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A *Drosophila* Homolog of Cyclase-Associated Proteins Collaborates with the Abl Tyrosine Kinase to Control Midline Axon Pathfinding

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Summary

We demonstrate that *Drosophila capulet* (*capt*), a homolog of the adenylyl cyclase-associated protein that binds and regulates actin in yeast, associates with Abl in *Drosophila* cells, suggesting a functional relationship in vivo. We find a robust and specific genetic interaction between *capt* and *Abl* at the midline choice point where the growth cone repellent Slit functions to restrict axon crossing. Genetic interactions between *capt* and *slit* support a model where Capt and Abl collaborate as part of the repellent response. Further support for this model is provided by genetic interactions that both *capt* and *Abl* display with multiple members of the Roundabout receptor family. These studies identify Capulet as part of an emerging pathway linking guidance signals to regulation of cytoskeletal dynamics and suggest that the Abl pathway mediates signals downstream of multiple Roundabout receptors.

Introduction

The accurate navigation of neuronal growth cones through the embryonic nervous system is essential for the formation of a functional network of axonal connections. Rapid progress has been made in the identification of extracellular factors and cell surface receptors that control growth cone behavior to achieve the highly specific patterns of innervation observed in vivo (reviewed by Tessier-Lavigne and Goodman, 1996; Mueller, 1999; Harris and Holt, 1999). However, less is known about the signaling machinery that interprets axon guidance information and translates it into directional cell motility.

In recent years, it has become clear that growth cone behaviors are regulated by protein phosphorylation under the control of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) (see reviews by Flanagan and Vanderhaegen, 1998; Stoker and Dutta, 1998; Gallo and Letourneau, 1999). In addition to receptor class proteins that directly link PTK or PTP catalysis to highly conserved extracellular domains, a number of intracellular enzymes have been implicated in axon guidance decisions as signaling partners of separate receptor proteins. For example, the mammalian PTKs Src and Fyn appear to play a role in axon outgrowth mediated by the adhesion molecules L1 and N-CAM, respectively (Beggs et al., 1994; Ignelzi et al., 1994).

In *Drosophila*, the Abelson PTK (Abl) is required for the formation of multiple axon pathways (Wills et al., 1999a). Combined genetic and biochemical experiments implicate Abl in the function of multiple axon guidance receptors, such as Dlar and Roundabout (e.g., Wills et al., 1999b; Bashaw et al., 2000). However, much more dramatic phenotypes are revealed in mutants that simultaneously lack *Abl* and a small group of intracellular proteins that appear to function in the *Abl* signaling pathway (reviewed by Lanier and Gertler, 2000). Studies of these Abl interactors and their orthologs in other species, such as the actin binding proteins Enabled and Profilin, and the guanine-nucleotide exchange factor Trio, suggest that Abl's primary role in axonogenesis involves the regulation of cytoskeletal dynamics (see Lanier and Gertler, 2000; Bateman and Van Vactor, 2001).

Many observations suggest that the navigational response of the growth cone to both attractive and repellent cues is mediated by rapid remodeling of actin networks (e.g., Bentley and Toroian-Raymond, 1986; Fan et al., 1993; Lin and Forscher, 1993; Luo et al., 1993; O'Connor and Bentley, 1993). While rapid progress has been made in linking the activities of certain actin regulatory proteins to guidance receptors (reviewed by Patel and Van Vactor, 2002), substantial mysteries remain. For example, the signaling output of the repellent receptor Roundabout (Robo) seems to involve the Abl substrate protein Enabled (Ena) (Bashaw et al., 2000). However, while mutations in *robo* display strong guidance defects at the midline choice point (Seeger et al., 1993), *ena* null alleles show only weak phenotypes in this context, suggesting that there is much more to the Robo pathway (Bashaw et al., 2000). Indeed, our continuing studies of the Abl pathway suggest that additional proteins act to link Abl to actin dynamics and that Abl's role in midline guidance is more complex than previously anticipated.

Several lines of convergent information focused our attention on the cyclase-associated protein (CAP) family as a potential link between Abl and actin. Although CAP was originally identified as a coactivator of yeast adenylyl cyclase (Vojtek et al., 1991), CAP proteins have also been shown to bind to the SH3 domain of the Abl kinase in vitro (Freeman et al., 1996). CAP family members are intracellular proteins characterized by an N-terminal domain that binds to adenylyl cyclase, a central proline-rich region, and a C-terminal domain that binds directly to monomeric actin (Gerst et al., 1991; Kawamukai et al., 1992; Freeman et al., 1995, 1996; Yu et al., 1999). Consistent with a vital role in regulating actin structures, loss of CAP activity results in cytoskeletal defects in yeast, Dictyostelium, and *Drosophila* (Vojtek et al., 1991; Benlali et al., 2000; Gottwald et al., 1996; Baum et al., 2000). Interestingly, the phenotype due to

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loss of the CAP actin binding domain in yeast can be compensated by overexpression of the actin binding protein Profilin, suggesting a functional relationship between CAP and Profilin (Haarer et al., 1993). Since we had previously shown a functional interaction between Abl and Profilin during axonogenesis in *Drosophila* (Wills et al., 1999a), we became interested in the function of the CAP homolog in *Drosophila* (known as *capulet* [*capt*; see http://flybase.bio.indiana.edu/] and *act up* [*acu*]; Benlali et al., 2000).

Results

Capulet Protein Accumulates in the Embryonic Nervous System

Previous studies in *Drosophila* have shown that although Abl, Ena, and Profilin proteins are expressed broadly during embryogenesis, at later embryonic stages they accumulate at highest levels within the developing nervous system (Henkemeyer et al., 1987; Verheyen and Cooley, 1994; Gertler et al., 1995). When we examined Capt expression with different antibodies, we found it abundantly expressed in early stage embryos (e.g., stage 4; Figure 1A), consistent with its documented role in oogenesis (Perrimon et al., 1996; Baum et al., 2000). In addition to expression in mesoderm and developing gut epithelia, Capt is abundant in the ventral nerve cord (VNC) at stages 12 and 13 when axon pathways are pioneered within the CNS (Figure 1B). At late stages (stages 16 and 17), when the last axon pathways are maturing and synaptogenesis is beginning, Capt preferentially accumulates within the VNC (Figure 1C). Therefore, Capt, Abl, Ena, and Profilin are coexpressed in neurons at stages important for axonal development.

Capulet Protein Associates with Abl in *Drosophila* Cells

Although in vitro peptide binding experiments suggest that Capt and Abl orthologs can associate directly (Freeman et al., 1996), no one had demonstrated this interaction in a physiological setting. Using monoclonal antibodies (mAbs) specific to Abl, we were able to immunoprecipitate (IP) endogenous Abl from *Drosophila* S2 cell lysates (see Experimental Procedures). When IPs of Abl were analyzed by SDS-PAGE and subsequent Western blot with anti-Capt antisera, we detected endogenous Capt as a protein that coprecipitated with Abl (Figure 2, lanes 2); no Capt was seen when primary antibody was omitted (Figure 2, lanes 1) or when either anti-Armadillo (Arm or *Drosophila* β-catenin; Figure 2, lanes 3) or anti-tubulin antibodies (data not shown) were used for the IP. For further confirmation of Capt protein association, we transfected S2 cells with a cDNA construct encoding full-length *capt* with a hemoagglutinin (HA)-epitope tag (see Experimental Procedures). Anti-HA antibody Western blots of Abl IPs detected a protein of the molecular weight expected for the tagged version of Capt (data not shown); no HA signal was observed in IPs from nontransfected cells or cells transfected with untagged cDNAs.

