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Rhomboid cleaves Star to regulate the levels of secreted Spitz

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Intracellular trafficking of the precursor of Spitz (Spi), the major *Drosophila* EGF receptor (EGFR) ligand, is facilitated by the chaperone Star, a type II transmembrane protein. This study identifies a novel mechanism for modulating the activity of Star, thereby influencing the levels of active Spi ligand produced. We demonstrate that Star can efficiently traffic Spi even when present at sub-stoichiometric levels, and that in *Drosophila* S_2R^+ cells, Spi is trafficked from the endoplasmic reticulum to the late endosome compartment, also enriched for Rhomboid, an intramembrane protease. Rhomboid, which cleaves the Spi precursor, is now shown to also cleave Star within its transmembrane domain both in cell culture and in flies, expanding the repertoire of known Rhomboid substrates to include both type I and type II transmembrane proteins. Cleavage of Star restricts the amount of Spi that is trafficked, and may explain the exceptional dosage sensitivity of the *Star* locus in flies.

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Introduction

The EGF receptor (EGFR) pathway defines one of the five pathways guiding most of the developmental decisions in *Drosophila*. It is used repeatedly at various developmental stages, and leads to induction of distinct cell fates depending upon the cell context of activation (Shilo, 2003). In accordance with the central role of this pathway, mutations in the different components give rise to severe developmental defects at every junction where the pathway is used. Conversely, ectopic activation leads to deleterious consequences as well. Thus, tight regulation of the spatial and temporal pattern of EGFR activation is crucial for all phases of development.

The primary EGFR ligand is Spi, a homologue of mammalian TGF-α (Rutledge *et al*, 1992). In contrast to other *Drosophila* patterning cascades, regulation of EGFR activation does not rely on restricted expression of this activating ligand, but rather on post-translational modification (Schweitzer *et al*, 1995a). Spi is broadly expressed throughout development, albeit as an inactive transmembrane precursor (Rutledge *et al*, 1992). The mechanism by which this precursor is processed to generate an active, secreted ligand lies at the heart of spatial and temporal regulation of EGFR activation.

A central aspect of Spi processing involves proteolytic cleavage by Rhomboid, a conserved seven-transmembrane domain protein. The catalytic domain of Rhomboid is embedded within the transmembrane domains, leading to intramembrane cleavage of Spi, and release of its extracellular portion from the membrane (Urban *et al*, 2001). Proteolytic cleavage by Rhomboid is specific; to date, the three transmembrane ligands of EGFR (Spi, Keren and Gurken) are the only known Rhomboid substrates in *Drosophila* (Ghiglione *et al*, 2002; Urban *et al*, 2002). Rhomboid uses distinct features in the transmembrane domain of the ligand for recognition and cleavage, most notably helix-breaking residue(s) at the outer third of the transmembrane helix (Urban and Freeman, 2003). Rhomboid expression is highly dynamic (Bier *et al*, 1990), and precisely precedes the pattern of activated MAPK that is induced by EGFR (Gabay *et al*, 1997). Ectopic expression of Rhomboid leads to the corresponding activation of EGFR in every tissue, indicating that Rhomboid expression is the limiting component in pathway activation (Golembo *et al*, 1996). In addition to Rhomboid (also referred to as Rhomboid-1), which carries out ligand processing in most tissues, two other members of the family have been characterized: Rhomboid-2/Brho/Stet is required in the oocyte and in signaling from germ cells (Guichard *et al*, 2000; Schulz *et al*, 2002; Gilboa and Lehmann, 2006), and Rhomboid-3/Roughoid is required in the eye (Wasserman *et al*, 2000).

The recent structural elucidation of the bacterial Rhomboid homologue, GlpG, has shed light on the mechanistic basis for intramembrane proteolysis (Wang *et al*, 2006; Wu *et al*, 2006; Ben-Shem *et al*, 2007). The catalytic serine is at the top of the fourth transmembrane helix, which does not traverse the bilayer completely. A hydrophilic cavity facing the extracellular side is created, to allow the access of water molecules. It also requires the substrate transmembrane domain to break its helical structure at the top third of the transmembrane domain, in order to fit the protease active site.

Modulating the levels of secreted Spi generated by the signal-producing cells provides a potential mechanism for restricting EGFR activation. Rhomboid levels are unlikely to

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contribute to such a mechanism, as Rhomboid functions catalytically to cleave Spi (Urban *et al*, 2001). Indeed, although ectopic expression of Rhomboid leads to expansion of the EGFR activation domains, the activation levels are comparable to the endogenous ones. Regulation of intracellular trafficking provides an alternative aspect of Spi processing by which active ligand levels can be controlled. The Spi precursor is produced in excess, but retained in the endoplasmic reticulum (ER) in every cell where it is synthesized. ER retention is an essential aspect, because in its absence, the Spi precursor can reach the plasma membrane, where it is cleaved by nonspecific metalloproteases, which ectopically release an active secreted moiety (Lee *et al*, 2001; Reich and Shilo, 2002). However, maintaining Spi in the ER imposes a constraint on the cells in which normal processing should take place, as Rhomboid resides in a different compartment. When expressed in mammalian cells, Rhomboid proteins localized to the Golgi (Lee *et al*, 2001; Urban *et al*, 2002). Trafficking of Spi is thus essential for cleavage.

Intracellular trafficking of Spi before cleavage is mediated by Star, a type II, single-pass transmembrane protein. Star associates with Spi and efficiently mediates its trafficking from the ER to the Rhomboid compartment (Lee *et al*, 2001; Tsruya *et al*, 2002). Star is an exceptionally dosage-sensitive component of the EGFR pathway. Mutations in the *Star* locus were first identified in 1919 by virtue of the dominant, haploinsufficient phenotype they induce in the eye, leading to rough eyes with a reduced number of photoreceptors in each ommatidium (Bridges and Morgan, 1919; Heberlein and Rubin, 1991; Brown and Freeman, 2003). Conversely, overexpression of Star leads to extra photoreceptor cells (Hsiung *et al*, 2001; Tsruya *et al*, 2002). Genetic interactions confirmed that these phenotypes are a result of hypo- or hyperactivation of the EGFR pathway, respectively (Kolodkin *et al*, 1994).

In the present study, we demonstrate that Star activity is subject to regulation, resulting in restriction of the levels of active Spi ligand generated by the signal-producing cells. Star appears to traffic the Spi precursor while cycling between the ER and late endosomes of *Drosophila* S_2R^+ cells. A key observation is that Rhomboid, a late endosome resident in these cells, cleaves both the Spi precursor and Star. Cleavage of Star, which occurs within the transmembrane domain, leads to its inactivation as a Spi chaperone. Star cleavage allows for quantitative regulation of Spi trafficking, modulating the level of ligand that is cleaved and secreted.

Results

The trafficked Spi precursor and Rhomboid localize to late endosomes of Drosophila S_2R^+ cells

Compartmentalization, coupled to tight control of intracellular trafficking, was shown to be crucial in the control of Spi processing. Following translation, the Spi precursor is retained in the ER, by COPI-dependent retrograde trafficking (Schlesinger *et al*, 2004). Cleavage by the transmembrane protease Rhomboid, generating the active form of Spi, usually takes place in a distinct cellular compartment. Trafficking between the ER and the Rhomboid compartment is mediated by the Star chaperone. In the heterologous COS cells, Spi is trafficked to the Golgi, where it is cleaved by Rhomboid.

