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The Transmembrane Protein Off-Track Associates with Plexins and Functions Downstream of Semaphorin Signaling during Axon Guidance

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Summary

The Plexin family of transmembrane proteins appears to function as repulsive receptors for most if not all Semaphorins. Here, we use genetic and biochemical analysis in *Drosophila* to show that the transmembrane protein Off-track (OTK) associates with Plexin A, the receptor for Sema 1a, and that OTK is a component of the repulsive signaling response to Semaphorin ligands. In vitro, OTK associates with Plexins. In vivo, mutations in the *otk* gene lead to phenotypes resembling those of loss-of-function mutations of either *Sema1a* or *PlexA*. The *otk* gene displays strong genetic interactions with *Sema1a* and *PlexA*, suggesting that OTK and Plexin A function downstream of Sema 1a.

Introduction

Axon guidance is a dynamic process by which environmental cues—attractive and repulsive, diffusible and fixed—are integrated within an extending growth cone to produce directed outgrowth. How environmental cues come to be interpreted by the growth cone's motility apparatus remains largely unknown, although much progress has been made from the standpoint of identifying surface receptors that bind the extracellular signals and initiate the internal process (e.g., Tessier-Lavigne and Goodman, 1996).

Four families of repulsive axon guidance receptors have been identified thus far. Eph tyrosine kinases are receptors for Ephrins (Brückner and Klein, 1998; Frisén et al., 1999). UNC-5 family proteins are repulsive receptors for Netrins (Leonardo et al., 1997; Leung-Hagesteijn et al., 1992). Plexins are receptors for secreted and transmembrane Semaphorins (Comeau et al., 1998; Takahashi et al., 1999; Tamagnone et al., 1999; Winberg et al., 1998). Roundabout family proteins are receptors for secreted Slits (Brose et al., 1999; Kidd et al., 1999; Li et al., 1999). Though these proteins may have similar overall effects on axon growth, able to induce steering, defasciculation, or outright collapse, they share little in the way of sequence similarity or common protein motifs. It is possible that each family of receptors has a separate biochemical signaling pathway by which it independently influences guidance. Alternatively, the cytoplasmic domains of the different receptors might be able to couple through a shared set of adaptor proteins (Bashaw and Goodman, 1999).

Many receptors for extracellular ligands become phosphorylated upon ligand binding, creating docking sites for adaptor proteins which go on to build local signaling complexes. Consistent with this type of signaling mechanism, under appropriate circumstances Plexins become tyrosine phosphorylated (Tamagnone et al., 1999), though Plexins themselves do not have intrinsic kinase activity (Maestrini et al., 1996). Perhaps this phosphorylation is important in coupling to downstream effectors. Thus, Plexin signaling may be regulated by associated proteins, including at least one active kinase. Indeed, we observed previously that mammalian Plexins B1 and A3 copurify an endogenous tyrosine-phosphorylated protein of around 160 kDa, suggesting that Plexin binding partners, including a kinase activity, are endogenously expressed by cultured cells (Tamagnone et al., 1999).

What is the identity of the 160 kDa Plexin binding protein? A candidate gene approach in *Drosophila* has led us to investigate Dtrk, previously identified based on a low-stringency screen using the tyrosine kinase domain of the mammalian TrkA receptor (Pulido et al., 1992). Many new receptor tyrosine kinase-related proteins have been characterized in the years since Dtrk was first described, and it has become increasingly apparent that Dtrk is not a homolog of the mammalian neurotrophin receptor TrkA, as was originally proposed (for example, Suga et al., 1997; van Kesteren et al., 1998). Here, we present phenotypic analysis demonstrating that this receptor controls certain aspects of axon guidance. Due to the disruption of axon tract morphology observed in mutant embryos, we have renamed the gene *off-track*, or *otk* (officially approved by Flybase curation).

Biochemical data show that OTK specifically associates with Plexins in vitro. Genetic disruption of *otk* leads to specific defects resembling those due to lesions in either Sema 1a, a transmembrane Semaphorin that mediates axon defasciculation (Yu et al., 1998), or its receptor, Plexin A (Winberg et al., 1998), suggesting that they may all act in the same pathway. Finally, genetic interactions between the three mutations (*sema1a*, *plexinA*, and *otk*) suggest that they function coordinately, with OTK and Plexin A acting downstream of Sema 1a. Thus, it appears that OTK and Plexin A can associate as components of a receptor complex and that OTK is likely to

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be an important component for repulsive signaling in response to Semaphorin ligands.

Results

Off-Track Is a Candidate Plexin-Associated Protein

Immunoprecipitated human Plexins A3 and B1 were previously shown to copurify a number of proteins from BOSC-23 cell extracts, some of which became tyrosine phosphorylated in an in vitro kinase assay (Tamagnone et al., 1999). Western blotting indicated that this activity was not due to the presence of Met, Ron, Abl, or Src tyrosine kinases. The most prominent labeled band other than Plexins was approximately of 160 kDa (Tamagnone et al., 1999).

To identify the putative Plexin-associated protein, we considered candidates in *Drosophila*. Several proteins with homology to receptor-tyrosine kinases have been identified that are expressed in the CNS and could potentially interact with Plexins. However, in the cases where the loss-of-function phenotypes have been assayed, there is not a notable similarity with those described for Semaphorins or Plexins, suggesting an unrelated function (e.g., EGFR, FGFR, Derailed). In other cases, in vivo functional data are yet lacking, but some of these candidates may be considered less probable on the basis of molecular weight (e.g., Dror, Nrk). A leading remaining contender is the *Drosophila* protein Off-track (OTK; previously called Dtrk; see Introduction).

OTK is a glycoprotein of apparent molecular weight 160 kDa whose extracellular domain, with its six immunoglobulin (Ig) repeats, shows similarity to cell adhesion proteins. In vitro studies have shown that OTK can mediate homophilic adhesion, which results in tyrosine phosphorylation of the intracellular domain (Pulido et al., 1992). In early *Drosophila* embryos, OTK transcript is broadly distributed, consistent with both maternal loading and zygotic expression. In later stages, the protein is detected on neuronal cell bodies and axons within the CNS and in the projections of motor neurons as they extend to muscle fibers in the periphery. Because of this axonal localization and in vitro adhesion, OTK has been suggested previously to play a role in selective fasciculation and axon guidance (Pulido et al., 1992). Based on its molecular weight, the observation that it can be tyrosine phosphorylated, and its expression on axons at the appropriate time in development to play a role in axon guidance, OTK seemed like a good candidate for possible interaction with Plexin.

