribution manifested by a low level of fluorescence that is detected primarily in vesicles, with no ER accumulation (Figures 2E and 2F). These results demonstrate that ER retention of the secreted form of the ligand is not a general feature of EGFR ligand biogenesis, but is rather a property specific to Spi and Keren.

cSpi Retention Is Not Mediated by COPI

Retrograde trafficking of proteins from the Golgi to the ER that is mediated by the COPI complex is a common strategy for ER retention (Sannerud et al., 2003). By incubating the cells with dsRNAs to different subunits of the COPI complex, we examined if ER retention of cSpi was compromised. The transmembrane precursor of Spi (mSpi) is also retained in the ER (Lee et al., 2001; Tsruya et al., 2002). Incubation with dsRNA to the COPIβ′ or COPIγ subunits (which are required for ER retention of BiP) compromised mSpi retention and led to the accumulation of mSpi in punctate structures (Figures 2M, 2N, 2P, and 2Q), indicating that the localization of mSpi in the ER depends on retrograde trafficking mediated by COPI. In contrast, these dsRNAs had no effect on the ER accumulation of cSpi (Figures 2J and 2K). Thus, a novel mechanism that is independent of retrograde trafficking by the COPI complex is implied for retention of cSpi in the ER.

A Screen for Genes Involved in cSpi Retention

In view of the involvement of a novel mechanism for the ER retention of cSpi, we sought to identify some of the necessary components, using an inhibitory RNA screen including 1035 distinct dsRNAs. Briefly, S_2R^+ cells expressing cSpi-GFP were plated on 384-well plates containing different dsRNAs (Kiger et al., 2003). Microscopic examination of the intracellular distribution of cSpi-GFP was carried out 3–4 days after incubation. Specifically, we looked for wells in which the perinuclear pattern of cSpi would be replaced by a typical secretory pattern, indicating a loss of function important for ER retention.

In 11 independent instances out of the 1035 genes, cSpi-GFP was not retained in an ER pattern, as summarized in Supplemental Table S1. The list includes kinases, signal transduction elements, and intracellular transport components. None of these genes altered the retention of mSpi, lending further support to the finding that a COPI-independent mechanism is involved in the

ER accumulation of cSpi. The effects of the different dsRNAs on retention of a resident ER protein, BiP, were also examined. Four of the dsRNAs altered the localization of BiP and gave rise to a punctate distribution, suggesting that they do not specifically function to retain cSpi.

Within the remaining seven genes, we were particularly struck by the identification of *small wing* (*sl*), which was previously shown to be a negative regulator of EGFR signaling in the eye (Thackeray et al., 1998). dsRNA for *sl* abolished the ER retention of cSpi, which instead appeared in punctate structures (Figure 2L). Conversely, it had no effect on the distribution of mSpi or BiP (Figures 2O and 2R).

sl encodes a phospholipase Cγ (PLC-γ) and is in fact the only member of this class of PLCs in the *Drosophila* genome. The Sl protein contains, in addition to the catalytic domains, different adaptor modules such as SH2, SH3, and PH domains. *sl* mutations were isolated as early as 1915 by Bridges. Null alleles are homozygous viable and exhibit two distinct defects: wings that are reduced in size and rough eyes. The wing phenotype is caused by the absence of PLC-enzymatic activity. However, the rough eye phenotype, which exhibited genetic interactions with EGFR signaling, relies on other scaffold domains of Sl, as it was not observed in point mutations that specifically abolish the catalytic activity (Mankidy et al., 2003). In view of the published results pointing to an interaction between Sl and EGFR, our subsequent analysis focused on the role of Sl in ER retention of cSpi and its biological role in attenuating EGFR signaling.

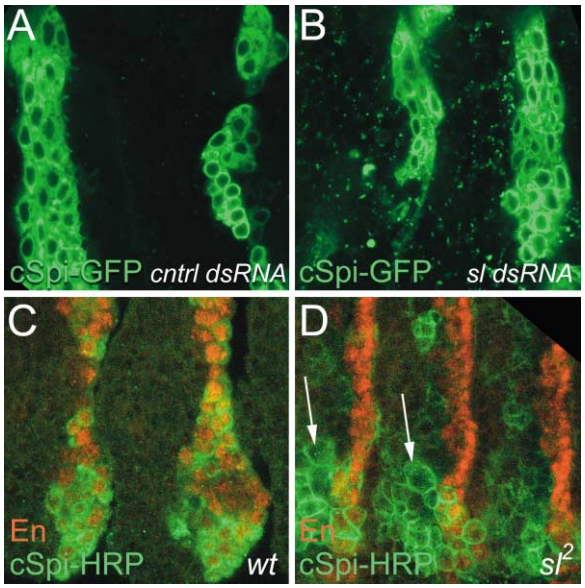


Figure 3. Sl Is Required for Retention of cSpi in Embryos

The requirement of Sl for retention was monitored in embryos expressing UAS-cSpi-GFP driven by *en-Gal4*.

(A) In live embryos, cSpi-GFP was retained in the typical perinuclear distribution after injection of a control dsRNA for the CG2005 gene.

(B) Following injection of *sl* dsRNA, the appearance of cSpi-GFP in vesicles within cells positioned between the *en* stripes was indicative of secretion from the producing cells.

(C) cSpi-HRP induced by *en-Gal4* was also restricted to the cells in which it was expressed (green, HRP; red, En).

(D) In *sl* null embryos, the level of cSpi-HRP in the producing cells was diminished, and instead cSpi-HRP was detected in the extracellular space and in intracellular vesicles (arrows).

Sl Is Required for ER Retention of cSpi in Embryos

To extend the initial findings in cell culture, we examined the role of Sl in ER retention of cSpi in embryos. Sl was shown to be uniformly expressed throughout development (Emori et al., 1994). We inactivated Sl by two ways. First, dsRNA for *sl* was injected into embryos expressing *UAS-cSpi-GFP* under the control of *en-Gal4*, and the distribution of cSpi followed in live embryos. Typically, cSpi is detected only in the expressing cells, with no fluorescence in the extracellular space or in endocytic vesicles in the adjacent cells (Figure 3A). However, following injection of *sl* dsRNA, some of the cSpi protein was detected in vesicles within the producing cells. Especially pronounced was the appearance of cSpi in endocytic vesicles in all cells positioned between the stripes of *en* expression, indicating that cSpi was indeed secreted from the producing cells and taken up by neighboring cells (Figure 3B).

