Developmental Cell *10*, 167–176, February, 2006 ©2006 Elsevier Inc. DOI 10.1016/j.devcel.2005.11.017

Palmitoylation of the EGFR Ligand Spitz by Rasp Increases Spitz Activity by Restricting Its Diffusion

Grant I. Miura,[1] John Buglino,[2] Diego Alvarado,[3] Mark A. Lemmon,[3] Marilyn D. Resh,[2] and Jessica E. Treisman[1,4,*]

[1] Skirball Institute for Biomolecular Medicine and Department of Cell Biology
New York University School of Medicine
540 First Avenue
New York, New York 10016

[2] Cell Biology Program
Memorial Sloan-Kettering Cancer Center
1275 York Avenue
New York, New York 10021

[3] Department of Biochemistry and Biophysics
University of Pennsylvania School of Medicine
809C Stellar-Chance Laboratories
422 Curie Boulevard
Philadelphia, Pennsylvania 19104

Summary

Lipid modifications such as palmitoylation or myristoylation target intracellular proteins to cell membranes. Secreted ligands of the Hedgehog and Wnt families are also palmitoylated; this modification, which requires the related transmembrane acyltransferases Rasp and Porcupine, can enhance their secretion, transport, or activity. We show here that *rasp* is also essential for the developmental functions of Spitz, a ligand for the *Drosophila* epidermal growth factor receptor (EGFR). In cultured cells, Rasp promotes palmitate addition to the N-terminal cysteine residue of Spitz, and this cysteine is required for Spitz activity in vivo. Palmitoylation reduces Spitz secretion and enhances its plasma membrane association, but does not alter its ability to activate the EGFR in vitro. In vivo, overexpressed unpalmitoylated Spitz has an increased range of action but reduced activity. These data suggest a role for palmitoylation in restricting Spitz diffusion, allowing its local concentration to reach the threshold required for biological function.

Introduction

The epidermal growth factor receptor (EGFR) is required for multiple developmental processes, including cell fate specification, differentiation, proliferation, and survival (Shilo, 2003; Sternberg and Horvitz, 1991; Wong, 2003). The human EGFR homologs, ErbB1–4, are overexpressed in a variety of cancers and play an important role in tumor progression, making them the target of numerous anticancer therapies (Hynes and Lane, 2005). In *Drosophila*, three EGFR ligands, Spitz (Spi), Gurken (Grk), and Keren (Krn), are produced as inactive transmembrane precursors that must be cleaved intramembranously by proteases of the Rhomboid family (Lee et al., 2001b; Reich and Shilo, 2002; Tsruya et al., 2002; Urban et al., 2002). *spi* is required for normal development of the embryo and the eye, wing, and leg imaginal discs, while *grk* functions to establish the embryonic body axes during oogenesis; the mutant phenotype of *krn* has not yet been described (Shilo, 2003). A fourth ligand, Vein, which resembles mammalian neuregulins, does not undergo cleavage (Schnepp et al., 1996). Argos is a secreted feedback inhibitor of the pathway which acts by binding and sequestering Spi, preventing it from activating the EGFR (Freeman et al., 1992; Golembo et al., 1996; Klein et al., 2004). As a result, Spi acts as a short-range ligand in vivo (Freeman, 1997; Shilo, 2003).

Palmitoylation is a common modification of intracellular proteins that influences their membrane association, protein trafficking, and lipid raft targeting (Bijlmakers and Marsh, 2003). However, few palmitoyltransferases have been identified. It was recently shown that Porcupine (Porc) and Rasp (also known as Sightless, Skinny hedgehog, and Central missing), members of a family of transmembrane proteins that act within the secretory pathway, are necessary for the palmitoylation of proteins of the Wnt and Hedgehog (Hh) families, respectively (Amanai and Jiang, 2001; Chamoun et al., 2001; Chen et al., 2004; Kadowaki et al., 1996; Lee and Treisman, 2001; Micchelli et al., 2002). Although this hydrophobic modification would be expected to decrease solubility, palmitoylation is essential for the function of these secreted morphogens (Amanai and Jiang, 2001; Chamoun et al., 2001; Kadowaki et al., 1996; Lee et al., 2001a; Lee and Treisman, 2001; Micchelli et al., 2002; Willert et al., 2003; Zhai et al., 2004).

In the case of *Drosophila* Wingless (Wg), palmitoylation on an internal cysteine residue is required for its lipid raft targeting, N-glycosylation in the endoplasmic reticulum, and secretion (Kadowaki et al., 1996; Tanaka et al., 2002; Willert et al., 2003; Zhai et al., 2004). In contrast, unpalmitoylated mouse Wnt3a can be secreted normally from 293 cells, but fails to promote β-catenin accumulation in target cells (Willert et al., 2003). Hh proteins carry a C-terminal cholesterol modification in addition to the N-terminal palmitate group; either modification suffices for lipid raft targeting, and acylation is not required for secretion (Chamoun et al., 2001; Chen et al., 2004; Lee and Treisman, 2001; Porter et al., 1996). Both Hh and Wg are packaged into large protein complexes containing lipophorins that promote their long-range transport, perhaps by interacting with heparan sulfate proteoglycans (Chen et al., 2004; Gallet et al., 2003; Lin, 2004; Panakova et al., 2005). Palmitoylation of mouse Sonic hedgehog (Shh), but only cholesterol modification of *Drosophila* Hh, is required for association with these complexes and therefore for long-range signaling in vivo (Chen et al., 2004; Gallet et al., 2003). *Drosophila* Hh requires palmitoylation for its activity rather than its transport; membrane-tethered Hh-CD2 is inactive in a *rasp* mutant background, and unpalmitoylated Hh is inactive in a cell-based assay that does not require transport (Chamoun et al., 2001).

*Correspondence: treisman@saturn.med.nyu.edu
[4] Lab address: http://saturn.med.nyu.edu/research/dg/treismanlab/

We show here that Rasp, which is required for Hh modification, also promotes palmitoylation of the EGFR ligand Spi on its N-terminal cysteine. *rasp* and the palmitoylation site are essential for Spi function in vivo and for its retention at the plasma membrane; unpalmitoylated Spi is more efficiently released from cells and activates target gene expression more weakly over a longer range. However, palmitoylation does not alter the ability of Spi to bind to or activate the EGFR in vitro. We suggest that in contrast to its role in enhancing long-range transport of Shh, palmitoylation restricts the diffusion of Spi. This would allow the local concentration of Spi to reach the threshold level required for sufficient EGFR activation.

Results

rasp Is Required for Spi-Dependent Photoreceptor Recruitment

Mutations in the *Drosophila rasp* gene have been shown to disrupt processes that require Hh signaling, including differentiation of photoreceptors in the eye disc (Amanai and Jiang, 2001; Chamoun et al., 2001; Lee and Treisman, 2001; Micchelli et al., 2002). Activation of *atonal* expression by Hh results in the differentiation of the R8 photoreceptor, the first to form within each cluster (Dominguez, 1999); differentiation of R1–R7 is then induced by Spi secreted from R8 (Freeman, 1997). Although no photoreceptors form in homozygous *rasp* mutant eye discs (Lee and Treisman, 2001) (see below), we observed that R8 differentiation was normal in small *rasp* mutant clones, indicating that the mutant cells were rescued by Hh diffusion from surrounding wild-type cells (Figures 1B and 1C). However, differentiation of R1–R7 was defective in these clones (Figures 1A and 1C).

To show that this phenotype was not an indirect consequence of loss of Hh function, we activated the Hh pathway downstream of the ligand by generating clones lacking the inhibitory receptor Patched (Ptc). In a wild-type background, *ptc* mutant clones in the anterior of the eye disc differentiated ectopic photoreceptor clusters (Figure 1D). In a *rasp* mutant background, however, *ptc* mutant clones contained predominantly single cells expressing the neuronal marker Elav, most of which also expressed the R8-specific marker Senseless (Sens) (Frankfort et al., 2001) (Figures 1E and 1F). Thus, even when the Hh pathway is maximally active, *rasp* is required for the differentiation of photoreceptors other than R8, a process mediated by Spi signaling through the EGFR.