BLAST searches of protein databases, using either the cytoplasmic kinase (Suga et al., 1997) or extracellular domain (this study, data not shown), indicate that the closest relatives of OTK are the chick protein KLG (Chou and Hayman, 1991) and its human homolog CCK4/PTK7 (Mossie et al., 1995; Park et al., 1996) (Figure 1).

OTK Associates with Plexins In Vitro

As a first test of OTK protein function, we turned to COS cells to check for molecular association. Epitope-tagged versions of both OTK and a variety of *Drosophila* and mammalian Plexins (DPlexA, PlexA3, and PlexB1) were generated and tested for expression. The cytoplasmic domains of Plexins are highly conserved, and, thus, binding relationships are likely to be conserved across phylogeny. Cells were cotransfected to express both proteins, and the formation of complexes was analyzed by immunopurification and Western blotting.

As shown in Figure 2A, *Drosophila* PlexA (HA tagged) is copurified with immunoprecipitated OTK (myc tagged). Moreover, mammalian PlexA3 and PlexB1 (VSV tagged) also are copurified with immunoprecipitated OTK (myc tagged) (Figure 2Bi). OTK can copurify all three Plexins but not an unrelated protein, the netrin receptor DCC (Keino-Masu et al., 1996). In addition, OTK (myc tagged) is copurified with immunoprecipitated mammalian Plexin A3 and B1 (VSV tagged) (Figure 2Bii). OTK is copurified in a similar fashion with immunoprecipitated *Drosophila* Plexin A (HA tagged; data not shown).

These experiments identify OTK as a transmembrane protein that can constitutively associate with both *Drosophila* and mammalian Plexins in transfected cells, raising the possibility that OTK might play a role in either up- or downregulating Plexin activity or mediating Semaphorin-Plexin signaling. To determine whether this association reflects a true functional interaction, we turned to genetic analysis of OTK in *Drosophila*.

Generation and Characterization of *otk* Loss-of-Function and Antisense Alleles

A direct in vivo test of OTK function was aided by the discovery of a P element insertional mutation near the *otk* gene, designated EP2017. This mutant strain was obtained from the collection of the Berkeley *Drosophila* Genome Project and was examined for axon guidance defects in homozygous embryos. Indeed, some defects were found, but they were subtle in nature and poorly penetrant. However, the element is located upstream of the coding sequence (see below and Figure 3) and may not completely disrupt gene function. We sought to generate complete loss-of-function *otk* alleles through imprecise excision of the P element (Voelker et al., 1984).

The sequence of the *otk* genomic locus and cDNA were assembled from public database entries along with the location of the EP2017 insert. The element is inserted 30 bp upstream of the 5′ end of the published *otk* cDNA. Since the *OTK* transcript is ~900 bp longer than the cDNA (Pulido et al., 1992), it is likely that the insert is in the 5′ UTR. Ten excision lines were genetically characterized; eight were homozygous lethal and two homozygous viable (the starting strain is semilethal), suggesting that *otk* is an essential gene. Molecular analysis indicated that the viable strains otk^2 and otk^8 are precise excisions. In contrast, the lethal strain otk^3 carries a 3 kb deletion that extends downstream of the EP2017 element, apparently disrupting *otk* but not upstream genes. The otk^3 lesion removes the putative translational start codon and part of the signal peptide and thus likely represents a complete loss-of-function allele. Subsequent examination of axon guidance defects has shown that otk^3 and three other lethal alleles are similar to one another in the variety and severity of their phenotypes, which are more pronounced than those displayed by the original EP2017 strain. In comparison, otk^2 is in the range of wild-type (Table 1).

To verify that the axon guidance phenotypes seen in these mutant strains are due specifically to the loss of OTK protein, we restored OTK function using a trans-

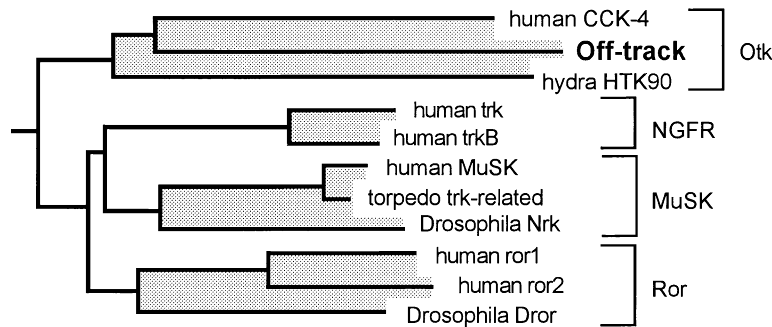

Figure 1. Neural Receptor Tyrosine Kinases

Phylogenetic relationships of tyrosine kinase receptors related to OTK. Alignments of the kinase domains were calculated using CLUSTAL V (DNASTAR, Lasergene). Accession numbers: human CCK-4 (U33635); Off-track (X63453); hydra htk90 (U59448); human trk (M23102); human trkB (U12140); human MuSK (AAB63044); Torpedo trk-related (L11311); Drosophila Nrk (AAF58420); human ror1 (M97675); human ror2 (M97639); Drosophila Dror (L20297). Similar phylogeny was previously reported by Suga et al., 1997.

genic construct, *UAS-otk*, under the control of the neuron-specific driver *elav-GAL4* (Luo et al., 1994). Indeed, this gene replacement was sufficient to rescue the guidance defects of the homozygous otk^3 mutants (Table 1; Figures 5D and 6C).

These reagents allowed us to test for another property of the EP2017 insert. The EP series of P elements contain a UAS gene-regulatory sequence that, in combination with a GAL4 driver, permits transcription of sequences flanking the insertion site of the P element (Rørth, 1996). In the present case, EP2017 is oriented such that GAL4-regulated expression yields short antisense *OTK* transcripts. In conjunction with *elav-GAL4*, one copy of EP2017 produces axon guidance abnormalities comparable with homozygous mutant otk^1 or otk^3 strains, suggesting that this antisense transcription from EP2017 confers a neuron-specific dominant loss-of-function phenotype (Table 1; Figures 3 and 5C).