Another approach to follow the role of Sl in cSpi retention was to collect embryos that are completely devoid of Sl. These embryos are zygotically mutant for *sl* and also do not carry a maternal contribution. cSpi that was tagged with HRP was expressed in these embryos by *en-Gal4*. Again, in wt embryos, cSpi was retained (Figure 3C), while in a *sl* mutant background, the level of cSpi in the producing cells was reduced, and instead the protein was found within and outside the adjacent cells (Figure 3D). In this case, cSpi was detected in the extracellular space, as well as in endocytic vesicles. The higher levels of cSpi-HRP in the extracellular space compared to cSpi-GFP may stem from a more efficient elimination of all Sl activity in the *sl* mutant background, compared to dsRNA injection.

The above experiments demonstrate that under conditions of cSpi overexpression, Sl is required in embryos for ER retention. Yet, in *sl* null mutants, no detectable EGFR hyperactivation phenotypes are observed and the mutant embryos are viable. In fact, an EGFR hyperactivation phenotype is consistently observed only in the eye (Thackeray et al., 1998). To understand the physiological significance of cSpi retention, we thus concentrated on the eye, where *sl* mutants exert a phenocopy of EGFR hyperactivation.

Sl Negatively Regulates EGFR Signaling in the Eye

We sought to characterize in detail the involvement of Sl in eye development. The Sl protein is comprised of 1235 aa. Three alleles of *sl* were used for the analyses. sl^1 is a hypomorphic allele, representing a P element insertion after aa 1040. sl^2 and sl^9 are regarded as null alleles, with a stop codon at residue 696 and 53, respectively (Mankidy et al., 2003; Thackeray et al., 1998). The molecular basis for the null phenotype is obvious for sl^9 in which all recognizable functional domains of the protein are eliminated.

Scanning electron micrographs of eyes of flies homozygous for the hypomorphic allele sl^1 demonstrated that

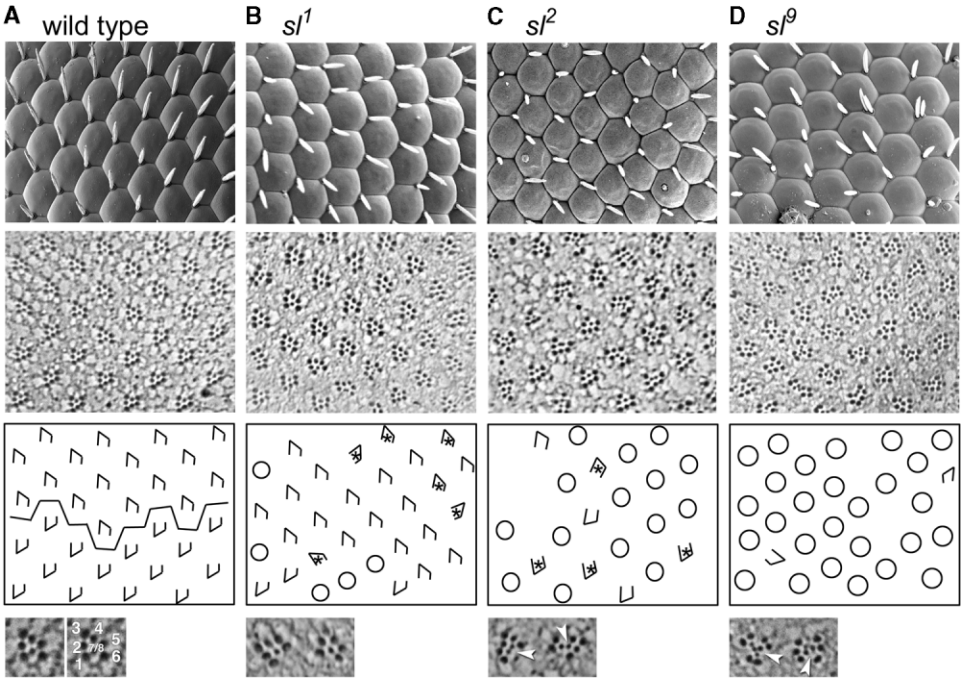


Figure 4. *sl* Eye Phenotypes

(A) Wild-type eye. Top panel, SEM; second panel, cross-section; third panel, scheme of section indicating the orientation of each ommatidium and the dorsoventral equator; and bottom panel, enlargement of representative ommatidia. Note that ommatidia are organized as mirror images on both sides of the equator.

(B) Flies homozygous for the hypomorphic allele sl^1 exhibit ommatidial rotation defects (asterisks) in 25% of the ommatidia (n = 154).

(C and D) For the null alleles sl^2 and sl^9, many ommatidia show recruitment of extra R7 cells (circles and arrowheads). sl^2, 22% rotation defects and 48% extra photoreceptor cells (n = 108), sl^9, 11% rotation defects and 61% extra photoreceptor cells (n = 196).

the ommatidial organization in ordered rows was slightly disrupted and the position of the bristles was altered. A cross-section of the eyes of these mutants revealed that ~25% of ommatidia were misrotated, implying that Sl is required for the proper rotation of ommatidia (Figure 4B).

Next, we analyzed the sl^2 and sl^9 alleles. In addition to altered rotation in some of the ommatidia (sl^2, 22%; sl^9, 11%), in the remaining ommatidia the number of photoreceptor cells was increased (sl^2, 48%; sl^9, 61%), and the overall trapezoid structure of the adult ommatidia was distorted. The number of outer photoreceptor cells remained normal, but the number of R7 cells ranged between 1 and 4 (Figures 4C and 4D). No elevation in the number of R8 cells was observed, as judged by staining of third instar larval eye discs with anti-Senseless (not shown). According to these data, Sl is required for proper rotation of ommatidia, as well as for accurate photoreceptor cell recruitment.

