Characterizing the Role of Crk in Central Nervous System Development in *Drosophila*

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Abstract

While determining the roles that different functional domains of Abelson tyrosine kinase (Abl), a key developmental regulator and oncogene, during normal development, our lab was surprised to find that a short, conserved motif (PXXP) in the linker region was more important for Abl's morphogenic roles than *both* kinase activity and F-actin binding. This finding led us to hypothesize that Crk binding to Abl's PXXP motif is critical for mediating Abl's developmental functions. Crk family proteins, including Crk and Crk-like (Crk-L), are a well-conserved family of small adaptor proteins that play a role in cell adhesion, migration, and other biological processes during normal development. Crk also plays a role in various cancers, including invasive bladder cancer, and Crk-L is a key mediator of oncogenic forms of Abelson tyrosine kinase (Abl) in Leukemia. Based on Abl's well-defined roles in central nervous system (CNS) patterning, we hypothesized that Crk might be required for proper CNS patterning. To test this hypothesis, I used RNAi to deplete Crk both maternally and zygotically in embryos and asked what effect, if any, this had on embryonic viability and CNS patterning. Using this approach, I found that Crk is required for embryonic viability and that loss of Crk results in CNS patterning defects. To better understand the mechanism(s) by which Crk may alter CNS patterning, I am looking at the Robo/Slit repulsive axon guidance cues in the Crk knock down embryos to determine if the CNS patterning defects observed are the result of loss of Crk affecting this pathway. I found that localization of these proteins remains largely normal and their function appears unaltered, indicating that this pathway is intact and does not require Crk. Preliminary analysis suggests that zygotic loss of *crk* also results in partially penetrant CNS patterning defects, mimicking what we see using an RNAi approach. We are continuing to characterize morphogenic defects associated with loss of Crk to determine its Abl-dependent and –independent roles. This work will help provide better insight into Crk's roles during normal development and disease states.

I. Introduction

Development and tissue homeostasis require tight coordination of cell adhesion with actin remodeling to allow cells to change shape and migrate. Both processes require the assembly and activity of multi-protein signaling complexes, which include small adaptor proteins[1]. Crk family proteins, including Crk and Crk-like (Crk-L), are a well-conserved family of small adaptor proteins that play a role in cell adhesion, cell migration, and other biological processes during normal development[2]. The mammalian Crk family is comprised of three proteins-- Crk I, Crk II and Crk-L-- expressed from two gene loci[3]. *crk* is alternatively spliced into *crk I* (minor form) and *crk II* (predominant form), while *crk-L* produces a single isoform[3]. Crk proteins include a Src Homology 2 (SH2) domain and either one or two Src Homology 3 (SH3) domains connected by linker sequences[1,3] (Fig. 1). Their SH2 domain allows for interactions with upstream binding partners such as phosphorylated receptor tyrosine kinases and focal adhesion complexes, while their SH3 domains mediate downstream interactions, typically with effector molecules or other adaptor proteins[4]. Both upstream and downstream interactions are controlled by phospho-regulation by tyrosine kinases, including Abelson tyrosine kinase (Abl)[3,5,6,7]. Both Crk and Crk-L also play critical roles in development and cancer. Crk drives epithelial-to-mesenchymal transitions in normal development, and is overexpressed in many cancers, including breast, ovarian, and bladder cancers, while Crk-L is a key mediator of oncogenic forms of Abl in Leukemia[6-11].

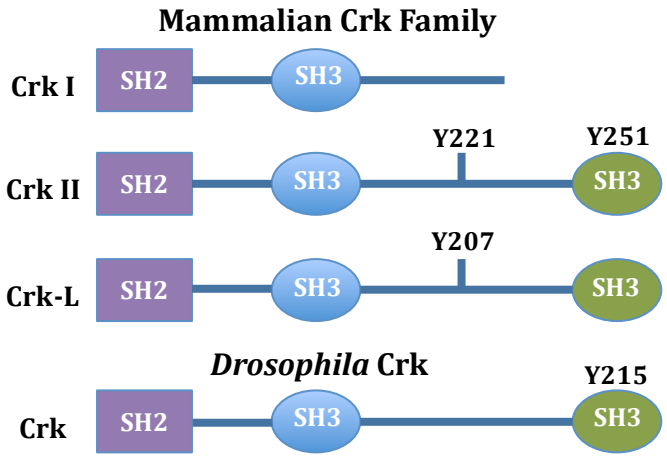

Figure 1. Crk is highly conserved between mammals and *Drosophila*. Regulatory phosphorylation sites indicated by Ys (e.g. Y221).

Crk I, Crk II, and Crk-L in mammals have many overlapping functions due to the conserved structure and functional domains between the proteins. This redundancy makes it difficult to study Crk function in mammals and cultured cells, because all three gene products would have to be knocked down to demonstrate complete loss-of-function phenotypes[12]. Knockout mouse models for Crk or Crk-L both die embryonically, but with different developmental defects, indicating that the different gene products also have distinct, non-overlapping roles[13-17]. As of yet, no double knockout has been conducted in mice. *Drosophila melanogaster* has only one *crk* gene, resulting in one Crk protein, and thus is ideal for studying the conserved function of Crk family proteins[18].

While determining the roles that different functional domains of Abelson tyrosine kinase (Abl), a key developmental regulator and oncogene, play during normal development, our lab was surprised to find that a short, conserved motif (PXXP) in the linker region was more important for Abl's morphogenic roles than *both* kinase activity and F-actin binding in certain contexts[19]. In mammals three proteins are known to bind Abl's PXXP motif—Abi[20,21], Crk[22], and Nck[22]—suggesting they may play a crucial role in Abl's function during morphogenesis. Of these three proteins, we wanted to know which, if any, work with Abl or are essential for Abl function during morphogenesis. I used RNAi to knockdown *abi, crk*, or *nck* expression, and ask if this resulted in phenotypes similar to maternal/zygotic Abl loss. Reducing *abi* or *crk* results in increased embryonic lethality similar to Abl loss, while *nck* knockdown does not (Fig. 3). Given that little is known about Crk function in *Drosophila* and Crk-L is the major downstream mediator of oncogenic effects of BCR-Abl, we decided to focus in on Crk. We hypothesized that Crk binding to Abl's PXXP motif is critical for mediating Abl's developmental effects. Based on Abl's well-defined roles in central nervous system patterning[19,23], I specifically focused on

whether Crk is required for proper CNS patterning and, if so, on more extensively characterizing Crk's role in CNS patterning.

