Dynein and Star interact in EGFR signaling and ligand trafficking

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Summary

Intracellular transport and processing of ligands is critical to the activation of signal transduction pathways that guide development. Star is an essential gene in Drosophila that has been implicated in the trafficking of ligands for epidermal growth factor (EGF) receptor signaling. The role of cytoplasmic motors in the endocytic and secretory pathways is well known, but the specific requirement of motors in EGF receptor transport has not been investigated. We identified Star in a screen designed to recover second-site modifiers of the dominant rough eye phenotype of the Glued mutation Gl1. The Glued (Gl) locus encodes the p150 subunit of the dynactin complex, an activator of cytoplasmic dynein-driven motility. We show that alleles of Gl and dynein genetically interact with both Star and EGFR alleles. Similarly to mutations in Star, the Gl1 mutation is capable of modifying the phenotypes of the EGFR mutation Ellipse. These genetic interactions suggest a model in which Star, dynactin and dynein cooperate in the trafficking of EGF ligands. In support of this model, overexpression of the cleaved, active Spitz ligand can partially bypass defective trafficking and suppress the genetic interactions. Our direct observations of live S2 cells show that export of Spitz-GFP from the endoplasmic reticulum, as well as the trafficking of Spitz-GFP vesicles, depends on both Star and dynein.

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Key words: Star, Dynein, Spitz, Drosophila

Introduction

Intracellular transport is an essential function of the microtubule motors, dynein and kinesin. In order to carry out this function, the cytoplasmic motors must be attached to, and released from, a variety of cellular cargoes at the right time and place. How cytoplasmic motors are linked to specific cargoes and how these linkages are regulated is still unclear. Dynactin (dynein activator protein) is one complex thought to be involved in linking membrane vesicles to dynein (Karki and Holzbaur, 1999; Muresan et al., 2001; Schroer, 2004; Waterman-Storer et al., 1997). However, whether dynactin is required for the binding of cargo or instead acts in the regulation of binding and/or motor activity is still controversial (Haghnia et al., 2007; Kim et al., 2007; Berezuk and Schroer, 2007). The dynactin, or Glued complex was originally identified as a stimulator of dynein-mediated vesicle motility in vitro (Gill et al., 1991; Schroer et al., 1996; Schroer and Sheetz, 1991). It consists of at least 10 different polypeptides ranging in size from a 24 kDa subunit to the p150/160 polypeptide, also known as the p150/160Glued polypeptide (Gill et al., 1991; Holleran et al., 1996; Lees-Miller et al., 1992; Paschal et al., 1993; Schroer and Sheetz, 1991). p150/160Glued binds directly to the dynein intermediate chain (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995) and is proposed to facilitate the association of the dynein motor with its cellular cargoes, which include Golgi vesicles, endosomal vesicles, synaptic vesicles and kinetochores (Burkhardt, 1998; Gill et al., 1991; Holleran et al., 1998; Holzbaur et al., 1991; King and Schroer, 2000). Other components of the dynactin complex have been shown to associate with membranous vesicles through an interaction with the spectrin membrane skeleton (Holleran et al., 2001; Holleran et al., 1996; Muresan et al., 1996; Muresan et al., 2001), and with kinetochores during mitosis via the cytoplasmic linker protein CLIP-170 (Dujardin et al., 1998; Vaughan and Vallee, 1995).

In order to further characterize pathways that require dynein function, we conducted a screen for P-element insertion mutations that dominantly modify the eye phenotype of the Glued allele Gl1. In Drosophila, the Gl1 mutation causes a dominant rough eye phenotype, with ommatidial disarrangements and defects in optic lobe connections (Plough and Ives, 1935). Gl1 encodes a truncated product because of the insertion of a B104 retrotransposon in its coding sequence (Swaroop et al., 1985). We previously showed that the truncated Gl1 product no longer assembles into the dynactin complex, but does functionally interact with certain dynein heavy chain (Dhc) mutants (McGrail et al., 1995). Mutations in Dhc also modify (either suppress or enhance) the dominant rough eye phenotype of Gl1, and a previously identified suppressor of the Gl1 phenotype, Su(Gl)102, is an allele of Dhc (McGrail et al., 1995). Here, we report that mutations in Star act as dominant modifiers of the Gl1 rough eye. Star is an essential gene involved in the proper processing of the EGF receptor ligand Spitz (Bang and Kintner, 2000; Golembo et al., 1996; Guichard et al., 1999). Spitz activation of EGF receptor signaling is critical throughout development, and its requirement during eye morphogenesis is well established (Klambt, 2002; Shilo, 2005). The dominant Star mutation S0 results in a rough eye phenotype similar to that of Gl1 (Kolodkin et al., 1994; Ruden et al., 1999). Star encodes a type II single transmembrane domain protein (Kolodkin et al., 1994) that concentrates at the nuclear periphery and is contiguous with the endoplasmic reticulum (ER) (Pickup and Banerjee, 1999). Star...
facilitates trafficking of inactive, membrane-bound Spitz precursor from the ER to an endosomal or Golgi compartment where it is cleaved by the protease Rhomboid (Lee et al., 2001; Tsuaya et al., 2002; Urban et al., 2002). Cleavage is required to transform Spitz into active ligand. Thus, understanding the regulation of intracellular Spitz transport is critical to understanding the activation of EGF signaling. Our observations provide evidence that the Star-dependent export of Spitz ligand from the ER requires cytoplasmic dynein.