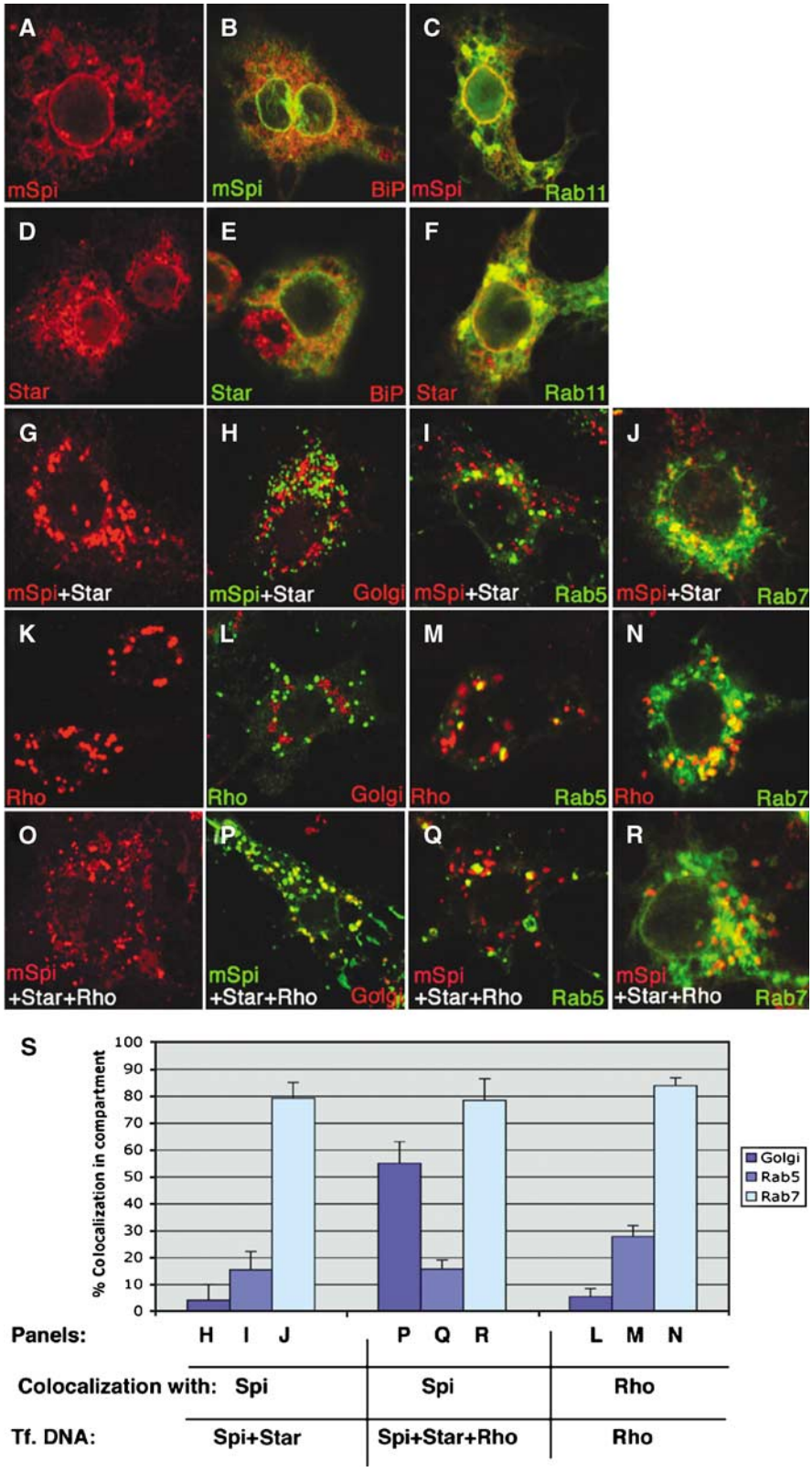
To follow this process in native *Drosophila* S_2R^+ cells, we first set out to define the cellular compartment(s) to which Spi is trafficked by Star. Compartment-specific markers used for this purpose included anti-BiP for the ER, anti-p120 for the Golgi, Rab5-GFP for early endosomes, Rab7-GFP for late endosomes and Rab11-GFP for recycling endosomes. As expected from previous studies, when expressed separately in S_2R^+ *Drosophila* cells, Spi and Star exhibited a perinuclear and punctate distribution. The perinuclear distribution colocalized with BiP (Figure 1B and E), and both patterns colocalized with Rab11 (Figure 1C and F). Upon coexpression with Star, Spi is efficiently translocated from the ER, exhibiting a punctate distribution, with no detectable accumulation on the plasma membrane (Figure 1G). The structures harboring trafficked Spi showed significant colocalization with Rab7, a lower level of overlap with Rab5 and no overlap with the Golgi marker (Figure 1H–J and S). We conclude that Star facilitates trafficking of Spi from the ER to late endosomes in S_2R^+ cells.

Significantly, Rhomboid-1 also localized predominantly to the Rab7 compartment, following its expression in S_2R^+ cells (Figure 1L–N and S), suggesting that Spi cleavage in *Drosophila* cells takes place in late endosomes. How does the presence of Rhomboid affect the intracellular distribution of Spi? Comparison of cells expressing Spi and Star in the absence or presence of Rhomboid showed significant accumulation of Spi in the late endosome in both cases (Figure 1J, R and S). However, in the presence of Rhomboid, low levels of Spi could also be identified in the Golgi (Figure 1P and S). This may indicate that Spi is secreted by trafficking to the Golgi, following cleavage in the late endosome. Additional trafficking routes through the recycling endosome or directly to the plasma membrane may also be envisaged (Zerial and McBride, 2001; Seachrist and Ferguson, 2003).

Figure 1 Intracellular localization of Spi processing machinery. The components of the Spi processing machinery were localized in *Drosophila* S_2R^+ cells. (**A**) mSpi is found in a perinuclear distribution as well as in a punctate pattern. (**B**) The perinuclear pattern colocalizes with the ER marker BiP, whereas the perinuclear and punctate patterns colocalize with Rab11 in the recycling endosome (**C**). (**D–F**) A similar distribution and colocalization was found for Star. (**G**) Coexpression of Spi and Star altered the distribution of Spi which was no longer detected in the perinuclear pattern, but instead accumulated in a punctate distribution. The punctate pattern does not localize with the Golgi marker (**H**) or Rab11 (not shown). Instead, it showed some colocalization with Rab5 marking the early endosome (**I**) and a pronounced colocalization with Rab7 marking the late endosome (**J**). (**K**) Rhomboid-1 is distributed in a punctate pattern, showing no colocalization with the Golgi (**L**), the recycling endosome (not shown) or the early endosome (**M**). A significant colocalization is observed with the late endosome marker (**N**). (**O–R**) Coexpression of Rhomboid with Spi and Star altered the distribution of Spi. Although significant colocalization in the Rab7 compartment was still detected (R), some of the weaker punctate structures containing Spi overlapped with the Golgi (P). This alteration may reflect trafficking of cleaved Spi, following processing by Rhomboid in the late endosome. Note: the following constructs were used. (A–C) mSpi-HA, (D–F) Star-HA, (G–J) mSpi-HA and Star-FLAG, (K–N) Rho-HA, (O–R) mSpi-HA, Star and Rho-FLAG. The different proteins were detected by anti-HA staining, anti-BiP for the ER, and anti-p120 for the Golgi. Rab5,Rab7 and Rab11 are GFP fusion proteins. (**S**) In each cell, vesicles with a given compartment marker were counted, and the percentage of those colocalizing with Spi or Rho monitored. For each bar, data were collected from 5 to 10 cells, and s.d.s are shown.