Loss-of-Function Phenotypes of *otk* Resemble Those of *PlexA* and *Sema1a*

If OTK is important for Plexin A function, then loss-of-function mutations in *otk* might show similar guidance phenotypes as other mutations in the pathway. Specifically, if OTK is a positive activator or effector of Plexin A, then loss-of-function phenotypes of one should resemble loss-of-function phenotypes of the other. However, if OTK is a negative regulator of Plexin A, then the loss of OTK might lead to similar phenotypes as the overproduction of Plexin A protein. Indeed, embryos mutant for *otk* display axon guidance defects in the CNS and in the projections of the motor nerves, with abnormalities that are similar to those previously reported for *PlexA* and *Sema1a* loss-of-function mutants (Winberg et al., 1998; Yu et al., 1998). The projections of motor neurons to their muscle targets are more obviously affected, disrupted in a way that suggests individual growth cones are not always able to defasciculate from pioneer neurons when they should. The most telling examples are provided by the dorsal projections of the segmental nerve (SN) and the ventrolateral or "b" branch of the intersegmental nerve (ISNb).

The major projection of the segmental nerve, the SNa, normally extends along the body wall to a lateral position, where it divides into a dorsal and a lateral branch. The dorsal branch then extends further, dividing again

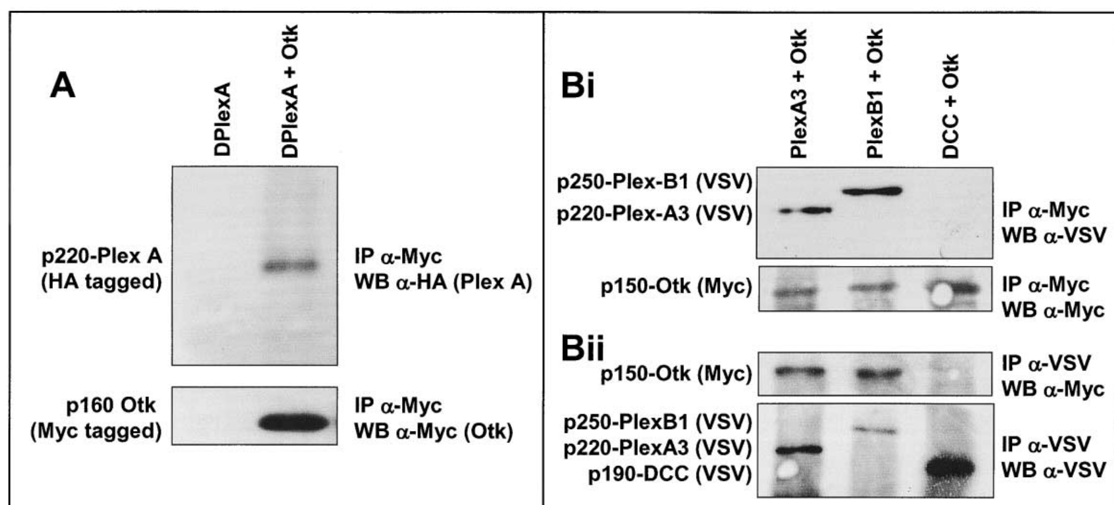

Figure 2. OTK Can Associate with Plexins in Living Cells

COS cells were transfected with OTK alone or in association with *Drosophila* Plexin A or mammalian Plexin A3 and B1 expression constructs. The two molecules are differently tagged, allowing selective immunopurification and Western blot analysis. (A) *Drosophila* PlexA (HA tagged) is copurified with immunoprecipitated OTK (myc tagged). (Bi) Mammalian PlexA3 and PlexB1 (VSV tagged) also are copurified with immunoprecipitated OTK (myc tagged). OTK can copurify all three Plexins but not an unrelated protein, the netrin receptor DCC (Keino-Masu et al., 1996). (Bii) OTK (myc tagged) is copurified with immunoprecipitated mammalian Plexin A3 and B1 (VSV tagged). OTK is copurified in a similar fashion with immunoprecipitated *Drosophila* Plexin A (HA tagged; data not shown).

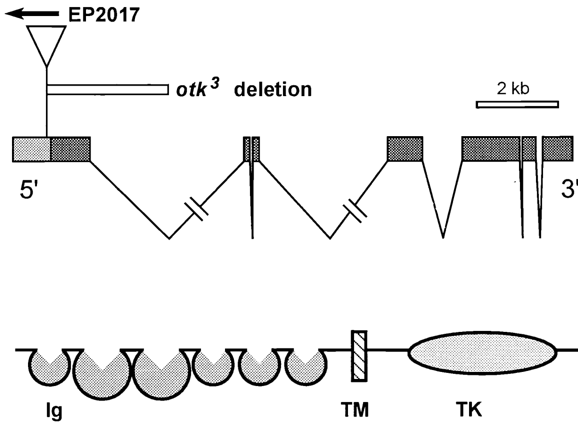

Figure 3. Genomic Locus of *otk*

(Above) Locus spans nearly 20 kb of genomic DNA. Dark boxes, exons included in published cDNA (4.6 kb). First exon includes ATG start codon. Lightly shaded box, additional 5′UTR inferred from transcript size on Northern (5.7 kb; Pulido et al., 1992). Triangle, EP2017 insert. Clear box, 3 kb deletion in *otk*^{3} allele. (Below) Schematic of OTK protein organization. The extracellular domain contains four type C2 (smaller) and two type V (larger) immunoglobulin (Ig) repeats, separated from the intracellular tyrosine kinase (TK) moiety by a transmembrane (TM) stretch.

and sending fine projections to innervate a group of transverse muscle fibers. In wild-type late stage 16 embryos, the dorsal SNa thus acquires a characteristic "pitchfork" appearance (Figure 4A). In *otk* loss-of-function or antisense mutants of the same age, these most dorsal growth cones remain fasciculated together in over 60% of segments and extend as a single thicker branch. This is highly similar to the aberrant SNa morphology displayed in *Sema1a* and *PlexA* loss-of-function mutant embryos (Figures 4B and 4C) (Winberg et al., 1998; Yu et al., 1998). In contrast, overexpressing Plexin A causes SNa axons to defasciculate prematurely (Winberg et al., 1998) (data not shown).