Results
A lethal P-element insertion in Star enhances the Gl¹ eye phenotype
To identify potential genes that regulate dynein-based functions in Drosophila, we screened for dominant modifiers of the rough eye phenotype exhibited by the dynactin mutation Gl¹. A collection of ~300 lethal P-element insertion lines spanning all four chromosomes was tested. One of the P-element insertion lines, P2036, enhanced the Gl¹ rough eye phenotype (Fig. 1). Although the P2036 line had no obvious eye phenotype by itself, in combination with Gl¹ it produced a significant reduction in eye size and disrupted the hexagonal packing of ommatidia. This enhancement of the Gl¹ phenotype was indeed linked to the P-element insertion, since it was reverted by excision of the P-element.

The gene disrupted by P2036 was identified as Star, which produces a protein that regulates the intracellular trafficking of the EGF receptor ligand Spitz in several developmental pathways (Kolodkin et al., 1994; Lee et al., 2001; Tsuaya et al., 2002). Southern blot and sequence analysis showed that only a single EGF receptor ligand Spitz in several developmental pathways produces a protein that regulates the intracellular trafficking of the Gl¹ allele, genetic crosses were set up using flies that carried Star with Gl¹ eye phenotype. A defect that removes the Star locus, Df(2L)S3, was tested for its ability to modify the Gl¹ dominant eye phenotype. Gl¹ flies showed a mild perturbation of the ommatidia (Fig. 2A), whereas Df(2L)S3 flies were near wild type in appearance (Fig. 2B). In flies carrying Gl¹ in combination with the Df(2L)S3, the eye was small, very narrow, and rough, with fewer ommatidia compared with the deficiency alone (Fig. 2C). To determine whether the interaction was specific to the Gl¹ dominant allele, genetic crosses were set up using flies that carried a deficiency for the Gl¹ locus (Gl¹-R2) or a recessive lethal mutation in the Gl¹ locus (Gl¹-1). Unlike Gl¹, these loss-of-function alleles of Gl did not exhibit dominant eye phenotypes. We found that they showed little or no interaction with S¹ (e.g. Fig. 2D) or the other three Star alleles (data not shown). Moreover, the enhancement of the Gl¹ rough eye phenotype by S¹ (Fig. 2E) was reverted by the introduction of a full-length Star transgene, hsStar-HA (Fig. 2F). We conclude that the interaction of Star with the Gl¹ locus is specific to the Gl¹ allele, and that reduction of Star gene dosage by 50% strongly enhances the Gl¹ eye phenotype.

Fig. 1. P2036, an allele of Star, enhances the Gl¹ eye phenotype. Scanning electron micrographs of Drosophila eyes. (A) In the wild-type eye, ommatidia are arranged in an orderly fashion. (B) Eyes derived from Gl¹ flies show disorganization in the arrangement of the ommatidia and bristles, giving a ‘rough’ appearance to the eye. (C) The parental line P2036 is not distinguishable from the wild type. (D) Eyes expressing both P2036 and Gl¹ are reduced in size and the general surface of the eye is very rough, showing a dominant enhancement of the Gl¹ rough eye. Genotypes shown: (A) wild type +/+; +/+, (B) +/+; Gl¹/+, (C) P2036/++; +/+, (D) P2036/++; Gl¹/+. Interaction of Star with Gl¹ is dosage sensitive
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Fig. 2. The interaction of Star with Gl¹ is dosage sensitive. The chromosomal deficiency Df(2L)S3, which removes the Star locus, enhances the Gl¹ eye phenotype. (A) Gl¹ flies have a mild but distinct disarrangement of ommatidia. (B) Df(2L)S3 flies are near wild type in appearance. (C) By contrast, flies expressing both the deficiency and the Gl¹ mutation display an extreme rough-eye phenotype. The eye is small, narrow and very rough with a reduced number of ommatidia. (D) A recessive lethal allele of Glued, Gl¹-3 shows little or no interaction with S¹ (e.g. Fig. 2D) or the other three Star alleles (data not shown). Moreover, the enhancement of the Gl¹ rough eye phenotype by S¹ (Fig. 2E) was reverted by the introduction of a full-length Star transgene, hsStar-HA (Fig. 2F). We conclude that the interaction of Star with the Gl¹ locus is specific to the Gl¹ allele, and that reduction of Star gene dosage by 50% strongly enhances the Gl¹ eye phenotype.
Mutations in Dhc modify Star