Sl Attenuates the EGFR Pathway

The recruitment of extra photoreceptor cells in *sl* mutants is consistent with hyperactivation of the EGFR pathway. The role of the EGFR pathway in correct ommatidial rotation was also demonstrated recently (Brown and Freeman, 2003; Gaengel and Mlodzik, 2003; Strutt and Strutt, 2003). Both over- and underactivation of EGFR signaling were shown to lead to misrotation of ommatidia. A genetic interaction between *sl* and *EGFR* heterozygotes has been previously observed with respect to photoreceptor cell recruitment (Thackeray et al., 1998). We wanted to examine these interactions further to include mutations representing additional elements in EGFR signaling and to monitor ommatidial rotation as well.

sl null flies carrying only one functional allele of *Star* or *EGFR* (*flb*) were examined. The *sl* phenotype was almost completely suppressed, with most ommatidia displaying the normal number of photoreceptor cells and correct rotation (Figures 5A and 5B). Note that *Star* heterozygous flies exhibit a phenotype of ommatidial misrotation (Brown and Freeman, 2003). This phenotype was suppressed in the absence of Sl. Conversely, when *sl* null flies carried a single copy of *argos*, encoding a negative regulator of EGFR signaling (Schweitzer et al., 1995a), the severity of the eye phenotype was enhanced (Figure 5C). Taken together, these results demonstrate that in the absence of Sl, the activity of EGFR signaling is enhanced, indicating that Sl normally acts as a negative regulator of EGFR signaling in the eye.

The Rhomboid-3/Roughoid and Sl Connection

While we showed that Sl is required for ER retention of cSpi when the latter is artificially produced in embryos (Figure 3), *sl* mutants do not exhibit any EGFR hyperactivation phenotypes in the embryo. The reason may be that in the embryo, cSpi is normally generated in the processing cells after it is trafficked from the ER, by the

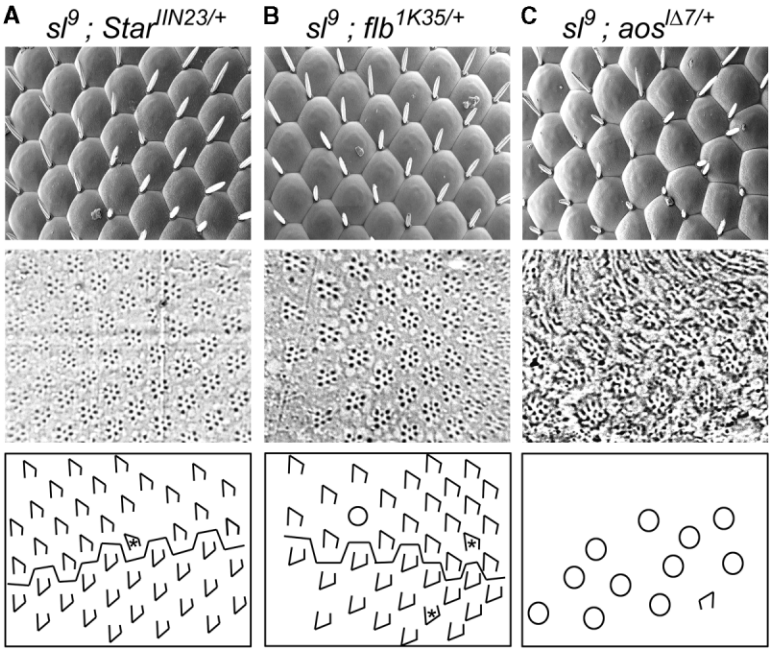


Figure 5. Sl Negatively Regulates EGFR Signaling in the Eye

(A) *sl* null flies carrying only one functional allele of *Star* have almost normal eyes with respect to ommatidial rotation and photoreceptor recruitment. This phenotype represents a rescue of the *sl* null phenotype, as well as the *Star* heterozygous dominant phenotype.

(B) *sl* null flies carrying only one functional allele of *EGFR/flb* also show rescue of the *sl* eye phenotype.

(C) In *sl* null flies carrying one functional allele of the EGFR inhibitor *argos*, the phenotype is more severe, and most ommatidia show excessive recruitment of photoreceptor cells.

compartmentalized activity of Rhomboid-1, the prominent protease cleaving Spi. Is there a tissue where cSpi is normally generated already in the ER? Since the properties of the protease determine the intracellular compartment where Spi cleavage takes place, we considered tissues where proteases other than Rhomboid-1 may be operating.

Rhomboid-1 is the founding member of a gene family in *Drosophila* (Wasserman et al., 2000). At least two additional members of the family were linked to distinct subsets of EGFR signaling. Rhomboid-2/Brho is expressed in the oocyte, where it may be responsible for cleavage of the EGFR ligand Gurken (Guichard et al., 2000). Rhomboid-3/Roughoid (Ru) is expressed in the eye, and its role in the eye is partially redundant with Rhomboid-1. Ru is the prominent protease in this tissue, however, since *rhomboid-1* mutant eyes have no phenotype, while *ru* mutant eyes display a phenotype consistent with compromised EGFR activation (Figure 6; Wasserman et al., 2000).

Immunohistochemical staining has indicated that all three Rhomboid proteins reside in the Golgi when expressed in mammalian cells (Urban et al., 2002). However, when their biological activity with respect to Spi cleavage was monitored in several cell types, a functional difference between Rhomboid-1 and the other members was noted. Rhomboid-1 is not active in the ER. Thus, coexpression of Rhomboid-1 and the Spi precursor (mSpi) that resides in the ER did not lead to ligand cleavage, since the two proteins do not occupy the same compartment (Lee et al., 2001; Tsruya et al., 2002). In contrast, Rhomboid-2 and Ru allowed efficient cleavage of mSpi even in the absence of Star (Ghiglione et al., 2002; Urban et al., 2002), taking place possibly during the transport of Rhomboid-2 or Ru from the ER to the Golgi. However, cSpi could not be secreted efficiently, due to the ER retention property described in this work. Only in the presence of Star was the cSpi molecule exported from the ER and secreted.

In view of the capacity of Ru to generate cSpi in the ER in cell culture, a similar property could be manifested in flies. Thus, tissues where Ru is normally expressed may represent a setting where cSpi is generated in the ER, and its proper retention would be a crucial biological process. While Sl is broadly expressed (Emori et al., 1994), the restricted distribution of Ru to the eye (Wasserman et al., 2000) could account for the limited range of tissues where the *sl* phenotype is manifested.