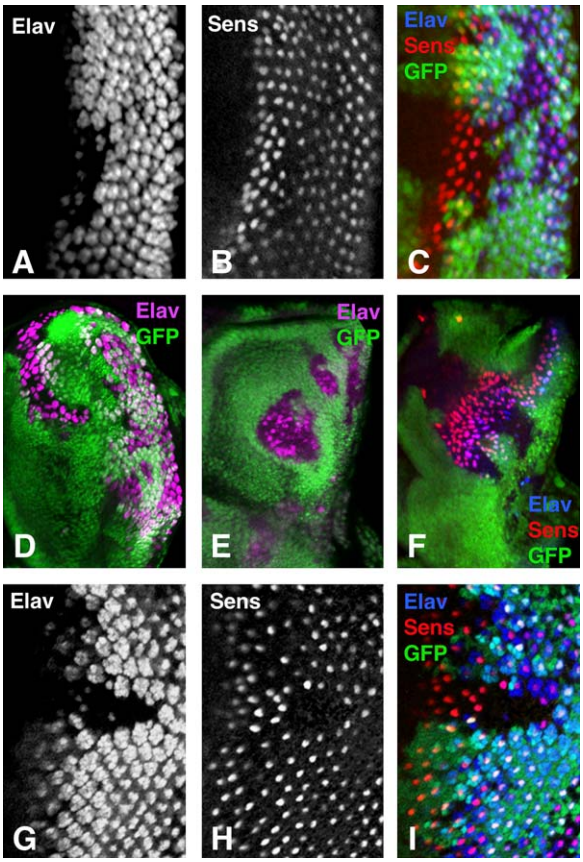

Figure 1. *rasp* Is Required for the Spi-Dependent Recruitment of Photoreceptors R1–R7

All panels show third instar eye imaginal discs with anterior to the left.

(A–C) $rasp^{T392}$ mutant clones marked by the absence of GFP (green in [C]). Photoreceptors are stained with anti-Elav ([A], blue in [C]) and R8 is stained with anti-Sens ([B], red in [C]). Although R8 develops almost normally in the absence of *rasp*, few other photoreceptors are recruited.

(D) ptc^{S2} mutant clones marked by the lack of GFP (green); Elav is stained in magenta. Ectopic photoreceptor clusters differentiate in and around the *ptc* clone.

(E and F) ptc^{S2} mutant clones marked by the lack of GFP (green) generated in $rasp^{T392}/rasp^{T802}$ eye discs. Sens (red in [F]) labels R8 cells and Elav (magenta in [E], blue in [F]) labels all photoreceptors. The *ptc* clones contain predominantly single Sens-expressing cells.

(G–I) $rasp^{T392}$ clones marked by the absence of GFP (green in [I]) generated in an $argos^{\Delta 7}$ mutant eye disc. Photoreceptors are stained with anti-Elav ([G], blue in [I]) and R8 is stained with anti-Sens ([H], red in [I]). Recruitment of R1–R7 to ommatidia containing an R8 cell is not rescued in the absence of *argos*.

rasp Is Required for EGFR Functions in the Wing Disc and Ovary

This result led us to test whether *rasp* was required for additional EGFR-dependent events. Wing vein development is induced by EGFR signaling, and in wild-type wing discs the EGFR target gene *argos* is expressed along the wing vein primordia (Gabay et al., 1997; Guichard et al., 1999) (Figure 2A). *rasp* mutant wing discs lacked *argos* expression (Figure 2B), and adult wings that contained *rasp* mutant clones often had missing wing veins at positions distant from the Hh-producing cells (Figure 2C). However, *rasp* was not required for Wg to induce differentiation of the wing margin sensilla, labeled by Sens expression (Figure 2B). *rasp* did not affect all EGFR target genes; *mirror* (*mirr*), a target of the neuregulin-related ligand Vein (Zecca and Struhl, 2002), was expressed normally in the notum primordia of *rasp* mutant wing discs (Figures 2D and 2E).

Embryos lacking both maternal and zygotic *rasp* failed to activate the Spi target genes *fasciclin III* and *argos* at the ventral midline (Golembo et al., 1996) (Figures 2F, 2G, 2I, and 2J). In addition, the eggshells of embryos formed from *rasp* mutant germline clones were ventralized, as shown by the fusion of the two dorsal appendages (Figures 2H and 2K). Dorsal follicle cell fates are induced by Grk, an EGFR ligand produced in the oocyte

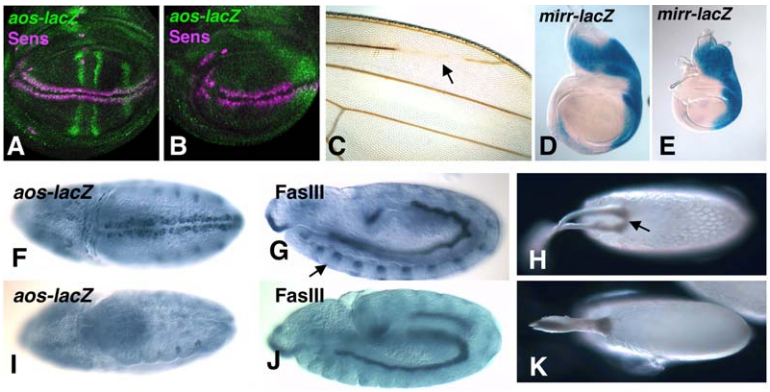

Figure 2. *rasp* Acts in Multiple EGFR-Dependent Processes

(A and B) Third instar wing discs stained with anti-β-galactosidase reflecting *argos-lacZ* expression in green, and with anti-Sens in magenta.
(A) Wild-type.
(B) $rasp^{T392}/rasp^{T802}$. *argos* is not expressed in the wing vein primordia in the absence of *rasp*, although Wg-dependent Sens expression is normal.
(C) An adult wing containing unmarked $rasp^{T392}$ mutant clones. Part of vein 2 is missing (arrow).
(D and E) Wing discs expressing *mirr-lacZ* stained with X-gal.
(D) Wild-type.
(E) $rasp^{T392}/rasp^{T802}$. *mirr*, a Vein target gene, is expressed normally in the absence of *rasp*.
(F–H) Wild-type embryos.
(I and K) Embryos derived from $rasp^{T802}$ germline clones.
(J) Embryo derived from $rasp^{T392}$ germline clone.
(F and I) These carry *argos-lacZ* and are stained with anti-β-galactosidase. *argos* is not expressed in the absence of *rasp*.
(G and J) Stained for Fasciclin III; striped expression at the ventral midline (arrow, [G]) is absent in *rasp* mutant embryos. The two dorsal appendages of the chorion (arrow, [H]) are fused when *rasp* is missing from the germline (K).

(Schupbach, 1987). Ventralization was consistently observed when the oocyte, but not the responding follicle cells, was mutant for *rasp*, placing the function of *rasp* within the ligand-producing cell and suggesting that it may be required for the activity of multiple EGFR ligands.

Rasp Acts Downstream of Spi Processing and Upstream of EGFR Activation

To locate the point at which Rasp functions within the EGFR pathway, we tested whether photoreceptor differentiation could be restored to *rasp* mutant eye discs by activating other components of the pathway. Spi is produced as an inactive transmembrane precursor that is chaperoned to the Golgi by Star, where it is cleaved in its transmembrane domain by the protease Rhomboid (Rho), allowing secretion of the biologically active extracellular domain (Lee et al., 2001b; Tsruya et al., 2002). As Spi is expressed ubiquitously (Rutledge et al., 1992), the distribution of Rho, in the presence of sufficient Star, controls the production of active ligand. Misexpression of Star and Rho together resulted in ectopic photoreceptor differentiation in wild-type eye discs (Figures 3A and 3B), but had no effect on *rasp* mutant eye discs (Figures 3C and 3D). Similarly, a truncated form of Spi that is constitutively secreted (sSpi) (Schweitzer et al., 1995) induced photoreceptor differentiation in a wild-type background, but its activity was greatly reduced in the absence of *rasp* (Figures 3E–3H). However, photoreceptor differentiation could be induced in *rasp* mutant eye discs by expressing an activated form of the EGFR (Queenan et al., 1997) (Figures 3I and 3J). These results place the requirement for *rasp* downstream of Rho and upstream of the EGFR, and suggest that the most likely target for Rasp activity is Spi itself.