The interactions described above, between Star and Gl1, resembled previously observed genetic interactions between Dhc and Gl1 (McGrail et al., 1995). To address whether this similarity reflects a common function, we asked whether Star also interacts with Dhc. The recessive allele, Dhc1-1 (Gepner et al., 1996), enhances the S' rough eye phenotype (Fig. 3A,B). In S'/+; Dhc1-1/+ flies, the hexagonal packing of ommatidia was more disrupted than in the S' background alone, and the size of the eye was reduced. This interaction is reverted back to the S' eye phenotype by the introduction of a wild-type Dhc transgene (data not shown). In addition, triple heterozygotes containing the S', Gl1 and Dhc1-1 alleles (S'/+; Dhc1-1/+ Gl1/) exhibited a more severe eye phenotype than the S'/+; Gl1/+ double heterozygotes (Fig. 3C,D). Other Dhc alleles tested did not significantly modify the S' eye phenotype, but did interact with Star to produce a wing vein phenotype. Both Dhc6-10 and Dhc1-1, in transheterozygous combinations with the Star allele S05671, produce a wing phenotype in which the L5 vein was incomplete and did not reach the wing margin (supplementary material Fig. S1). This interaction appeared to be specific to the S05671 allele, because S' in combination with Dhc alleles did not show any wing vein phenotype (data not shown). Although the Gl1 eye phenotype was enhanced by S05671, a wing vein phenotype was not produced (data not shown).

Star is epistatic to Dhc in its interaction with Gl1

Having found that both Star and Dhc interact with Gl1, we assessed the epistasis between the three gene products by analyzing eye phenotypes in different combinations of mutations. We have previously reported that certain Dhc mutations enhance the Gl1 rough eye, whereas other Dhc alleles suppress it (McGrail et al., 1995). More recently, we have established that another mutation originally isolated as a suppressor of the Gl1 rough eye phenotype, Su(Gl)77 (Harte and Kankel, 1982), is a Dhc allele (see Materials and Methods). Flies expressing both Gl1 and a Dhc mutation that suppresses Gl1 were crossed to S' flies, and the eye phenotypes of the progeny were examined (Fig. 4). As expected, flies carrying either of the Dhc alleles (Su(Gl)77 or Dhc6-10) that suppress the Gl1 rough eye had wild-type eye morphology (Fig. 4C). With the addition of the S' mutation, the Gl1 eye is enhanced, despite the presence of a suppressor (Fig. 4D). Even in the presence of both Dhc mutations that suppress Gl1, the rough eye phenotype was still enhanced by S' (compare Fig. 4E,F). These results suggest that Star function is required for the suppression of Gl1 eye phenotype by the Dhc mutations, and provide additional evidence that Star, dynein and dynactin act in a common pathway.

Biochemical assays of Star-dynein interactions

The association between the dynein complex and Star was first examined with a partitioning assay. Flies expressing a functional

Fig. 3. Dhc alleles also interact with Star. (A) The S' allele generates a dominant, mild, rough eye phenotype with slightly abnormal ommatidia. (B) Dhc1-1 dominantly enhances the S' eye phenotype. The eyes are narrow, small and the eye surface is rougher. Dhc1-1/+/Gl1 by itself does not have any dominant phenotypes. (C) S' and Gl1 interact to enhance the rough eye. (D) Dhc1-1 further enhances the S'-Gl1 eye interaction. Wing phenotypes are also produced (see supplementary material Fig. S1). Genotypes shown: (A) S'/+; +/+, (B) S'/+; Dhc1-1/+, (C) S'/+; +/Gl1, (D) S'/+; Dhc1-1+/+ Gl1.