Rhomboid limits trafficking of Spi by Star

How do different stoichimetric relationships between Spi and Star impinge on trafficking? We transiently transfected varying DNA amounts of Spi and Star into *Drosophila* S_2R^+ cells, and monitored their intracellular localization. A GFP-Spi fusion construct and HA-tagged Star were used to allow for simultaneous detection of both elements. We found that cotransfection of Spi altered the intracellular distribution of Star in a dose-dependent manner. When transfected at a five-fold excess over Spi, Star remained predominantly in the



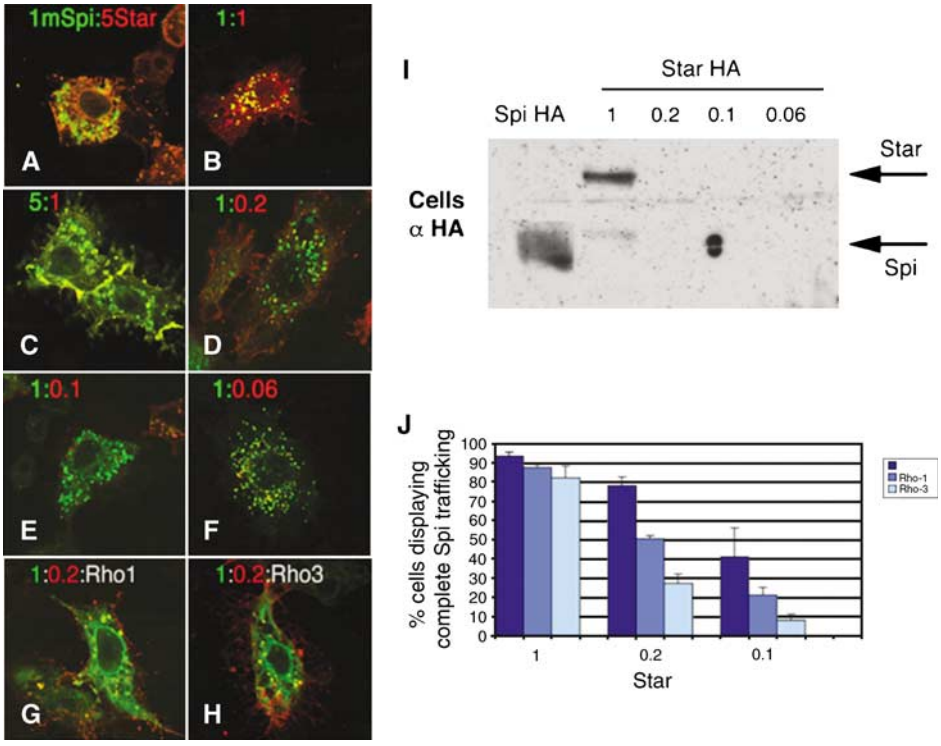


Figure 2 Rhomboid impairs Spi trafficking by Star. The ratio between Star and Spi expressed in S_2R^+ cells was manipulated to examine the effect of Star on Spi trafficking and vice versa. (**A**) When Star-HA was transfected at a five-fold excess relative to Spi-GFP, all molecules of Spi (green) were detected in a punctate pattern, whereas most Star molecules (red) were located in the ER. Star was detected by anti-HA staining. (**B**) Equal levels of Star/Spi displayed complete trafficking of Spi and colocalization of the majority of Star staining. (**C**) A five-fold excess of Spi still displayed complete trafficking of Spi. Colocalization of Star was complete. (**D**) When the same five-fold excess of Spi was retained but the total level of DNA was reduced by a factor of five, a similar distribution was observed in ~80% of the cells. In the remaining cells, only partial trafficking of Spi was observed. All subsequent experiments were carried out with these lower levels of *Spi* DNA. (**E, F**) A 10- or 15-fold excess of Spi over Star still displayed complete Spi trafficking in ~40% of the cells. The effect of Rhomboid on trafficking of Spi by Star was examined under conditions in which Star levels were limiting. (**G**) Coexpression of Rhomboid when Spi was present at a five-fold excess over Star resulted in localization of Spi to the ER, as well as in punctate structures, in a significant portion of the cells (compare with D). (**H**) A similar result was obtained when Rhomboid-3 was coexpressed with Spi and Star. (**I**) Relative levels of Spi and Star were compared when both were tagged with HA and transfected at equal DNA levels. Star protein levels were lower than Spi, implying that Star can efficiently traffic Spi when its relative protein level is even lower than what was predicted by transfected DNA ratios. (**J**) Quantification of the efficiency of Spi trafficking in the presence of Rhomboid or Rhomboid-3, at declining levels of Star. Although both Rhomboid proteins significantly impaired Spi trafficking by Star, Rhomboid-3 did so more efficiently.

ER (Figure 2A). Raising the relative levels of transfected Spi resulted, however, in a gradual depletion of Star from the ER, and Star was found to colocalize with Spi in a punctate pattern. Intermediate levels of Star were observed in the ER when Star and Spi were transfected in equivalent levels (Figure 2B), whereas little or no Star remained in the ER when Spi was transfected at a five-fold excess over Star (Figure 2C). These experiments demonstrate that high levels of Spi can alter the intracellular distribution of Star.

Complete trafficking of Spi was observed in these cotransfection experiments, even when Spi was in excess. To further characterize the capacity of low levels of Star to traffic Spi, a fixed level of Spi was expressed along with decreasing concentrations of cotransfected Star. Many of the cells continued to exhibit full trafficking of Spi, even when Spi DNA was transfected at a 15-fold excess over Star (Figure 2D–F). To verify the stoichiometric ratios between Spi and Star proteins, their levels were compared when both proteins were tagged with HA, and Star-HA was transfected at decreasing DNA concentrations. Star protein levels were lower than those of Spitz when equal DNA amounts were transfected, and were hardly visible upon decreasing the concentration of its DNA (Figure 2I). The relative levels of Star protein appear to be even lower than predicted from the DNA ratios. Thus, markedly sub-stoichiometric levels of Star can efficiently traffic Spi. A plausible mechanism may be the recycling of Star from the late endosome to the ER, to traffic additional molecules of Spi.

A marked change in the efficiency of Spi trafficking by low levels of Star was observed upon coexpression of Rhomboid. In many cells, only partial trafficking of Spi was detected, and the majority of Spi molecules were retained in the ER (Figure 2G). This effect of Rhomboid on Spi trafficking was quantified (Figure 2J). A five-fold excess of Spi over Star could be efficiently trafficked in the absence of Rhomboid, such that in ~80% of the cells Spi was fully cleared from the ER. However, in the presence of Rhomboid, efficient trafficking of Spi was achieved in only ~50% of the cells. The attenuating effect of Rhomboid was observed only when Star levels were limiting. When Spi and Star were expressed at an equimolar ratio, highly efficient trafficking was obtained, both in the absence or presence of Rhomboid.