Genetic experiments suggest that Abl interacts with a number of actin regulatory proteins to control cytoskeletal assembly (e.g., Gertler et al., 1995; Wills et al., 1999a; Liebl et al., 2000). Given the functional redundancy observed between CAP and Profilin in yeast (Haarer et al., 1993), we wondered if Capt and Profilin might participate in some form of protein complex regulated by the Abl kinase. We transfected S2 cells with full-length *Drosophila* Abl (dAbl), *Drosophila* Src64 (dSrc), or the truncated mammalian v-Abl and then assayed Capt immunoprecipitations with anti-Profilin (Chic) and anti-actin antibodies. We saw no significant binding of Capt and Profilin in cells transfected with dSrc or v-Abl (Figure 2B) or in untransfected controls where endogenous dAbl is expressed at very low levels (data not shown). However, an association of Capt with Profilin and with actin was observed when dAbl was elevated (Figure 2B), suggesting a model where Abl, Capt, and Profilin function together in a cytoskeletal protein complex.

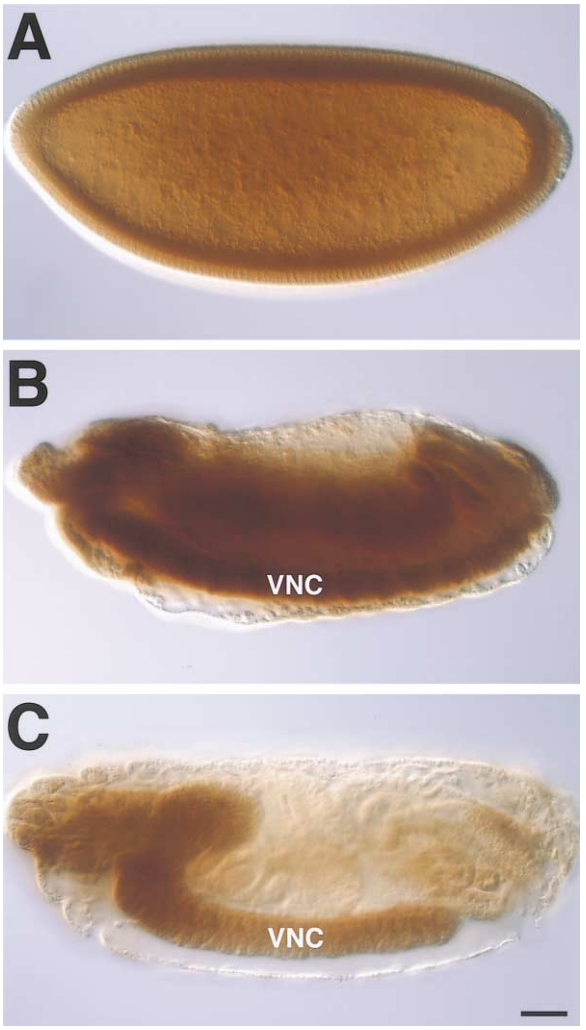

Figure 1. Capulet Is Expressed in the Embryonic CNS

(A) Capt protein is ubiquitous and highly expressed in stage 4 embryos. Whole-mount staining of fixed embryos with an affinity purified anti-Capt antibody is shown (see Experimental Procedures).

(B) Capt protein is broadly distributed at stage 12/13 and is abundant in mesoderm, the developing gut epithelium, and the ventral nerve cord (VNC). This is the stage at which axon pathways are pioneered within the CNS.

(C) Capt protein preferentially accumulates within the VNC at stage 16/17, when the last axon pathways are maturing and synaptogenesis is beginning. Scale bar is approximately 30 μm.

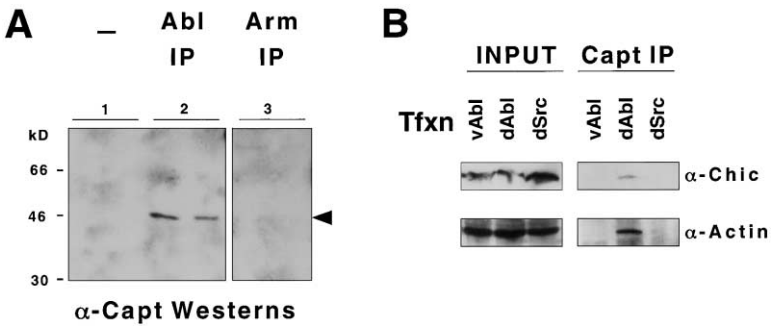

Figure 2. Capulet Associates with Abl and Profilin in *Drosophila* Cells

(A) Immunoprecipitation (IP) with anti-Abl monoclonal antibodies reveals that the Abl and Capt proteins endogenous to S2 cells associate, as detected by anti-Capt antibody Western blot. Each IP is shown in pairs of lanes where the left-hand lane contains 66% of the IP and the right-hand lane contains 33% of the IP. The Capt protein that IPs with Abl in lanes 2 is the expected molecular weight (45 kDa, arrowhead), as determined by comparison to molecular weight standards (shown), consistent with published data. No Capt signal is detected when primary antibody is omitted in lanes 1 (−) or when antibodies to *Drosophila* β-Catenin (Arm) are used to IP in lanes 3.

(B) S2 cells were transfected with cDNAs encoding either v-Abl, full-length *Drosophila* Abl (dAbl), or full-length *Drosophila* Src64 (dSrc) (see Experimental Procedures). Extracts from each cell population were either applied directly to SDS-PAGE and Western blotted with antibodies against *Drosophila* Profilin (α-Chic) or *Drosophila* actin (α-actin) to control for baseline expression of these proteins (INPUT), or they were immunoprecipitated (IP) with anti-Capt antibodies and then analyzed to determine if Profilin and actin associate with Capt under each condition. Transfection of dAbl, but not vAbl or dSrc, induced a significant increase in the association between Capt and both Profilin and actin.

capulet and *Abl* Interact during Midline Axon Guidance

The expression and interactions of Capt protein raised the question of whether Capt contributes to the function of the Abl pathway during nervous system development. However, examination of many independent *capt* allelic combinations that remove zygotic expression without affecting other genes nearby revealed no defects in the embryonic CNS (see Experimental Procedures). We attribute this to the large maternal supply of Capt protein visible in the early embryo (Figure 1A; see Experimental Procedures). Unfortunately, like Profilin null mutations (*chickadee*) (Cooley et al., 1992; Verheyen and Cooley, 1994), *capulet* null alleles completely block oogenesis, preventing the use of germline mosaics for the study of zygotic phenotypes in the absence of maternal expression (Perrimon et al., 1996; Baum et al., 2000). However, because strong zygotic phenotypes can be induced when mutations in various Abl pathway components are combined with mutations in *Abl* (e.g., *disabled*) (Gertler et al., 1993), we reasoned that zygotic functions of *capt* might be revealed through genetic interactions.

Among the strongest genetic interactions are synthetic phenotypes that arise in transheterozygotes, which lack only one copy of each interacting locus (e.g., Kidd et al., 1999). Heterozygotes that lack one copy of *capt* or *Abl* alone show no detectable CNS phenotypes when compared to wild-type strains (Figures 3A and 3D). However, combination of one *capt* and one *Abl* allele results in a distinct axon pathfinding defect (see below).