Fig. 4. Star is required for suppression of Gl1 by Dhc alleles. Epistasis tests were carried out by examining different mutant combinations using SEM. Representative examples of (A) the Gl1 eye, and (B) the Gl1 eye enhanced by S', are shown to allow comparisons. (C) Su(Gl)77, a mutation in the Dhc locus, partially suppresses the Gl1 dominant eye phenotype. Dhc6-10 similarly suppresses the Gl1 rough eye (not shown). (D) The mutation in Star overcomes the suppression effect of the Dhc allele Su(Gl)77, and results in an enhanced Gl1 phenotype. The same result (not shown) is seen with Dhc6-10 in the presence of Gl1 and S'. (E) Su(Gl)77 in combination with Dhc6-10 completely suppresses the Gl1 eye phenotype. (F) Despite the presence of two Dhc mutations that suppress Gl1, S' still shows enhancement of the Gl1 rough eye. Genotypes shown: (A) +/+; Gl1/+; (B) S'/+; Gl1/+, (C) Su(Gl)77 Gl1/+, (D) S'/+; Su(Gl)77 Gl1/+, (E) +/+; Su(Gl)77 Gl1/Dhc6-1, (F) S'/+; Su(Gl)77 Gl1/Dhc6-1.
hemagglutinin (HA)-tagged Star transgene, hsStar-HA (Pickup and Banerjee, 1999), were used to analyze the relative amounts of Dhc and Star present in fractions enriched for vesicles. A crude preparation of vesicles was clarified by high-speed centrifugation to yield vesicle membranes in the pellet and soluble proteins in the supernatant. As expected, the transmembrane protein Star-HA partitioned into the vesicle pellet fraction (Fig. 5A). Although much of the dynein was soluble, some was also present in the membrane pellet, consistent with an association with vesicles. Dynein and Star also exhibited overlapping, but not identical, sedimentation profiles on Nycodenz density gradients. This result could indicate that a subpopulation of Star-containing vesicles also associates with dynein (Fig. 5B). Dynein is known to bind microtubules with high affinity in the absence of ATP and low affinity in the presence of ATP. This property has been used previously to co-sediment rhodopsin-bearing vesicles with microtubules, in the presence of dynein and in an ATP-sensitive manner (Tai et al., 1999). Similarly, if dynein and Star are present on the same vesicles, then Star should also show an ATP-sensitive association with microtubules. We polymerized microtubules in vesicle preparations derived from hsStar-HA flies and looked for Star in the microtubule pellet fraction. An increased amount of both Dhc and Star were found to pellet with microtubules in the absence of ATP, suggesting that the association of Star-containing vesicles with microtubules is mediated by dynein (Fig. 5C).

We also conducted chemical crosslinking experiments to investigate the interaction between Star and dynein. S2 cells transfected with Star-HA were used to prepare membranes by flotation on step gradients (Haghnia et al., 2007). Fractions containing both Star-HA and dynein were treated with EDC [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride], a zero-length chemical crosslinker. Immunoblot analysis showed that the reaction products include an increasing amount of a high molecular mass complex that was recognized by antibodies to both Dhc and Star-HA (Fig. 5D). A corresponding decrease in the amounts of noncrosslinked Dhc and Star-HA is observed.

Dhc and Glued interact with other components of the EGFR signaling pathway

Ellipse1 (Elp1) is a hyperactivating mutation in the EGFR receptor. Elp1 flies have small eyes with a reduced number of ommatidia, as shown in Fig. 6A (Baker and Rubin, 1989). Alleles of Dhc (Dhc6-10, Dhc6-6, Dhc6-19, Dhc14-1), as well as the Gl1 allele, enhance the Elp1 eye phenotype (Fig. 6B-D, and data not shown). In addition to the dominant eye phenotypes, Elp1 produced wing vein phenotypes (Fig. 7B,E) (Baker and Rubin, 1989; Lindsley and Zimm, 1992). Mutations in Star suppress the wing phenotypes produced by Elp1 (Sturtevant et al., 1993), and also suppress wing phenotypes produced by mutations in Delta (Dl), a Notch receptor ligand (Heberlein et al., 1993; Sturtevant and Bier, 1995). To further test the contribution of dynein to these pathways, we asked whether Gl and Dhc alleles also modify wing phenotypes in Elp1 and Dl mutants. We found that Gl suppressed the wing vein phenotype exhibited by Elp1 (Fig. 7F) and by Dl alleles (Fig. 7G,H). Gl also interacts with Rhomboid (rho), which operates in concert with Star and the EGF receptor during wing development (Sturtevant et al., 1993). The overexpression of rho produced an extra wing vein phenotype that was suppressed by Gl (Fig. 7L). These observations indicate that dynein function has a role in EGF receptor signaling during both wing and eye development.