II. Methods

Fly stocks used

All stocks were maintained at on standard cornmeal agar media at room temperature or 25°C. All RNAi crosses were maintained at 25°C.

The following stocks, obtained from the Bloomington Drosophila Stock Center (NIH P400D018537), were used in this study:

y^1, sc^*, v^1; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.HMC03964}attP40 (*crk* HMC RNAi);

y^1, sc^*, v^1; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.HMS01597}attP2 (*abi* RNAi);

y^1, v^1; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.GL01519}attP2/TM3, Sb^1 (*nck* RNAi)

We obtained y^1, sc^* v^1; P{$y^{+t7.7}$ $v^{+t1.8}$=TRiP.HMJ2295}attP40/*CyO* (*crk* HMJ RNAi) from the National Institute of Genetics (Mishima, Japan).

Additional stocks used include *y,w* (used as our wild-type laboratory strain); and *w;* P(mat-tub-Gal4)mat67*;* P(mat-tub-Gal4)mat15 (subsequently referred to as *matII; matIII)*; and *w*; elav-Gal4/CyO.

RNAi experiments

For all RNAi experiments, except CNS-specific knockdown, we wanted to knockdown both maternal and zygotic mRNA and protein, as described by Staller *et al*[24] (summarized in Fig. 2). To do so, we generated mothers carrying two copies of a strong, maternally contributed GAL4 (*matII; matIII*) and a single copy of the UAS-shRNA targeting the transcript of interest. In general, the genotype of the mothers used in the RNAi experiments was:

$$\frac{UAS\text{-}shRNA\ targeting\ transcript\ of\ interest}{matII}; \frac{matIII}{+}$$

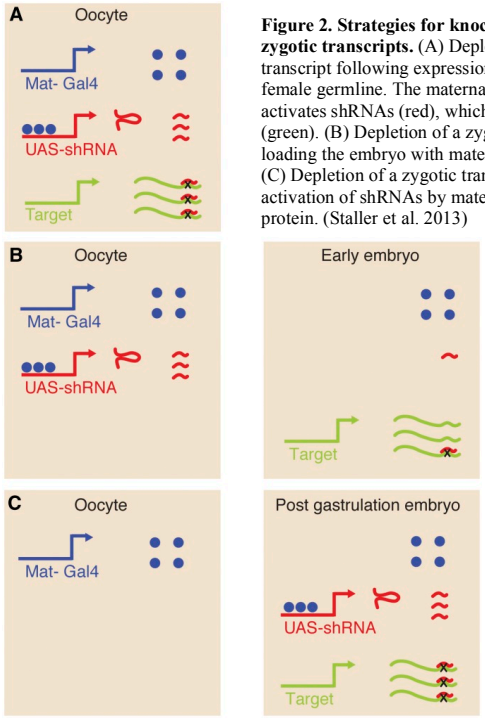

Figure 2. Strategies for knockdown of maternal and zygotic transcripts. (A) Depletion of a maternal transcript following expression of shRNAs in the female germline. The maternal Gal4 driver (blue) activates shRNAs (red), which deplete target transcripts (green). (B) Depletion of a zygotic transcript by loading the embryo with maternally derived shRNAs. (C) Depletion of a zygotic transcript following zygotic activation of shRNAs by maternally loaded Gal4 protein. (Staller et al. 2013)

We then crossed virgins of this genotype to males carrying zero, one, or two copies of the UAS-shRNA targeting the transcript of interest, depending on whether the insert was homozygous viable. Using this approach, all embryos had reduced maternal contribution of the transcript of interest. Additionally, we were able to alter the level of zygotic knockdown by varying the number of copies of UAS-shRNA present.

Measuring embryonic viability

To measure embryonic viability, crosses were set up in cups and allowed to lay eggs on apple juice agar plates overnight (16-24 hours) at 25°C. Individual embryos were then transferred to new plates, and the total number of embryos recorded (~200 embryos/individual experiment). After forty-eight hours at 25°C, the number of embryos that have hatched and the number of embryos that did not hatch were recorded. Embryos are expected to hatch after 24 hours, so after 48 hours, any eggs that have not hatched are considered dead. Percent viability was then determined by dividing the number of embryos that hatched by the total number of embryos placed on the plate. An un-popped cuticle prep was done on any unhatched eggs to determine how many were unfertilized and the percent viability was adjusted accordingly.

Confocal microscopy of Central Nervous System

Embryos were dechorionated in 50% bleach for 5 minutes at room temperature. They were then fixed in 1:1 4% formaldehyde:heptane for 20 minutes at room temperature and the vitelline membrane removed by shaking embryos in 1:1 methanol:heptane. The embryos were then rinsed three times in phosphate buffered saline (PBS) containing 0.1% Triton-X100 (PBT), and blocked by incubating in PBT containing 1% normal goat serum (PNT) for 30 minutes at room temperature. They were then incubated in primary antibody diluted in PNT either at room temperature for 4 hours or overnight at 4°C. Following washing three times with PBT, embryos were incubated in secondary antibody diluted in PNT either at room temperature for 2 hours or overnight at 4°C.

Primary antibodies used were anti-BP102 (1:200), anti-FasII (1:100), anti-robo (1:100), and anti-slit (1:10) and were obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIF and maintained at the University of Iowa, Department of Biology, Iowa City, IA, 52242. Secondary antibodies used were anti-mouse immunoglobulin G2a (IgG2a), anti-mouse immunoglobulin G2b (IgG2b), anti-mouse immunoglobulin G1 (IgG1), and anti-rat IgG conjugated to Alexa Fluor (AF) 488, AF568, or AF647 as indicated. The embryos were mounted on glass slides in Aqua-Poly/Mount (Polysciences) and imaged on a Zeiss LSM-5 PASCAL confocal microscope. Images were processed using ImageJ[25].