We sought to examine the possible functional connection between Ru and Sl in eye development. A hypomorphic allele, ru^{1}, and a null allele, ru^{PLLb}, are available (Wasserman et al., 2000). In homozygous ru^{1} flies, photoreceptor cells are normally recruited. However, 64% of the ommatidia show rotation defects (Figure 6A). The phenotype of ru^{PLLb} homozygous flies is more severe; less photoreceptor cells are recruited in 84% of the ommatidia (Figure 6D).

Since Ru is likely to generate cSpi in the ER, which is subsequently retained by Sl, we tested for possible genetic interactions between *sl* and *ru*. In the ru^{1} hypomorphic background, the amount of cSpi is compromised. The expectation was that in combination with a *sl* background, this defect may be corrected due to more efficient secretion of the low levels of cSpi that are produced. Indeed, homozygous double mutant combinations of ru^{1} with hypomorphic or null *sl* alleles gave rise to almost complete phenotypic rescue (Figures 6B and 6C).

In contrast to the *ru* hypomorph, which produces lower levels of cSpi, in the ru^{PLLb} null background, we expected no cSpi to be produced in the ER and hence no rescue by *sl* mutants. Surprisingly, some rescue was observed in sl^{9} null flies, manifested primarily in the normal number of photoreceptor cells in most ommatidia. The rescue was incomplete, however, as 50% of the ommatidia still exhibited rotation defects (Figure 6F). In the absence of Ru, Spi is cleaved by Rhomboid-1. This

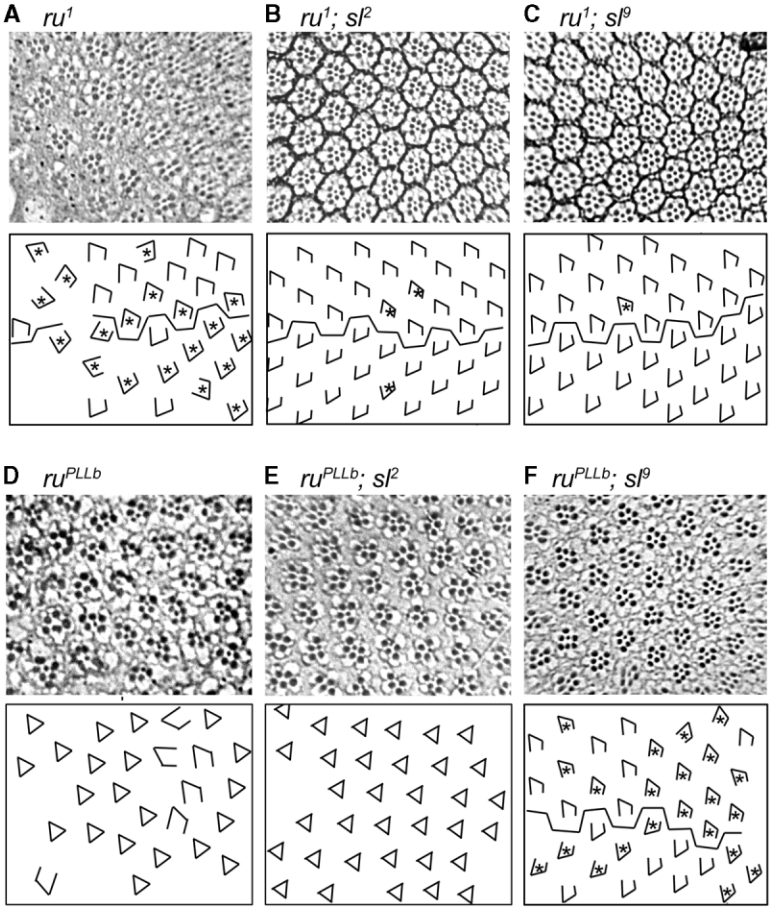


Figure 6. Genetic Interactions between *ru* and *sl*

If Ru generates cSpi in the ER, which is subsequently retained by Sl, we expected to identify genetic interactions between the two loci.

(A–C) The ru^1 hypomorphic mutation, which exhibits ommatidial rotation defects (64%; n = 45)), was rescued in *sl* mutant flies (triangles, misrotated ommatidia: sl^2, 11%; n = 103; sl^9, 5%; n = 177).

(D–F) The ru^{pLLb} null mutation was not rescued in sl^2 mutant flies (ommatidia with fewer photoreceptors, 79%; n = 108) and was fully rescued, in terms of photoreceptor recruitment but not in terms of ommatidial rotation (misrotated ommatidia, 50%, n = 106), by the sl^9 mutant background. We suggest that residual levels of cSpi that are generated by Rhomboid-1 in the ER may become more effective once ER retention is compromised.

is evident from the observation that the defects in photoreceptor recruitment in *rhomboid-1/ru* double mutants are more severe than in *ru* null mutants alone (Baonza et al., 2001). One possible explanation for the partial rescue of a *ru* null by *sl* is that while Rhomboid-1 cleaves mSpi after exit from the ER, it generates very low levels of cSpi in the ER. Efficient release of these molecules in the *sl* background could account for the partial rescue.