Rasp Induces Spi Palmitoylation in Cultured Cells

If the Spi protein is modified by the Rasp acyltransferase, its extracellular domain should be more hydrophobic than predicted from its amino acid sequence. We used Triton X-114 phase separation (Bordier, 1981) to assess the hydrophobicity of sSpi, which lacks the transmembrane domain, in *Drosophila* S2 cells, which have been shown to express *rasp* (Chamoun et al., 2001). Lysates of cells transfected with sSpi were extracted with Triton X-114, and the aqueous and Triton phases were blotted with Spi antibody. The hydrophilic protein proliferating cell nuclear antigen (PCNA) and a myristoylated form of green fluorescent protein (GAP-GFP)

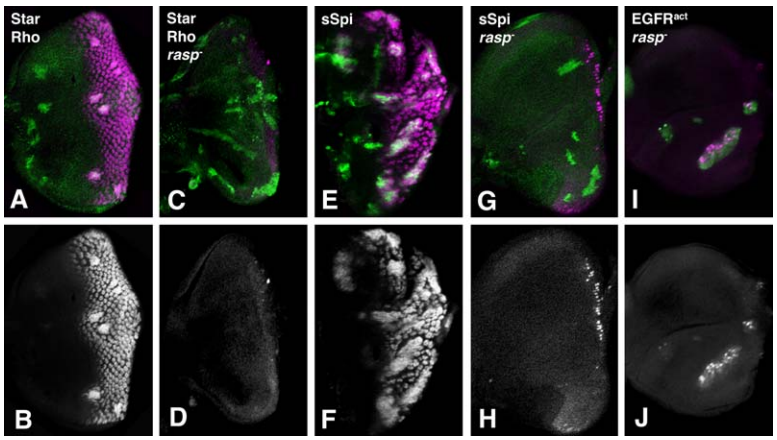

Figure 3. *rasp* Is Required for Spi Activity Upstream of the EGFR

All panels show third instar eye discs with anterior to the left. Clones of cells misexpressing Star and Rho (A–D), sSpi (E–H), or activated EGFR (I and J) are positively marked by coexpression of β-galactosidase (green in [A], [C], [G], and [I]) or GFP (green in [E]). Elav is stained in magenta (A, C, E, G, and I) and shown alone (B, D, F, H, and J).
(A, B, E, and F) Wild-type eye discs.
(C, D, and G–J) $rasp^{T392}/rasp^{T802}$ eye discs.
In *rasp* mutant eye discs, photoreceptor differentiation cannot be induced by Star and Rhomboid or by sSpi, but it can be induced by activated EGFR.

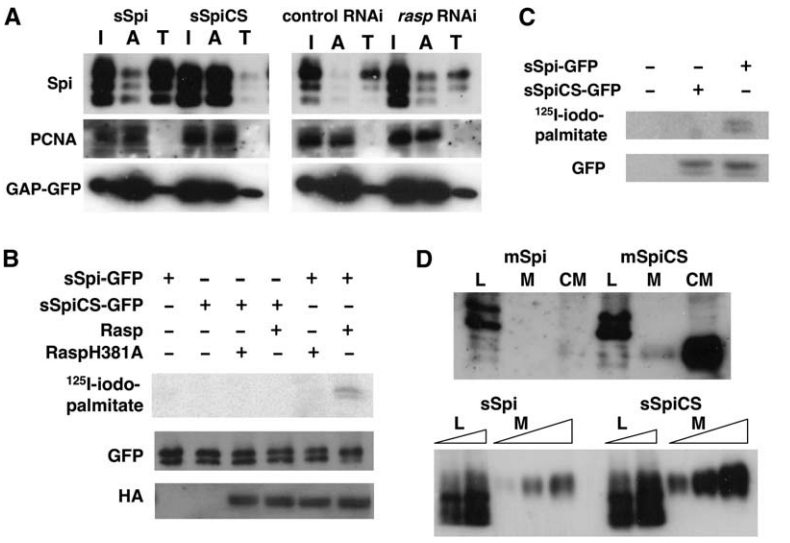

Figure 4. Rasp Promotes Palmitoylation of the N-Terminal Cysteine of Spi

(A) Triton X-114 phase separation of lysates of S2 cells transfected with GAP-GFP and with sSpi or sSpiCS as indicated (left panel) or of S2 cells transfected with sSpi and treated with double-stranded RNA homologous to *kohtalo* (control RNAi) or *rasp* (right panel). Western blots with anti-Spi, anti-PCNA, and anti-GFP are shown. I, input; A, aqueous phase; T, Triton X-114 phase. sSpi partitions into the detergent phase, while sSpiCS partitions into the aqueous phase. Reducing *rasp* levels increases the proportion of sSpi partitioning into the aqueous phase. A 64% reduction of *rasp* RNA was observed in this experiment using RT-PCR to measure *rasp* levels (not shown).

(B) Extracts from COS cells transfected with sSpi-GFP, sSpiCS-GFP, wild-type HA-tagged Rasp, or HA-RaspH381A as indicated, and labeled with [^{125}I]IC16. The upper gel is an autoradiograph of anti-GFP immunoprecipitates. The lower gels show Western blots of the same extracts with anti-GFP and anti-HA. Only wild-type sSpi incorporates labeled palmitate, and labeling is observed only in the presence of wild-type Rasp.

(C) Extracts from untransfected S2 cells or S2 cells transfected with sSpi-GFP or sSpiCS-GFP as indicated, and labeled with [^{125}I]IC16. The upper gel is an autoradiograph of anti-GFP immunoprecipitates, and the lower gel is a Western blot of the same extracts with anti-GFP. Labeled palmitate can be incorporated into wild-type sSpi but not sSpiCS in S2 cells.

(D) Western blots of Spi in lysates (L), media (M), and 20-fold concentrated media (CM) from S2 cells transfected with Star, Rhomboid, and either mSpi or mSpiCS (upper panel) or from S2 cells transfected with either sSpi or sSpiCS (lower panel). Under conditions of low Star expression, in which very little secreted wild-type Spi can be detected, mSpiCS is efficiently cleaved and recovered from the media. Increasing volumes of lysates (10 and 20 μl) and media (5, 10, and 20 μl) containing sSpi or sSpiCS were loaded to show that approximately 4-fold more sSpiCS than sSpi is present in the media. Blots of cotransfected GFP in the lysates demonstrated equivalent transfection efficiencies for wild-type and mutant Spi (not shown).

(Ritzenthaler et al., 2000) were used as controls for the fractionation. sSpi partitioned predominantly into the Triton layer (Figure 4A), appearing more hydrophobic than myristoylated GFP. Reducing the levels of Rasp in S2 cells by RNA interference increased the proportion of Spi that partitioned into the aqueous layer (Figure 4A), indicating that Rasp is required for Spi hydrophobicity.

To confirm that its hydrophobicity was due to acylation, we transfected COS cells with a GFP-tagged sSpi protein (Tsruya et al., 2002), with or without cotransfection of HA-tagged Rasp. The cells were labeled with [^{125}I]IC16, a radio-iodinated palmitate analog (Alland et al., 1994), and lysates were immunoprecipitated with anti-GFP antibody. Radiolabeled palmitate was incorporated into sSpi-GFP only in the presence of Rasp (Figure 4B), confirming that Spi is palmitoylated in a Rasp-dependent manner. Mutation of the active site histidine of Rasp to alanine (Chamoun et al., 2001) rendered it unable to promote incorporation of labeled palmitate into Spi (Figure 4B), supporting the model that Rasp itself is the acyltransferase for Spi. In *Drosophila* S2 cells, we were able to detect [^{125}I]IC16 incorporation into sSpi-GFP even in the absence of cotransfected Rasp (Figure 4C), suggesting that endogenous Rasp is sufficient for Spi palmitoylation and that this modification is the likely cause of its hydrophobicity.

The Palmitoylated Cysteine Is Essential for Spi Function

Rasp promotes palmitoylation of Hh on the N-terminal cysteine of the secreted signaling domain (Chamoun et al., 2001; Lee et al., 2001a; Pepinsky et al., 1998). The amino acid immediately following the signal peptide of Spi is also a cysteine, and all the other cysteine residues in the extracellular domain are predicted to be involved in disulfide bonds in the EGF repeat. We mutated this N-terminal cysteine (C29) to serine, a change that has been shown to abolish Hh palmitoylation (Pepinsky et al., 1998). This mutant form of Spi (sSpiCS) partitioned entirely into the aqueous phase in Triton X-114 fractionation (Figure 4A), and did not incorporate labeled palmitate in COS cells expressing Rasp (Figure 4B) or in S2 cells (Figure 4C). The N-terminal cysteine is thus essential for Spi palmitoylation and is likely to be the site of palmitate attachment. To test the importance of this residue for Spi function in vivo, we generated a full-length Spi transgene in which cysteine 29 was mutated to serine (mSpiCS). When expressed ubiquitously, wild-type full-length Spi (mSpi) completely rescued the lethality of *spi* mutations; however, mSpiCS had no rescuing activity (Table 1). Both proteins were expressed at similar levels and were equally stable in vivo (Figures 5A–5C). We also tested the ability of mSpiCS to rescue photoreceptor differentiation in *spi* mutant clones. Whereas *spi* clones expressing wild-type mSpi differentiated normally, *spi* clones expressing mSpiCS were indistinguishable from *spi* clones with no rescue construct (Figures 5D–5I). Taken together with the *rasp* mutant phenotype, these results indicate that palmitoylation is essential for Spi activity in vivo.