Axons in the *Drosophila* embryonic CNS are organized into two major groups: longitudinal pathways that extend along the anterior-posterior axis and commissural pathways that carry contralateral projections across the midline (Goodman and Doe, 1993). The midline, composed of specialized glial cells, acts as an organizing center that provides secreted growth cone attractants (Netrins) to build commissural pathways and a secreted repellent (Slit) to prevent inappropriate midline crossing (reviewed by Tessier-Lavigne and Goodman, 1996; Van Vactor and Flanagan, 1999). Subsets of longitudinal axons that depend on Slit to maintain their ipsilateral trajectories can be visualized specifically at late embryonic stages (stage 17) with the anti-Fasciclin II (FasII) antibody mAb 1D4 (Van Vactor et al., 1993; Lin et al., 1994); these FasII-positive axons never cross the midline in wild-type embryos (Figures 3A and 3D).

In contrast to wild-type, *capt-Abl* transheterozygotes display consistent axon guidance defects at the CNS midline. In these double mutants, ipsilateral axon fascicles now ectopically cross, primarily from the most dorsal-medial MP1 pathway (Figures 3B and 3C). An allelic series of this *capt-Abl* synthetic phenotype is seen across many different transallelic combinations, showing that the effect is independent of genetic background (Figure 3D). No gross defects in the number or fates of postmitotic neurons were detected in any *capt-Abl* mutants (data not shown). To our surprise, although temporal delays were sometimes observed, *capt-Abl* transheterozygotes did not show any lasting defects in embryonic motor axon pathways (data not shown).

To test whether the midline axon guidance function for *capulet* is dependent on Capt expression in postmitotic neurons, we expressed a wild-type *capt* transgene under control of P[elav-GAL4] in a strong *capt-Abl* background; a 15-fold rescue of the *capt-Abl* phenotype was observed (Figure 3E). Interestingly, a parallel rescue experiment using an N-terminal deletion removing the putative Capt adenylyl cyclase-associated domain provides only a 2.7-fold rescue under the same conditions (Figure 3E; see Discussion), despite the fact that the same transgene fully rescues *capt*^{acuE636} to viability (Benlali et al., 2000). To test the specificity of *capt* genetic interactions, we also examined combinations of *capt* and mutations in two Src PTK genes, *Dsrc64* and *Dsrc42*. No significant interaction was seen between *capt* and either Src homolog during CNS development (Figure 3F). Thus, *capulet* and *Abl* cooperate specifically during midline axon guidance.

capulet Interacts with the Midline Repellent Pathway

The failure of the midline gatekeeper function in *capt-Abl* transheterozygote embryos suggested that Capt might function in the repellent pathway downstream of Slit. To test this genetically, we examined transheterozygotes lacking one allele of *capt* and one allele of *slit*. These

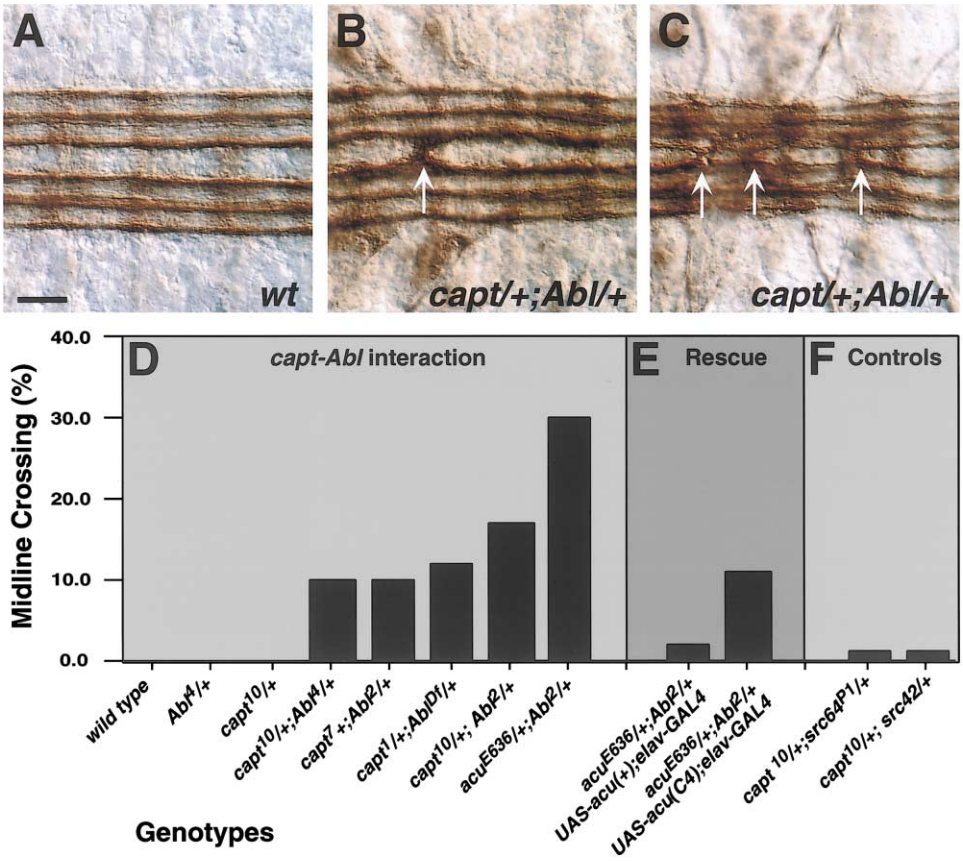

Figure 3. *capulet* Interacts with Abl during Midline Axon Guidance

(A) Several CNS segments in a wild-type embryo are shown stained with mAb 1D4 at stage 17, revealing three large parallel fascicles of longitudinal axons on each side of the midline. These fascicles never approach or cross the midline.

(B) A $capt^{10}/+;Abl^{4}/+$ mutant shows a synthetic phenotype characterized by a modest frequency of abnormal midline crossing events. Fascicles are observed crossing the midline (white arrow).

(C) A $capt^{10}/+;Abl^{2}/+$ mutant shows several midline crossing errors (white arrows).

(D) The frequency of midline crossing errors is shown in a variety of mutant combinations with several independent *capt* and *Abl* alleles in comparison to wild-type and heterozygous controls. The number of abdominal, embryonic stage 17 segments scored in each genotype (n) was 126, 120, 102, 126, 90, 84, and 72, respectively.

(E) Neural-specific rescue of the strongest *capt-Abl* phenotype (see last bar in [D]) is shown using either wild-type or an N-terminal truncation of a *capt* cDNA. While full-length *capt* provides complete rescue (n = 110), the deletion shows only partial rescue activity (n = 90).

(F) As controls for the specificity of the *capt-Abl* interaction, a strong *capt* allele was combined with mutations in two Src PTK genes. Neither *Dsrc64* nor *Dsrc42* showed any genetic interaction with *capt* (n = 84 and 78, respectively). Scale bar is approximately 5 μm. Anterior is to the left.

mutants showed a significant increase in the number of midline crossing errors compared to controls (e.g., $slit^{2}/+$ alone displays 4% ectopic midline crossing [n = 90 A2-7 segments] whereas $capt^{10}/+;slit^{2}/+$ embryos show 16% [n = 126]; Figures 3D, 4B, 4C, and 4I). This genetic interaction was seen consistently with multiple alleles of *capt*. Thus, *capt* and *slit* cooperate during midline guidance.