Sl Is Required in the R8 Cells

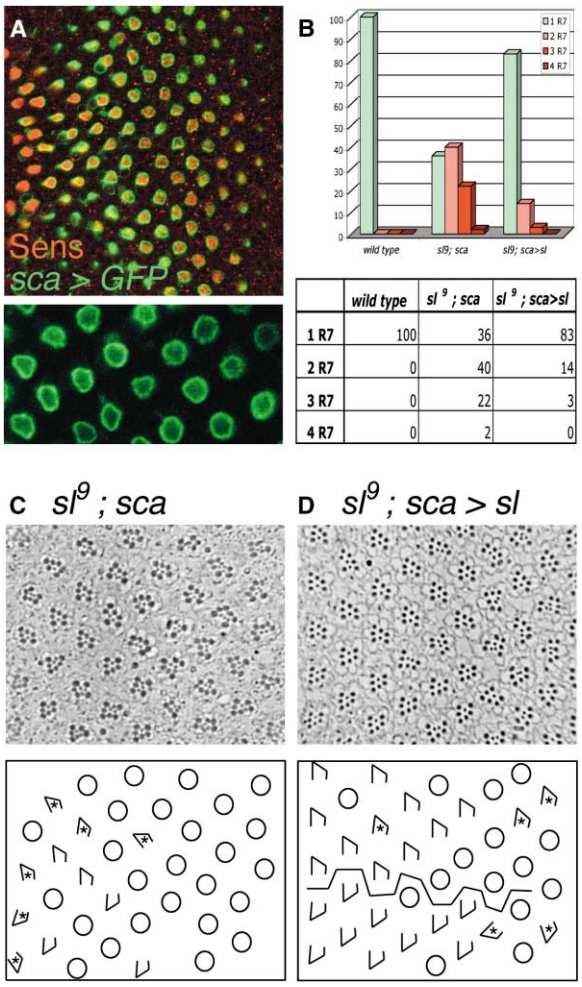
The results presented thus far suggest that Sl is required for ER retention in the cells where cleavage of Spi takes place, rather than in cells receiving the EGFR signal. This is different from the known paradigm of PLCγ in mammalian cells, which functions downstream to the activated receptor (Rhee, 2001). To test this prediction, we expressed the normal Sl protein in *sl* null flies only in the R8 photoreceptor cells. During eye development, R8 cells are the first to be specified at the morphogenetic furrow (Banerjee and Zipursky, 1990). Their recruitment is not dependent upon EGFR activation (Tio and Moses, 1997). These cells provide the source of cSpi that, in turn, induces the recruitment of the other photoreceptor cells (Freeman, 1996).

The *sca-Gal4* driver is expressed specifically in the R8 cells. Induction of membrane-tethered GFP by this driver shows expression only in the R8 cells, which are marked by nuclear Senseless staining (Figure 7A). When Sl was expressed by this driver in *sl* null flies, a reduction in the excessive recruitment of R7 cells, as well as a significant rescue of the rotation defects, was observed (Figures 7B–7D).

Discussion

Different signaling pathways exhibit distinct solutions to restrict the range of activation by ligands. In the Notch pathway, Delta and Serrate function as membrane bound ligands, thus restricting the range of activation to the cells immediately adjacent to the source (Lai, 2004; Schweisguth, 2004). Hedgehog operates over a limited number of cell diameters via an attachment of a cholesterol molecule to the protein (Ingham, 2001). Enhanced levels of Wg degradation were also shown to confine the range of activation (Sanson et al., 1999). In addition to restrictions at the level of the different ligands, a variety of inducible negative feedback responses were uncovered for each of the pathways. These components include diffusible inhibitors as well as elements that function in a cell-autonomous manner (Freeman, 2000; Perrimon and McMahon, 1999).

The EGFR pathway induces a wide range of cell fates during *Drosophila* development (Shilo, 2003). In most cases, activation of the pathway dictates a binary choice, and the range of activation is restricted to one



	wild type	sl 9 ; sca	sl 9 ; sca>sl
1 R7	100	36	83
2 R7	0	40	14
3 R7	0	22	3
4 R7	0	2	0

Figure 7. Sl Functions in the R8 Cells

To examine if Sl function is executed in the R8 cells producing cSpi, Sl was specifically expressed in the R8 cells of the larval eye imaginal disc in the background of *sl* null flies.

(A) The *sca-Gal4* driver is expressed in the R8 cells (marked by nuclear Senseless staining, red), as monitored by the expression of *UAS-CD8-GFP*. The anterior part of the eye imaginal disc is to the left.

(B–D) *sl* adult flies carrying only the *sca-Gal4* driver were compared to flies that also carried the *UAS-sl* rescue construct. While in the *sl* mutant only 36% of the ommatidia had one R7 cell (n = 170), in the rescued flies 83% of the ommatidia had one R7 cell (n = 162). Significant rescue of the rotation defects of *sl* was also observed. Of the ommatidia with the correct R7 number, 26% were misrotated in the *sl* mutant (n = 61), and only 6% in the rescued flies (n = 134). Note: no phenotypes were observed in the eye when *sl* was expressed by *sca-Gal4* in *wt* flies.

or few cell diameters from the active ligand source. One strategy to restrict the range of activation involves the induction of several inhibitory feedback responses (Shilo, 2003). This work explores a complementary mechanism whereby the range of EGFR activation is limited by restricting the amount of active ligand that is secreted by the producing cells.

cSpi Is Specifically Retained in the ER

We have discovered an unexpected feature exhibited by the cleaved form of Spitz, namely its capacity to be retained in the ER. This retention seems to be a general property, as it was observed in several tissues in the fly and in cell culture. Even when high levels of cSpi were expressed, the retention capacity in vivo was not saturated. Normally, the Spi precursor is cleaved by Rhomboid (after trafficking from the ER) to generate the active, secreted form of the ligand. Since expression of the cleaved form represents an artificial manipulation, we examined the possible relevance of this ER retention.

Several observations indicate that cSpi is biologically active and does not accumulate in the ER due to misfolding. In cell culture, a significant portion of the ligand is eventually secreted to the medium and is a potent activator of EGFR. In flies, the low levels of cSpi that are secreted were capable of triggering MAPK phosphorylation in the cells expressing cSpi and occasionally in one row of adjacent cells. Low levels of cSpi, too weak to be detected by GFP fluorescence, may leak outside the producing cells to trigger EGFR on the cell surface.

The accumulation of cSpi in the ER appears to reflect a novel mechanism for ER retention. In contrast to ER retention of the full-length Spi precursor, cSpi remains in the ER when the retrograde trafficking machinery from the Golgi to the ER is compromised following incubation with dsRNA for COPI (Figure 2). Utilization of a novel ER retention and export machinery has been previously identified for the SREBP protein-regulating cholesterol synthesis (Yang et al., 2002).

Sl Is Required for cSpi Retention in Cells and Flies

To identify the mechanism responsible for cSpi retention and assess its biological significance, we conducted a screen for dsRNAs that would compromise this property. We focused our analysis on the *sl* gene, in view of previous observations demonstrating that it is a negative regulator of EGFR signaling in the eye (Thackeray et al., 1998).