III. Results

Based on our lab's finding that the PXXP motif within conserved region 1 (CR1) is more essential for morphogenesis than *both* kinase activity and F-acting binding[19], we wanted to look at which of Abl's PXXP-binding partners are required during embryonic morphogenesis. In mammals, it is known that three SH3 domain containing proteins, Abi[20,21], Crk[22], and Nck[22],

interact with Abl via this conserved PXXP motif. To determine if any of these PXXP-binding partners is essential for embryonic morphogenesis, I conducted a first pass RNAi screen that should allow us to knockdown both maternal and zygotic mRNA and protein levels. In wild-type flies, it is not unusual for up to 10% to die embryonically (in our wild-type control, 5.5% died, Fig. 3). The knockdown of *nck* resulted in 5.5% lethality, identical to the wild-type control (Fig. 3), though we did not determine if *nck* RNAi effectively reduced Nck protein levels. Consequently, we decided not to continue further with *nck*. In contrast, *crk* RNAi (HMC03964) showed a lethality of 87%, while *abi* RNAi showed a lethality of 99% (Fig. 3). Here, I am focused on the effects of loss of *crk* on embryonic morphogenesis.

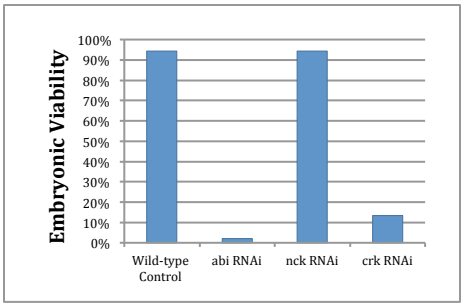

Figure 3: A first pass RNAi-based screen revealed that reduction of *abi* or *crk* severely reduced larval viability, while *nck* had no effect on viability.

Because of its location on the 4th chromosome, we lacked many of the sophisticated genetic tools that would allow us to generate embryos lacking both maternal and zygotic *crk*. There was only one extant *crk* allele (*crk*^{KG00336}; a P-element insertion between the transcription start site and start codon), and it has not been fully characterized. Because of this limitation, we initially relied on transgenic RNAi lines to reduce maternal and zygotic *crk* levels. I

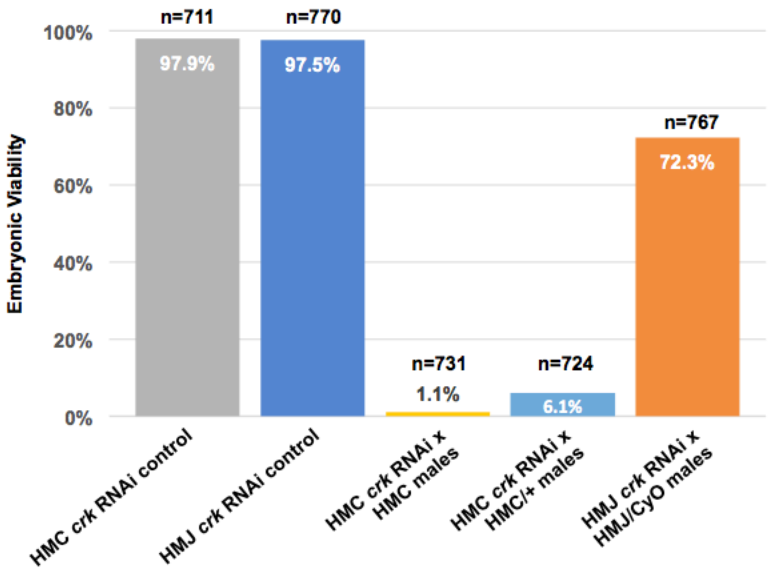

Figure 4. *crk* HMC RNAi results in strong embryonic lethality, while *crk* HMJ RNAi also results in significant, albeit weaker, embryonic lethality.

used two *crk* RNAi lines- HMC03964 (from here on known as *crk* HMC RNAi) and HMJ2995 (from here on known as *crk* HMJ RNAi), each of which targets a different region of the *crk* transcript, to ensure the phenotypes I observed were caused by knockdown of *crk* and not the result of non-specific off-target effects.

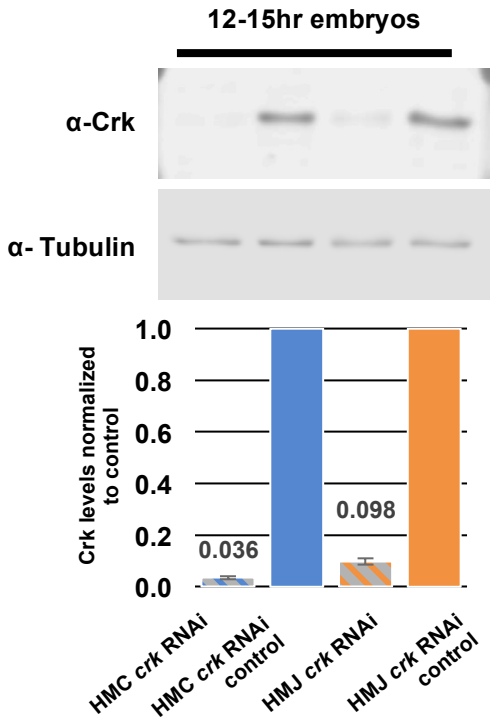

Figure 5. Both RNAi lines result in strong knockdown of *crk* protein, but to different degrees.

The controls used for viability experiments carried the RNAi construct for the respective RNAi lines, but did not have the Mat-GAL4, so the RNAi was not produced. The HMC control and HMJ control result in 97.9% viability and 97.5% viability, respectively (Fig. 4), which is unchanged from *wildtype* where up to 10% die embryonically. Using the *crk* HMC RNAi line to deplete maternal/zygotic *crk* strongly reduces embryonic viability. Crossing $\frac{UAS-HMC03964\,(crk\,RNAi)}{matII}$; $\frac{matIII}{+}$ virgins to males that are heterozygous for the *crk* RNAi (HMC03964) decreases viability to 6.1%, while crossing these virgins to *crk* RNAi homozygous males further reduces viability to 1.1% (Fig. 4). Using the second *crk* HMJ RNAi line, I observed 72.3% embryonic viability, which is decreased from *wildtype*, but less severe than the HMC line (Fig. 4). This compares to 9.4% viability in maternal/zygotic loss of *abl*[19].

To ensure this increase in lethality compared to wild-type is the result of knockdown of Crk protein levels, we conducted a Western blot to measure Crk protein levels in both RNAi lines. The HMC line virtually eliminates Crk, while the HMJ line substantially reduces it (Fig.