Palmitoylation Restricts Spi Movement

We considered several possible mechanisms by which palmitoylation might affect Spi function. First, palmitoylation could be required for Spi secretion; palmitoylation of Wg has been shown to target the protein to lipid rafts

Table 1. The N-Terminal Cysteine of Spi Is Essential for Its Function In Vivo

Genotype	% Survival (*n*)
spi^{1}/spi^{SC1}	0 (125)
spi^{1}/spi^{SC1}; UAS-*mSpi*; *da*-Gal4	107 (169)
spi^{1}/spi^{SC1}; UAS-*mSpiCST7*; *da*-Gal4	0 (186)
spi^{1}/spi^{SC1}; UAS-*mSpiCST13*; *da*-Gal4	0 (167)

Percentage survival is calculated based on the number of adult flies carrying the balancer chromosome; the expected survival rate is half this number. Total number of flies counted is given in parentheses. T7 and T13 are two independent insertions of the UAS-*mSpiCS* transgene.

and promote its secretion (Kadowaki et al., 1996; Zhai et al., 2004). In contrast, mouse Shh does not require palmitoylation for either lipid raft targeting or secretion (Chen et al., 2004). We found that mSpiCS could be cleaved in and secreted from S2 cells cotransfected with Star and Rho (Figure 4D). This agrees with previous findings that COS cells transfected with Star and Rho are able to cleave and secrete Spi (Lee et al., 2001b), despite the lack of Spi palmitoylation in these cells (Figure 4B).

A second possibility is that palmitoylation could be required for binding to the EGFR or for its activation. However, we found that sSpiCS purified from transfected S2 cells bound to immobilized EGFR and induced its autophosphorylation as effectively as wild-type sSpi (see Figures S1A and S1C in the Supplemental Data available with this article online). This is not surprising, as binding to the EGFR has been shown to require only the EGF domain of Spitz (Klein et al., 2004), which is distant from the N-terminal palmitate modification.

A third possibility is that palmitoylation might regulate Spi transport in vivo. Palmitoylation is required for Shh to form a multimeric complex that has a longer range of movement in vivo than monomeric Shh (Chen et al., 2004). However, sSpi induces photoreceptor differentiation only in immediately adjacent cells, suggesting that it does not act over a long range in vivo (Figures 3E and 3F). Our data suggest the opposite hypothesis, that palmitoylation restricts Spi movement. We found that wild-type sSpi was less efficiently recovered from S2 cell culture media than sSpiCS, both when derived from full-length Spi coexpressed with Star and Rho and when expressed as the truncated form (Figure 4D). These results suggest that palmitoylated sSpi is primarily cell associated. To confirm this, we stained live S2 cells expressing His-tagged sSpi or sSpiCS with an antibody to the C-terminal epitope tag. Strong plasma membrane staining was observed for wild-type sSpi, whereas very little sSpiCS was present at the membrane (Figures 6A and 6B). Staining of permeabilized cells revealed similar expression levels for the two proteins (Figures 6C and 6D), suggesting that most sSpiCS is released into the culture medium. Palmitoylation appears to have the same effect on Spi distribution in vivo. GFP-tagged forms of sSpi (Schlesinger et al., 2004) or mSpi (Tsruya et al., 2002) coexpressed with Star and Rho were tightly localized to the cells expressing them in wild-type eye discs, but in *rasp* mutant eye discs, GFP was visible on particles distant from the expressing cells (Figures 6E–6H).

The long-range distribution of nonpalmitoylated sSpi correlated with an increase in the range of its activity

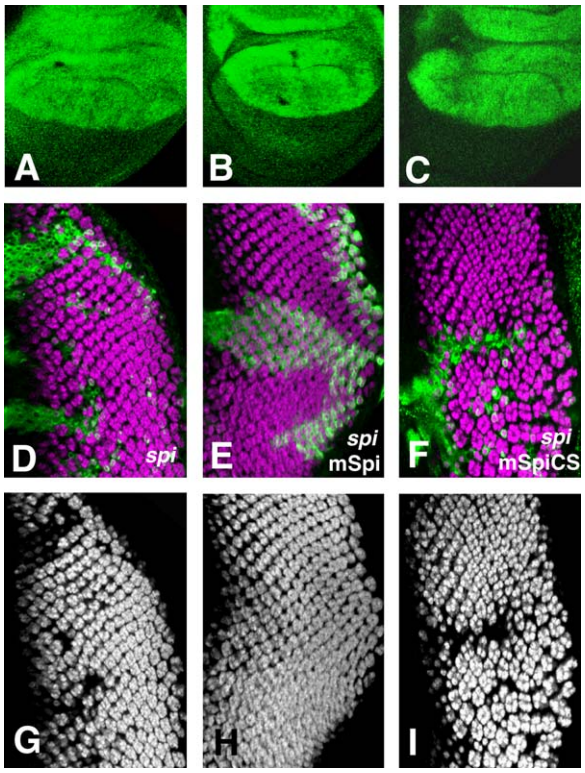

Figure 5. mSpi and mSpiCS Are Equivalently Expressed In Vivo, but mSpiCS Is Unable to Rescue *spi* Mutant Phenotypes

(A–C) Wing discs stained with anti-Spi antibody (green).
(A) *ap*-GAL4/UAS-*mSpi*.
(B) *ap*-GAL4/UAS-*mSpiCST7*.
(C) *ap*-GAL4/UAS-*mSpiCST13*.
ap-GAL4 drives expression in the dorsal compartment of the wing disc.
(D–F) Eye discs stained with anti-Elav (magenta); clones are positively labeled with GFP (green). Elav staining is shown alone in (G–I).
(D and G) spi^{SC1} clones.
(E and H) spi^{SC1} clones expressing UAS-*mSpi*.
(F and I) spi^{SC1} clones expressing UAS-*mSpiCST13*.

in vivo. When expressed with *vestigial* (*vg*)-GAL4 in a stripe at the dorsal-ventral boundary of the wing disc, wild-type sSpi induced a narrow stripe of expression of the target gene *argos-lacZ* at the presumptive wing margin (Figure 6I). However, sSpiCS expressed in the same pattern was able to induce *argos-lacZ* in a much broader domain (Figures 6M–6O). Several transgenic lines were tested for each construct, and these gave different intensities but similar patterns of *argos-lacZ* expression (Figure 6 and data not shown). In order to directly compare the activity of sSpi expressed at equal levels with and without a palmitate modification, we expressed the same sSpi transgene in wild-type and *rasp* mutant discs. In the absence of *rasp*, induction of *argos* was weaker but longer range than in wild-type wing discs (Figure 6J). This was not due to an effect of the *rasp* mutation on *vg*-GAL4 expression, as an activated Ras transgene (Karim and Rubin, 1998) induced a similar intensity and range of *argos-lacZ* expression in wild-type and *rasp* mutant wing discs (Figures 6K and 6L).

We observed the same difference in range when sSpi and sSpiCS were expressed in segmentally repeated stripes in embryos using *engrailed* (*en*)-GAL4. Activation

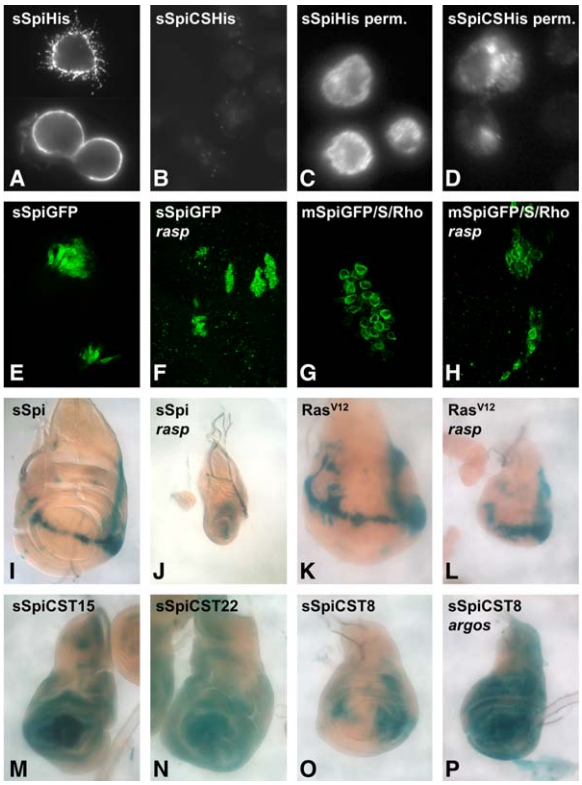

Figure 6. Palmitoylation Promotes Membrane Association of Spi, Increasing Its Activity but Reducing Its Range

(A–D) S2 cells stained with anti-His antibody. (A) and (B) are stained live, while (C) and (D) are fixed and permeabilized. (A) and (C) are transfected with His-tagged sSpi and (B) and (D) with His-tagged sSpiCS. Plasma membrane association is abolished by the C29S mutation, while the intracellular distribution is not affected.