The ISNb normally diverges from the main branch of the ISN in a ventral position, termed "choice point #1" (Figure 5A). Within the ventral muscle domain axons of the ISNb then defasciculate from one another: at choice point #2, a single axon splits off to innervate muscle fibers 6 and 7, and at choice point #3, axons either stop and innervate muscle 13 or extend further to muscle 12. By late embryonic stage 16, these growth cones have typically reached their targets and formed rudimentary synaptic contacts along the edges of these muscle fibers. In *otk* loss-of-function or antisense mutants, growth cones may fail to defasciculate at any of the three choice points. ISNb axons occasionally fail to exit the ISN at choice point #1, instead bypassing their muscle targets completely or else extending small aberrant projections directly from the main branch of the ISN. More often, choice point #1 is navigated correctly but then axons are unable to defasciculate at choice points #2 or #3, resulting in a thickened, stalled nerve and a failure to innervate one or more of the muscles in this domain (Figures 5B and 5C; Table 1).

Within the CNS, additional abnormalities are observed. A subset of longitudinal axons is highlighted by monoclonal antibody labeling; in the wild-type, they form neat parallel tracks. In *otk* mutant embryos, these tracks are variably wavy and defasciculated and occasionally discontinuous (Figure 6). The incidence of "broken" axon tracks is greater in the antisense embryos than in the loss-of-function embryos (35% versus 15%, n = 105 and 126).

The abnormalities seen in the SNa and ISNb of embryos lacking *otk* are qualitatively and quantitatively highly reminiscent of those described for both *Sema1a* and *PlexA* mutants (Figures 4C, 5E, and 5F) (Winberg et al., 1998; Yu et al., 1998). All of these mutants also show qualitatively similar defects in the major axon tracts within the CNS, but, in the case of *otk*, these defects are less pronounced. Still, the strong resemblance among the phenotypes of all these mutations suggests that these three genes may all be acting in the same genetic pathway, consistent with the hypothesis that OTK positively influences Plexin A function.

Table 1. Genetic Interactions between Loss-of-Function Mutations in *Off-Track*, *Sema1a*, and *PlexA*

	SNa Abnormal	ISNb Abnormal Branching at Choice Point:		
Genotype	Branching	#1	#2	#3
Controls				
Wild-type	19.0% (n = 105)	0% (105)	4.8% (105)	8.6% (105)
otk^1 −/+	22.8% (119)	2.5% (119)	4.4% (113)	6.7% (119)
otk^3 −/+	18.9% (58)	3.6% (55)	5.4% (55)	5.4% (55)
Sema1a −/+	25.1% (135)	0.7% (138)	7.2% (138)	8.7% (138)
PlexA Df −/+	19.0% (105)	1.8% (108)	4.6% (108)	9.2% (108)
Loss-of-function phenotypes				
otk^1 −/−	61.7% (107)	13.5% (104)	22.1% (104)	17.3% (104)
otk^3 −/−	55.8% (52)	19.0% (58)	37.9% (58)	17.2% (58)
otk antisense *EP2017/+; elav- GAL4/+*	56.4% (101)	2.9% (103)	26.4% (106)	19.0% (105)
Sema1a −/−	67.0% (100)	11.8% (102)	52.0% (102)	46.1% (102)
PlexA Df −/−	74.7% (158)	19.9% (161)	43.5% (161)	49.5% (161)
Transheterozygous phenotypes				
otk^3 −/+; *Sema 1a* −/+	38.2% (68)	5.8% (69)	16.9% (71)	15.3% (72)
otk^3 −/+; *PlexA Df* −/+	55.3% (85)	13.2% (91)	20.9% (91)	29.7% (91)
Sema 1a −/+; *PlexA Df* −/+	72.1% (154)	8.6% (151)	56.3% (151)	30.5% (151)
Transgenic rescue				
otk^3 −/−, *UAS-otk, elav-GAL4*	27.0% (89)	1.1% (89)	10.1% (89)	9.0% (89)

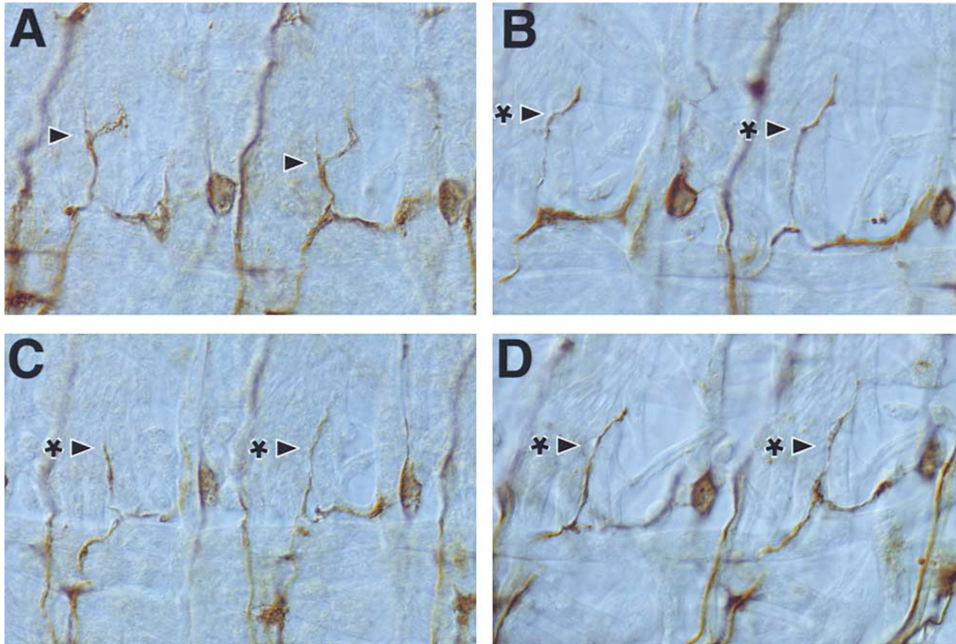

Figure 4. Abnormal Projection of the SNa Motor Nerve in *otk* and *PlexA* Mutants

Photomontages of late stage 16 embryos, showing two hemisegments per panel and focusing on the lateral muscles. The motor projection is revealed by staining with mAb 1D4 (anti-Fas II). Anterior is to the left and dorsal is up. (A) Wild-type. The dorsal branch divides to innervate multiple muscle fibers (arrowhead). (B) otk^3 loss-of-function. The dorsal branch does not divide in the proper location (asterisks) but rather extends as a single projection. (C) *PlexA*-deficient mutant. The dorsal branch fails to divide (asterisks) and either stalls (left) or extends as one projection (right). (D) $otk^3/+$; *PlexA*/+ transheterozygote. The dorsal branch fails to divide (asterisks).

otk Loss-of-Function Mutations Interact Genetically with *Sema1a* and *PlexA*

Another way to investigate whether these proteins may work together is to test for dominant genetic interactions. For most proteins, reducing gene dose to a single copy (thus reducing the protein level by 50%) produces mild or undetectable defects. However, reducing the gene dose of two different proteins may generate a phenotype if the two proteins normally function together. This "transheterozygous" genetic test has been applied to several pairs of proteins that have also been shown to interact biochemically: Notch and Delta (Artavanis-Tsakonas et al., 1995), Boss and Sevenless (Zipursky and Rubin, 1994), Sema 1a and Plexin A (Winberg et al., 1998), and Slit and Robo (Kidd et al., 1999).