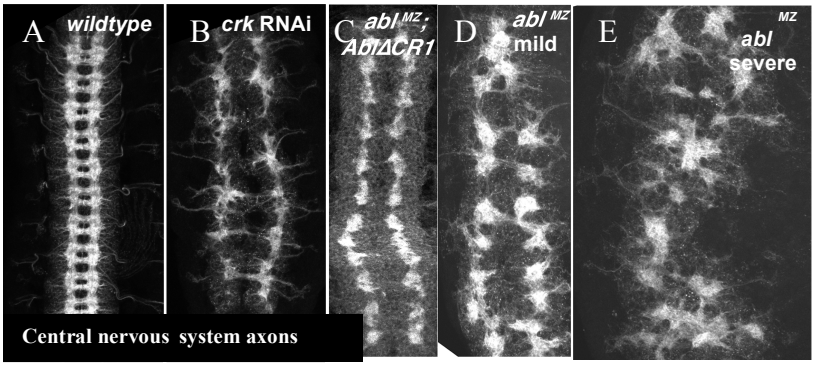

Figure 6. *crk* RNAi results in CNS patterning defects similar to the minor class of mild phenotypes seen in of *abl* maternal/zygotic mutants or upon deletion of CR1. (A) wildtype. (B) *crk* RNAi (HMC03964). (C) *abl* with a deletion of the CR1 region (AblΔCR1), (D-E) *abl* maternal/zygotic mutants. Wild-type central nervous systems exhibit a ladder-like appearance with longitudinal axon bundles making up the sides and commissural axon bundles making up the rungs (A). *crk* RNAi results in a range of phenotypes, including disorganized longitudinal axon bundles and failure to form commissural axon bundles (B). AblΔCR1 mutants fail to form commissural axon bundles, though the longitudinal axon bundles appear to form normally (C). abl^{MZ} exhibit a less frequently observed, milder phenotype with disorganized longitudinal axons bundles and no commissural axon bundles (D), but the more frequent phenotype exhibits a severely disrupted CNS (E). (Panels A, C, D, and E reproduced from Rogers et al. 2016)

5). This demonstrates that both RNAi lines result in decreased levels of *crk*, but to different extents, which may explain the observed differences in lethality.

Using these RNAi reagents, I next looked at Crk's role in central nervous system (CNS) patterning to compare its role with Abl's well-defined role in CNS patterning[19,23]. I used antibodies against BP102, which labels all CNS axons[26], and FasII, which labels a subset of axons in the longitudinal axon bundles[27,28]. The *crk* HMC RNAi line results in severely disrupted CNS patterning, similar to 70% of *ablΔCR1* mutants and the milder phenotypes seen in 44% of *abl* null mutants (Fig. 6B compared to 6C-D and Fig. 7). This similar phenotype, in which longitudinal axons form (albeit

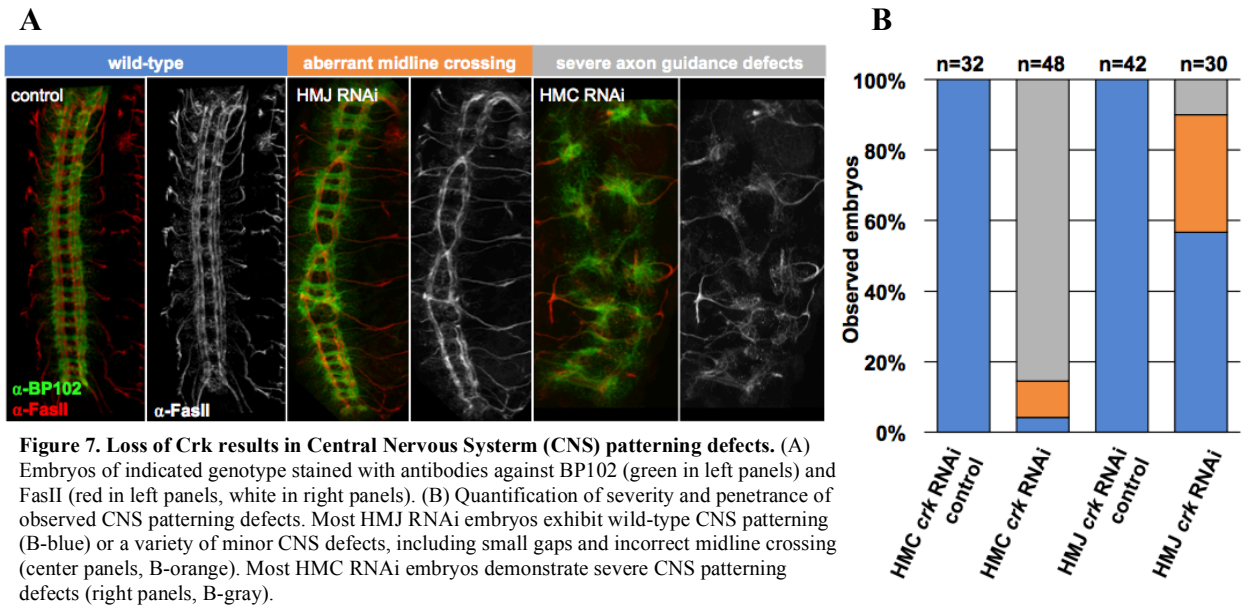

Figure 7. Loss of Crk results in Central Nervous Systerm (CNS) patterning defects. (A) Embryos of indicated genotype stained with antibodies against BP102 (green in left panels) and FasII (red in left panels, white in right panels). (B) Quantification of severity and penetrance of observed CNS patterning defects. Most HMJ RNAi embryos exhibit wild-type CNS patterning (B-blue) or a variety of minor CNS defects, including small gaps and incorrect midline crossing (center panels, B-orange). Most HMC RNAi embryos demonstrate severe CNS patterning defects (right panels, B-gray).

abnormally) and the commissural axons fail to form entirely, is more mild when compared to the severe CNS patterning phenotypes seen in 56% of maternal and zygotic *abl* mutants (Fig. 6C compared to 6E).