To further test the model that *capt* acts in the repellent pathway, we turned to the receptors. However, we realized that single gene mutations might not be sufficient. This is because the response to Slit is mediated by multiple receptors: Robo, Robo2, and Robo3 (Rajagopalan et al., 2000a, 2000b; Simpson et al., 2000a, 2000b). Indeed, *capt* transheterozygotes lacking single alleles in *robo*, *robo2*, or *robo3* alone showed little if any midline phenotype (Figure 4K). Yet, when *capt* alleles were combined with double mutations lacking one copy of *robo* and *robo2* simultaneously, we observed a phenotype almost 2-fold greater than that seen in the *robo,robo2* heterozygous embryos (compare Figure 4L to Figure 4I). Interestingly, *capt*/+ did not enhance the phenotype of *robo,robo3* heterozygotes (compare Figure 4L to Figure 4I), which is already quite strong.

As *capt* activity is further reduced, the interaction with *robo2* gets stronger; mutants lacking two copies of *capt* and one copy each of *robo2* and *robo3* (see Experimental Proceedures) displayed penetrant midline phenotypes (e.g., $capt^{10}/Df(2L)ast2$ showed 54% ectopic midline crossing, n = 72, and $capt^{1}/Df(2L)ast5$ showed 71% midline crossing, n = 84). Since these allelic combinations were the most severe, we used them for more detailed phenotypic analysis. For example, since the repulsion of growth cones at the midline is dependent

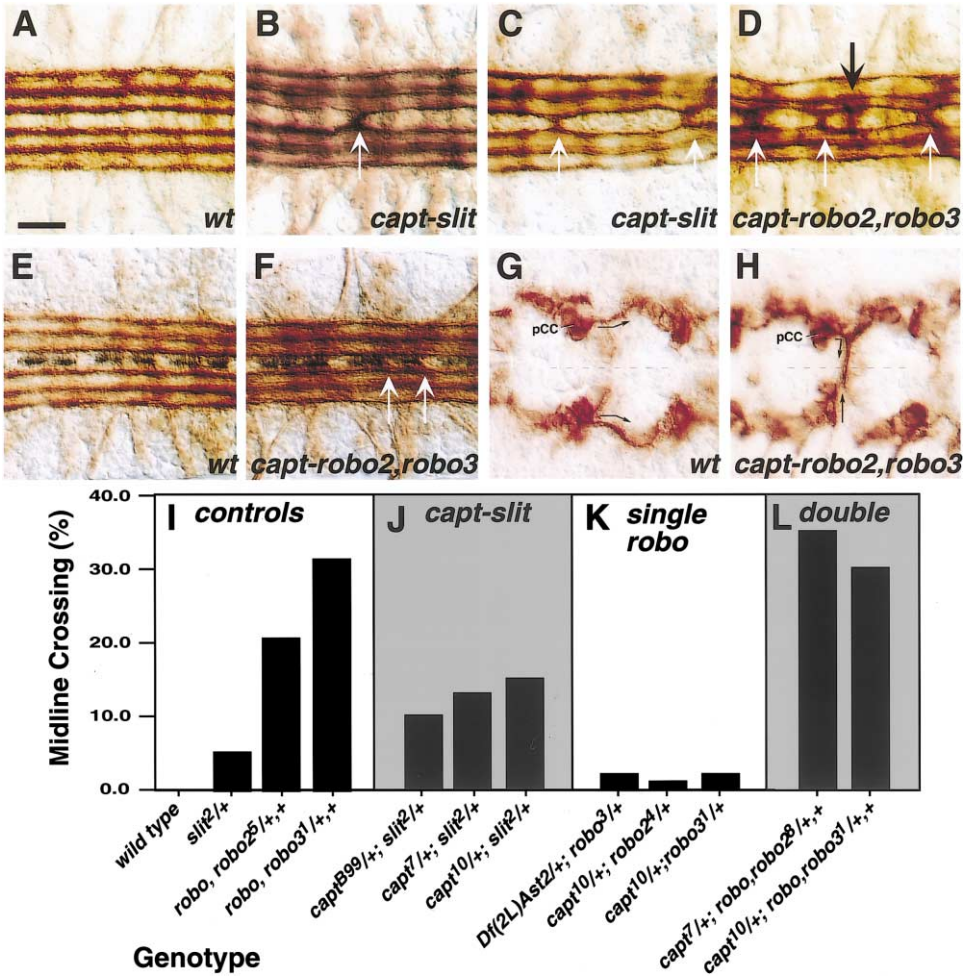

Figure 4. *capulet* Interacts with *slit* and Multiple *robo* Genes at the Midline

(A) Several CNS segments in a wild-type (wt) embryo are shown stained with mAb 1D4 at stage 17, revealing three large parallel fascicles of longitudinal axons on each side of the midline. These fascicles never approach or cross the midline (see [I] for quantification).

(B and C) $capt^{10}$, +/+, $slit^{2}$ transheterozygotes shows ectopic midline crossing events. The dose-sensitive nature of the interaction suggests these genes act in cooperation.

(D) Multiple midline crossing defects are observed in $capt^{10}$/*Df (2L) AST2*. Both medial (white arrows) and intermediate (black arrow) longitudinal pathways show inappropriate midline crossing.

(E) A wild-type (wt) CNS segment is double stained here with mAb 1D4 in brown (di-amino-benzidine, DAB) and anti-Wrapper in blue (DAB + nickel). Anti-Wrapper (Noordermeer et al., 1998) marks midline glia, which express the repellant Slit and act here as cell fate markers for the midline cells.

(F) $capt^{7}$/*Df(2L)ast2* is stained as in (E). Inappropriate midline crossing (white arrows) takes place in the presence of Wrapper-positive (blue-black) midline glia. Thus, the *capt* mutant phenotype does not result from a loss of midline cell fate.

(G) Two CNS segments in a wild-type (wt) embryo are shown stained with mAb 1D4 at embryonic stage late 12/early 13. At this time, the sibling anterior corner cell (aCC) and posterior corner cell (pCC; marked) are pioneering ipsilateral peripheral and central pathways, respectively. In each segment, pCC axons extend anterior to pioneer the MP1 fascicle—a group of about eight axons that runs in a trajectory medial and parallel to the midline glial boundary (arrows indicate the pCC trajectory in one segment).

(H) In strong *capt-robo2,robo3* mutant combinations at this stage, we sometimes find an abnormal orientation of pCC growth cones toward the midline. The pCC axon trajectories are highlighted with black arrows; the midline is marked with a dashed line.

(I) Midline crossing is quantified in wild-type embryos in comparison to *slit* and *robo* double heterozygotes as controls for genetic interactions; moderate levels of midline crossing are seen in these embryos (n = 126, 90, 216, and 60, respectively).

(J) Multiple *capt-slit* transheterozygotes display consistent midline crossing errors that are 2.5- to 4-fold higher than seen in *slit*/+ alone (n = 138, 114, and 192, respectively).

(K) *capt* fails to show a strong genetic interaction with mutations in single *robo* genes (n = 66, 84, and 60 for *robo*, *robo2*, and *robo3*, respectively).

(L) Moderate genetic interactions are seen in transheterozygotes lacking one copy each of *capt* and $robo^{GA285}$ and *robo2* simultaneously (n = 93); however, no significant interaction is seen with $robo^{GA285}$,*robo3* compared to control (n = 65, control in [I]). Scale bar is approximately 8 μm. Anterior is to the left, except in (G) and (H) (right).