We examined embryos singly and doubly heterozygous for *otk* and *PlexA* and observed strong phenotypic effects due to the combination. Embryos lacking one copy each of both *otk* and *PlexA* exhibit the same variety of SNa and ISNb defects as seen in the single homozygous mutants, to nearly the same degree of severity (Figures 4D, 5G, 5H, and Table 1). This provides strong genetic support for the hypothesis that Otk and Plexin A proteins function positively together through direct contact.

Likewise, embryos doubly heterozygous for *otk* and *Sema1a* also show phenotypic enhancement beyond additive effects of the single heterozygotes, supporting the idea of a ternary complex of Sema 1a-Plexin A-OTK proteins (Figures 5I and 5J). However, the severity of phenotypes in the *otk*, *Sema1a* combination is somewhat less than in the others (Table 1). The discrepancy may reflect a true difference between the association of OTK with Sema 1a compared to Plexin A. Alternatively, it may arise from differences in the normal expression levels of the various proteins: if Plexin A were the least abundant component under normal circumstances, then reducing the levels of the other two would be less consequential in this test. Similar genetic interaction tests were performed with Sema 1a and Derailed (another receptor tyrosine kinase expressed in the nervous system), and no interaction was observed (data not shown).

otk Loss-of-Function Suppresses *Sema1a* Gain-of-Function Effects

Our bias has been to suppose that OTK somehow affects the ability of Plexin A to mediate Sema 1a signaling. However, because all three proteins are expressed by many of the same neurons, the genetic tests above are also consistent with the possibility that OTK may interact directly with Sema 1a in *cis*. To verify that OTK can act genetically downstream of the signal, we made use of the GAL4 system to misexpress Sema 1a in muscles, thus offering an excess of repulsive target-derived ligand. Ectopic presentation of Sema 1a on specific muscles using *UAS-Sema1a* and *H94-GAL4* turns these muscles into nonpermissive substrates and prevents motoneurons from innervating them correctly (Figure 7A). Consistent with previously published results (Winberg et al., 1998), we find that the abnormal innervation of muscle 13 increases from 22% (n = 101; with *H94-GAL4* driver alone) to 49% (n = 99; with addition of *UAS-Sema1a*) in this Sema1a gain-of-function experiment. This phenotype is suppressed by removing one copy

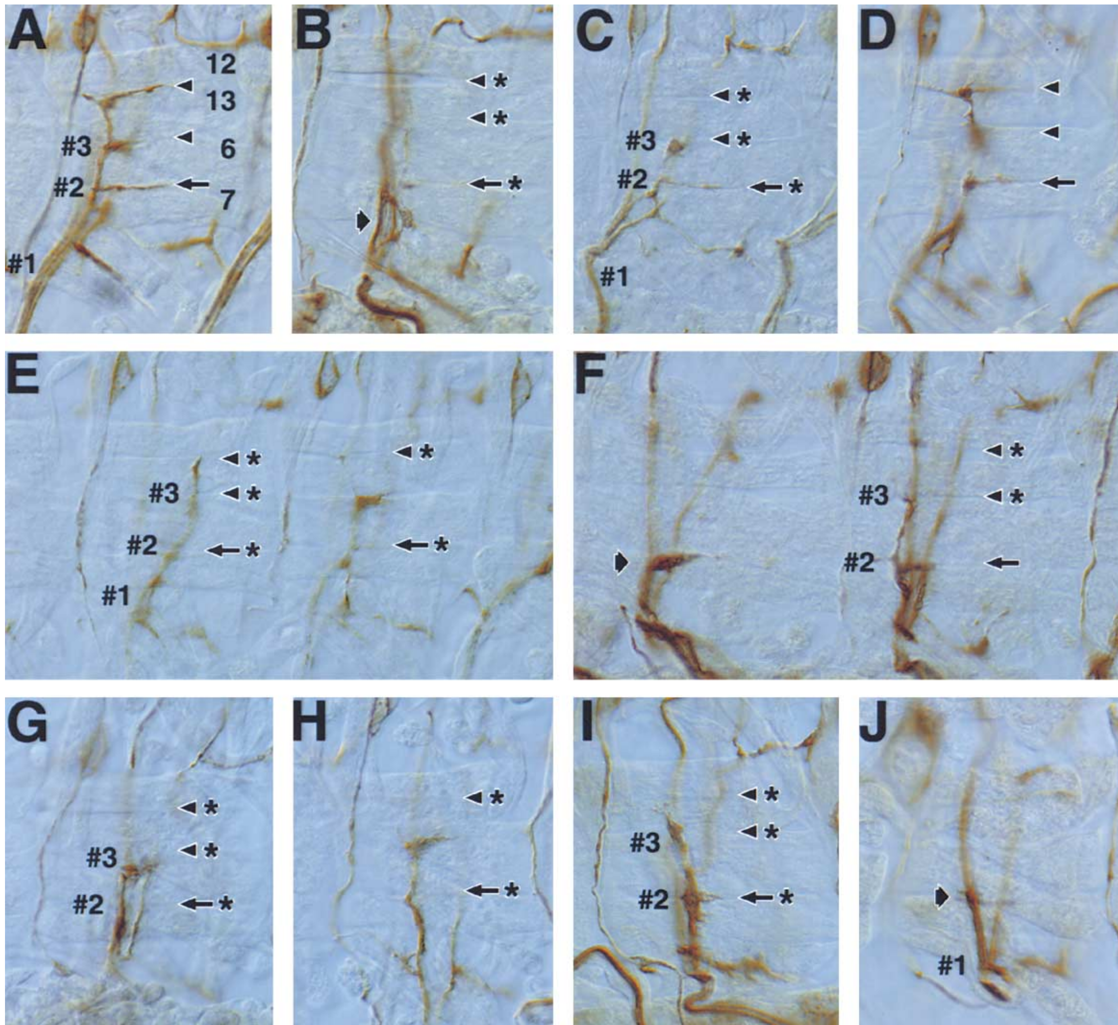

Figure 5. Abnormal Projection of the ISNb Motor Nerve in *otk*, *PlexA*, and *Sema1a* Mutants