The *crk* HMC RNAi line most commonly results in severely disrupted CNS patterning (85%), with many embryos lacking commissural axon formation and with abnormal longitudinal axons (Fig. 7). A minority of *crk* HMC RNAi embryos (15%) develop minor CNS patterning defects or have wild-type CNS patterning (Fig. 7). The *crk* HMJ RNAi line results in more mild defects in CNS patterning (35% abnormal), mostly incorrect midline crossings, but also including loss of commissures and small breaks in the longitudinal axon bundles of the ventral nerve cord (Fig. 7). A small percentage (5%) of HMJ RNAi embryos exhibit severe CNS patterning. However, the majority (55%) develop wild-type CNS patterning (Fig. 7). Despite the difference in phenotype, the RNAi lines are consistent in that they both result in embryonic lethality and affect CNS patterning, indicating that the defects are likely due to *crk* knockdown as opposed to off-target effects in one of the RNAi lines.

Because the one extant *crk* mutant ($crk^{KG00336}$)[29] is a P-element insertion and has not been fully characterized, we sought to create a null allele using CRISPR. In addition to deleting the entire *crk* locus, this approach allowed us to incorporate an attP landing site, so we can target rescue constructs back into the genomic locus, thereby conferring endogenous regulation of these rescue constructs, including fluorescent protein tagged and FRT-flanked versions. We have recently begun characterizing this mutant ($crk^{\Delta attP}$), using the approaches we used with the RNAi lines. We never see $crk^{\Delta attP}/crk^{\Delta attP}$ adults, which suggests homozygous loss of *crk* is lethal. To maintain this allele as a stock, we put it over a GFP-marked inverted fourth chromosome (In(4) $ci^{D}ci^{D}pan^{ciD}$) that is also homozygous lethal. To define the lethal period for the homozygous

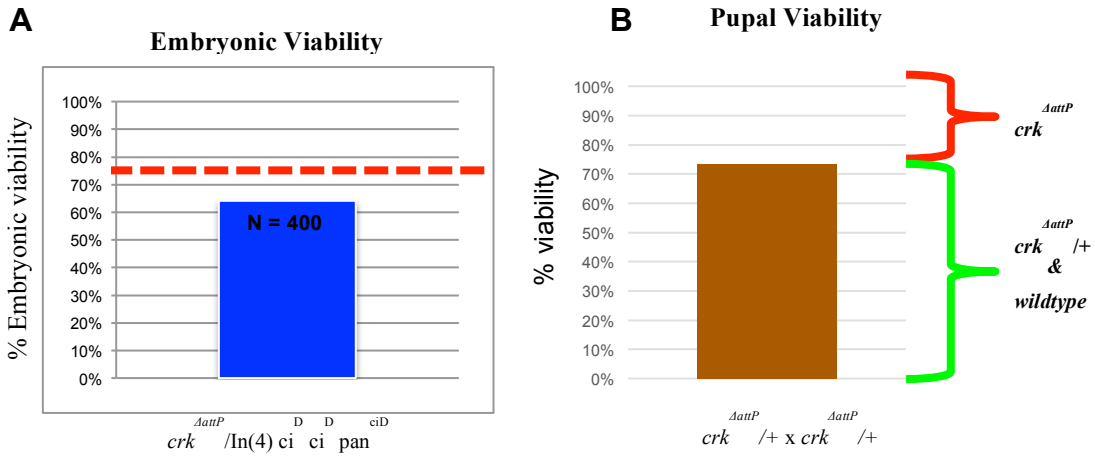

Figure 8. *crk*$^{\Delta attP}$ zygotic mutants do not die embryonically, but do exhibit pupal lethality. (A) Quantification of embryonic viability. The GFP-marked inverted fourth chromosome (In(4) $ci^{D}ci^{D}pan^{ciD}$) is homozygous lethal, so the expected baseline embryonic viability is 75% (red dotted line). Embryonic viability of *crk*$^{\Delta attP}$ zygotic mutants was 65%. (B) Quantification of pupal viability. Pupal viability of *crk*$^{\Delta attP}$ zygotic mutants was 75%.

crk$^{\Delta attP}$ mutants, we first assessed embryonic viability of this stock. Because homozygosity for the In(4) $ci^{D}ci^{D}pan^{ciD}$ chromosome is lethal, our base-line viability will be 75% as 25% of the embryos will die from homozygosity of In(4) $ci^{D}ci^{D}pan^{ciD}$ (Fig. 8A, dotted red line). If the *crk*$^{\Delta attP}$ mutation is embryonic lethal, we would expect 25% of the embryos to die from homozygosity of *crk*$^{\Delta attP}$. However in this assay, we observe only 35% embryonic lethality as opposed to the 50% we would expect if *crk*$^{\Delta attP}$ mutants are embryonically inviable, indicating that at least some, if not all, of the homozygous *crk* mutants survived embryogenesis (Fig. 8A). Next, we measured pupal lethality; percent viability was determined by dividing the number of adults that eclose by the total number of pupae that formed on the sides of the vial. Pupal lethality experiments were done using *crk*$^{\Delta attP}$ heterozygotes (over a true wildtype chromosome) crossed to themselves, so we would expect 25% to be wild-type, 50% to be heterozygotes, and 25% to be *crk*$^{\Delta attP}$ homozygotes. Here we observed that 25% of the pupae die, which corresponds to the expected fraction that would be homozygous for *crk*$^{\Delta attP}$ (Fig. 8B). This suggests that