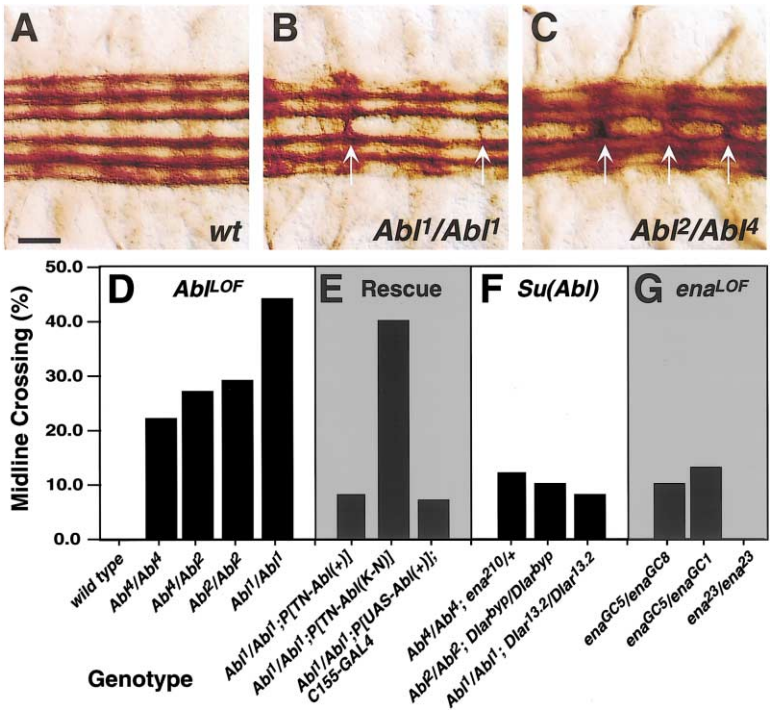

Figure 5. Abl and Ena Play Roles in Midline Axon Guidance

(A) Several CNS segments in a wild-type (wt) embryo are shown stained with mAb 1D4 at stage 17, revealing three large parallel fascicles of longitudinal axons on each side of the midline. These fascicles never approach or cross the midline.

(B) An *Abl*^{1}/*Abl*^{1} mutant shows prominent midline crossing defects (white arrows). In addition, the most lateral FasII-positive fascicle is often thin or missing (see Wills et al., 1999a).

(C) An *Abl*^{2}/*Abl*^{4} mutant shows the same midline crossing phenotype (white arrows).

(D) The frequency of midline crossing errors is shown in wild-type (n = 126) compared to several Abl mutant combinations (n = 96, 132, 102, and 216, respectively).

(E) Rescue experiments show that while wild-type Abl transgenes attenuate the Abl midline phenotype when expressed under either an endogenous promotor (n = 132) or under neural-specific GAL4 (C155; n = 132), a kinase-dead transgene fails to rescue (n = 102).

(F) Mutations in both *ena* and *Dlar* reduce the penetrance of different Abl allelic combinations by 2-fold or more (compare to [D]; n = 144, 108, and 90, respectively).

(G) Interestingly, strong *ena* loss-of-function alleles display modest levels of midline crossing errors, while a truncation that removes the C-terminal EVH2 domain displays no midline phenotype at all (n = 120, 126, and 126, respectively). Scale bar is approximately 5 μm. Anterior is to the left.

on the presence of the midline glia, which secrete the Slit repellent (Rothberg et al., 1990), we examined the midline glia in these mutants with anti-Wrapper antibody, which specifically stains the surface of these glial cells (Noordermeer et al., 1998) (Figure 4E). Midline glia were present in *capt-robo2,robo3* mutants, even where axons inappropriately crossed the midline (Figure 4F). We also looked at the first axons in the MP1 fascicle just as they are pioneering the ipsilateral pathway early in CNS development. At stage 12, the posterior corner cell (pCC) extends its axon along an anterior trajectory parallel to the midline in order to pioneer the most medial Fasciclin II-positive (MP1) pathway (Figure 4G). In *capt-robo2,robo3* mutants, we sometimes found pCC axons that turned toward and crossed the midline at this early stage (Figure 4H). This phenotype is similar to that seen in *robo* alleles (Seeger et al., 1993).

Abl Is Required to Restrict Axon Passage across the Midline Choice Point

Previous studies of Abl function during midline guidance led to a model where Abl acts to antagonize Robo signaling (Bashaw et al., 2000). However, our analysis of *capt-Abl* transheterozygotes suggests that Abl might play a dual role and also be required for restriction of midline crossing. Consistent with this prediction, examination of several *Abl* homozygotes reveals an allelic series of midline crossing phenotypes identical to those seen in *capt-Abl* transheterozygotes (Figures 5B–5D). Expression of a wild-type *Abl* transgene under its endogenous promotor in a strong mutant background rescued the midline crossing phenotype, as did expression of *Abl* specifically in neurons (under control of P[1407-GAL4]) (Luo et al., 1994); however, a kinase-dead transgene was unable to rescue the defect (Figure 5E). Like other aspects of Abl function, the midline crossing defects in *Abl* mutants could be suppressed by dose reduction of it's substrate protein Ena or by loss of the receptor protein tyrosine phosphatase Dlar (Figure 5F). These observations demonstrate that Abl is required for inhibiting the passage of ipsilateral axons across the midline and suggest that the role of Abl is more complex than previously appreciated.

Since analysis of *Abl* loss-of-function would predict cooperation between Abl and other genes in the repellent pathway, we assayed for genetic interactions in embryos transheterozygous for *Abl* and either *slit* or combinations of mutations in different *roundabout* genes (ie. *slit*/+;*Abl*/+ or *robo,robo2*/+,+;*Abl*/+). To our surprise, these embryos displayed striking midline phenotypes far stronger than control genotypes (compare Figures 6E and 6F). For example, *slit*^{2}/+;*Abl*^{2}/+ transheterozygotes show a 24-fold enhancement of the *slit*^{2}/+ phenotype. This experiment strongly supports the model that Abl acts positively in the Slit pathway, consistent with the phenotypes of *Abl* homozygotes and of all the *capulet* genetic interactions that we observed.

Abl Interacts with Multiple Robo Genes

The network of genetic interactions that we observe suggests that the Abl pathway is involved in signaling downstream of multiple Robo-family receptors. However, previous studies showed Abl binding to the Robo cytoplasmic domain in vitro to be dependent on a peptide motif (CC3) that is not present in Robo2 or Robo3 (Bashaw et al., 2000). We wanted an in vivo test for

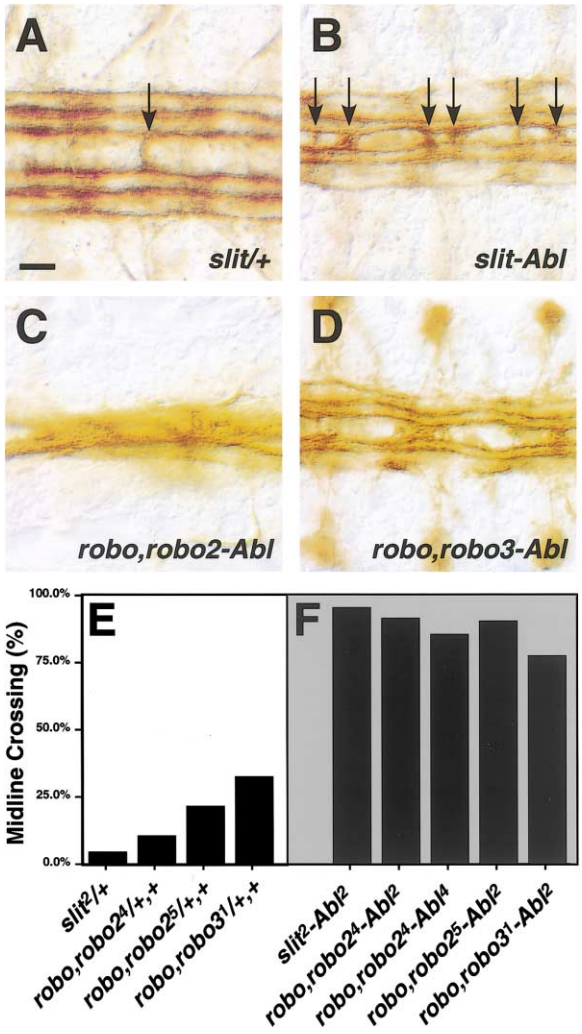

Figure 6. Abl Is a Potent Enhancer of Repellent Pathway Phenotypes

(A) *slit*/+ heterozygotes show a very low penetrant midline crossing phenotype (arrow), as assessed with mAb 1D4 at stage 17 (quantified in [E]).