Late stage 16 embryos stained with mAb 1D4, focusing on the ventral longitudinal muscles. Anterior left, dorsal up. (A) Wild-type. The ISNb splits off the major trunk of the ISN at choice point #1 prior to entering the muscle domain. At choice point #2, some growth cones continue dorsally, but one growth cone exits the ISNb, sending a projection into the cleft between muscles 6 and 7 (arrow). At choice point #3, different growth cones split off to innervate muscles 13 and 12 (arrowheads). (B and C) Loss of *otk* function disrupts defasciculation at these three choice points. (B) otk^3 loss-of-function mutant. The ISNb has not completely defasciculated from the ISN at choice point #1 (fat arrow), resulting in weak or missing innervation of the muscles in this area (asterisks). (C) *otk* antisense mutant: *EP2017/+*; *elav-GAL4/+*. Growth cones are stalled at choice point #3, leading to failed innervation of the last two muscles (asterisks). (D) Rescue of otk^3 with *elav-GAL4* and *UAS-otk*. ISNb axons have navigated all three choice points correctly and restored normal innervation. (E) *PlexA*-deficient mutant. The ISNb defasciculates abnormally. At right, failures at choice points #2 and #3 result in failure to innervate any of these muscles (asterisks). At left, only very fine filopodial projections have been extended from choice points #2 and #3 (asterisks). (F) *Sema1a* loss-of-function. The ISNb defasciculates abnormally. At right, the branch has remained with the ISN at choice point #1 and exited abnormally in the area of choice point #2 (fat arrow). At left, choice points #1 and #2 have been passed normally, but axons are stalled at choice point #3; the furthest muscles are not innervated (asterisks). (G and H) otk^3/+; *PlexA*/+ transheterozygotes. Axons fail to defasciculate at choice points #2 and #3, leading to missing innervation of ventral muscles (asterisks). (I and J) otk^3/*Sema1a* transheterozygotes. ISNb axons fail to defasciculate. (I) Choice points #2 and #3 are passed abnormally, muscles are not innervated (asterisks). (J) Axons fail to exit the ISN at choice point #1 and instead stall on the ISN (fat arrow).

of *otk*, reducing neuronal expression levels. Abnormal innervation of muscle 13 returns down to 26% (n = 95). The previous study (Winberg et al., 1998) showed that the addition of Sema1a increased the percent abnormal from 19% to 53% (very similar to what we reconfirm here) and that removing a single copy of *PlexA* reduced this frequency of abnormal innervation down to 21%. Thus, removal of one copy of *otk* is nearly as effective in reducing the Sema1a gain-of-function as is removal of one copy of PlexA. As neuronal OTK is sensitive to muscle-derived Sema 1a, this experiment confirms that OTK is able to act downstream of Sema 1a (Figure 7B).

Discussion

Plexins are a family of transmembrane proteins that have been shown to be receptors for both secreted and transmembrane Semaphorins (Comeau et al., 1998; Winberg

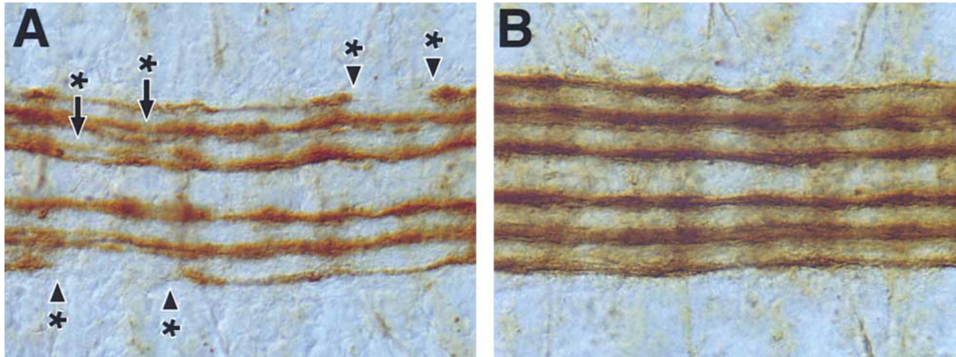

Figure 6. Abnormal CNS Axon Tracts Due to *otk* Loss-of-Function

CNS axons expressing the mAb 1D4 epitope. Dorsal view, anterior to the left. (A) *otk*^{3} loss-of-function. Outer tracts are discontinuous, with clumped appearance where axons have stalled (arrowheads). Inner tracts are somewhat "unraveled" (arrow). (B) Rescue of *otk*^{3} with *elav-GAL4* and *UAS-otk* restores approximately normal CNS morphology with parallel, unbroken tracts.

et al., 1998; Takahashi et al., 1999; Tamagnone et al., 1999). In this paper, we identify a transmembrane glycoprotein that appears to function as a component of Plexin signaling during axon guidance (Figure 8).

Previous studies have shown that Plexins can be tyrosine phosphorylated (Tamagnone et al., 1999), although an intrinsic kinase activity of Plexins was excluded (Maestrini et al., 1996). It was shown that mammalian Plexins B1 and A3 copurify tyrosine-phosphorylated proteins endogenously expressed by cultured cells (Tamagnone et al., 1999). Among other tyrosine-phosphorylated proteins associated with Plexins, there is a prominent band of around 160 kDa which could represent the active kinase itself or, alternatively, a phosphorylated protein specifically associating with Plexins and involved in the signal transduction pathway triggered by Semaphorins (Tamagnone et al., 1999).

In this paper, we have shown that Otk, a transmembrane protein of about 160 kDa, with homology to receptor tyrosine kinases, both associates with Plexins in vitro and appears to function in a Semaphorin-Plexin signaling pathway in vivo to control certain aspects of axon guidance. Biochemical data show that OTK specifically associates with Plexins in vitro. Genetic disruption of *otk* leads to specific defects resembling those due to lesions in either Sema 1a, a transmembrane Semaphorin that mediates axon defasciculation (Yu et al., 1998), or its receptor Plexin A (Winberg et al., 1998). These data suggest that all three proteins—Sema 1a, Plexin A, and OTK—may function in the same pathway. Finally, genetic interactions suggest that OTK and Plexin A acting downstream of Sema 1a. Thus, it appears that OTK and Plexin A can associate as components of a receptor complex that mediates the repulsive signaling in response to Semaphorin ligands.