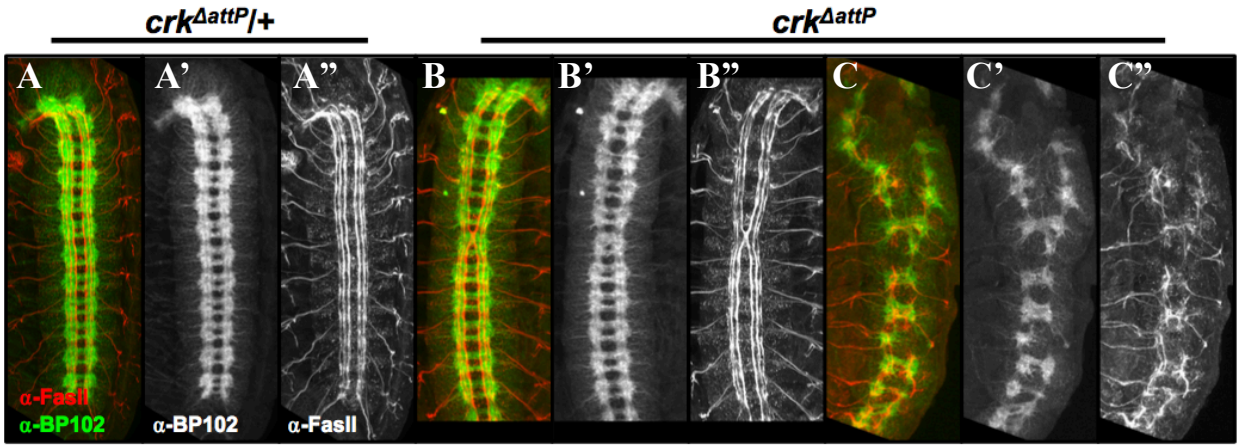

Figure 9. Heterozygotes for the *crk* deletion exhibit wild-type central nervous systems, while homozygotes for the deletion show partially penetrant CNS phenotypes. Embryos of the indicated genotype stained with antibodies against BP102 (green, A'-C') and FasII (red, A"-C"). $crk^{\Delta attP}$ heterozygotes exhibit normal CNS patterning (A-A"). $crk^{\Delta attP}$ homozygotes show minor CNS defects, such as inappropriate midline crossing (B-B"), while some embryos exhibit severely disrupted CNS patterning (C-C").

$crk^{\Delta attP}$ homozygotes primarily die as pupae, similar to *abl* zygotic mutants[30].

Because of the CNS patterning phenotype observed in the *crk* RNAi embryos, I next asked if $crk^{\Delta attP}$ mutants also exhibit CNS patterning defects. Here, I was able to distinguish $crk^{\Delta attP}$ heterozygotes from $crk^{\Delta attP}$ homozygotes using a homozygous lethal, GFP marked fourth chromosome (P{w[+mC]=ActGFP}unc-13[GJ]). Using this strategy, $crk^{\Delta attP}$ homozygotes are readily identified by the absence of GFP. Preliminary examination of these embryos shows that, while CNS patterning in $crk^{\Delta attP}$ heterozygotes and the majority (81%, n=17) of $crk^{\Delta attP}$ homozygotes appear normal (Fig. 9A), homozygous mutants exhibit partially penetrant CNS phenotypes (Fig. 9 B, C). Some embryos (6%, n=17) show defects similar to the more mild defects seen when using *crk* RNAi line HMJ2995, including inappropriate midline crossing or small breaks (Fig. 9). However, 12% (n=17) of embryos showed much more severe defects that resembled the stronger *crk* RNAi line (HMC03964) or *abl* maternal zygotic mutants (Fig. 9).

The CNS phenotypes seen in both RNAi lines and our *crk* deletion mutant led us to ask how Crk is participating in CNS patterning. During the development of the CNS, axons extend

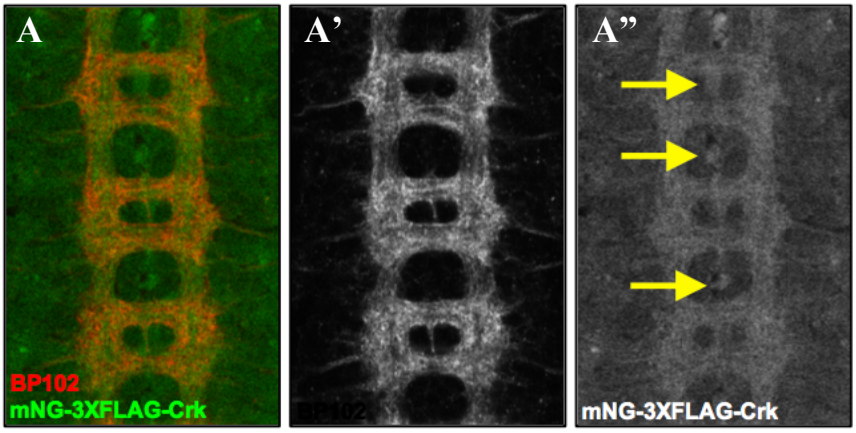

Figure 10. Endogenously tagged Crk shows that Crk in enriched in axons and cells at the midline. mNG-3XFLAG-Crk embryos with tagged Crk (green A, A") and stained for BP102 (red A, A'). These embryos show localization of endogenous Crk in the embryonic CNS. Crk is enriched in axons and in cells at the midline (A", yellow arrows).

from the cell bodies along either side of the longitudinal tracks, and either remain on the ipsilateral side and extend longitudinally or cross the midline and extend along the contralateral tract[31].

Mediating this decision are a number of attractive and repulsive guidance cues along the midline, which direct the axons to cross in certain places[31]. We started by asking whether Crk localizes to the CNS, using a rescue construct tagged with monomeric Neon Green and a 3XFLAG tag (mNG-3XFLAG-Crk), which is expressed from the endogenous locus. Using this line, we looked at Crk localization in the CNS and found that Crk was enriched in axons and in cells at the midline (Fig. 10- yellow arrows, presumably midline glia). The presence of Crk in axons indicates that it is in the right location and cell type to participate in interpretation of axon guidance cues.

I am currently looking at one type of axon guidance cue, a repulsive midline cue mediated by the interaction between Slit (a repulsive ligand) and Robo (its receptor)[32], to determine if Crk plays a role in this signaling pathway. Previous work by other groups has shown that Abl works downstream of Robo to negatively regulate Slit/Robo signaling[33]. Based on these findings and our hypothesis that Crk may work with Abl to regulate CNS patterning, we would expect Crk to also act downstream of Robo. However, given that Robo is also a substrate for Abl kinase activity[33], I sought to rule out the possibility that Crk could work upstream of

Robo to regulate Robo or Slit localization by asking if Robo and Slit localization is altered by loss of Crk.

