STUDY OF DISABLED ADAPTOR PROTEIN IN THE ABELSON KINASE SIGNALING PATHWAY IN DROSOPHILA

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ABSTRACT

Lyudmila Kotlyanskaya Cherner: Study of Disabled adaptor protein
in the Abelson kinase signaling pathway in Drosophila
(Under the direction of Edward Giniger)

In the development of tissues such as the nervous system, the actin cytoskeleton is remodeled in cells by protein signaling cascades that allow cells to change shape, move and sense their environment by sending out projections. At the center of one such signaling cascade, important during neuronal migration and axon guidance in both vertebrates and invertebrates, is the Abelson tyrosine kinase. Abl interacts with a set of receptors, adaptors, and actin binding and regulating proteins. In *Drosophila abl* was specifically found to genetically interact with *disabled* (*dab*), an adaptor that also binds to Notch, a receptor that genetically interacts with *abl*. Dab is the link that transmits extracellular signals to the kinase signaling module. In this study we sought to dissect how the protein structure of Dab contributes to the mechanism of Abl function in the nervous system both *in vivo* and *in vitro*.

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Abl	Abelson nonreceptor tyrosine kinase
Arg	Abl-related gene
CML	human chronic myelogenous leukemia
CNS	Central Nervous System
Dab	Disabled adaptor protein
Dab^{mz}	Disabled maternal and zygotic mutant
Ena	Enabled actin binding protein
GEF	Guanine exchange factor
ISN	Intersegmental Nerve
ISNb	Intersegmental Nerve b
N^{ts}	Notch temperature sensitive allele
PTB	phosphotyrosine-binding domain
SH2	Src Homology 2 domain
SH3	Src Homology 3 domain
SNa	Segmental Nerve a

CHAPTER 1: ABELSON KINASE AND DISABLED IN NEURONAL DEVELOPMENT

Introduction

During embryonic development the building of tissues requires that cells orchestrate a wide array of physiological processes. The nervous system in particular is a uniquely complex model to study different morphogenetic stages that a cell undergoes during development. An undifferentiated cell divides until the cell stops mitosis and a neuronal identity is established. The cell differentiates and changes shape. In the developing vertebrate brain neuronal cells may then migrate long distances alone or in coordination with many other cells to locations where ultimately they will reside and function. Subsequently they send out membrane projections, axons and dendrites that navigate along complex and long paths. This stage is referred to as the axon guidance stage. Finally the cell forms synapses with other neurons or muscles. In invertebrates such as *Drosophila*, neurons do not need to migrate due to the short distances involved, but they do undergo axonal migration and guidance necessary for future synapse formation. This also makes the fruit fly a simpler model to study neuronal development.

The fundamental process underlying changes in cell shape, cell migration and axon guidance is the remodeling of the actin cytoskeleton and the associated membranes. Signals from the environment that are transduced through signaling networks inside the cell ultimately lead to rearrangements of G-actin monomers into different types of F-actin filament networks.

Depending on the type of actin structures, the membrane may be reshaped, may lead to membrane protrusion and cell movement, or growth of a neurite projection.

Different types of protrusive plasma membrane structures can co-exist at the leading edge of the cell. These structures will change the shape of the cell, determine the speed of cell motility, the ability of the cell to communicate with the environment or to adhere to a substrate. Lamellipodia are thin sheet-like regions which contain a "dendritic" branched F- actin filament network. The actin in this region is constantly being polymerized in the front and depolymerized in the back, driving forward protrusion of the plasma membrane (Ridley et al, 2003). Behind the dynamic lamellipodium is the more stable lamella region, where actin is linked to the contractile and adhesive mechanisms which also aid in protrusion (Ponti et al., 2004). A second type of structure that can exist at the leading edge is filopodia. Filopodia are finger-like projections that contain parallel bundles of actin filaments. Filopodia explore the environment by interacting with extracellular cues and are especially used by growth cones in axonal guidance.

Aided by the leading edge protrusions, the neuron can undergo several types of cell migration in the developing vertebrate neocortex. Cortical neurons that will eventually become glutamatergic tend to migrate radially from the ventricular zone to the pial surface. Early in development, newly born postmitotic cells undergo somal translocation by extending a single long basal process to the pial surface, followed by nuclear translocation called nucleokinesis and shortening of the basal process. The neuron may then switch to locomotion, a process in which the leading edge becomes free and the cell body moves in a saltatory manner in close apposition to the long basal processes of radial glial cells, which serve as the scaffold (Ghashghaei et al., 2007). On the other hand GABA (γ-aminobutyric acid)-containing interneurons tend to migrate tangentially using the marginal zone neurons, corticofugal fibers or the pial membrane as the

scaffold. All neurons can also switch between radial and tangential migration (Valiente et al., 2010).