(E–H) Eye-antennal discs with clones expressing sSpiGFP (E and F) or mSpiGFP, Star, and Rho (G and H).

(E and G) Wild-type.

(F and H) $rasp^{T392}/rasp^{T802}$.

GFP visualized in unfixed discs is present on particles distant from the clones in *rasp* mutant discs but not in wild-type discs.

(I–P) X-gal-stained wing discs expressing *argos-lacZ*. *vg*-GAL4 drives the expression of UAS-sSpi (I and J), Ras^{V12} (K and L), sSpiCS line T15 (M), sSpiCS line T22 (N), or sSpiCS line T8 (O and P). Discs are wild-type (I, K, and M–O), $rasp^{T392}/rasp^{T802}$ (J and L), or $argos^{\Delta 7}/argos^{W11}$ (P). Wild-type discs were stained in parallel with *rasp* mutant or *argos* mutant discs. Loss of *rasp* or mutation of Spi C29 reduces the intensity but increases the range of *argos* expression. Ras^{V12} activates *argos* in a localized pattern in *rasp* mutant wing discs. Removal of *argos* enhances the activity of sSpiCS.

of *argos-lacZ* was limited to the cells expressing sSpi and those immediately adjacent to them, whereas sSpiCS induced a lower level of *argos-lacZ* expression that extended to cells distant from the *en* stripes (Figures S2A–S2D). The reduced activity of sSpiCS was also apparent from the reduced dorsal expansion of denticle belts on the cuticles of embryos expressing sSpiCS compared to those expressing sSpi (data not shown). In eye discs, both sSpi expressed in clones of *rasp* mutant cells and sSpiCS expressed in clones of wild-type cells produced a long-range inhibition of photoreceptor differentiation (Figures S2E–S2H), rather than inducing ectopic photoreceptors like wild-type sSpi (Figures 3E and 3F). This was probably due to induction of *argos* expression (Figures S2G and S2H), which then inhibited photoreceptor differentiation in surrounding cells. A similar phenotype seen in the *Ellipse* mutant allele of the EGFR has been attributed to a reduced level of activity sufficient to activate *argos* but not to induce photoreceptor differentiation (Lesokhin et al., 1999). The combination of reduced biological activity and increased range of action of unpalmitoylated Spi suggests that the primary role of palmitoylation is to concentrate Spi close to its site of production.

Local concentration of Spi might be necessary to allow it to exceed the concentration of Argos, a secreted inhibitor that binds and sequesters Spi (Klein et al., 2004). Indeed, the activity of sSpiCS in the wing disc was enhanced when we removed the remaining wild-type copy of *argos* (Figure 6P). However, when we generated *rasp* mutant clones in eye discs homozygous for a null allele of *argos*, we found that R1–R7 photoreceptors were still not recruited to ommatidia in which R8 differentiated normally (Figures 1G–1I). Removing wild-type *argos* also failed to restore normal *argos-lacZ* expression to *rasp* mutant wing discs (data not shown). These results show that *rasp* is necessary for Spi-dependent processes even in the absence of *argos*. EGFR activation sufficient for target gene expression in vivo is therefore likely to require a threshold Spi concentration that is achieved through palmitoylation-mediated restriction of Spi diffusion.

Discussion

Rasp Is Required to Palmitoylate Two Secreted Ligands

We have shown that the acyltransferase Rasp promotes palmitoylation of the EGFR ligand Spi in addition to its previously reported substrate Hh. *rasp* mutants show phenotypes similar to *spi* mutants, and *rasp* is required for the activity of ectopic sSpi produced either by cleavage of endogenous Spi or by expression of a truncated protein. Rasp is also necessary for the hydrophobic character of Spi expressed in S2 cells. Palmitoylation of Spi by Rasp can be reproduced in COS cells, which do not contain any endogenous Spi palmitoyltransferase activity; either these cells do not express a Rasp homolog, or it is too divergent to recognize *Drosophila* Spi. Mutation of the predicted active site histidine of Rasp blocks palmitate incorporation into Spi, suggesting that Spi may be a direct target of Rasp. However, we cannot exclude the possibility that other proteins present within COS cells contribute to the acyltransferase activity.

The basis for substrate recognition by Rasp is not obvious. There is little sequence homology between Hh and Spi following the palmitoylated cysteine, although both proteins have several basic amino acids in the vicinity (Figure S3A); basic amino acids follow the palmitoylation site of some classes of intracellular proteins (Bijlmakers and Marsh, 2003). Myc-tagged Skn, the mouse homolog of Rasp, was reported to localize to the endoplasmic reticulum (ER) in CHO cells (Chen et al., 2004); in S2 cells, we have seen colocalization of HA-Rasp with markers of the Golgi apparatus (data not shown). If Hh and Spi are palmitoylated in the same cellular compartment, they later follow different paths; Hh is released from the cell through the activity of the membrane protein Dispatched (Burke et al., 1999), whereas Spi requires Star for export from the ER and is then

activated by Rho-mediated cleavage (Lee et al., 2001b; Tsruya et al., 2002). Because sSpi can be palmitoylated, cleavage by Rho is not a prerequisite for palmitoylation. However, the effect of palmitoylation on secretion is more dramatic for full-length Spi than for sSpi (Figure 4D), suggesting that palmitoylation may be more efficient when Spi undergoes its normal processing.

Grk and Krn, but Not Vein, Are Likely Targets for Rasp

rasp is also required for processes mediated by EGFR ligands other than Spi (Figure 2). The observation that lack of *rasp* in the germline causes ventralization of the follicle cells (Figure 2K) suggests that Grk might be palmitoylated. Consistent with this possibility, we have observed that the extracellular domain of Grk also fractionates into the Triton X-114 layer when expressed in S2 cells, although to a lesser extent than Spi (Figure S3B). The *rasp* phenotype is relatively mild compared to loss of *grk* (Schupbach, 1987), suggesting that Grk has a less stringent requirement for palmitoylation than Spi.

Wing vein development, which is affected in *rasp* mutants (Figures 2A–2C), requires both Rho and Vein, but not Spi (Guichard et al., 1999). As Vein is not synthesized as a transmembrane precursor, the requirement for Rho may suggest the involvement of Krn, a ligand closely related to Spi (Reich and Shilo, 2002). Grk and Krn have cysteine residues immediately following the signal peptide, making them likely substrates for Rasp, but Vein does not (Figure S3A), consistent with the observation that *rasp* is not required for the expression of the Vein target gene *mirr*. It is unclear whether vertebrate EGFR ligands undergo a similar palmitoylation, as none of the known ligands has an N-terminal cysteine residue; TGF-α is palmitoylated on two cysteines in the cytoplasmic domain of the transmembrane precursor, but this is likely to involve a different mechanism (Shum et al., 1996). It will be interesting to determine whether EGFR signaling is affected in mice mutant for the *rasp* homolog *Skn* (Chen et al., 2004).

Palmitoylation Increases the Local Concentration of Spi

We have found that both the acyltransferase Rasp and cysteine 29 are essential for the activity in vivo of endogenous or overexpressed full-length Spi, and significantly enhance the activity of overexpressed truncated Spi. By contrast, in vitro studies with sSpiCS clearly argue that loss of palmitoylation has no effect on EGFR binding or activation, or on Argos binding (Figure S1). Thus, it is likely that palmitoylation defines biologically critical spatial or temporal aspects of Spi distribution, rather than affecting its inherent binding properties. Indeed, mutating cysteine 29 in either full-length or truncated Spi allows greater recovery of secreted Spi from cell culture media. In addition, wild-type tagged sSpi shows strong membrane localization both in S2 cells and in imaginal discs, while unpalmitoylated sSpi is not membrane associated in S2 cells and can reach and act on distant cells in vivo. We therefore suggest that palmitoylation is required to maintain a high local concentration of Spi, perhaps by directly tethering Spi to the plasma membrane or allowing it to form a complex with other factors that restrict its diffusion (Figure 7).