In wildtype embryos, Robo is restricted to the longitudinal axons, which do not cross the midline (Fig. 11A-A"). In 16/17 *crk* HMC RNAi embryos, where we get the strongest CNS phenotypes, Robo restriction and enrichment in longitudinal axons is unchanged (Fig. 11B-C"), suggesting that Crk is not required for proper localization of Robo within the CNS. I next wanted to ask whether loss of Crk had any effect on localization of Robo's ligand, Slit. In *wildtype*, Slit is localized at the midline where it is secreted by midline glia (Fig. 11D-D"). In *crk* HMC RNAi embryos, Slit localization to the midline is also largely normal, although I do observe minor

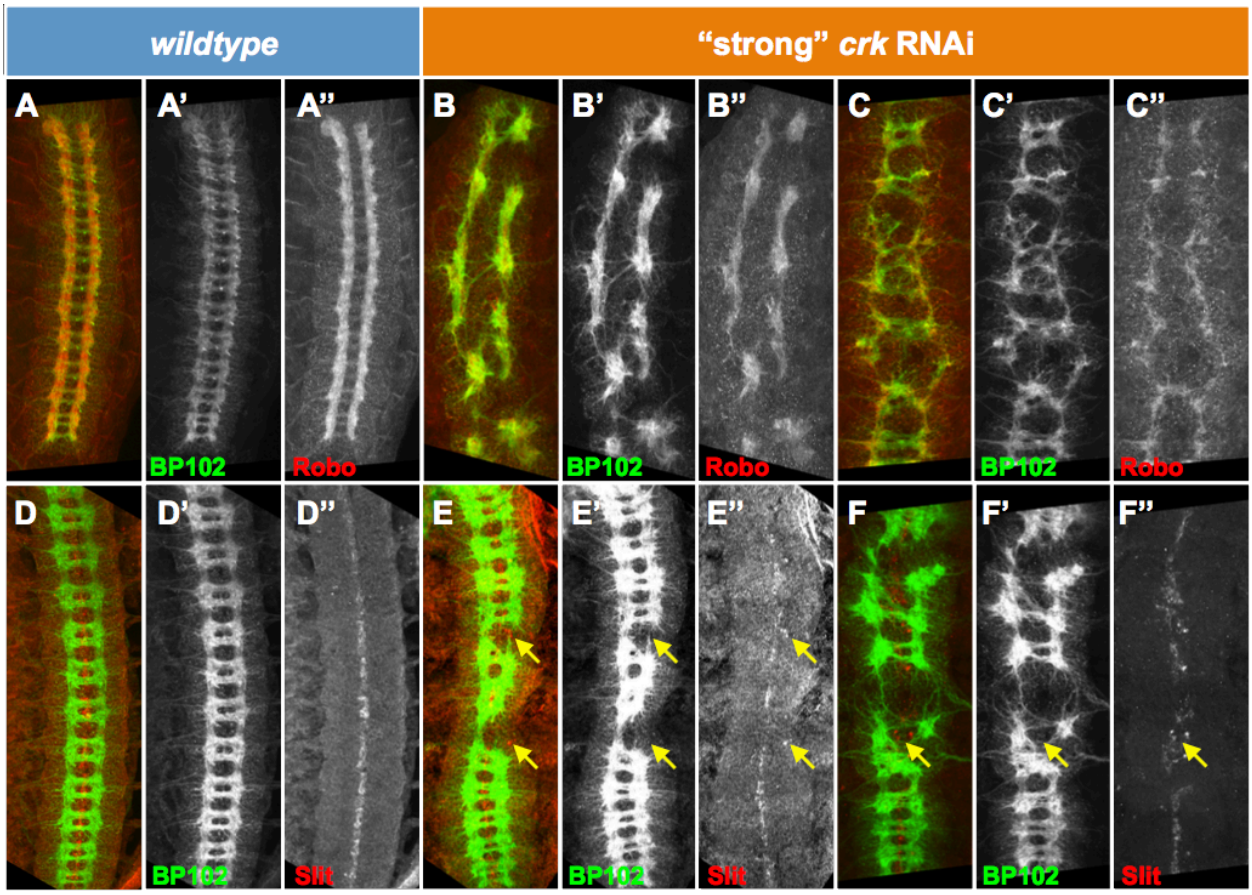

Figure 11. Robo localization in the *crk* HMC RNAi line is similar to that in wildtype embryos, and while Slit localization is largely normal, there are some midline disruptions. (A-C) Embryos stained with BP102 (green, A'-C') and Robo (red, A"-C"). Wild-type embryos show Robo is enriched in the longitudinal axons (A-A"). Robo in HMC RNAi embryos is localized to the longitudinal axons that do form, albeit abnormally (B-C"). (D-F) Embryos stained with BP102 (green, D'-F') and Slit (red, D"-F"). Slit in HMC RNAi embryos localizes primarily to cells at the midline, but Slit is also found in some cells outside the midline (E-F, yellow arrows). These abnormal localizations of Slit correspond with strong repulsion of axon bundles (E-F, arrows).

midline disruptions in 7/11 embryos (Fig. 11E-F"). This suggests that Crk is largely dispensable for proper Slit localization. Interestingly, where I observe midline disruptions causing Slit to be misplaced, I observed strong repulsion of axon bundles (arrows, Fig. 11E-F"), suggesting that Slit-Robo- mediated repulsive signaling is intact and does not require Crk.

Next, I attempted central nervous system- specific knockdown of Crk using a GAL4 driver expressed in neurons, *elav*-GAL4, crossed to the *crk* HMC RNAi line or the *crk* HMJ RNAi line. Embryos from both crosses exhibited central nervous systems with no noticeable defects (data not shown). I also used an overexpression construct crossed to *elav*-GAL4 to overexpress Crk in a CNS-specific manner. This also resulted in embryos with wild-type central nervous systems (data not shown). Since Crk knockdown or overexpression in the axons did not show an obvious phenotype, I began to look at whether the CNS phenotypes seen in Crk knockdown embryos might be the result of Crk depletion in midline cells. I used *slit*-Gal4 to deplete Crk in midline cells, which did not show an increase in embryonic lethality compared to *wildtype* (data not shown). However, with tissue-specific knockdown, we have no way of determining if Crk protein levels are actually reduced in that tissue, which could explain the lack of noticeable phenotype. It is also possible that the knockdown achieved using this strategy is occurring too late in morphogenesis to have an effect on CNS patterning.

IV. Discussion

Studies in cell culture and in mouse mutants suggest Crk is essential for cell adhesion, cell migration, and other biological processes during normal development[4]. Loss of Crk can result in disruption of these processes leading to developmental defects or cancer[6-10]. By understanding how Crk works, both in normal development and in disease states, we can begin to understand what specifically causes these disease states and how we might go about fixing

them. We started studying Crk after our lab's work on Abl revealed that Abl's CR1, which contains a conserved PXXP motif, is more essential for some aspects of development than both the F-actin binding domain and kinase activity[19]. This finding led us to focus on better understanding the role of Abl's PXXP-binding partners during morphogenesis.