Axon guidance also relies on leading edge protrusions. It is mediated by the growth cone; the leading edge of the axon of the neuron. The growth cone contains three major compartments. The P (peripheral) region is located near the membrane and contains lamellipodia and filopodia. The C (central) region is connected to the axonal shaft and contains bundles of microtubules that extend from the axon. Microtubules are used to deliver vesicles and organelles into this region. Some microtubules extend into the P region to interact with the F-actin. The T region, the transitional zone, is located between the P and C regions and contains acto-myosin contractile structures. These are important for regulation of actin flow and microtubule lattice stabilization. The balance of protrusion and adhesion to the extracellular matrix via the coordination of these structures in the growth cone provides the force for the neurite outgrowth and motility (Vitriol and Zheng, 2012).

Although many guidance cues, receptors, kinases, phosphatases and actin binding molecules have been identified in regulation of the actin cytoskeleton during different developmental stages of the cell, one challenge in the field has been how all these different molecules communicate with each other and balance each other to transduce an extracellular signal into cytoskeletal change. A thorough knowledge of the signaling pathways that control actin cytoskeletal rearrangement are essential to understanding how cells build tissues such as the central nervous system in embryonic vertebrate and invertebrate development.

Section 1.1 Abelson kinase

In the introduction to Abelson kinase (Abl) I will first give a brief overview, then talk about the domain structure of the protein and the mechanism of kinase activation and finally discuss a variety of roles Abl plays in development, particularly in mammalian and *Drosophila* nervous system development. Subsequent sections will describe the interactions of Abl with its affiliated cofactors.

In the regulation of the actin cytoskeleton many signaling pathways have been discovered to be involved. Our lab focuses on the study of one particular pathway, at the center of which is the Abelson (Abl) non receptor tyrosine kinase. Abl is an extremely interesting kinase because it interacts both genetically and biochemically with a large number of molecules both upstream at the cell membrane and downstream, directly regulating or binding to the actin cytoskeleton in both vertebrates and invertebrates. Abl has been convincingly linked to the action of most of the common, phylogenetically conserved families of ligands and receptors that control axon growth and guidance throughout the animal kingdom, including Netrin receptors, slit receptors, semaphorin receptors, integrins, and others. Our lab and many other labs have shown in the *Drosophila* model system that Abl interacts with a set of proteins we refer to as the Abl signaling module, discussed below. These interactions allow Abl to act as a molecular switch, bringing together multiple extracellular inputs and balancing the outputs of multiple signaling pathways to produce different types of actin structures, such as lamellipodia and filopodia or regulating the cell's propensity for motility or adhesion. Furthermore, by understanding how Abl is regulated to act as a molecular switch, in the future we can create a general model for such switches that can help explain actin dynamics in other organisms. The same Abl-associated proteins and protein

interactions that direct axon patterning in flies play the same roles in *C. elegans*, and also in vertebrates.

Many of the proteins that interact with Abl were originally discovered in *Drosophila*. *Abl* mutants displayed such a mild phenotype that screens were designed to find mutants that enhance or suppress this phenotype. In spite of *abl* zygotic mutants having a number of defects at different stages of development, many animals are viable to adulthood. There is a low level of recessive lethality at the pharate adult pupal stage. Mutant adults have reduced longevity and fertility and have rough eye phenotype in which the retinal cells' pattern is disrupted (Henkemeyer MJ et al., 1987). Dominant enhancer mutations in genes found via mutagenesis screens conducted by various groups became known as haploinsufficiency dependent on an a*bl* mutant background (HDA). Some of these genes are upstream receptors that receive signals from the extracellular environment. For example, in *Drosophila* neurons *abl* interacts with *dlar* transmembrane tyrosine phosphatase, *notch*, a receptor that regulates neuronal cell fate, *neurotactin* (*nrt*), a transmembrane glycoprotein that promotes cell adhesion, *roundabout (robo)* axon guidance receptor and many others. Upstream, *abl* also interacts with *disabled* (*dab*), expressed as an adapter scaffolding protein. Downstream, *abl* interacts with *failed axon connections* (*fax*), an axon guidance molecule, *armadillo/b-catenin*, that is important in the formation of adhesive junctions, *chickadee/profilin*, a protein involved in actin polymerization, *trio*, a guanine exchange factor (GEF) for Rac and Rho small GTPases, and others (Bradley and Koleske 2009, Moresco and Koleske 2003). Interestingly, *abl* mutant phenotypes have been also found to be suppressed by an *ena*, an F-actin binding and polymerization factor. Furthermore Abl family kinases also bind and presumably regulate F-actin and microtubules directly.