We do not know whether OTK and Plexins normally associate in vivo in growth cones or whether they might only be brought together by ligand binding. In the absence of ligand in vitro, we find a tight association between the two transmembrane proteins. If transmembrane Semaphorins, like their secreted relatives, function as dimers, then binding of Sema 1a to Plexin A might provide a mechanism for clustering receptor complexes, which by analogy might activate one or more associated kinases and lead to the phosphorylation of Plexin and OTK. Testing such speculations will have to await an appropriate system for testing ligand activation.

Interestingly, despite its homology with receptor tyrosine kinases and the observation that immunoprecipitates of *Drosophila* OTK possess tyrosine kinase activity

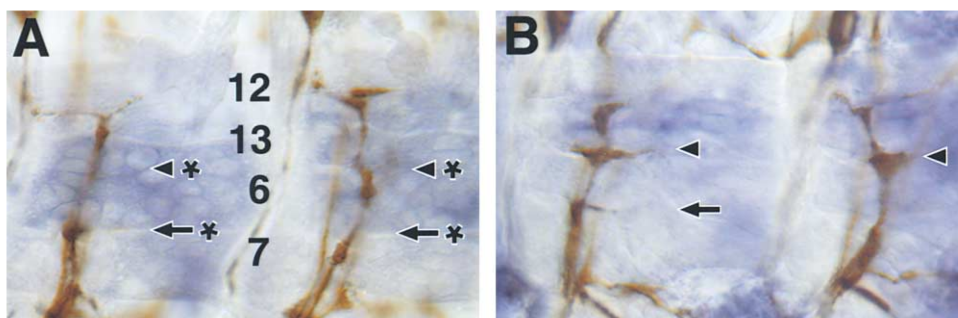

Figure 7. Genetic Suppression of *Sema1a* Gain-of-Function Due to Reduced *otk* Expression

Late stage 16 embryos focusing on the ventral longitudinal muscles. Blue staining is ectopic Sema 1a transcript, under the control of *H94-GAL4*, most highly expressed by muscles 6 and 13. See text for percentages and numbers examined. (A) Muscle expression prevents efficient innervation, especially of muscles 6, 7, and 13 (asterisks). (B) In an *otk*^{3}/+ partial loss-of-function embryo, spreading nerve-muscle contacts are restored to some muscles (arrow, arrowheads).

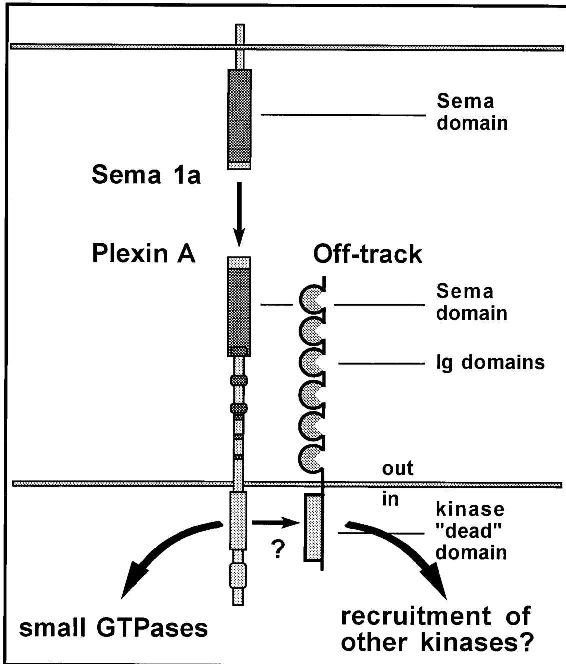

Figure 8. Model of Potential Protein Interactions Involved in Plexin Signaling

Off-track and Plexin A associate to form a receptor complex. Since OTK appears to have no or only modest kinase activity, we propose that some other active kinase is present in or recruited to the OTK/Plexin complex in order to account for the observed tyrosine phosphorylation of these proteins. The cytoplasmic domain of another Plexin in *Drosophila*—Plexin B (PlexB)—binds directly to the active, GTP-bound form of the Rac GTPase and, in addition, that a different region of PlexB binds to RhoA (Hu et al., 2001). The genetic and biochemical evidence suggests a model whereby PlexB mediates repulsion in part by coordinately regulating two small GTPases in opposite directions: PlexB binds to Rac^{GTP} and downregulates its output by blocking its access to PAK and, at the same time, binds to and increases the output of RhoA. The model in this figure suggests that Plexin A does the same. While the contribution of OTK to this signaling pathway has not yet been investigated, by analogy with other tyrosine-phosphorylated receptor complexes, one hypothesis is that a Rho exchange factor is recruited to the activated Plex/OTK complex, providing local activation of Rho.

(Pulido et al., 1992), OTK itself is probably not an active tyrosine kinase. The OTK sequence suggests that it belongs to a family of kinase "dead" receptors. The catalytic domain of OTK, like other members of this family, is altered in a few key conserved residues that are implicated in autophosphorylation (the conserved DFG motif substituted by YPA). Vertebrate family members bear similar alterations in the DFG motif and apparently do not have kinase activity (Chou and Hayman, 1991; reviewed by Kroiher et al., 2000). We have observed modest tyrosine phosphorylation of OTK in 293T cells but no significant increase in Plexin phosphorylation upon coexpression with OTK (our unpublished data). Thus, OTK either possesses a weak catalytic activity, which is barely detectable in the tested experimental conditions, or like other members of the CCK-4 subfamily of receptor tyrosine kinases, OTK might be kinase dead. In the latter case, some other active kinase would be expected to be present in or recruited to the OTK/Plexin complex in order to account for the observed tyrosine phosphorylation of these proteins. This situation is reminiscent of the interleukin receptors, which are heterodimers composed of a ligand binding subunit and a signal transducing subunit known as gp130. Neither subunit possesses a catalytic activity; rather, gp130 associates with the Janus kinases. Upon ligand binding, the receptors multimerize, resulting in activation of the Janus kinases and tyrosine phosphorylation of the receptor.