(B) The frequency of midline crossing errors (arrows) increases nearly 20-fold when *slit* and *Abl* mutations are combined in one transheterozygous embryo ($slit^2$/+;Abl^2/+). Some of these double mutant embryos are so severe as to resemble *slit*/*slit* homozygotes (see [C]).

(C and D) Although $robo^{GA285}$,$robo2^5$/+,+ and $robo^{GA285}$,$robo3^1$/+,+ heterozygotes display midline crossing alone, they are clearly enhanced by loss of one dose of Abl.

(E) Quantification of heterozygous control genotypes shown in frequency of ectopic midline crossing seen in abdominal segments (n = 90, 120, 216, and 60, respectively).

(F) Quantification of the midline crossing phenotypes in compound mutant genotypes reveals a potent genetic interaction between *Abl* and *slit* or multiple *robo* genes (n = 132, 132, 54, 63, and 66, respectively).

Abl-Robo interactions to explore this issue. Since Abl appears to act in both positive and negative capacities at the embryonic midline, we turned to an alternative genetic assay to evaluate *Abl* interaction with the *robo* gene family. When wild-type Abl is overexpressed in the developing compound eye, under the control of a synthetic *glass* promotor (GMR-GAL4), we observe a mild rough-eye phenotype (Figure 7A; see figure legend for details). This phenotype is not seen in adults expressing only GAL4 (Figure 7F) or a kinase-dead mutation in *Abl* (data not shown). Thus, we tested this Abl phenotype for interactions with various UAS-Robo transgenes.

As predicted from our loss-of-function analysis, while expression of wild-type Robo alone had little, if any, effect on retinal patterning (Figure 7G), the combination of Abl and Robo caused a striking increase in the severity of the Abl gain-of-function eye phenotype (Figure 7B). Thus, Robo serves as an enhancer of Abl activity in this kinase-dependent assay. This was also true of Robo2 (compare Figure 7I, control, to Figure 7D) and of Robo3 (compare Figure 7J, control, to Figure 7E). These data support the hypothesis that all Robo receptors can engage the Abl signaling pathway. So, is this in vivo interaction dependent on the Robo domains previously shown to recruit Abl and Ena proteins? Interestingly, neither deletion of CC2 nor deletion of CC3 was found to attenuate the *Abl-Robo* interaction (Figures 6C and 6H, respectively). A UAS-*robo* transgene lacking the motif CC1 did show a reduction in eye phenotype when combined with UAS-*Abl*, but the difference was slight (data not shown).

To confirm that Abl can interact with Robo in a CC3 domain-independent fashion during axon guidance, we examined embryos that overexpress Abl and either wild-type Robo(+) or mutant Robo(ΔCC3) in postmitotic neurons. Abl gain-of-function alone generates two axon guidance phenotypes: (1) ISNb motor axon bypass of ventral target muscles (Wills et al., 1999b) and (2) ectopic midline crossing (Bashaw et al., 2000). Interestingly, coexpression of Abl and either Robo(+) or Robo(ΔCC3) dramatically enhanced the ISNb axon phenotype (Figures 7K–7N); however, there was no effect on midline crossing in any of these genotypes (data not shown). Thus, in vivo, Abl is capable of a functional interaction with all three Robo receptors via some novel mechanism. However, the midline guidance system is specifically refractory to a simultaneous increase in Abl and Robo activities, perhaps due to the dual role of Abl in this context.

Discussion

Our major goal is to understand the signaling pathways that allow growth cones to accurately interpret axon guidance cues and translate this information into directional movement. The midline of the central nervous system is an important axon guidance choice point in vertebrates and invertebrates alike, and it has emerged as a powerful model system to study axon guidance behavior. During embryonic development, specialized midline cells act as a global organizing center, generating both secreted attractants and growth cone repellents. Elegant functional studies indicate that the repellent gatekeeper role depends on the action of axonal receptors in the Roundabout (Robo) family and their repellent ligands in the Slit family of secreted proteins (reviewed by Flanagan and Van Vactor, 1998; Van Vactor and Flanagan, 1999; Rusch and Van Vactor, 2000). In fact, growth cone repulsion is a major force in the pat-