Overexpression of secreted Spitz rescues the rough eye phenotype

It has been proposed that Star acts to chaperone Spitz precursor from the ER to the Golgi, where cleavage by Rho produces the
Dynein and Star interact in EGFR pathway

The transgenic expression of a truncated form of Spitz mimics the secreted ligand (sSpitz), and activates the Drosophila EGFR pathway in embryos mutant for Star and/or rho (Schweitzer et al., 1995). We reasoned that Star mutations might enhance the Gl1 rough eye phenotype because of the role of dynein in transporting Spitz. To test this hypothesis, we asked whether overexpression of UAS-sSpitz could rescue the Gl1 rough eye phenotype. Instead of the original Gl1 line, we used an inducible Gl construct, UAS-\(\alpha\)-GAL4\(\times\) js (Mische et al., 2007). Expression of js driven by actin-GAL4 produced small eyes with disruptions in the hexagonal packing of the ommatidia (Fig. 8A). This rough eye phenotype was indeed suppressed by expression of UAS-sSpitz (Fig. 8B). Our result is in agreement with other data showing that Spitz requires transport from the ER to another compartment before cleavage and activation can occur (Lee et al., 2001; Tsruya et al., 2002, 2007), and suggests that this trafficking is defective in the Gl1 mutant.

**Spitz-GFP is actively transported by dynein in Drosophila S2 cells**

To directly visualize the transport of Spitz, we transfected S2 cells with Spitz-GFP. Spitz-GFP accumulated in the lattice of the endoplasmic reticulum (ER) that encompasses the nucleus and extends into the cytoplasm (Fig. 9A) (Lee et al., 2001; Tsruya et al., 2002). Previous studies have shown that in the presence of Star, Spitz-GFP exits the ER in vesicles that are trafficked to the Golgi and/or endosomal compartments (Lee et al., 2001; Tsruya et al., 2002; Tsruya et al., 2007). We used live imaging techniques to examine the transport of Spitz-GFP following the coexpression of Star, and quantified the changes in transport following the reduction of dynein levels by RNAi.

**In cells coexpressing both Spitz-GFP and Star, the distribution of Spitz-GFP was not limited to the ER lattice, but accumulated in numerous small vesicles that transiently moved through the cytoplasm in a linear fashion (Fig. 9B; Table 1; supplementary material Movie 1). This movement was characteristic of microtubule-based transport of cytoplasmic organelles, and was blocked by the microtubule inhibitor colcemid (data not shown). In fixed immunocytological preparations, dynein was present...**
We provide evidence that components of the dynein-dynactin pathway interact with Star to regulate transport and signaling by Spitz. First, mutations in Star dominantly interact with the Gl^1^ mutation. Reduction of Star gene dosage by 50% severely enhances the Gl^1^ eye phenotype. This interaction between Gl^1^ and the Star allele is specific to the loss of Star function, since the altered eye phenotype is reverted by the presence of a Star transgene. The rescue suggests that the wild-type proteins interact in vivo, and that the phenotype does not reflect neomorphic protein interactions. Second, Star interacts with mutations in dynein itself. The observed interactions for both Star and Dhc are allele-specific, suggesting that specific domains within the Star and Dhc products mediate the interactions. Third, the suppression of the Gl^1^ eye phenotype by certain Dhc alleles (e.g. Su(gl)^77^), requires Star function. The suppression is reversed in the presence of a Star mutation, emphasizing the common pathway in which these gene products function. Finally, genetic interactions between the Dhc and Star loci are observed in both the eye and the wing, supporting a bona fide interaction, and suggesting that a common pathway operates within different tissues.

What do the functional interactions between components of the dynein motor and EGFR signaling pathway mean? One intriguing possibility is that the dynein-dynactin complex is bound through Star to ER vesicles that contain EGFR ligands. Previous work has throughout the cytoplasm and could be observed to colocalize on a subpopulation of the Spitz-GFP vesicles (Fig. 9D). Next, we asked whether dynein is involved in the transport of Spitz-GFP from the ER. We used two sets of dsRNA to effectively deplete dynein heavy chain to levels undetectable by western blot (data not shown). Following the elimination of dynein activity, the number of vesicles per cell was reduced by 60% compared with control cells (Fig. 9C,E). In addition, the motility of Spitz-GFP vesicles was significantly inhibited (Fig. 9C,F; Table 1). The velocity of motile vesicles is reduced, and at least half of the RNAi-treated cells show no transport of Spitz-GFP vesicles. The microtubule organization of the interphase cells was undisturbed after Dhc RNAi treatment (data not shown). Our results show that dynein acts together with Star to transport the Spitz-GFP ligand in S2 cells.