I screened three known CR1 binding partners in mammals, Abi[20,21], Nck[22], and Crk[22], for embryonic lethality and found that knockdown of *abi* and *crk*, but not *nck*, results in strong embryonic lethality. *nck* knockdown resulted in 5.5% lethality (same as wild-type control), whereas *abi* RNAi showed 99% lethality and *crk* RNAi showed 87% lethality. The efficacy of the *nck* RNAi line in reducing *nck* transcript levels was not assessed, so we cannot rule out the possibility that Nck has a role here. Given that little is known about Crk function in *Drosophila* and Crk-L is the major downstream mediator of oncogenic effects of BCR-Abl, we decided to focus on Crk's roles in development, working with and independently of Abl. To study *crk* function I have used two *crk* RNAi lines and a *crk* null allele we recently developed using CRISPR.

The broadest assessment of phenotype is measuring lethality caused by knockdown of the protein, so I started there. The first *crk* RNAi line (TRiP line HMC03964) exhibited an embryonic lethality of 93.9% when crossed to males carrying one copy of the RNAi and 98.9% when crossed to males with two copies of the RNAi. To check for off-target effects, I also looked at a second *crk* RNAi line (TRiP line HMJ2995), which shows a lower, but still elevated, embryonic lethality of 26.7%. Western data showed that the *crk* HMC line virtually eliminates Crk, while the *crk* HMJ line substantially reduces it, but does not completely eliminate it, which may explain the difference in lethality. Our zygotic *crk* CRISPR mutants ($crk^{\Delta attP}$) did not show a significant embryonic lethality, but were found to die later, in the pupal stages. This is different

from the *crk* RNAi lines, indicating maternal contribution of *crk* is sufficient for embryonic development, but not for survival to adulthood. We are currently also eliminating maternal contribution by generating germline clones using a FLPout strategy[34] with our $crk^{\Delta attP}$ allele that will allow us further assess how complete elimination of Crk affects embryogenesis.

Based on Abl's well-defined role in CNS patterning[19,24], I next asked if Crk has a role in CNS patterning by looking at the CNS of *crk* RNAi embryos and $crk^{\Delta attP}$ homozygotes. The *crk* HMC RNAi line shows CNS patterning defects where the longitudinal axons form abnormally, and the commissural axons do not form at all. This is similar to the phenotype of AblΔCR1 mutants or the less frequent, milder phenotypic class observed in *abl* maternal/zygotic mutants. The weaker *crk* HMJ RNAi line shows less severe CNS patterning defects where the CNS has only small gaps or breaks or inappropriate midline crosses, resembling similar phenotypes seen in *abl* zygotic mutants. The similarity of phenotypes (though not of severity) between the two RNAi lines suggests that knockdown of *crk* is the cause of these phenotypes and they are likely not the result of off-target effects. Preliminary examination of $crk^{\Delta attP}$ mutants shows phenotypes more similar to the weaker *crk* RNAi line (small breaks and midline crosses); however, some mutants exhibit severe CNS patterning defects similar to loss of Abl or deletion of CR1. The similarity of these phenotypes to those of maternal/zygotic loss of *abl* suggests Crk and Abl may be working together to modulate cell behavior during CNS patterning. However, more data is needed to more definitively characterize the CNS patterning phenotypes seen in these animals. Maternal contribution of *crk* in $crk^{\Delta attP}$ zygotic mutants may mask the defects caused by loss of *crk*, so it would be helpful to look at CNS patterning after eliminating maternal contribution in these mutants, where we would expect to see stronger phenotypes. However, preliminary

examination of embryos lacking maternal contribution of Crk shows severe developmental defects that result in severe disruption of the embryos before the CNS begins to develop.

I next asked how Crk is mechanistically affecting CNS patterning during development. During the development of the CNS, axons either remain on the ipsilateral side of the CNS and extend longitudinally or cross the midline and extend along the contralateral tract, a decision that is mediated by a number of attractive and repulsive guidance cues. Using an endogenous rescue construct tagged at the N-terminus with monomeric Neon Green and a 3XFLAG tag (mNG-3XFLAG-Crk), we were able to look at Crk localization in the CNS. We found that Crk localizes both to the axons and to cells at the midline (presumably midline glia), which indicates that Crk is in the right location to potentially play a role in axon guidance decision making. I am currently asking if one of these midline guidance cues, a repulsive cue mediated by the interaction between Slit and its receptor Robo, is altered by loss of Crk. Robo localization in the *crk* HMC line appears to be normal, restricted to the longitudinal axons. Slit localization is also largely normal with Slit localized to the midline, but does demonstrate some midline disruptions. These disruptions correspond with strong axon bundle repulsion, indicating that Slit-Robo signaling is likely intact, and that Crk is not required to mediate Slit/Robo-dependent repulsive guidance at the midline. I am currently collecting more data to more definitively characterize these phenotypes. Future work will need to be done to assess whether loss of Crk alters localization or function of mediators of additional repulsive axon guidance cues as well as attractive axon guidance cues at the midline.

To further assess this, we attempted to knock down *crk* in a CNS-specific manner, which showed no effect on CNS patterning (data not shown). Overexpression of *crk* in a CNS-specific manner also exhibited no noticeable CNS patterning defects (data not shown). Additionally,

knock down of Crk in midline cells did not result in an increase in embryonic lethality, but the CNS patterning in these embryos has yet to be observed. However, we are unable to definitively determine if Crk levels are reduced in these specific tissues, so the lack of phenotype observed may be the result of insufficient knockdown or reduction of Crk too late in development to have an effect.

We are also continuing to characterize the effects of loss of *crk* on overall morphogenesis as well as what role *crk* plays in other developmental events in *Drosophila*. We have developed a number of rescue constructs to be incorporated into our *crk* deletion mutant and are working to characterize and quantify their ability to rescue loss of *crk.* Additionally, we are using the mNG-3XFLAG-Crk rescue construct to look at Crk localization throughout the embryo during various stages of morphogenesis. We also plan to assess how phenotypes seen upon loss of Crk are modified by altering levels of Abl, and vice versa. By fully characterizing Crk's role in development, we can gain a better understanding of Crk's role in cancer and other disease states and create better treatments for these conditions.

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