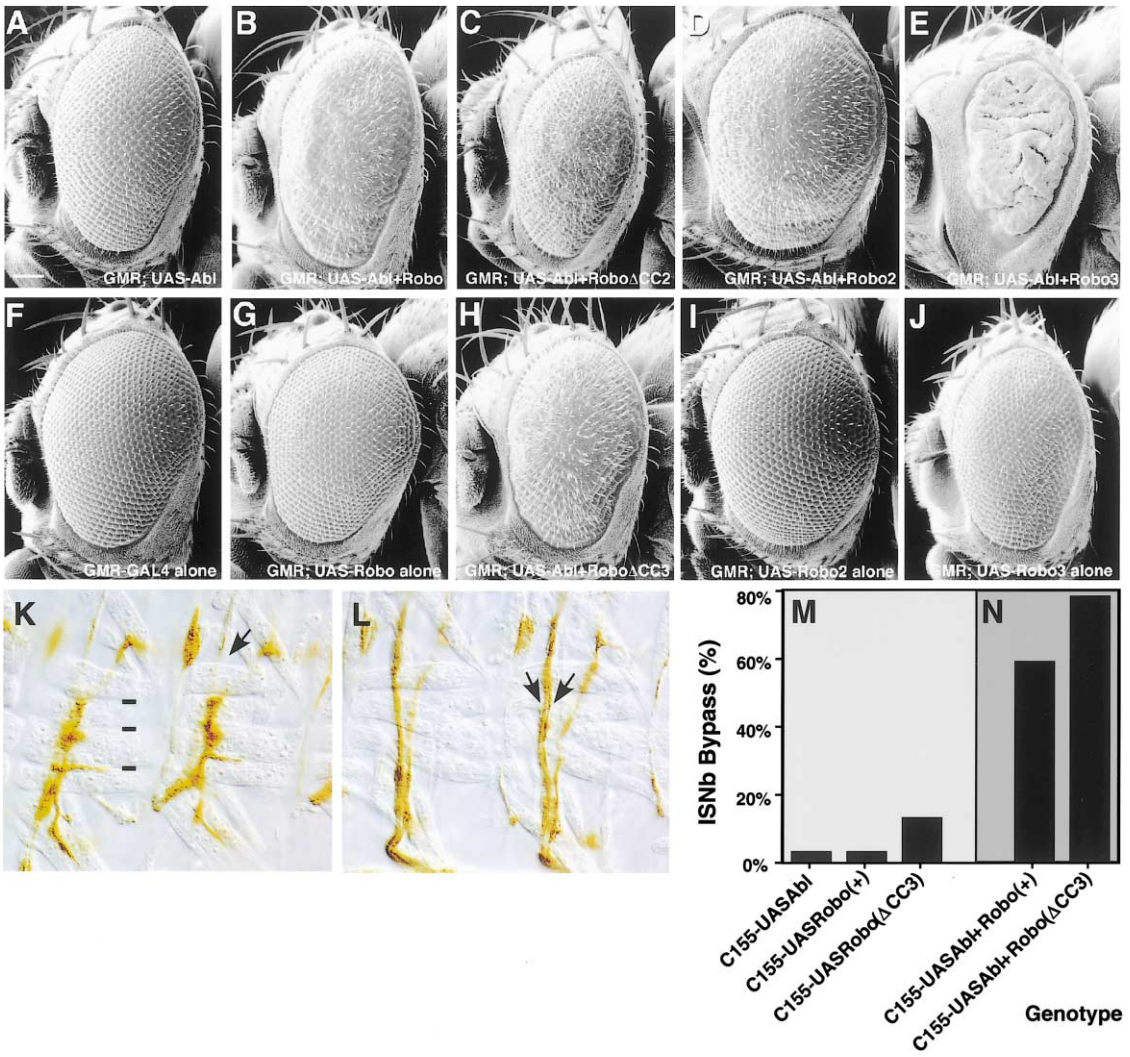

Figure 7. Multiple *robo* Genes Potentiate an Abl Gain-of-Function Phenotype

(A) A scanning electron micrograph of an adult *Drosophila* shows the mild pattern defect caused by overexpression of wild-type *UAS-Abl* under the control of a single copy of *GMR-GAL4*. This phenotype is dependent on Abl kinase activity, because overexpression of $Abl^{(K\text{-}N)}$ does not disrupt the retinal pattern (data not shown). All subsequent genotypes in this figure include one copy of *GMR-GAL4*.

(B) Coexpression of *Abl* and *robo* consistently potentiates the *Abl* gain-of-function defect in retinal patterning (compare to [A] and [G]). This phenotype was seen in all adults of this genotype examined (see Experimental Procedures).

(C) Cooverexpression of *Abl* and *robo(ΔCC2)* is shown. Deletion of the CC2 motif in the *robo* cytoplasmic domain does not alter the genetic interaction between *Abl* and *robo* in this assay system (compare to [B]).

(D) Cooverexpression of *Abl* and *robo2* consistently potentiates the Abl gain-of-function retinal phenotype (compare to [A] and [I]).

(E) Cooverexpression of *Abl* and *robo3* creates a dramatic, synergistic defect in retinal development (compare to [A] and [J]).

(F) A control shows that a single copy of GMR-GAL4 alone shows no defect in retinal patterning (compare to [A]).

(G) A control shows that a single copy of GMR;UAS-Robo alone shows no defect in retinal patterning.

(H) Cooverexpression of *Abl* and *robo(ΔCC3)* is shown. Deletion of the CC3 motif in the *robo* cytoplasmic domain does not alter the genetic interaction between *Abl* and *robo* in this assay system (compare to [B]).

(I) A control shows that overexpression of *UAS-robo2* alone has no effect on retinal patterning (compare to [D]).

(J) A control shows that overexpression of *UAS-robo3* alone has only a minor effect on retinal patterning compared to (E).

(K) Normal ISNb motor axon innervation of the ventral muscles observed with mAb 1D4 in an embryo expressing UAS-Abl under control of C155-GAL4. ISNb makes synaptic contacts at the clefts between the ventral fibers (dashes), whereas the ISN continues on an external path to reach dorsal muscles (arrow).

(L) An ISNb "bypass" phenotype seen in a *C155-GAL4;UAS-Abl;UAS-Robo(ΔCC3)* embryo. ISNb extends past the ventral muscles in an external trajectory following the ISN and can be seen as a separate fascicle in one of these segments (arrows).

(M and N) Quantification of the ISNb bypass phenotype in embryos that overexpress Abl, Robo(+), or Robo(DCC3) alone reveal a low level of guidance errors ([M]; n = 266, 95, and 70); however, coexpression of Abl and either form of the receptor results in a highly synergistic axon guidance phenotype (n = 85 and 125). Scale bar is approximately 50 μm. Anterior is to the left.

terning of axonal connections throughout the nervous system and is thought to restrict the ability of axons to regenerate after neural injury (reviewed by Goldberg and Barres, 2000; Schwab, 2000; Tessier-Lavigne and Goodman, 2000). For this reason, the mechanism of the repellent response is of great interest.

Through our studies of embryonic axon guidance, we provide here compelling evidence that a member of the adenylyl cyclase-associated protein (CAP) family plays a role in the accurate navigation of developing axons. Phenotypic analysis of double mutant embryos demonstrates that Capt cooperates with Abl, Slit, and multiple Roundabout receptors to prevent the inappropriate traffic of axons across the midline choice point. Consistent with published data on the relative contribution of Robo2 and Robo3 to midline repulsion (Rajagopalan et al., 2000a, 2000b; Simpson et al., 2000a, 2000b), we find that *capt* and *Abl* show stronger interactions with *robo,robo2* double mutants; however, Abl does appear to interact with all three receptors. The genetic and biochemical interactions we observe suggest both that Capt functions directly in the Abl pathway and that this cytoskeletal regulatory pathway is involved in the repellent response to Slit.

The Role of Capulet in Growth Cone Repulsion

Detailed studies of the prototypical growth cone repellent CollapsinI/Semaphorin3A have shown that the repellent response involves a collapse of the leading edge structures supported by actin cytoskeleton (Fan et al., 1993; Luo et al., 1993; Fan and Raper, 1995). Similar results have been seen for members of the Ephrin and Slit protein families (Meima et al., 1997a, 1997b; Nguyen Ba-Charvet et al., 1999). The fact that repellents promote a net disassembly of actin polymer arrays favors the simple model that repellent signaling antagonizes the actin assembly process.

Studies of CAP homologs from yeast, *Dictyostelium*, mouse, pig, and human suggest that the C-terminal actin binding domain acts to sequester monomers to prevent actin polymerization (Freeman et al., 1996; Gieselmann and Mann, 1992; Gottwald et al., 1996; Zelicof et al., 1996). More recent studies also suggest that human CAP promotes actin disassembly and monomer recycling through interactions with the actin-depolymerizing factor Cofilin (Moriyama and Yahara, 2002). Consistent with an inhibitory role for CAP-family members, studies of epithelial development and oogenesis in *Drosophila* demonstrate that Capt functions to suppress the hyperassembly of actin microfilaments (Baum et al., 2000; Benlali et al., 2000). Interestingly, a similar function has been ascribed to Abl and Arg during neurogenesis in the mouse (Koleske et al., 1998). Thus, we favor a model where Abl helps to recruit and regulate CAP activity to inhibit net actin assembly downstream of Robo family receptors.

New Models for Abl in Midline Axon Guidance

Previous data supported a simple model where Robo recruits Abl and Ena as components in the repellent pathway (Bashaw et al., 2000). In this model, Ena acts as an effector molecule to link Robo to actin assembly and Abl acts purely to antagonize and/or downregulate Robo. While we have confirmed that Abl gain-of-function creates ectopic midline crossing (Z.W. and D.V.V., unpublished data), our additional discovery that Capt and Abl cooperate to support the repellent response and that Abl loss-of-function generates ectopic midline crossing suggests that new models are necessary.