**Discussion**

Activation of the *Drosophila* EGF receptor is primarily regulated through the controlled intracellular trafficking and proteolytic activation of its ligand, Spitz (Klambt, 2002; Shilo, 2005). Spitz is critical for mediating EGF receptor signaling during many aspects of development, including eye development. Spitz ligand is produced as an inactive transmembrane precursor and requires Star for its transport from the ER to the site of proteolytic cleavage in the Golgi and/or endosomal compartment (Lee et al., 2001; Tsruya et al., 2002). Proteolytic cleavage by Rho, an intramembrane serine protease, activates the Spitz ligand (Lee et al., 2001; Tsruya et al., 2002; Tsruya et al., 2007; Urban et al., 2001). Our results extend these observations to suggest that Star-mediated trafficking of the EGF ligands and the consequent activation of EGF signaling depend on dynein function.

We provide evidence that components of the dynein-dynactin pathway interact with Star to regulate transport and signaling by Spitz. First, mutations in Star dominantly interact with the Gl^1^ mutation. Reduction of Star gene dosage by 50% severely enhances the Gl^1^ eye phenotype. This interaction between Gl^1^ and the Star allele is specific to the loss of Star function, since the altered eye phenotype is reverted by the presence of a Star transgene. The rescue suggests that the wild-type proteins interact in vivo, and that the phenotype does not reflect neomorphic protein interactions. Second, Star interacts with mutations in dynein itself. The observed interactions for both Star and Dhc are allele-specific, suggesting that specific domains within the Star and Dhc products mediate the interactions. Third, the suppression of the Gl^1^ eye phenotype by certain Dhc alleles (e.g. Su(gl)^77^), requires Star function. The suppression is reversed in the presence of a Star mutation, emphasizing the common pathway in which these gene products function. Finally, genetic interactions between the Dhc and Star loci are observed in both the eye and the wing, supporting a bona fide interaction, and suggesting that a common pathway operates within different tissues.

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suggested an essential role for Star as an adapter in the trafficking of ER vesicles (Lee et al., 2001; Tsruya et al., 2002). In Drosophila embryos, Star protein is enriched in the nuclear membrane and contiguous ER (Pickup and Banerjee, 1999). In the present study, we show that vesicle membrane preparations enriched for Star also contain dynein, and associate with microtubules in an ATP-sensitive fashion. Our chemical crosslinking experiments provide additional evidence for the physical association of Star with the dynein complex, and support a model in which dynein mediates the trafficking and processing of the Spitz ligand through its association with Star. Our data are consistent with a direct interaction, but do not exclude the possibility that other proteins mediate the interaction between Star and dynein.

Our analysis of Spitz transport in living S2 cells extends previous studies that show Star, Spitz and Rho are each transported from the ER to Golgi following heterologous expression in COS cells (Lee et al., 2001) or S2 cells (Tsruya et al., 2002). Our results confirm that the export of Spitz from the ER, and its accumulation in Golgi vesicles, require Star. We further show that the number of Spitz-GFP-labeled vesicles formed, as well as their transport along microtubules, is dynein dependent. This result is consistent with previous studies suggesting that dynein and dynactin associate with ER- and Golgi-derived vesicles, and mediate their transport along microtubules (Burkhardt et al., 1997; Presley et al., 1997; Watson et al., 2005). In mammalian cells, exit of newly synthesized cargo from the ER is driven by the sequential assembly of vesicles (Aridor et al., 2001; Scales et al., 1997); cargo is initially concentrated into COPII-coated vesicles and then subsequently moved to the Golgi in transport vesicles in which COPII coamater is replaced by COPI. Recent studies have provided evidence that the association of dynactin with COPII vesicles is coupled to ER exit (Watson et al., 2005). Further observations suggest that Cdc42 temporally regulates dynein association with COPI vesicles and the retrograde transport of vesicles from Golgi to ER (Chen et al., 2005).