Expression of another member of the Rhomboid family, Rhomboid-3, also attenuated the capacity of

sub-stoichiometric levels of Star to traffic Spi (Figure 2H). Rhomboid-3 was even more efficient than Rhomboid-1 in this regard, reducing for example the percentage of cells displaying complete Spi trafficking at a 5:1 Spi/Star ratio from 80 to 25% (Figure 2J). These results demonstrate a significant influence of Rhomboid proteases on the capacity of Star to traffic Spi.

Rhomboid-dependent cleavage of Star

We sought to determine whether the diminished trafficking capacity of Star in Rhomboid-expressing cells was associated with post-translational modifications of the Star protein. Extracts prepared from *Drosophila* S2 cells coexpressing Spi, Star and Rhomboid were subjected to Western blot analysis. A Star construct tagged C-terminally with an HA moiety was used and Star expression was monitored using anti-HA antibodies. Star-HA is detected in cell extracts primarily as an ~90 kDa band. Coexpression of Rhomboid, however, led to the appearance of a prominent lower molecular weight (~35 kDa) form of Star in the cells, in the presence or absence of Spi (Figure 3). As this form was detected by anti-HA, it represents a C-terminal fragment of Star, and may correspond to the cleaved extracellular domain of this type II transmembrane protein.

Consistent with this interpretation, a similarly sized fragment was also secreted into the medium and detected by anti-HA (Figure 3). An efficient cleavage of Star was similarly observed when Rhomboid-3/Roughoid was coexpressed with Star instead of Rhomboid-1 (Figure 3). Low levels of the 35 kDa fragment were detected in the absence of transfected Rhomboid, perhaps owing to low levels of endogenous Rhomboid in S2 cells. Interestingly, the apparent Rhomboid-dependent cleavage of Star did not require the presence of Spi (Figure 3).

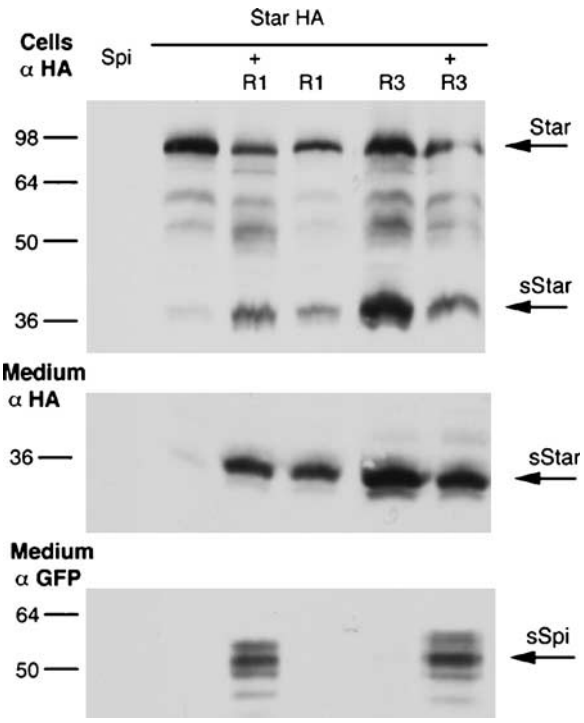


Figure 3 Rhomboid-dependent cleavage of Star. Modification of full-length Star tagged at the C-terminus (Star-HA) was examined. In the presence of Rhomboid (R1), a lower 35 kDa form, representing the extracellular domain of Star (sStar), was detected by anti-HA antibodies. This cleaved form is also secreted to the medium. Cleavage of Star was independent of the presence of Spi. Spi-GFP coexpressed with Star and Rhomboid underwent cleavage and secretion to the medium (sSpi), in accordance with previous studies. Similar results were obtained when Star was coexpressed with Rhomboid-3. Note the higher efficiency of Star cleavage in this instance.

Rhomboid cleaves Star directly

Is Rhomboid the protease directly responsible for Star cleavage? Several approaches were taken to address this critical issue. To assess whether Rhomboid acts catalytically in this context, we examined the level of Star cleavage following dilution of cotransfected Rhomboid. Secreted Star could still be detected in the medium even when the levels of Rhomboid DNA were 5000-fold lower than those of Star (Figure 4A). Similar findings were previously reported for cleavage of Spi by Rhomboid (Urban *et al*, 2001). To verify the relative levels of Star and Rhomboid proteins, their expression levels were compared when both were tagged with HA. Although Rhomboid levels were higher than Star following transfection of equal DNA amounts, Rhomboid protein was undetectable (yet functional) when its DNA was 1/50 or less than that of Star (Figure 4B).

We repeated the cell-based cleavage assay using a Rhomboid variant in which the active site serine 217 has been mutated (Urban *et al*, 2001). Neither secreted Spi nor secreted Star could be detected in the medium of cells coexpressing Spi and Star with this mutant form of Rhomboid (Figure 4C). This set of results is consistent with Rhomboid acting as a proteolytic enzyme in bringing about the cleavage of Star.

Finally, we carried out the Star cleavage assay in a heterologous cell line, reasoning that components required for Star cleavage in addition to Rhomboid may not be conserved. Star tagged with FLAG at the C-terminus was expressed alone or in the presence of Rhomboid-1 or Rhomboid-3 in human 293 cells. The appearance of the cleaved form of Star following coexpression with Rhomboid proteins was observed, both in the cells and in the medium (Figure 4D). Taken together, these experiments strongly suggest that cleavage of Star is a direct consequence of proteolysis by Rhomboid.

We carried out mass spectrometric analysis to determine the cleavage site within Star that gives rise to the secreted, 35 kDa form. The cleaved form of Star was purified from the medium of S_2R^+ cells expressing both Rhomboid-3 and a Star construct FLAG-tagged at its C-terminus (Figure 5A). Mass spectrometry of proteolytic fragments generated by treatment with chymotrypsin identified several peptides corresponding to the Star extracellular region. The most N-terminal peptide contained residues ^{298}IVYMoxDTTEIRHQQF^{311} corresponding to part of the predicted transmembrane domain of Star (Figure 5B). We conclude that the secreted form of Star is generated by intramembrane proteolysis, further supporting the notion of direct cleavage by Rhomboid proteins.

In vivo cleavage of Star by Rhomboids

To monitor the cleavage of Star by Rhomboids in an *in vivo* setting, we fused the transcriptional reporter LexA-VP16 to the extreme N-terminus of Star. This reporter contains a nuclear-localization signal, and thus, upon cleavage of Star