Sl is broadly expressed (Emori et al., 1994). Compromising the levels of Sl in embryos, either by dsRNA injection or in a *sl* mutant background, led to efficient release of cSpi. Thus, Sl is also required in vivo for the retention of cSpi. The actual retention mechanism remains unknown. Sl is a cytoplasmic protein, while cSpi is a secreted protein that is retained within the ER lumen. Additional proteins must participate to form a physical link. While *sl* encodes a PLCγ, we do not believe that its enzymatic activity is necessary for the retention process. *sl* interacts genetically with EGFR signaling only in the eye. *sl* mutants that were defective in the catalytic domain did not give rise to an eye phenotype (Mankidy et al., 2003). In addition to the catalytic domain, Sl also contains several motifs that may mediate protein-protein interactions, including SH2, SH3, and PH domains. It is thus possible that in addition to its enzymatic role, Sl serves as a scaffold protein in other contexts. In mammalian cells, PLCγ has been shown to function in the cells receiving the signal, downstream to receptor-tyrosine kinases (Rhee, 2001). The implicated role of Sl/PLCγ in the cells producing the signal points to a novel function of this protein.

cSpi Retention Is Required Specifically in the Eye

While Sl is broadly expressed, the EGFR hyperactivation phenotype of *sl* null flies is manifested only in the eye. This phenotype entails recruitment of extra R7 photoreceptor cells and misrotation of ommatidia. The restricted effect led us to examine the possibility that cSpi is normally generated in the ER only in the eye. The cleavage of EGFR ligands depends upon a distinct family of serine proteases that carry out intramembrane proteolysis. Rhomboid-1 is the primary player, and hence mutations in this gene give rise to embryonic phenotypes that are similar to *spi* or *Star* (Mayer and Nusslein-Volhard, 1988).

Two additional members of the family, Rhomboid-2/Brho and Rhomboid-3/Ru, are expressed in the oocyte and the eye, respectively (Guichard et al., 2000; Wasserman et al., 2000). Recently, expression of Ru was also detected in the embryonic VUM neurons, where it plays a role in guidance of tracheal migration in the CNS (Gallio et al., 2004). Homozygous null mutations for *ru* demonstrated that it is essential for normal eye development, but its role is partially redundant with Rhomboid-1, as some photoreceptor cells are recruited. The question is whether there are properties of Ru that are distinct from those of Rhomboid-1 and may account for the generation of cSpi in the ER during eye development.

As far as substrate specificity is concerned, the Rhomboid 1-3 proteins are all capable of cleaving the membrane precursors of the EGFR ligands Spi, Keren, and Gurken. In addition, all three proteins are enriched in the Golgi when expressed in mammalian cells (Urban et al., 2002). However, functional assays in cell culture, including both *Drosophila* and mammalian cells, suggest that in contrast to Rhomboid-1, Ru may be capable of cleaving the Spi precursor already in the ER (Urban et al., 2002). However, cSpi is secreted only upon coexpression of Star. While Ru is located primarily in the Golgi, this cleavage may take place en route to the Golgi. The failure to secrete cSpi in the absence of Star likely represents the property of ER retention that was uncovered in this work.

ru and *sl* give rise to opposite phenotypes in the eye. We assume that they act in a sequential manner, i.e., Ru generates cSpi in the ER and Sl mediates the retention of this ligand, to avoid excessive secretion. The genetic interaction experiments between *ru* and *sl* can be interpreted within this context. Indeed, the eye phenotype of hypomorphic mutations in *ru* could be efficiently rescued by mutations in *sl*. While the level of cSpi in the ER was compromised, more efficient secretion was facilitated in the *sl* background, thus compensating for the initial defect. Surprisingly, even null mutations in *ru* were partially rescued by *sl* mutants. In a *ru* null background, Rhomboid-1 is the only other known Rhomboid family protease that is functional in the eye. We suggest that residual levels of cSpi may also be generated in the ER by Rhomboid-1. An efficient release of these low levels in the absence of Sl may lead to the partial rescue we observed in *ru* null mutants.

Finally, the requirement for Sl specifically in the cells processing the ligand was demonstrated by the capacity to rescue *sl* null flies by expressing Sl in the R8 cells. Normally, expression of Rhomboid-1 and Ru in the differentiating photoreceptor cells is induced by EGFR activation, thus making these cells a source for subsequent rounds of photoreceptor cell recruitment. Incomplete rescue by expression of Sl in R8 cells may be explained by the failure to restore ER retention of cSpi in the other photoreceptor cells expressing Ru.

In conclusion, we have demonstrated that the cleaved form of Spi is efficiently retained in the ER through a novel mechanism. This retention is significant only in the developing eye, where the Rhomboid-3/Ru protein may normally generate the cleaved ligand in the ER. Thus, in spite of efficient cleavage of mSpi in the ER, only the molecules that will overcome retention by association with Star will be secreted to activate EGFR in the neighboring cells. *small wing*, encoding a PLCγ, provides a link to the retention mechanism, and *sl* mutants exhibit EGFR hyperactivation phenotypes mainly in the eye. The eye is a tissue where the restricted range of EGFR activation is particularly crucial. The number of undifferentiated precursor cells is limited. EGFR activation is responsible for sequential inductions of the different cell types comprising the mature ommatidia (Flores et al., 2000; Freeman, 1996). It is thus imperative to restrict the number of cells that are induced at every cycle. Negative feedback loops that are transcriptionally induced by EGFR activation in the cells receiving the signal were previously shown to be central to this restriction. We demonstrate that fine tuning the level of ligand that is released by the cells providing the signal represents another cardinal tier of regulation.

Experimental Procedures

DNA Constructs

The following constructs were generated by PCR and inserted into the pUAST vector. cSpi-GFP was formed by inserting eGFP after T54 of Spi and a stop codon after P128 prior to the transmembrane domain. cSpiΔEGF was formed by a deletion of C78–C117. cSpi-HRP was generated by fusing the Wg signal peptide followed by HRP (obtained from J.P Vincent; Dubois et al., 2001) to R28 of Spi and inserting a termination codon within the transmembrane domain after A144. sGFP was produced by fusing the signal peptide of Spi (residues 1–54) to eGFP. cKeren was generated by fusing the signal peptide of Spi (residues 1–54) to eGFP and Keren (residues I39–T133). A stop codon was inserted following T133, prior to the transmembrane domain. Vein-GFP was obtained from T. Volk. Argos-GFP was generated by insertion of eGFP following E155. *UAS-sl* was generated by cloning the full-length *sl* cDNA (RE62235) into pUAST.