The diversity of vesicular cargo raises the question of how the binding of dynein, as well as other motors, is targeted to distinct vesicle populations and how transport is regulated. Dynein is known to participate in secretory vesicle trafficking, but whether there are specific transmembrane proteins that mediate the trafficking of specific receptor ligands is not understood. Although direct interaction of dynactin and the Sec23p component of the COPII complex has been reported, coamater-independent recruitment of dynein to vesicles has also been proposed (Matanis et al., 2002). Our observations are consistent with the possibility that Star acts in the attachment of the dynein-dynactin motor complex to ensure the transport of Spitz-GFP vesicles. However, Star may alternatively interact with dynein indirectly, through other vesicle-associated proteins that mediate its connection to the dynein-dynactin complex. In either case, transport of Spitz from the ER by dynein would permit its proteolytic cleavage and activation in another cytoplasmic compartment. Dynein is also reported to facilitate vesicle transport between endosomal compartments (Lebrand et al., 2002). Recycling of Star protein appears to be important for the maintenance of signaling and may also involve dynein-based transport. Recent work has suggested that Star itself is cleaved by Rho (Tsruya et al., 2007). Cleaved Star fails to recycle to the ER and thus the trafficking of additional Spitz ligand is restricted. The cleavage of Star may modulate the amount of active ligand and the level of signaling. The interactions described – both genetic and biochemical – indicate that Star, Rho, dynein and dynactin function cooperatively to achieve the proper regulation of Spitz trafficking and signaling.

Star might also serve as a common link in the trafficking pathways of multiple ligands, as previously suggested by Lee and co-workers (Lee et al., 2001). Two other EGF ligands found in Drosophila, Keren and Gurken, are also activated by proteolytic release and require Star for trafficking from the ER, albeit to different extents (Ghiglione et al., 2002; Urban et al., 2002). The binding of Star to ligands within the ER lumen may promote motor-dependent transport from the ER to the Golgi complex by revealing an ER export signal, or masking an ER retention signal (Lee et al., 2001). Notch, EGF and sevenless mutants interact with Star mutants (Heberlein et al., 1993; Kolodkin et al., 1994), as well as with Dhc and Gl mutants (our unpublished data). Yet, beyond these signaling pathways, mutations in Star do not appear to affect general vesicle transport. We propose that the Gl and Dhc mutations enhance the Star phenotype by disrupting Spitz transport, thereby inhibiting the cleavage and secretion of active Spitz ligand. It is known that the Gl dominant mutation produces a truncated product that competes with wild-type protein for binding to the dynein motor complex (McGrail et al., 1995; Waterman-Storer et al., 1995). We speculate that in the double heterozygous mutant backgrounds, the reduced level of transport activity is unable to deliver sufficient Spitz ligand for processing, and thereby compromises signaling at a critical period during development. In a test of this hypothesis, we found that transgenic expression of the active form of Spitz (sSpitz) can partially bypass the requirement for dynein-based transport of inactive Spitz. Our results demonstrate that dynein specifically contributes to the trafficking of the Spitz ligand from the ER, and to its activation by proteolytic cleavage. It will be important to discover exactly how dynein associates with the putative adapter, Star, and whether this association is regulated in a developmental context to control EGF signaling. Future experiments will need to elucidate whether diverse adapters specify the attachment of specific transport machineries to vesicles containing distinct ligands.

### Materials and Methods

#### Fly stocks

Dhc and Gl mutations have been described previously (Gepner et al., 1996; McGrail et al., 1995; Silvanovich et al., 2003). The mutations Gl and SuGl77 are described by Harte and Kankel (Harte and Kankel, 1982). We established that SuGl77 is a hypomorphic allele of Dhc; females expressing the SuGl77 mutation in combination with a deficiency that removes Dhc are sterile, but the sterile phenotype is completely rescued by introduction of a Dhc transgene. A recombinant SuGl77 Gl chromosome containing both SuGl77 and Gl was generated by meiotic recombination. S05671 was obtained from the Berkeley Drosophila Genome Project. Flies that carry a recessive lethal mutation in the Gl locus (Gl or Gl) or deficiencies that remove the Gl locus (Df(3L) f-GF3b and Df(3L) Gf) were gifts from Douglas Kankel (Yale University, New Haven, CT). UASp-ΔGl was described previously (Mische et al., 2007). hsStar-HA and hsrho30A were described (Pickup and Banerjee, 1999; Sturtevant et al., 1993). UAST-Spitz was a gift from Ben-Zion Shilo (Weizmann Institute of Science, Rehovot, Israel) (Tsruya et al., 2002). All other lines were obtained from the Bloomington Stock Center.