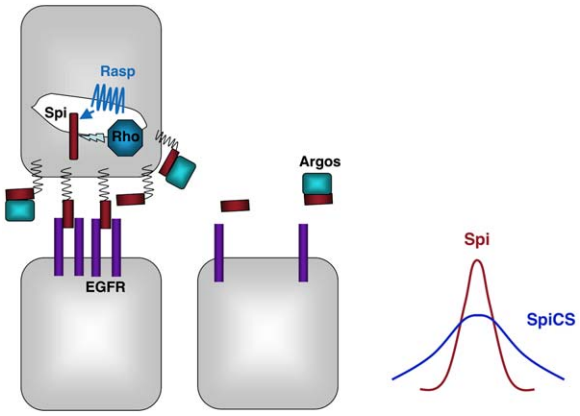

Figure 7. Model for the Effect of Palmitoylation on Spi Signaling

Palmitoylation of Spi by Rasp in the secretory pathway may promote its tethering to the plasma membrane following Rho-mediated cleavage. This would increase its concentration close to the Spi-producing cells, allowing it to activate the EGFR either in its membrane-bound state, as shown, or following release. Unpalmitoylated Spi would diffuse away and would not reach the threshold concentration required for activation. Argos would reduce the effective concentration of both palmitoylated and unpalmitoylated Spi. On the right is a representation of the gradients formed by palmitoylated and unpalmitoylated Spi.

Palmitoylation might have additional effects on Spi signaling; its strong effect on secretion of mSpi could be partially due to an inhibitory effect on Spi cleavage, although this would be unlikely to lead to increased Spi activity. It is also possible that palmitoylation contributes to endocytosis and recycling of Spi, a mechanism that has been reported to enhance Wg signaling (Pfeiffer et al., 2002). Palmitoylation is unlikely to affect ER retention of sSpi, as this occurs in both COS cells and S2 cells (Schlesinger et al., 2004; Urban et al., 2002). In addition, we have not observed any effect of palmitoylation on the intracellular distribution of tagged sSpi in S2 or COS cells (Figure 6 and data not shown).

Spi acts as a short-range signal in vivo, in part due to its induction of the secreted feedback inhibitor Argos (Freeman, 1997). Palmitoylation of Spi does not affect its binding to Argos (Figure S1B), as expected because this binding is mediated by the EGF domain of Spi (Klein et al., 2004). In addition, we have found that *rasp* is required for Spi function even in the complete absence of *argos*. We therefore suggest that a high concentration of Spi is necessary simply to reach the level of EGFR activation required for biological function, irrespective of the presence of Argos. Our results suggest that palmitoylation is the mechanism used to achieve this local accumulation of Spi.

Different Roles for Palmitoylation of Secreted Ligands

Although Hh, Wg, and Spi all carry palmitate modifications essential for their function, palmitoylation appears to have different effects on each molecule. Wg, though not Wnt3a, requires palmitoylation for its secretion (Kadowaki et al., 1996; Willert et al., 2003; Zhai et al., 2004). Shh requires palmitoylation for incorporation into a lipoprotein complex that enhances its transport; Wg is also found in a similar complex (Chen et al., 2004; Panakova

et al., 2005). In addition to its effects on transport, palmitoylation enhances Hh activity in assays that do not require transport (Chamoun et al., 2001; Chen et al., 2004). We note that sSpiCS does not show the dominant-negative effects described for HhC84S (Chamoun et al., 2001; Lee et al., 2001a), suggesting that palmitoylation does not affect Spi activity in the same way.

Palmitoylation of intracellular proteins frequently promotes membrane association, though it usually does so in conjunction with a second lipid modification (Bijlmakers and Marsh, 2003). This raises the possibility that palmitoylated Spi is associated with the plasma membrane, rather than binding to lipoprotein particles like those that transport Hh and Wg. If so, it will be interesting to learn whether membrane-tethered sSpi can directly bind the EGFR. Full-length transmembrane Spi, in which the EGF domain is adjacent to the membrane, is inactive in the absence of Rhomboid, but membrane association of sSpi through its N-terminal palmitate group would place the EGF domain at a distance from the membrane. If membrane-bound Spi cannot activate the EGFR, Spi may be released from the membrane by depalmitoylation. Cycles of palmitoylation and depalmitoylation have been shown to regulate the intracellular localization of Ras (Rocks et al., 2005). However, the N-terminal palmitate modification is likely to form a stable amide linkage as in Hh (Pepinsky et al., 1998), rather than a labile thioester bond. Alternatively, release of Spi could be accomplished by proteolytic processing. Interestingly, we have found that the sequence of wild-type sSpi released into the media from S2 cells begins at methionine 45, whereas sSpiCS begins with the serine at position 29, immediately after the signal peptide (data not shown).

Our observation that palmitoylation of Spi is essential in vivo extends the importance of this modification of extracellular secreted proteins to a third class of ligands. However, its function appears to vary between different molecules and across species. Further study of membrane-bound palmitoyltransferases and their substrates is likely to yield new insights into the regulation of ligand secretion, transport, and activity.

Experimental Procedures

Fly Stocks and Genetics

Stocks used were *rasp*^{T392}, *rasp*^{T802}, *ptc*^{S2}, *aos*^{W11}, *aos*$^{\Delta 7}$, *mirr*^{cre2}, UAS-*Star*, UAS-*rho*, UASp-*sSpi*, UAS-*EGFRλtop*, UAS-*mspi*, UAS-*sSpiGFP*, UAS-*mSpiGFP*, UAS-*ras*^{V12}, *da*-GAL4, *apterous* (*ap*)-GAL4, *en*-GAL4, and *vg*-GAL4 (all described in Flybase). Figures 1A–1C were generated by crossing FRT80, *rasp*^{T392}/TM6B to *ey*FLP1; FRT80, *Ubi*-GFP/TM6B. Figure 1D was generated by crossing FRT42, *ptc*^{S2}/SM6-TM6B to *ey*FLP1; FRT42, *Ubi*-GFP. Figures 1E and 1F were generated by crossing FRT42, *ptc*^{S2}; *rasp*^{T392}/SM6-TM6B to *ey*FLP1; FRT42, *Ubi*-GFP; *rasp*^{T802}/SM6-TM6B. Figures 1G–1I were generated by crossing *ey*FLP1; FRT80, *rasp*^{T392}, *aos*$^{\Delta 7}$/TM6B to FRT80, *aos*$^{\Delta 7}$, Ubi-GFP/TM6B. Germline clones were made by crossing *hs*FLP122/Y; FRT2A, P(*ovo*^{D})/TM3 to FRT2A, *rasp*/TM6B and heat shocking first and second instar larvae 1 hr at 38ºC. The resulting *hs*FLP122; FRT2A, *rasp*/FRT2A, P(*ovo*^{D}) females were crossed to *rasp*, *aos-lacZ*/TM6B males. Dorsal appendage fusion was observed in all eggs derived from *rasp* germline clones, making it very unlikely to result from clones in the follicle cells. Figures 3A–3D and 3G–3J and Figures 6E–6H were generated by crossing *rasp*^{T392} (UAS-*Star*); UAS-*rho* (or UASp-*sSpi* or UAS-*EGFRλtop* or UAS-*sSpiGFP* or UAS-*rho*, UAS-*mSpiGFP*)/SM6-TM6B to *hs*FLP122, *act*>CD2>GAL4; *rasp*^{T802}, UAS-*lacZ*/TM6B and heat shocking first and second instar larvae 1 hr at 38ºC. Figures 3E and 3F and Figures S2E–S2H were generated by crossing FRT80, *aos-lacZ* (*rasp*^{T392}); UASp-*sSpi* (or UAS-*sSpiCS*)/SM6-TM6B to *ey*FLP or *hs*FLP, UAS-*GFP*; *Tub*-GAL4; FRT80, *Tub*-GAL80. Figures 5D–5I were generated by crossing FRT40, *spi*^{SC1}; (UAS-*mSpi* or UAS-*mSpiCS*)/SM6-TM6B to *hs*FLP122, UAS-*GFP*; FRT40, *Tub*-GAL80; *Tub*-GAL4/TM6B and heat shocking first and second instar larvae 1 hr at 38ºC. Figures 6J and 6L were generated by crossing *vg*-GAL4; *rasp*^{T802}/SM6-TM6B to *aos-lacZ*, *rasp*^{T392}; UASp-*sSpi* or UAS-*ras*^{V12}/SM6-TM6B.