To better understand Abl function it is important to introduce Abl protein structure and kinase activation mechanism. Abl family kinases are highly conserved in DNA sequence and protein structure. The N terminus is alternatively spliced and has two major isoforms in vertebrates. Human isoform 1a/murine isoform 1 is non myristoylated. Human isoform 1b/isoform IV in mice are "capped" or bound to a myristoyl fatty acid. On the N terminus the vertebrate Abl and *Drosophila* D-Abl have tandem Src Homology 3 (SH3), Src Homology 2 (SH2), tyrosine kinase domains and four proline-rich domains (Pro-X-X-Pro). Polyproline motifs interact with SH3 domains (Feller et al., 1994; Ren et al., 1994). Murine Abl also contains three nuclear localization signals, DNA-binding domains, a nuclear export sequence, and G and F actin-binding domains, while D-Abl has an F-actin binding domain only. Arg (Abl-related gene), an Abl homologue, does not have nucleic acid or G-actin binding functions, but interestingly has a microtubule binding region.

Abl kinase functions by the latch-clamp-switch mechanism (Harrison, 2003). When in inactive confirmation the SH3 and SH2 domains act as a "clamp" by folding onto the kinase domain, while the myristoyl group or hydrophobic residues at the N terminus or endogenous lipids in the cell act as a "latch" by docking into the hydrophobic pocket in the kinase domain. Abl kinase may get activated in a number of ways. A ligand in the form of an activated receptor or an adaptor protein, may bind to the SH3 or SH2 domains, releasing their "clamp" on the kinase domain. A cytoplasmic phosphopeptide can also bind to Abl SH2 to activate the kinase (Hantschel et al., 2003). To prevent the kinase from inactivation and to properly orient the catalytic site, Abl must be phosphorylated on two "switch" tyrosines Y245 and Y412 by Src-family kinases or autophosphorylated in trans by another Abl molecule (Brasher and Van Etten, 2000; Tanis et al., 2003).

Although we are specifically interested in Abl's roles in neuronal development, specifically axon growth and guidance, discussed below, notably Abl is important for actin regulation in many other developmental processes. Abl is best known as an oncogene involved in 95% of human chronic myelogenous leukemia (CML) cases and other cancers. In malignant cells the Abl kinase is constitutively hyperactivated via a chromosomal translocation that results in the fusion of BCR gene and Abl N terminus, known as the Philadelphia chromosome. In mammalian normal cells, Abl is important in many morphogenetic events including epithelial polarity and integrity, spermatogenesis, hematopoeisis, and immune system formation, as well as mediating cellular DNA damage responses to genotoxic or oxidative stress via its nucleic function unique to mammals. In *Drosophila*, Abl signaling has been shown to be important during cellularization, in germband retraction, dorsal closure, and photoreceptor and retinal epithelium patterning.

Although Abl participates in the development of many tissues in both vertebrates and invertebrates, arguably its key role is in the development of the nervous system. In the mouse, Abl and its homologue Arg are both expressed in most neurons of the developing and adult brain (Koleske et al., 1998). Although a*bl* homozygous mutants and *arg* homozygous mutants' brains look WT, $abl^{-/-}$ $arg^{-/-}$ animals die at stage E10.5 and have defects in neural tube formation stemming from actin cytoskeleton defects in the neuroepithelial cells (Koleske et al 1998). This suggests that *abl* and *arg* may have redundant functions in the mouse brain. One possibility is that Abl may regulate neuronal migration. In support of this, Jossin et al in 2003 showed that in *in vitro* embryonic slice culture inhibitors of Abl, together with Src family kinase inhibitors, but not individually, cause neuronal migration defects in which neurons migrate into incorrect layers, similar to a well known phenotype *reeler* (discussed further in Section 1.2 on Dab). Abl may also be important in mammalian axon guidance. Cortical neurons in culture that are overexpressing

WT or constitutively activated mutant *abl* have increased neurite outgrowth (Zukerberg et al 2000) and dendritic microspikes (Woodring et al., 2002).

In *Drosophila* there is overwhelming evidence of the importance of Abl in nervous system development. *Drosophila* Abl (D-Abl) expression is particularly pronounced in the axons of the nervous system (Gertler et al., 1989). Zygotic *abl* homozygous mutants or gain-of-function mutants both have ectopic longitudinal axons that cross the midline. Furthermore in *D-abl*$^{-/-}$ mutants peripheral axons called Intersegmental Nerve b (ISNb) that innervate the body wall do not reach their target muscle 12, do not make a neuromuscular junction there and "stall" at muscle 13 (Wills et al., 1999b). On the other hand, overexpression of Abl leads to ISNb "bypassing" its trajectory of muscles 6, 7, 13 and 12 altogether and instead growing along the nearby Intersegmental Nerve (ISN) axon bundle (Wills et al., 1999a). A maternal and zygotic *abl* mutant has similar and even more severe CNS defects (Grevengoed et al., 2001).