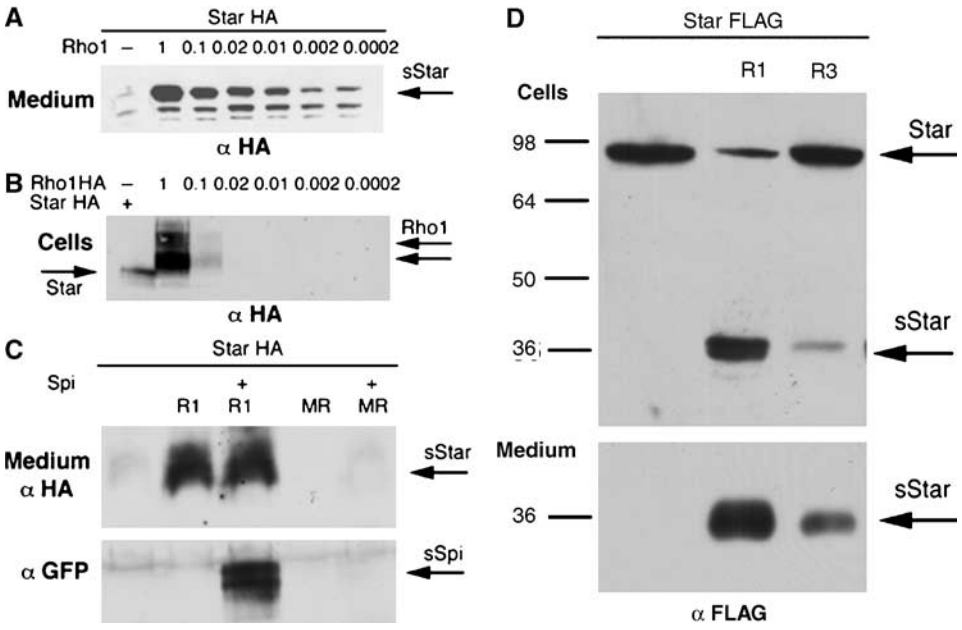


Figure 4 Rhomboid cleaves Star directly. (**A**) The capacity of Rhomboid to act catalytically in promoting Star cleavage was examined under conditions of low concentrations of Rhomboid. Even a 5000-fold DNA dilution could mediate efficient Star cleavage and secretion. (**B**) Protein levels of Star and Rho, both tagged with HA, were compared following transfection of equal DNA levels. Rho protein levels were higher under these conditions. Nevertheless, when Rho DNA was diluted 1/50 or more, it was no longer detected. Rho appeared as a higher molecular weight doublet owing to multimerization in the sample of the protein, which contains multiple TM domains. (**C**) To test if the enzymatic properties of Rhomboid are necessary for Star cleavage, we expressed Star with a mutated form of Rhomboid (MR), that is deficient in proteolytic activity. The cleaved and secreted forms of Spi and Star, which are readily generated in the presence of intact Rhomboid-1 (R1), could not be detected under these circumstances. (**D**) To examine the possibility of direct cleavage of Star by Rhomboid, Star-FLAG was expressed in human 293 cells. In the presence of Rhomboid-1 (R1) or Rhomboid-3 (R3), Star underwent cleavage and secretion into the medium of this heterologous cell line, implying direct cleavage by Rhomboid.

should be targeted to the nucleus. This process can be followed by transcriptional induction of a LexA reporter. Star-LV was expressed in the fly eye by the *GMR-Gal4* driver, and Western blot analysis was carried out on adult head extracts. In addition to the full-length protein, a smaller band of ~70 kDa was detected by an anti-LexA antibody. The smaller band corresponds in size to the entire cytoplasmic domain of Star with the LexA protein, as the fragment comigrated with a Star-LV construct that was truncated at the putative cleavage site of the Star transmembrane domain (Figure 6A).

To test if cleavage of Star could be driven by Rhomboids *in vivo*, the Star-LV construct was expressed by *prd-Gal4*, in embryos carrying a *lacZ* reporter for nuclear LexA-VP16. No reporter expression was detected in the absence of Rhomboid expression (Figure 6B). However, when either Rhomboid-1 or Rhomboid-3 was coexpressed with Star-LV in the *prd* stripe pattern, prominent expression of the reporter was detected (Figure 6C and D). Thus, Rhomboid-dependent cleavage of Star can be demonstrated in embryos.

Cleavage disrupts the trafficking capacity of Star

Is there a causal relationship between processing of Star by Rhomboid and attenuation of its ability to efficiently traffic Spi in Rhomboid-expressing cells? To address this issue, we generated HA-tagged constructs that represent the two forms of Star that are produced by cleavage: spStar, in which a signal peptide is attached to the extracellular portion of Star; and Star-NTM, which is composed of the transmembrane and cytoplasmic portions of the protein (Figure 7A; Tsruya *et al*, 2002).

Both constructs were assayed for their subcellular localization and for their ability to traffic Spi. spStar is secreted to the medium (not shown). Examination of the intracellular localization of spStar demonstrated that it was found only in secretory vesicles and was unable to traffic the Spi precursor, even when expressed in excess (Figure 7B). Star-NTM was similarly incapable of trafficking Spi and did not accumulate in the ER (Figure 7C). These experiments suggest that the resulting forms of Star produced by Rhomboid cleavage are incapable of undergoing retrograde trafficking and cannot facilitate trafficking of Spi. Cleavage of Star by Rhomboid therefore leads to inactivation of Star.

Discussion

Dynamic and highly regulated activation of the EGFR pathway is essential for the normal development of most, if not all, *Drosophila* tissues (Shilo, 2003, 2005). Spatial and temporal control of ligand processing provides key regulatory mechanisms governing EGFR activation (Schweitzer *et al*, 1995b). Most factors involved in EGFR ligand biogenesis are expressed ubiquitously. In contrast, expression of the protease Rhomboid, which cleaves the ligand precursors to yield an active ligand, is tightly regulated and highly dynamic, and corresponds to the resulting pattern of EGFR activation (Gabay *et al*, 1997). Rhomboid thus serves as a limiting factor in this process. Rhomboid functions in an enzymatic, sub-stoichiometric manner. In cell culture, even minute

amounts of Rhomboid can efficiently cleave the EGFR ligands (Urban *et al*, 2001). In flies, however, overexpression of Rhomboid does not lead to levels of EGFR activation that are higher than the endogenous ones, as measured by dpERK accumulation (not shown). Thus, although Rhomboid expression strictly determines the spatial and temporal onset patterns of EGFR pathway activation, the extent of pathway activity cannot be controlled by regulating Rhomboid levels.

The instructive and pleiotropic roles of EGFR go hand in hand with the necessity to restrict the spatial spreading of this activation, to induce the correct number of cells in each instance. In the cells receiving the EGFR signal, several inducible mechanisms account for the spatial restriction of signal spreading. They include Argos, a secreted inhibitor that itself is a target gene for the pathway. Although Argos is induced only in cells receiving maximal EGFR activation, it is a secreted protein that may exert its effect several cell diameters away from its site of production. Two additional negative regulators function in a cell-autonomous manner, and are induced in a broader domain than *argos*. Kekkon is a transmembrane protein that forms nonfunctional heterodimers with EGFR, and Sprouty is a cytoplasmic protein that interferes with the Ras signaling pathway. Mutants for