The fact that Abl is required for midline restriction suggests that Abl plays a dual role in the Robo pathway. There are different models to explain this. As a key enzymatic component in the signaling pathway, Abl may support repellent signaling (by recruiting the necessary actin binding proteins) and also feed back on the receptor (by downregulating through phosphorylation) to adjust the sensitivity of the pathway. This model is attractive because it may explain how growth cones can adapt to different regions within a gradient of Slit. In order for a growth cone to perceive an extracellular gradient (attractive or repellent) over an extended distance, the dynamic range of the response must be continually adjusted. If the receptor system becomes saturated at any point in the gradient, the growth cone will be blind to the extracellular asymmetry at higher concentrations. Conversely, if receptor output is too low, then the signaling differential across the leading edge may be too small to detect the gradient. It has therefore been postulated that gradient guidance will require some form of adaptation to keep the signaling threshold within the appropriate dynamic range as the growth cone moves toward or away from the source (Goodhill, 1998). If Abl is part of the repellent response, it would also be an effective source of feedback to help match receptor sensitivity to the gradient conditions. A similar role has been postulated for MAP kinase in the Netrin signaling pathway (Forcet et al., 2002; Ming et al., 2002).

The question of exactly how Abl and its signaling partners interface with the Robo receptor family is still unclear. Our biochemical data suggest that Abl, Capt, and Profilin may form a large protein complex. However, the genetic interactions between *Abl* and *robo* indicate that the CC3 motif is not necessary for a functional link between Abl and Robo. This makes sense because *Abl* and *capulet* also interact with *robo2*, a receptor that lacks both CC2 and CC3 sequences. It is interesting that deletion of motif CC1, which is conserved in all the *Drosophila* Robo family members (Rajagopalan et al., 2000b; Simpson et al., 2000a), caused a slight attenuation of the *robo-Abl* interaction in our assay (see Results). CC1 is also the Robo sequence phosphorylated by Abl in vitro (Bashaw et al., 2000).

Convergent Signaling Pathways

The emerging picture of axon guidance signaling pathways is highly complex (e.g., see review by Patel and Van Vactor, 2002). While this may be required to coordinate the many cell biological events that underlie directional specificity during cell motility, it is also possible that this property provides greater opportunity for signal integration. In this light, the potential link between Capulet and adenyyl-cyclase is intriguing (see Vojtek et al., 1991; Yu et al., 1994; Shima et al., 2000). Cyclic nucleotides (cAMP and cGMP) have potent modulatory effects on axon guidance responses in vitro (Song et al., 1997, 1998). Although our rescue experiments show that the

N-terminal region of Capulet equivalent to the cyclase-interacting domain of other CAP family proteins is not absolutely required for axon guidance function, the reduced rescue activity of this mutant is consistent with cyclase playing a modulatory role in the repellent pathway.

Experimental Procedures

Cell Culture and Protein-Protein Interactions

S2 cells were cultured in Schneider's media supplemented with 10% FCS and 50 units/ml penicillin/streptomycin. For each immunoprecipitation, 1×10^7 cells were lysed in 1% NP40, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM Na_3VO_4, and protease inhibitors pepstatin, leupeptin, aprotinin (1 μg/ml), and Pefabloc SC (10 μg/ml; Boehringer Mannheim). Transfections were carried out using Cellfectin (Boehringer Mannheim). The manufacturer's protocol was followed for such experiments. Briefly, 20 μl of Cellfectin and 2 μg of DNA were preincubated for 45 min and subsequently added to 60 mm plates, followed by a 24 hr incubation time, washing, and a subsequent 24 hr incubation period. Lysis was facilitated by douncing followed by a 10 s vortex followed by a 30 min incubation on ice. After a 25 min microfuge spin at maximum speed, supernatents were precleared in protein A/G beads (Pierce) for 30 min at 4°C. All immunoprecipitations were carried out in 800 μl of lysis buffer rotating for 4 hr at 4°C. Two washes using lysis buffer followed precipitation. After sample buffer addition and 5 min boiling, extracts were analyzed by SDS-PAGE. Gels were transferred by wet transfer onto PVDF (Millipore) membrane in 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 15% MeOH. Membranes were blocked in 3% milk in PBST (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, and 0.1% Tween-20) for 1 hr. Western blot analysis was carried out dependent on the manufacturer's recommendations for anti-HA (Boehringer Mannheim) and anti-tubulin antibodies (Boehringer Mannheim) or protocols we developed for Abl and Capt antibodies (described below). The anti-Profilin antibody (Cooley et al., 1992) was used at 1:1000 dilution for Western blot analysis and 1:100 in S2 cell stainings.

All cDNAs were subcloned into pPAC expression vectors (Invitrogen) for transfection experiments in S2 cells. This vector drives constitutive expression of transgenes under the control of an actin promoter.

Genetics and Anatomical Analysis

The *capt* alleles used in these experiments include a lethal P element insertion (P3605) isolated from the Spradling Stocks referred to here as $capt^1$ as well as $capt^7$ and $capt^{10}$, two alleles generated by P element mobilization. These alleles were analyzed by Northern blot analysis for loss of RNA transcript in third instar larvae (Baum et al., 2000) as well as by Western and whole-mount staining of late-stage embryos with *capt* affinity-purified antibodies (described below) for protein expression. Other alleles were generated by chemical mutagenesis (Benlali et al., 2000; see Acknowledgments). Multiple deficiency stocks [Df(2L)AST2 and Df(2L)AST5] obtained from the Bloomington stock center were also utilized in these experiments. Both Dfs take out the *Star* gene, which we subsequently examined in transheterozygous embryos using multiple *Star* alleles (S^1, S^{54}), each in combination with *capt* mutants. No neuronal phenotypes were observed in these transheterozygotes (data not shown). We evaluated nervous system phenotypes with a panel of different antibody markers in a large number of *capulet* allelic combinations designed to avoid confounding effects of genetic background; no consistent axonal phenotypes were observed in these mutants. Analysis of CNS and motor axon projections, as well as glial cell identity, was carried out using antibodies (mAb ID4 and mAb Wrapper) and staining procedures previously described (Van Vactor and Kopczynski, 1999; Noordermeer et al., 1998). Double mutant stocks were generated by standard techniques using genetic markers, whole-mount antibody staining, as well as lethality in crosses to other alleles of the same gene to confirm mutant alleles. The *Abl* and *capt* neural-rescue experiment was carried out using the driver lines 1407-GAL4 and elav-GAL4 as described in Wills et al. (1999a). The TnAbl lines (both wt and kinase-dead) were initially described in Henkemeyer et al. (1990).

It should be noted that the zygotic phenotypes we observe are due to incomplete loss-of-function. Residual Capt protein is detectable by Western blot and whole-mount immunohistochemical staining even in homozygous *capt* embryos (data not shown), presumably due to maternally supplied protein.

Immunohistochemical Reagents

Abl antibodies were used at 1:200 in whole-mount immunohistchemical stainings of embryos, 1:500 in Western blots, and 2–4 μg per IP.

Polyclonal antibodies were generated against one synthetic peptide (B.B. and N.P., unpublished data) of Capt in rabbit. ELISA was used to confirm immunoreactivity to the appropriate peptide. Antibodies were subsequently affinity purified and used at 1:100 in whole-mount immunohistochemical staining, 1:2000 on Western blots, and 1 μg per IP.

Scanning Electron Microscopy

At least ten adults of each genotype mentioned in Figure 6 were examined by light microscopy (2–4 days post eclosion), and six were prepared for scanning electron microscopy (SEM). Adults were dehydrated through an EtOH series (25%, 50%, 75%, 100% × 2; each for 24 hr) and then critical point dried and sputter coated. The phenotypes were consistent from adult to adult at both light and SEM levels.

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