Antibodies, Immunohistochemistry, and Western Blotting

Staining of eye and wing discs and embryos was performed as described (Lee and Treisman, 2001). Antibodies used were rat anti-Elav (1:50; Developmental Studies Hybridoma Bank, Iowa City, IA), guinea pig anti-Sens (1:1000) (Frankfort et al., 2001), mouse anti-En (1:1; Developmental Studies Hybridoma Bank), and rabbit anti-β-galactosidase (1:5000; Cappel, Irvine, CA). sSpiGFP and mSpiGFP distribution were viewed in unfixed eye-antennal discs that were dissected, mounted in 80% glycerol/0.1 M phosphate buffer (pH 7.2), and scanned shortly thereafter. For S2 cell staining, untransfected S2 cells and S2 cells expressing sSpi or sSpiCS were induced with 0.5 mM $CuSO_4$ for 16 hr. Cells were washed with phosphate-buffered saline (PBS) and immobilized on poly-L-lysine-treated coverslips. Control cells were fixed with 1.8% formaldehyde for 10 min and permeabilized with 0.1% saponin (included with the antibody). Cells were incubated with anti C-terminal His antibody (Invitrogen, Carlsbad, CA) at 1:1000 with 1% BSA for 1 hr at room temperature, washed with PBS, and incubated with goat anti-mouse Alexa488 (Molecular Probes, Carlsbad, CA) at 1:500 with 1% BSA for 1 hr at room temperature. Images were obtained using a Leica (Wetzlar, Germany) DM IRBE microscope. For Western blotting, lysates and media were heated to 95ºC for 5 min and loaded onto a 15% SDS-PAGE gel. Gels were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA) and blocked overnight with TBST (20 mM Tris [pH 7.6], 137 mM NaCl, 0.2% Tween-20) supplemented with 10% low-fat milk. Membranes were incubated with TBST with 10% milk supplemented with antibodies for 1 hr at room temperature. Blots were washed with TBST for 1 hr and incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000; Jackson Immunoresearch, West Grove, PA or Amersham, Piscataway, NJ) for another hour. Blots were developed with enhanced chemiluminescence (Pierce, Rockford, IL or Amersham). Antibodies used were rat anti-Spi (1:100) (Schweitzer et al., 1995), mouse anti-Grk (1:50) (Ghiglione et al., 2002), mouse anti-PCNA (Novus Biologicals, Littleton, CO), mouse anti-GFP (1:1000; Roche, Indianapolis, IN or Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-HA (1:1000; Santa Cruz).

Constructs and Transfections

UAS-sSpiCS was generated by PCR, introducing the C29S mutation and a stop codon at K133. The fragment containing the C29S mutation was subsequently transferred into pcDNA-sSpiGFP (made by cloning the GFP-tagged sSpi of Tsruya et al. (2002) into pcDNA3.1 and into mSpi (Lee et al., 2001b). pcDNA-HARasp was made by PCR, adding an HA epitope tag to the C-terminal end of Rasp. UAS-*HArasp*, which contained the same tag, was able to rescue *rasp* lethality and photoreceptor development in *rasp* mutant eye discs (data not shown). Transgenic flies (UAS-*mSpiCS*, UAS-*sSpiCS*, and UAS-*HArasp*) were made by standard methods. UAS-sGrk was kindly provided by Erika Bach. pcDNA-HARaspH381A was made using the QuikChange kit (Stratagene, La Jolla, CA) with pcDNA-HARasp as a template and the following primers, 5′-GCCTTTGTGTTCGTCTGGGCAGGATGCTACACCTATGTG-3′, 5′-CACATAGGTGTAGCATCCTGCCCAGACGAACACAAAGGC-3′. S2 cells were maintained in Schneider's medium (GIBCO, Carlsbad, CA) supplemented with 10% fetal calf serum. Cells were transfected with Effectene (Qiagen, Valencia, CA) according to the manufacturer's instructions. All UAS plasmids were cotransfected with *actin*-GAL4. For protein purification, sSpi and sSpiC25S (residues 1–128) were cloned into the pMT/V5-His vector (Invitrogen) and stably cotransfected into S2 cells with pCoHygro, following the manufacturer's instructions. Protein production was induced with 0.5 mM $CuSO_4$, and protein purifications were carried out as described (Klein et al., 2004).

COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells were transfected with Lipofectamine (Invitrogen) according to the manufacturer's instructions. Introduction of dsRNA into S2 cells was performed as described (Clemens et al., 2000). S2 cells were transfected 48 hr after introduction of dsRNA and were harvested after an additional 48 hr and processed for Triton X-114 separation. RNA was isolated by Trizol extraction and cDNA synthesis was performed using the Superscript First Strand Synthesis system for RT-PCR (Invitrogen). *Actin 5C* was used as a control for the RT-PCR. *rasp* dsRNA spans a 660 bp region covering amino acids 281–500. Control dsRNA was homologous to the mediator complex subunit gene *kohtalo*. dsRNA was synthesized using the T3 Megascript kit (Ambion, Austin, TX).

Triton X-114 Phase Separation

Cells were harvested after 48 hr and lysed in 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Triton X-114. Phase separation was performed as described (Bordier, 1981). Equal volumes of each phase and the input were immunoblotted with anti-Spi, anti-GFP, and anti-PCNA antibodies.

mSpi Cleavage Assay

S2 cells were transfected with actin-GAL4, UAS-GFP, UAS-mSpi, or UAS-mSpiCS together with pRmHaStar and pRmHaRho (Lee et al., 2001b). Twenty-four hours after transfection, cells were placed in serum-free media containing 500 μM $CuSO_4$ for 72 hr. Cells and media were processed as described (Tsruya et al., 2002), and 1 ml of media was concentrated 20-fold using Centricon columns (Millipore, Billerica, MA). Figure 4D shows 40 μl of lysates, 50 μl of media, and 25 μl of concentrated media.

Palmitate Labeling

COS-1 cells or S2 cells expressing sSpi-GFP and HARasp constructs were starved for 1 hr in DMEM containing 2% dialyzed fetal bovine serum, followed by incubation with 10–20 μCi/ml [^{125}I]IC16, a radio-iodinated palmitate analog, for 4 hr at 37ºC. The cells were then washed two times with 5 ml of ice-cold STE (100 mM NaCl, 10 mM Tris, 1 mM EDTA [pH 7.4]) and lysed in 500 μl of RIPA buffer (150 mM NaCl, 50 mM Tris [pH 7.4], 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA). Cell lysates were clarified by ultracentrifugation at 100,000 × g for 15 min in a T100.2 rotor (Beckman, Fullerton, CA). The levels of sSpi-GFP and HARasp in the total cell lysates were determined by SDS-PAGE and Western blot analysis. Immunoprecipitation of sSpi-GFP and sSpiCS-GFP was performed as follows. Clarified lysate was incubated with 5 μl of rabbit anti-GFP (Santa Cruz) and 50 μl of protein A/G$^+$ agarose beads (Santa Cruz) at 4ºC for 16 hr in RIPA buffer. After incubation the beads were collected by centrifugation at 1000 × g and washed two times in 500 μl of fresh RIPA buffer. Final bead pellets were resuspended in 40 μl of 2× sample buffer without DTT or β-mercaptoethanol. Immunoprecipitated samples were run on a 12.5% SDS-PAGE gel, dried, and exposed by phosphorimaging for 4–7 days. Screens were analyzed on a Storm 840 phosphorimager (Molecular Dynamics, Piscataway, NJ). Labelings were performed in duplicate and repeated three times.

Supplemental Data

Supplemental Data include three figures and are available at http://www.developmentalcell.com/cgi/content/full/10/2/167/DC1/.

Acknowledgments

We thank Erika Bach, Nick Baker, Hugo Bellen, Matthew Freeman, Justin Kumar, Ilaria Rebay, Pernille Rorth, Benny Shilo, Gary Struhl, the Bloomington *Drosophila* Stock Center, and the Developmental Studies Hybridoma Bank for fly stocks and materials. We are also grateful to Erika Bach, Mark Philips, and Ruth Lehmann for experimental advice. The manuscript was improved by the critical comments of Dorian Anderson, Erika Bach, Nick Baker, Inés Carrera, Claude Desplan, Reza Farajian, Kerstin Hofmeyer, Jeremy Nance, and Jean-Yves Roignant. This work was supported by grants from the National Institutes of Health (EY13777 to J.E.T., GM57966 to M.D.R., and CA79992 to M.A.L.). D.A. is a Damon Runyon Fellow supported by the Damon Runyon Cancer Research Foundation (DRG-1884–05).