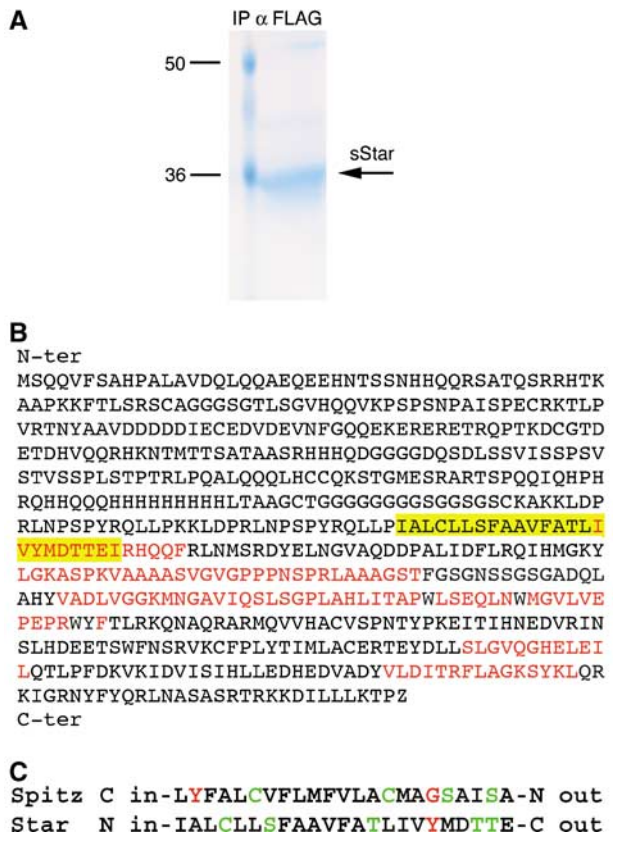


Figure 5 The site of Star cleavage within its transmembrane domain. (**A**) Star-FLAG was overexpressed together with Rhomboid-3 in *Drosophila* S2 cells. The medium was collected and purified by anti-FLAG immunoprecipitation. Following elution and SDS–PAGE electrophoresis, the purified secreted Star (sStar) was digested and the resulting peptides analyzed by mass spectrometry. (**B**) Within the protein sequence of Star, the predicted transmembrane domain is marked in yellow. The identified peptides are marked in red, including the most N-terminal one, which includes part of the transmembrane domain, indicating that the cleavage site lies within the transmembrane domain. (**C**) Alignment of the Spi and Star transmembrane domains, according to their orientation in the membrane. Similar to Spi, Star contains a helix-breaking residue (red) in the top third of its transmembrane domain, which is compatible with intramembrane proteolysis by Rhomboids. Helix-forming residues are marked in black, helix-breaking residues in red, and others in green.

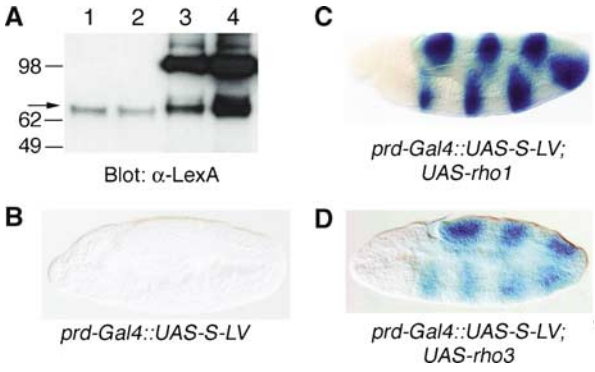


Figure 6 *In vivo* cleavage of Star by Rhomboids. (**A**) Extracts of adult fly heads in which the expression of the Star-LV fusion protein was induced by *GMR-Gal4* were examined for cleavage of Star, and Western blots were probed with a LexA antibody. Lanes 1, 2: fly lines expressing a Star-LV-C1 construct truncated within the transmembrane domain at the putative cleavage site, lanes 3, 4: fly lines expressing the full-length Star-LV. The appearance of an ~70 kDa band identical to the truncated Star-LV is observed (arrow), indicating that cleavage at this site takes place in fly heads. Line 8 depicted in lane 4 was used for subsequent experiments in embryos. (**B**) Full-length Star-LV was expressed by the *prd-Gal4* driver in embryos carrying the *LexA-lacZ* reporter. (**C**) When the embryos also carried a *UAS-rho1* or a *UAS-rho3* (**D**) construct, prominent expression of LacZ by the reporter was observed following incubation with X-Gal, indicating that Rhomboid-dependent cleavage of Star-LV takes place in embryos.

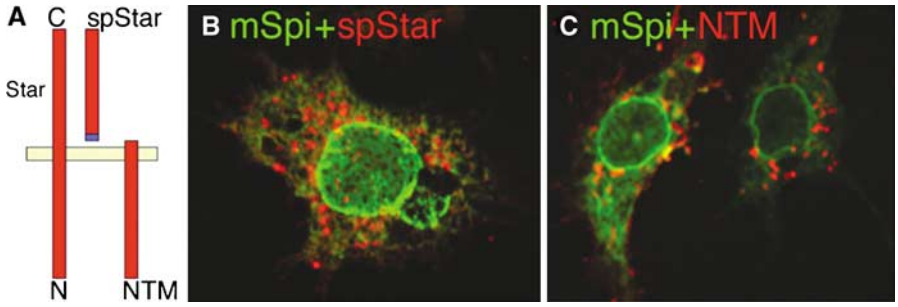


Figure 7 Inactivation of Star trafficking functions following cleavage. To test the potential biological activities of the truncated forms resulting from Star cleavage, we coexpressed with Spi-GFP the spStar-HA or NTM-HA truncated Star constructs, shown schematically in (**A**). (**B**) spStar was detected in secretory vesicles and did not mediate trafficking of Spi-GFP. (**C**) NTM did not accumulate in the ER, and again did not traffic Spi. We conclude that both forms are inactive in terms of Spi trafficking. The truncated Star-HA proteins were detected by anti-HA staining.

these negative regulators exhibit an expansion of the domain of EGFR activation (Shilo, 2003).

We examined whether the cells that produce the active ligand also contribute to restriction of the spatial domain of EGFR activation, by limiting the amount of secreted ligand produced. We focused on Spi, which is the primary ligand throughout development. The levels of Spi precursor that are produced do not seem to play a role in this regulation, as overexpression of Spi does not yield any phenotypes (Reich and Shilo, 2002). Several mechanisms that efficiently cope with excess levels of Spi have been identified. First, the Spi precursor is retained in the ER, preventing its unregulated cleavage by fortuitous proteases in cells which do not express Rhomboid. Second, Spi is labile, such that even high levels of expression of a GFP-tagged ligand were difficult to visualize in embryos (Tsruya *et al*, 2002).

The levels of the active ligand, cleaved Spi (cSpi), provide an alternative and attractive target for controlling the extent of pathway activation by the signal-producing cells. A likely candidate for controlling the level of cSpi is Star, as it forms protein–protein interactions with Spi and allows its trafficking to the site of processing (Lee *et al*, 2001; Tsruya *et al*, 2002). Furthermore, the levels of Spi protein are reduced in clones lacking Star, suggesting that Star stabilizes Spi (Hsiung *et al*, 2001). Finally, Star is known to be the element most sensitive to dosage alterations in the EGFR pathway. Mutations in *Star* have been identified multiple times in genetic screens, which rely on dosage sensitivity. In fact, the original dominant, haploinsufficient phenotype of *Star* in the eye relies on its dosage sensitivity. Conversely, overexpression of Star yields a dramatic EGFR hyperactivation phenotype in the same tissue (Hsiung *et al*, 2001; Tsruya *et al*, 2002). This work explored the involvement of Star in regulating the levels of cSpi that are released by the producing cells.

Star traffics Spi to a 'dead end' compartment

In contrast to the highly restricted and regulated expression of Rhomboid during development, the expression of Spi and Star is broad. Although the trafficking role of Star is crucial to allow Spi to reach the Rhomboid compartment, in cells that do not express Rhomboid, the ubiquitous coexpression of Spi and Star poses a problem. Once Spi is allowed to exit the ER, fortuitous cleavage on the plasma membrane may generate low levels of active ligand. Coexpression of Spi and Star in *Drosophila* cells, however, resulted in efficient accumulation of Spi in a punctate cytoplasmic distribution, but not on the plasma membrane (Tsruya *et al*, 2002). Examination of colocalization with several compartment markers identified this structure as the late endosome. Once trafficked to the late endosomal compartment, the degradation route of the Spi precursor is not known. We failed to see significant colocalization of Spi with a lysosomal marker (not shown), but this may stem from rapid degradation in the lysosome.