Fly Strains

The following lines were used: *prd-Gal4*, *en-Gal4*, *dpp-Gal4*, *sca-Gal4*, *UAS-lacZ* (nuclear), *UAS-CD8-GFP*, and PDI-GFP (Bobinnec et al., 2003) obtained from Y. Bobinnec; sl^{1}, sl^{2}, and sl^{9} from J. Thackeray; and flb^{1K35}, $Star^{IIN23}$, $argos^{l\Delta7}$, ru^{1}, and ru^{PLLb} obtained from M. Gallio.

Cells and dsRNA

S_2R^+ cells were used for all experiments. The cells were transiently transfected with the appropriate pUAST plasmid and *actin-Gal4* (calcium phosphate transfection kit, Invitrogen). dsRNA screen was performed according to Kiger et al. (2003). T7 promoter sequence was inserted on both 3′ and 5′ oligonucleotides. COPIγ primers: 5′ primer, ATAGCGATTGCCTGTAAGCTGA; 3′ primer, ATGGCCGTG ACCGCGGCTGCTC; Sl primers: 5′ primer, CTTCGTCGTGCTCCCT AAAC; 3′ primer, CTGCATAATGCGACAGTGCT. CG2005 (R-PTP 99A) primers: 5′ primer, CACTGGCACATACATCGTCC; 3′ primer, CTGCGGAGTACATTGGGATT.

Immunohistochemistry and Histology

For immunohistochemistry, the following antibodies were used: rabbit anti-β-gal (Cappel), anti-En 4D9 (obtained from the Developmental Studies Hybridoma Bank developed under the auspices of

the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA), mouse anti-dpERK (Sigma), Cy-2 conjugated goat anti-HRP (Jackson ImmunoResearch), guinea pig anti-Senseless from Hugo Bellen, anti-Troponin H to detect BiP (Babraham Bioscience Technologies), and mouse anti-*Drosophila* Golgi (p120) (Calbiochem).

For anti-dpERK staining of embryos, fixation was carried out in 4% formaldehyde/PBS for 20 min, primary antibody incubated overnight at 4°C in 0.1% Triton/PBS, and secondary antibody (goat anti-mouse biotin, Chemicon) incubated for 90 min at RT. It was amplified by Streptavidin-HRP for 30 min at RT, followed by incubation with Tyramide biotin for 20 min (both from Perkin Elmer TSA biotin system). Finally, embryos were incubated with Streptavidin-Cy3 for 30 min at RT (Jackson ImmunoResearch). Eye sections were according to Tomlinson and Ready (1987), and embedding was in JB-4 embedding kit (Electron Microscopy Sciences).

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References

Banerjee, U., and Zipursky, S.L. (1990). The role of cell-cell interaction in the development of the *Drosophila* visual system. Neuron *4*, 177–187.

Baonza, A., Casci, T., and Freeman, M. (2001). A primary role for the epidermal growth factor receptor in ommatidial spacing in the *Drosophila* eye. Curr. Biol. *11*, 396–404.

Bier, E., Jan, L.Y., and Jan, Y.N. (1990). rhomboid, a gene required for dorsoventral axis establishment and peripheral nervous system development in *Drosophila melanogaster*. Genes Dev. *4*, 190–203.

Bobinnec, Y., Marcaillou, C., Morin, X., and Debec, A. (2003). Dynamics of the endoplasmic reticulum during early development of *Drosophila melanogaster*. Cell Motil. Cytoskeleton *54*, 217–225.

Brown, K.E., and Freeman, M. (2003). Egfr signalling defines a protective function for ommatidial orientation in the *Drosophila* eye. Development *130*, 5401–5412.

Casci, T., Vinos, J., and Freeman, M. (1999). Sprouty, an intracellular inhibitor of Ras signaling. Cell *96*, 655–665.

Dubois, L., Lecourtois, M., Alexandre, C., Hirst, E., and Vincent, J.P. (2001). Regulated endocytic routing modulates wingless signaling in *Drosophila* embryos. Cell *105*, 613–624.

Emori, Y., Sugaya, R., Akimaru, H., Higashijima, S., Shishido, E., Saigo, K., and Homma, Y. (1994). *Drosophila* phospholipase C-gamma expressed predominantly in blastoderm cells at cellularization and in endodermal cells during later embryonic stages. J. Biol. Chem. *269*, 19474–19479.

Flores, G.V., Duan, H., Yan, H., Nagaraj, R., Fu, W., Zou, Y., Noll, M., and Banerjee, U. (2000). Combinatorial signaling in the specification of unique cell fates. Cell *103*, 75–85.

Freeman, M. (1996). Reiterative use of the EGF receptor triggers differentiation of all cell types in the *Drosophila* eye. Cell *87*, 651–660.

Freeman, M. (2000). Feedback control of intercellular signalling in development. Nature *408*, 313–319.

Freeman, M., Klambt, C., Goodman, C.S., and Rubin, G.M. (1992). The argos gene encodes a diffusible factor that regulates cell fate decisions in the *Drosophila* eye. Cell *69*, 963–975.

Gabay, L., Seger, R., and Shilo, B.Z. (1997). In situ activation pattern of *Drosophila* EGF receptor pathway during development. Science *277*, 1103–1106.

Gaengel, K., and Mlodzik, M. (2003). Egfr signaling regulates ommatidial rotation and cell motility in the *Drosophila* eye via MAPK/Pnt signaling and the Ras effector Canoe/AF6. Development *130*, 5413–5423.

Gallio, M., Englund, C., Kylsten, P., and Samakovlis, C. (2004). Rhomboid 3 orchestrates Slit-independent repulsion of tracheal branches at the CNS midline. Development *131*, 3605–3614.

Ghiglione, C., Carraway, K.L., 3rd, Amundadottir, L.T., Boswell, R.E., Perrimon, N., and Duffy, J.B. (1999). The transmembrane molecule kekkon 1 acts in a feedback loop to negatively regulate the activity of the *Drosophila* EGF receptor during oogenesis. Cell *96*, 847–856.

Ghiglione, C., Bach, E.A., Paraiso, Y., Carraway, K.L., 3rd, Noselli, S., and Perrimon, N. (2002). Mechanism of activation of the *Drosophila* EGF Receptor by the TGFalpha ligand Gurken during oogenesis. Development *129*, 175–186.