| Table 1. Comparison of motility of Spitz-GFP vesicles in wild-type and Dhc siRNA cells |
|-----------------|-----------------|-----------------|
|野型            | Dhc siRNA       |
| Velocity (μm/second ± s.d.) | Run length (μm ± s.d.) |
| 0.54±0.20       | 2.60±0.95       |
| 0.35±0.14       | <0.001          |

Average calculated velocities and run lengths of Spitz-GFP vesicles were directly compared in control and Dhc siRNA-treated S2 cells. Values represent mean ± s.d.
We conducted an F1 screen of a collection of lethal P-element insertion lines obtained from the Bloomington Stock Center. P(Balancer) males were crossed to virgin Glf Sh/Balancer females, and progeny carrying both the P insertion and Glf Sh were examined for modification of the Glf rough eye phenotype. In the case of lethal interactions, this class was absent. Eye phenotypes were evaluated by light and scanning electron microscopy (SEM).

DNA analysis
For plasmid rescue, DNA isolated from flies heterozygous for the P element was digested with Xhol and SpeI. The DNA was ligated and transformed into E. coli XL1-Blue cells. Plasmids that contained DNA flanking the Pelement were isolated and sequenced using a primer specific to the P element.

Scanning electron microscopy
Fly heads from three-day-old female flies were dissected and immediately dehydrated in an ethanol series as described previously (Carthew and Rubin, 1990), then prepared for SEM by critical point drying using liquid CO2. The dried heads were coated with gold-palladium in an Ernst Fullam Sputter Coater. The SEM images were collected using Hitachi SH50 scanning electron microscope and recorded onto film.

Biochemical methods
Files expressing the HA-tagged Star transgene (hs-Star-HA) were heat shocked at 37°C for 2 hours. Samples highly enriched in vesicles were prepared from head tissues according to a method based on a published procedure (Nakagawa et al., 2000). Briefly, fly heads were homogenized in PMEG buffer plus protease inhibitors, and centrifuged briefly at 10,000 g. The low-speed supernatant contains vesicles and membranes that are further enriched in the high-speed pellet. Vesicles were fractionated on a 20-60% nycodenz step gradient, run for 22 hours at 40,000 rpm in a SW50.1 rotor at 4°C.

Microtubule co-sedimentation assays were carried out as previously described (Hays et al., 1994). In brief, Star-HA vesicles from above were resuspended in wild-type embryo extracts. Microtubules were polymerized from endogenous tubulin and pelleted with associated MAPs. Parallel experiments either depleted or supplemented MgATP, and either included or omitted paclitaxel (taxol). Pellets were analyzed by western blotting.

Chemical crosslinking experiments used membranes prepared from S2 cells by centrifugation briefly at 10,000 g to remove debris. The low-speed pellets were analyzed by western blotting.

Results

References

Live imaging of S2 cells and analysis
Images were acquired using an Eclipse TE200 inverted microscope equipped with the PerkinElmer ConfoCalm Imaging System (PerkinElmer, Waltham, MA) and Hamamatsu’s Orca-ER digital camera. Spitz-GFP vesicle movements were captured at 1 second intervals using 2 x 2 binning with a 100 x planapo (NA 1.4) objective. The vesicle number and rate of transport were measured for control (n=9 cells), Dhc RNAi (n=23 cells) and colcemid-treated (n=9 cells). The number of vesicles in each sample was scored in the first frame of each time-lapse sequence analyzed. Since the movies were collected from a single focal plane, our analysis underestimates total vesicle numbers. Owing to the significant decrease in the number of vesicles present in the Dhc RNAi-treated cells, more of these cells were examined so that the total numbers were comparable to control and colcemid-treated cells. Moving vesicles that displayed linear movement for at least three consecutive frames were selected for analysis. Velocity and run-length of Spitz-GFP vesicles were manually tracked with Metamorph (Molecular Devices, Sunnyvale, CA) image analysis software ‘Track Points’ function as described previously (Mische et al., 2007). Stationary vesicles of similar spherical shape and Spitz-GFP intensity were identified based on a qualitative comparison to the moving vesicle population.

The average velocity and total run-length for each motile Spitz-GFP vesicle were calculated using Microsoft Excel, as was the standard deviation (s.d.) for velocity and run-lengths for all vesicles measured in control and dnRNA-treated cells. The velocity and run-length were directly correlated with those of the control cells. All statistical significance calculations were determined using the Student’s t-test on unpaired data. Significance was established if p<0.05.

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Dynein and Star interact in EGF R pathway

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