Expression of Rhomboid-1 in S_2R^+ cells demonstrated that the protein accumulates in a punctate structure that again corresponds to the late endosome. These results are different from the localization of Rhomboid in the Golgi upon expression in heterologous COS cells (Lee *et al*, 2001; Urban *et al*, 2002). As we have localized Rhomboid in *Drosophila* cells, the compartment identified here may be physiologically more relevant. In view of the potent enzymatic activity of Rhomboid, we cannot exclude, however, the possibility that the action of Rhomboid is effectively manifested in a compartment where it is not abundant, such as the Golgi. Cleavage of Spi by Rhomboid may thus be executed either in the Golgi en route to the late endosome, or in the late endosome, where the Spi/Star complex, as well as Rhomboid, are primarily localized.

If Spi is cleaved in the late endosome, there are several routes that may mediate its secretion, including Rab9-mediated trafficking to the Golgi, trafficking to the recycling endosome or direct Rab4-mediated trafficking to the plasma membrane (Zerial and McBride, 2001; Seachrist and Ferguson, 2003). The kinetics of each of these steps may be different.

Star is cleaved by Rhomboids

We have identified an efficient cleavage of Star that is mediated by Rhomboid. This process does not require Spi, and leads to the secretion of the entire extracellular domain of Star. Cleavage of Star is performed efficiently even when Rhomboid is present at highly sub-stoichiometric levels, it requires an intact active site of the Rhomboid protease, and is executed in the presence of either Rhomboid-1 or Rhomboid-3. The ability to recapitulate Star cleavage in the heterologous 293 human cell line and the identification of the cleavage site within the Star transmembrane domain strongly suggest that cleavage by Rhomboid is direct.

These findings are surprising for several reasons. All intramembrane proteases identified to date, including Presenillin, Site-2 protease, Rhomboid and Signal peptide peptidase, were thought to recognize either type I or type II transmembrane targets (Urban and Freeman, 2002; Wolfe and Kopan, 2004). The capacity of Rhomboids to cleave not only EGFR ligands, which are type I proteins, but also a type II protein such as Star significantly widens its substrate recognition spectrum. The recently published crystal structures of GlpG, a Rhomboid protease from *Escherichia coli*, suggest possible mechanisms by which Rhomboid substrates might gain access to the protease active site (Wang *et al*, 2006; Wu *et al*, 2006; Ben-Shem *et al*, 2007). The structures clearly show that only individual transmembrane helices can potentially enter the active site. However, no specific restriction can be derived from these structures as to the orientation of the substrate in the membrane. As the structures do not impose substrate directionality, it is plausible that type II transmembrane proteins can also enter the active site, provided that they contain appropriate residues, that confer flexibility at the external third part of their transmembrane helix, as has been demonstrated for the type I transmembrane proteins (Urban and Freeman, 2003). In this regard, both Spitz and Star contain residues that might confer helical fragility at the appropriate location (Figure 5C).

Biological consequences of Star cleavage

Modulation of chaperone proteins may provide an effective way to regulate protein trafficking. A prominent example is provided by the SCAP chaperone, which traffics the transcription factor SREBP between cellular compartments in response to changes in sterol levels (Yang *et al*, 2002). High sterol levels facilitate the association between SCAP and the INSIG-1 protein, and compromise the trafficking ability of SCAP. Cleavage of Star may represent an alternative mean to modulate chaperone activity. In this case, modulation is not

dependent on changing physiological conditions, but on the presence of the protease Rhomboid.

Is processing of Star by Rhomboid responsible for attenuating its ability to traffic Spi efficiently? One challenge posed by the sub-stoichiometric mode of Star trafficking is the capacity to restrict the number of Spi molecules that will undergo processing by Rhomboid. The efficient Rhomboid-dependent mechanism of Star cleavage may solve this problem. Once cleaved, Star cannot undergo retrograde trafficking to the ER. In cells expressing Rhomboid, the number of cycles mediated by Star is thus restricted. We have shown that upon Star cleavage, the N-terminal part, which retains the transmembrane domain, did not relocate to the ER, whereas the cleaved C-terminal domain was secreted from the cell. In view of the enzymatic nature of Rhomboid proteolytic activity, we expect this process to be highly proficient in inactivating and removing Star.

Another potential compartment in which Star may be cleaved by Rhomboids is the ER. In this case, the trafficking capacity of Star would be inactivated even before the first cycle of Spi trafficking is executed. In contrast to Rhomboid-1, some of the Rhomboid proteins (e.g. Rhomboid-3) also appear to be active in the ER, In cell culture assays, these Rhomboids were shown to cleave Spi in the absence of Star (Urban *et al*, 2002). This difference between Rhomboid-1 and Rhomboid-3 may account for the higher potency of Rhomboid-3 to attenuate Spi trafficking in the presence of limiting concentrations of Star (Figure 2J). It would be interesting to determine the mechanistic basis for the dosage sensitivity to *Star* that was observed during eye development.

In conclusion, we have identified an additional layer of regulation in the Spi processing circuitry, where the Rhomboid protease cleaves not only the ligand precursor but also the trafficking protein Star. This cleavage provides for tighter control of the level of cSpi that will be secreted to activate EGFR signaling in neighboring cells.

Materials and methods

DNA constructs

Spi-HA was generated by insertion of the HA-encoding oligonucleotide into the *Spe*I site of the Spi cDNA. spStar was generated by fusing the signal peptide of Argos (aa 1–27) to arginine 307 of Star. This construct does not contain a transmembrane domain. The constructs were cloned into the pUAST vector. All other constructs in pUAST were described previously (Tsruya *et al*, 2002). The Rhomboid-1, Rhomboid-3 and the mutant Rhomboid-1 cDNAs were obtained from S Urban and M Freeman and inserted into pUAST. Star-FLAG was generated by insertion of the FLAG tag at the C-terminus in pUAST and pCDNA3 vectors. In all cases, the actin-Gal4 plasmid was used in cotransfection to induce expression. The Rab5, Rab7 and Rab11 GFP fusion protein constructs were obtained from M Gonzalez-Gaitan.

The PCR-amplified sequence of LexA-VP16 (Loewer *et al*. 2004) was used to replace the HA-fusion part of the Gateway vector pTHW from Terence Murphy (http://www.ciwemb.edu/labs/murphy/Gateway%20vectors.html) resulting in the vector pTLVW. The PCR-amplified full-length or 3′-truncated (*Star-C1*) coding region of the *Star* cDNA was first cloned into pENTRY-D-Topo and then into pTLVW according to the manufacturer's protocols (Invitrogen). Primers used were as follows: *Star-N* CACCATGTCGCAGCAA GTGTTCTCG; *Star-C* GGGCGTCTTCAGCAGCAG; *Star-C1* TTATAAT GTGGCGAAGACTGC. Star-C1 is truncated within the transmembrane domain after L297, corresponding to the predicted cleavage site from mass spectroscopy. Expression of clones was tested in S2 cells before transgenic *Drosophila* were generated using standard procedures.