Golembo, M., Schweitzer, R., Freeman, M., and Shilo, B.Z. (1996). Argos transcription is induced by the *Drosophila* EGF receptor pathway to form an inhibitory feedback loop. Development *122*, 223–230.

Guichard, A., Roark, M., Ronshaugen, M., and Bier, E. (2000). brother of rhomboid, a rhomboid-related gene expressed during early *Drosophila* oogenesis, promotes EGF-R/MAPK signaling. Dev. Biol. *226*, 255–266.

Ingham, P.W. (2001). Hedgehog signaling: a tale of two lipids. Science *294*, 1879–1881.

Kiger, A., Baum, B., Jones, S., Jones, M., Coulson, A., Echeverri, C., and Perrimon, N. (2003). A functional genomic analysis of cell morphology using RNA interference. J. Biol. *2*, 27.

Kramer, S., Okabe, M., Hacohen, N., Krasnow, M.A., and Hiromi, Y. (1999). Sprouty: a common antagonist of FGF and EGF signaling pathways in *Drosophila*. Development *126*, 2515–2525.

Lai, E.C. (2004). Notch signaling: control of cell communication and cell fate. Development *131*, 965–973.

Lee, J.R., Urban, S., Garvey, C.F., and Freeman, M. (2001). Regulated intracellular ligand transport and proteolysis control EGF signal activation in *Drosophila*. Cell *107*, 161–171.

Mankidy, R., Hastings, J., and Thackeray, J.R. (2003). Distinct phospholipase C-gamma-dependent signaling pathways in the *Drosophila* eye and wing are revealed by a new small wing allele. Genetics *164*, 553–563.

Mayer, U., and Nusslein-Volhard, C. (1988). A group of genes required for pattern formation in the ventral ectoderm of the *Drosophila* embryo. Genes Dev. *2*, 1496–1511.

Neuman-Silberberg, F.S., and Schupbach, T. (1993). The *Drosophila* dorsoventral patterning gene gurken produces a dorsally localized RNA and encodes a TGF alpha-like protein. Cell *75*, 165–174.

Perrimon, N., and McMahon, A.P. (1999). Negative feedback mechanisms and their roles during pattern formation. Cell *97*, 13–16.

Reich, A., and Shilo, B.Z. (2002). Keren, a new ligand of the *Drosophila* epidermal growth factor receptor, undergoes two modes of cleavage. EMBO J. *21*, 4287–4296.

Reich, A., Sapir, A., and Shilo, B. (1999). Sprouty is a general inhibitor of receptor tyrosine kinase signaling. Development *126*, 4139–4147.

Rhee, S.G. (2001). Regulation of phosphoinositide-specific phospholipase C. Annu. Rev. Biochem. *70*, 281–312.

Rutledge, B.J., Zhang, K., Bier, E., Jan, Y.N., and 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.

Sannerud, R., Saraste, J., and Goud, B. (2003). Retrograde traffic in the biosynthetic-secretory route: pathways and machinery. Curr. Opin. Cell Biol. *15*, 438–445.

Sanson, B., Alexandre, C., Fascetti, N., and Vincent, J.P. (1999). Engrailed and hedgehog make the range of Wingless asymmetric in *Drosophila* embryos. Cell *98*, 207–216.

Schnepp, B., Grumbling, G., Donaldson, T., and Simcox, A. (1996). Vein is a novel component in the *Drosophila* epidermal growth factor

receptor pathway with similarity to the neuregulins. Genes Dev. *10*, 2302–2313.

Schweisguth, F. (2004). Notch signaling activity. Curr. Biol. *14*, R129–R138.

Schweitzer, R., Howes, R., Smith, R., Shilo, B.Z., and Freeman, M. (1995a). Inhibition of *Drosophila* EGF receptor activation by the secreted protein Argos. Nature *376*, 699–702.

Schweitzer, R., Shaharabany, M., Seger, R., and Shilo, B.Z. (1995b). Secreted Spitz triggers the DER signaling pathway and is a limiting component in embryonic ventral ectoderm determination. Genes Dev. *9*, 1518–1529.

Shilo, B.Z. (2003). Signaling by the *Drosophila* epidermal growth factor receptor pathway during development. Exp. Cell Res. *284*, 140–149.

Strutt, H., and Strutt, D. (2003). EGF signaling and ommatidial rotation in the *Drosophila* eye. Curr. Biol. *13*, 1451–1457.

Sturtevant, M.A., Roark, M., and Bier, E. (1993). The *Drosophila* rhomboid gene mediates the localized formation of wing veins and interacts genetically with components of the EGF-R signaling pathway. Genes Dev. *7*, 961–973.

Thackeray, J.R., Gaines, P.C., Ebert, P., and Carlson, J.R. (1998). small wing encodes a phospholipase C-(gamma) that acts as a negative regulator of R7 development in *Drosophila*. Development *125*, 5033–5042.

Tio, M., and Moses, K. (1997). The *Drosophila* TGF alpha homolog Spitz acts in photoreceptor recruitment in the developing retina. Development *124*, 343–351.

Tomlinson, A., and Ready, D.F. (1987). Cell fate in the *Drosophila* ommatidium. Dev. Biol. *123*, 264–275.

Tsruya, R., Schlesinger, A., Reich, A., Gabay, L., Sapir, A., and Shilo, B.Z. (2002). Intracellular trafficking by Star regulates cleavage of the *Drosophila* EGF receptor ligand Spitz. Genes Dev. *16*, 222–234.

Urban, S., Lee, J.R., and Freeman, M. (2001). *Drosophila* rhomboid-1 defines a family of putative intramembrane serine proteases. Cell *107*, 173–182.

Urban, S., Lee, J.R., and Freeman, M. (2002). A family of Rhomboid intramembrane proteases activates all *Drosophila* membrane-tethered EGF ligands. EMBO J. *21*, 4277–4286.

Wasserman, J.D., Urban, S., and Freeman, M. (2000). A family of rhomboid-like genes: *Drosophila* rhomboid-1 and roughoid/rhomboid-3 cooperate to activate EGF receptor signaling. Genes Dev. *14*, 1651–1663.

Yang, T., Espenshade, P.J., Wright, M.E., Yabe, D., Gong, Y., Aebersold, R., Goldstein, J.L., and Brown, M.S. (2002). Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER. Cell *110*, 489–500.