Another receptor tyrosine kinase carrying mutations in conserved DFG catalytic residues, h-Ryk/d-Derailed, appears also to be kinase inactive (Katso et al., 1999). Nevertheless, Ryk/Derailed is crucially involved in axon guidance (Callahan et al., 1995; Bonkowsky et al., 1999). Thus, at least two highly conserved receptor tyrosine kinases, both of which are members of families which are kinase dead—OTK (here) and Derailed (Callahan et al. 1995; Bonkowsky et al., 1999)—have been shown to function in axon guidance. In the case of OTK, it functions apparently by associating with Plexins and helping to mediate their output.

The signal transduction pathway activated by Semaphorins is beginning to be clarified (e.g., Tamagnone and Comoglio, 2000). The cytoplasmic domains of Plexins do not have any obvious signal transduction motif such as a kinase or phosphatase domain. However, the cytoplasmic domains of Plexin B receptors bind directly to the Rac GTPase in a GTP-dependent manner (Vikis et al., 2000; Driessens et al., 2001; Rohm et al., 2000). In a parallel study from one of our laboratories (Hu et al., 2001 [this issue of *Neuron*]), we confirm that the cytoplasmic domain of Plexin B (PlexB) binds directly to the active, GTP-bound form of the Rac GTPase and, in addition, that a different region of PlexB binds to RhoA. The genetic and biochemical evidence suggests a model whereby PlexB mediates repulsion in part by coordinately regulating two small GTPases in opposite directions: PlexB binds to Rac^{GTP} and downregulates its output by blocking its access to PAK and, at the same time, binds to and increases the output of RhoA. While the contribution of OTK to this signaling pathway has not yet been investigated, by analogy with other tyrosine-phosphorylated receptor complexes, one hypothesis to test is that a Rho exchange factor is recruited to the activated Plex/OTK complex, providing local activation of Rho.

Prior to the identification of Plexins as Semaphorin receptors (Comeau et al., 1998; Takahashi et al., 1999; Tamagnone et al., 1999; Winberg et al., 1998) and the implication of OTK as a Plexin-associated kinase (this study), both proteins were shown to be capable of mediating cell aggregation in vitro (Ohta et al., 1995; Pulido et al., 1992). These studies led to the suggestion that both Plexins and OTK might function as homophilic cell adhesion molecules. Whether either or both of them normally functions in a homophilic fashion in vivo is unknown.

We have come to associate Semaphorins as being ligands and Plexins as their receptors. But their roles in axon guidance may not be this simple. On the one hand, some Semaphorins are transmembrane proteins with cytoplasmic domains that appear as if they might be capable of transducing signals. Thus, some Semapho-

rins might themselves be receptors as well as ligands. On the other hand, Plexins, which are related to Semaphorins and have extracellular Semaphorin domains (Winberg et al., 1998), can bind to themselves. Thus, some Plexins might be both ligands and receptors. Finally, Plexins associate with OTK, which also can bind homophilically.

The data presented here demonstrate a role for OTK downstream from a Semaphorin on the receiving side of a signaling event. The best evidence for this conclusion is the genetic suppression data. Removing one copy of *otk* suppresses a Sema 1a gain-of-function phenotype. The most parsimonious interpretation of this result is that OTK functions downstream of Sema 1a. We do not know to what degree OTK binding and function is ligand gated. Moreover, it is not known whether OTK responds directly to Semaphorins, to some other ligand, or alternatively whether it simply binds to Plexins as part of a Semaphorin signaling complex. It will be interesting in the future to determine how these different Semaphorin, Plexin, and OTK proteins associate, modulate Semaphorin-mediated signal transduction, and thus control axon guidance.

Experimental Procedures

In Vitro Expression and Immunoprecipitation

COS cells were transfected by a standard DEAE-dextran method. In cotransfection experiments, to counteract a notable difference in protein expression levels, $\sim$5 μg of cDNA plasmids encoding Plexins were used for each μg of *otk* cDNA. All the expression constructs used included different in-frame epitope tags at the N terminus (HA, VSV) or C terminus (myc) of the proteins. Cells were harvested 48 hr after transfection; the extraction buffer contained 1% Triton-X and a cocktail of protease and phosphatase inhibitors, as described (Tamagnone et al., 1999). Immunopurification was performed using monoclonal antibodies directed against protein tags: anti-HA high affinity (3F10, Boehringer-Roche), anti-Myc (9E10, Boehringer-Roche), and anti-VSV (P5D4, Sigma). The same antibodies were used for Western blot analysis; final detection was done using ECL detection (Amersham).

Genetic Reagents

BLAST search starting from the published sequence of OTK recovered the genomic region as captured in Genbank clones AC007475 and AC007575, deposited by the *Drosophila* Genome Center (Lawrence Berkeley Laboratory). Using these for new searches identified the P element strain EP2017 as having an insertion near *otk*. Standard P remobilization was performed to generate white revertants; homozygous viable and lethal derivatives were additionally characterized by PCR-based sequencing using gene-specific and P-specific primers. Alignments were performed using DNASTAR (Lasergene). For transgenic rescue, DNA encoding the full-length OTK protein was amplified as two PCR fragments from *Drosophila* embryonic cDNA. The complete DNA sequences of several PCR subclones were determined; these coincided with published sequence of the coding sequence, plus 241 nucleotides of 5′UTR and 14 nucleotides of 3′UTR (Pulido et al., 1992). The fragments were combined and subcloned into the pUAST vector, and the resulting construct was coinjected into w^{1118} embryos with pπ27.1 helper plasmid yielding several independent transformants. Other strains were previously described: elav-GAL4 (Luo et al., 1994), H94-GAL4 (Lin and Goodman, 1994), Sema 1a and UAS-Sema 1a (Yu et al., 1998), Plexin A (Winberg et al., 1998). The *PlexA* mutant Df(4)C3 was the generous gift of S. Flister and W. Gehring.

Immunohistochemistry

Embryos were prepared according to standard protocols. Monoclonal antibody 1D4 (anti-Fasciclin II) was used at 1:5 (Van Vactor et al., 1993). Overexpressing embryos were identified using digoxigenin-labeled antisense probes (Kopczynski et al., 1996); homozygous mutant embryos were identified using anti-β-galactosidase antibody (Promega, 1:200) to label reporter gene expression from a "blue balancer" chromosome (*CyOwg*-β, gift of John Thomas).

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