Received: July 5, 2005
Revised: October 19, 2005
Accepted: November 17, 2005
Published: February 6, 2006

References

Alland, L., Peseckis, S.M., Atherton, R.E., Berthiaume, L., and Resh, M.D. (1994). Dual myristylation and palmitylation of Src family member p59fyn affects subcellular localization. J. Biol. Chem. *269*, 16701–16705.

Amanai, K., and Jiang, J. (2001). Distinct roles of Central missing and Dispatched in sending the Hedgehog signal. Development *128*, 5119–5127.

Bijlmakers, M.J., and Marsh, M. (2003). The on-off story of protein palmitoylation. Trends Cell Biol. *13*, 32–42.

Bordier, C. (1981). Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem. *256*, 1604–1607.

Burke, R., Nellen, D., Bellotto, M., Hafen, E., Senti, K.A., Dickson, B.J., and Basler, K. (1999). Dispatched, a novel sterol-sensing domain protein dedicated to the release of cholesterol-modified hedgehog from signaling cells. Cell *99*, 803–815.

Chamoun, Z., Mann, R.K., Nellen, D., von Kessler, D.P., Bellotto, M., Beachy, P.A., and Basler, K. (2001). Skinny hedgehog, an acyltransferase required for palmitoylation and activity of the Hedgehog signal. Science *293*, 2080–2084.

Chen, M.H., Li, Y.J., Kawakami, T., Xu, S.M., and Chuang, P.T. (2004). Palmitoylation is required for the production of a soluble multimeric Hedgehog protein complex and long-range signaling in vertebrates. Genes Dev. *18*, 641–659.

Clemens, J.C., Worby, C.A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B.A., and Dixon, J.E. (2000). Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. Proc. Natl. Acad. Sci. USA *97*, 6499–6503.

Dominguez, M. (1999). Dual role for *hedgehog* in the regulation of the proneural gene *atonal* during ommatidia development. Development *126*, 2345–2353.

Frankfort, B.J., Nolo, R., Zhang, Z., Bellen, H., and Mardon, G. (2001). *senseless* repression of *rough* is required for R8 photoreceptor differentiation in the developing *Drosophila* eye. Neuron *32*, 403–414.

Freeman, M. (1997). Cell determination strategies in the *Drosophila* eye. Development *124*, 261–270.

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 *1992*, 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.

Gallet, A., Rodriguez, R., Ruel, L., and Therond, P.P. (2003). Cholesterol modification of hedgehog is required for trafficking and movement, revealing an asymmetric cellular response to hedgehog. Dev. Cell *4*, 191–204.

Ghiglione, C., Bach, E.A., Paraiso, Y., Carraway, K.L. III, Noselli, S., and Perrimon, N. (2002). Mechanism of activation of the *Drosophila* EGF receptor by the TGFα 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., Biehs, B., Sturtevant, M.A., Wickline, L., Chacko, J., Howard, K., and Bier, E. (1999). *rhomboid* and *Star* interact synergistically to promote EGFR/MAPK signaling during *Drosophila* wing vein development. Development *126*, 2663–2676.

Hynes, N.E., and Lane, H.A. (2005). ERBB receptors and cancer: the complexity of targeted inhibitors. Nat. Rev. Cancer *5*, 341–354.

Kadowaki, T., Wilder, E., Klingensmith, J., Zachary, K., and Perrimon, N. (1996). The segment polarity gene *porcupine* encodes a putative

multitransmembrane protein involved in Wingless processing. Genes Dev. *10*, 3116–3128.

Karim, F.D., and Rubin, G.M. (1998). Ectopic expression of activated Ras1 induces hyperplastic growth and increased cell death in *Drosophila* imaginal tissues. Development *125*, 1–9.

Klein, D.E., Nappi, V.M., Reeves, G.T., Shvartsman, S.Y., and Lemmon, M.A. (2004). Argos inhibits epidermal growth factor receptor signalling by ligand sequestration. Nature *430*, 1040–1044.

Lee, J.D., and Treisman, J.E. (2001). Sightless has homology to transmembrane acyltransferases and is required to generate active Hedgehog protein. Curr. Biol. *11*, 1147–1152.

Lee, J.D., Kraus, P., Gaiano, N., Nery, S., Kohtz, J., Fishell, G., Loomis, C.A., and Treisman, J.E. (2001a). An acylatable residue of Hedgehog is differentially required in *Drosophila* and mouse limb development. Dev. Biol. *233*, 122–136.

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

Lesokhin, A.M., Yu, S.Y., Katz, J., and Baker, N.E. (1999). Several levels of EGF receptor signaling during photoreceptor specification in wild-type, *Ellipse*, and null mutant *Drosophila*. Dev. Biol. *205*, 129–144.

Lin, X. (2004). Functions of heparan sulfate proteoglycans in cell signaling during development. Development *131*, 6009–6021.

Micchelli, C.A., The, I., Selva, E., Mogila, V., and Perrimon, N. (2002). Rasp, a putative transmembrane acyltransferase, is required for Hedgehog signaling. Development *129*, 843–851.

Panakova, D., Sprong, H., Marois, E., Thiele, C., and Eaton, S. (2005). Lipoprotein particles are required for Hedgehog and Wingless signalling. Nature *435*, 58–65.

Pepinsky, R.B., Zeng, C., Wen, D., Rayhorn, P., Baker, D.P., Williams, K.P., Bixler, S.A., Ambrose, C.M., Garber, E.A., Miatkowski, K., et al. (1998). Identification of a palmitic acid-modified form of human Sonic hedgehog. J. Biol. Chem. *273*, 14037–14045.

Pfeiffer, S., Ricardo, S., Manneville, J.B., Alexandre, C., and Vincent, J.P. (2002). Producing cells retain and recycle Wingless in *Drosophila* embryos. Curr. Biol. *12*, 957–962.

Porter, J.A., Young, K.E., and Beachy, P.A. (1996). Cholesterol modification of hedgehog signaling proteins in animal development. Science *274*, 255–259.

Queenan, A.M., Ghabrial, A., and Schupbach, T. (1997). Ectopic activation of torpedo/Egfr, a *Drosophila* receptor tyrosine kinase, dorsalizes both the eggshell and the embryo. Development *124*, 3871–3880.

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.

Ritzenthaler, S., Suzuki, E., and Chiba, A. (2000). Postsynaptic filopodia in muscle cells interact with innervating motoneuron axons. Nat. Neurosci. *3*, 1012–1017.

Rocks, O., Peyker, A., Kahms, M., Verveer, P.J., Koerner, C., Lumbierres, M., Kuhlmann, J., Waldmann, H., Wittinghofer, A., and Bastiaens, P.I. (2005). An acylation cycle regulates localization and activity of palmitoylated Ras isoforms. Science *307*, 1746–1752.

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.

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

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.

Schupbach, T. (1987). Germ line and soma cooperate during oogenesis to establish the dorsoventral pattern of egg shell and embryo in *Drosophila melanogaster*. Cell *49*, 699–707.

Schweitzer, R., Shaharabany, M., Seger, R., and Shilo, B.-Z. (1995). 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.

Shum, L., Turck, C.W., and Derynck, R. (1996). Cysteines 153 and 154 of transmembrane transforming growth factor-α are palmitoylated and mediate cytoplasmic protein association. J. Biol. Chem. *271*, 28502–28508.

Sternberg, P.W., and Horvitz, H.R. (1991). Signal transduction during *C. elegans* vulval induction. Trends Genet. *7*, 366–371.

Tanaka, K., Kitagawa, Y., and Kadowaki, T. (2002). *Drosophila* segment polarity gene product porcupine stimulates the posttranslational N-glycosylation of wingless in the endoplasmic reticulum. J. Biol. Chem. *277*, 12816–12823.

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. (2002). A family of Rhomboid intramembrane proteases activates all *Drosophila* membrane-tethered EGF ligands. EMBO J. *21*, 4277–4286.

Willert, K., Brown, J.D., Danenberg, E., Duncan, A.W., Weissman, I.L., Reya, T., Yates, J.R. III, and Nusse, R. (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature *423*, 448–452.

Wong, R.W. (2003). Transgenic and knock-out mice for deciphering the roles of EGFR ligands. Cell. Mol. Life Sci. *60*, 113–118.

Zecca, M., and Struhl, G. (2002). Control of growth and patterning of the *Drosophila* wing imaginal disc by EGFR-mediated signaling. Development *129*, 1369–1376.

Zhai, L., Chaturvedi, D., and Cumberledge, S. (2004). *Drosophila* Wnt-1 undergoes a hydrophobic modification and is targeted to lipid rafts, a process that requires *porcupine*. J. Biol. Chem. *279*, 33220–33227.