Cell culture

Calcium phosphate precipitates were used for transfection: Invitrogen calcium phosphate transfection kit for S2R$^+$ cells and Clontech CalPhos mammalian transfection kit for S2 cells. Cell transfection and maintenance, cell staining, extract preparation and Western blotting were described previously (Tsruya *et al*, 2002). Calcium phosphate precipitates were used for transfection into HEK-293 cells.

The following antibodies were used for localization: mouse monoclonal anti-HA (BabCO), mouse anti-Golgi (Calbiochem), rat anti-Troponin H to detect BiP (Babraham Bioscience Technologies), mouse monoclonal anti-FLAG (Sigma) and mouse monoclonal anti-GFP (Roche). Star-FLAG was transfected together with Rhomboid-3 into *Drosophila* S2 cells. The medium was collected a week after the transfection, and concentrated on 10 000 MWCO Amicon Ultra (Millipore). The concentrated medium was used for immunoprecipitation with FLAG using the anti-FLAG M2 affinity gel (Sigma), followed by elution with a FLAG peptide (Sigma). The secreted Star band was cut out of a 12% SDS–PAGE gel.

In vivo assays for Star cleavage

Flies carrying the *UAS-Star-LV* or the *UAS-Star-C1-LV* construct and the *GMR-Gal4* driver were generated. For Western blot analysis, flies younger than 24 h and NuPAGE 4–12% Bis–Tris gels were used. For further details see Loewer *et al* (2004) and Merdes *et al* (2004). A mouse anti-LexA antibody (Santa Cruz) was used for detection.

For analysis of embryos, the *UAS-Star* line 8 was used, in conjunction with the *LexA-lacZ* reporter (Szuts and Bienz, 2000), *prd-Gal4* and *UAS-rho1-HA* or *UAS-rho3-GFP*.

Mass spectroscopy

To examine where Star is cleaved, we implemented different mass spectrometric approaches. The protein band was used for in-gel digestion with bovine trypsin, chymotrypsin (sequencing grade, Roche Diagnostics, Germany) or V8 (Boehringer Mannheim). Extracted peptides were subjected to peptide fingerprinting, which was performed on a Bruker Reflex IIITM MALDI-TOF mass spectrometer equipped with a delayed extraction ion source, a reflector and a 337 nm nitrogen laser. Nanoelectrospray tandem mass spectrometric analysis (nano-ESI-MS/MS) was performed on API Q-STAR Pulsari Electrospray-Quadrupole TOF tandem mass spectrometer (MDS-Sciex, Canada, ABI) containing a quadrupole collision cell (MDS-Sciex, Canada, ABI) and equipped with a nanoelectrospray source (MDS Proteomics, Odense, Denmark).

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References

Ben-Shem A, Fass D, Bibi E (2007) Structural basis for intramembrane proteolysis by rhomboid serine proteases. *Proc Natl Acad Sci USA* **104:** 462–466

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

Bridges C, Morgan TH (1919) *The Drosophila 'Red Book' of Lindsley and Zimm*. Vol 278, p 279. Carnegie Institution of Washington publ.

Brown KE, Freeman M (2003) EGFR signalling defines a protective function for ommatidial orientation in the *Drosophila* eye. *Development* **130:** 5401–5412

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

Ghiglione C, Bach EA, Paraiso Y, Carraway III KL, Noselli S, Perrimon N (2002) Mechanism of activation of the *Drosophila* EGF receptor by the TGFalpha ligand Gurken during oogenesis. *Development* **129:** 175–186

Gilboa L, Lehmann R (2006) Soma-germline interactions coordinate homeostasis and growth in the *Drosophila* gonad. *Nature* **443:** 97–100

Golembo M, Raz E, Shilo BZ (1996) The *Drosophila* embryonic midline is the site of Spitz processing, and induces activation of the EGF receptor in the ventral ectoderm. *Development* **122:** 3363–3370

Guichard A, Roark M, Ronshaugen M, 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

Heberlein U, Rubin GM (1991) Star is required in a subset of photoreceptor cells in the developing *Drosophila* retina and displays dosage sensitive interactions with rough. *Dev Biol* **144:** 353–361

Hsiung F, Griffis ER, Pickup A, Powers MA, Moses K (2001) Function of the *Drosophila* TGF-alpha homolog Spitz is controlled by Star and interacts directly with Star. *Mech Dev* **107:** 13–23

Kolodkin AL, Pickup AT, Lin DM, Goodman CS, Banerjee U (1994) Characterization of Star and its interactions with sevenless and EGF receptor during photoreceptor cell development in *Drosophila*. *Development* **120:** 1731–1745

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

Loewer A, Soba P, Beyreuther K, Paro R, Merdes G (2004) Cell-type-specific processing of the amyloid precursor protein by Presenilin during *Drosophila* development. *EMBO Rep* **5:** 405–411

Merdes G, Soba P, Loewer A, Bilic MV, Beyreuther K, Paro R (2004) Interference of human and *Drosophila* APP and APP-like proteins with PNS development in *Drosophila*. *EMBO J* **23:** 4082–4095

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

Rutledge BJ, Zhang K, Bier E, Jan YN, Perrimon N (1992) The *Drosophila* spitz gene encodes a putative EGF-like growth factor involved in dorsal–ventral axis formation and neurogenesis. *Genes Dev* **6:** 1503–1517

Schlesinger A, Kiger A, Perrimon N, Shilo BZ (2004) Small wing PLCgamma is required for ER retention of cleaved Spitz during eye development in *Drosophila*. *Dev Cell* **7:** 535–545

Schulz C, Wood CG, Jones DL, Tazuke SI, Fuller MT (2002) Signaling from germ cells mediated by the rhomboid homolog stet organizes encapsulation by somatic support cells. *Development* **129:** 4523–4534

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

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

Seachrist JL, Ferguson SS (2003) Regulation of G protein-coupled receptor endocytosis and trafficking by Rab GTPases. *Life Sci* **74:** 225–235

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

Shilo BZ (2005) Regulating the dynamics of EGF receptor signaling in space and time. *Development* **132:** 4017–4027

Szuts D, Bienz M (2000) LexA chimeras reveal the function of *Drosophila* Fos as a context-dependent transcriptional activator. *Proc Natl Acad Sci USA* **97:** 5351–5356

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

Urban S, Freeman M (2002) Intramembrane proteolysis controls diverse signalling pathways throughout evolution. *Curr Opin Genet Dev* **12:** 512–518

Urban S, Freeman M (2003) Substrate specificity of rhomboid intramembrane proteases is governed by helix-breaking residues in the substrate transmembrane domain. *Mol Cell* **11:** 1425–1434

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

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

Wang Y, Zhang Y, Ha Y (2006) Crystal structure of a rhomboid family intramembrane protease. *Nature* **444:** 179–180

Wasserman JD, Urban S, 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

Wolfe MS, Kopan R (2004) Intramembrane proteolysis: theme and variations. *Science* **305:** 1119–1123

Wu Z, Yan N, Feng L, Oberstein A, Yan H, Baker RP, Gu L, Jeffrey PD, Urban S, Shi Y (2006) Structural analysis of a rhomboid family intramembrane protease reveals a gating mechanism for substrate entry. *Nat Struct Mol Biol* **13:** 1084–1091

Yang T, Espenshade PJ, Wright ME, Yabe D, Gong Y, Aebersold R, Goldstein JL, Brown MS (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

Zerial M, McBride H (2001) Rab proteins as membrane organizers. *Nat Rev Mol Cell Biol* **2:** 107–117