Work in many labs has established that Abl interacts with a set of proteins that promote the ability of Abl to act as a molecular switch to regulate the actin structures in the neuronal growth cone during axon guidance in *Drosophila*. Here we will describe one example of how Abl links to a cell surface receptor that directs axon growth. Much evidence from the Giniger lab suggests that Abl interacts with the Notch receptor in axons to regulate axon growth but not neuronal identity. *Abl* and *notch* interact genetically. For example, in heteroallelic *abl* mutants with one mutant allele of *Notch,* in the CNS there are missing longitudinal tracts between neuromeres and in the PNS the lateral portion of the ISN axons are missing, while cell identities are unaffected (Giniger 1998). Furthermore, the reduction of Notch in a heteroallelic *abl*$^{-/-}$ background causes genetic lethality, thus Notch acts as an HDA locus. In Notch temperature sensitive mutants N^{ts}, if Notch expression is turned off via a temperature shift after neuronal

identities are established, ISNb axons have the same "bypass" phenotype as *abl* gain of function mutants (Crowner et al 2003). In fact, *Notch* is a suppressor of *abl*, because a combination of N^{ts} mutant and *abl* overexpression enhances bypass significantly compared to either by itself, while one mutant *abl* allele in a N^{ts} background suppresses the bypass phenotype. Conversely, overexpression of Notch and overexpression of Abl together suppresses the bypass phenotype compared to overexpression of Abl alone (Crowner et al 2003).

To better understand how Abl may be biochemically interacting with Notch, the Giniger lab has studied a candidate scaffolding protein Disabled (Dab), which is an adaptor protein that was found to genetically interact with *abl* (Song et al 2010) and simultaneously interacts genetically and co-IPs with Notch from embryo lysate (LeGall et al 2008). We describe the progress made on Dab's interaction with Abl in more detail in Section 1.2 on Dab and in Chapter 4.

In characterizing the Abl signaling pathway that is specifically responsible for axon guidance in the *Drosophila* nervous system, our lab has also looked at downstream actin cytoskeleton regulators that were found to interact with *abl* in genetic screens. For example, a chromosomal deficiency that removes the *trio* gene or a missense mutation in $trio^{M89}$ both shifted the lethality from adult to prepupal stage in the *abl* mutant background, thus establishing Trio as an HDA locus. Similarly to other HDA effects, embryos that were heterozygous mutant for *trio* in the $abl^{-/-}$ background displayed significantly increased defects in commissure and longitudinals formation in the CNS than the single mutants of *trio* or *abl* alone. As determined by *in situ* hybridization, Trio mRNA is indeed expressed in most neurons of the embryonic CNS, as well as other migrating cell types throughout embryogenesis, consistent with importance in actin cytoskeleton regulation (Liebl et al 2000). Interestingly, *trio* mutants, similarly to *abl* or

dab mutants have stalled ISNb motonerves that cannot reach the muscle 12/13 cleft in the embryonic body wall. Furthermore, *trio* genetically interacts with other *abl* pathway components. For example, a heterozygous *trio* mutation rescues the ISNb bypass defect in the $notch^{ts}$ mutant (Song et al 2011). Trio is a guanine exchange factor for the GTPases Rac1 and Rho. Our lab has shown previously that Trio GEF1, the specific domain for Rac (Newsome et al., 2000, Bellanger et al., 1998), but not the Trio GEF2, the specific domain for Rho (Spencer et al., 2001), is essential for Abl-dependent motor axon guidance in the *Drosophila* nervous system, by showing that mutation of GEF1 but not GEF2 causes stalling of ISNb. Consistent with this, Rac GTPase is indeed involved in Notch/Abl signaling pathway in *Drosophila* axons, because a *rac* heterozygous mutant, unlike a *rho* mutation, also suppressed the ISNb bypass defect of $Notch^{ts}$, similarly to a *trio* mutation. Previously, other labs have also demonstrated that *rac* in *Drosophila* is important for axon guidance (Luo et al., 1994).

Another molecule that was found to interact with *abl* in a genetic screen is *enabled* (*ena*), an F-actin binding protein. Interestingly, heterozygosity for ena was found to alleviate the embryonic lethality of flies that are homozygous mutant for *abl* and heterozygous for an HDA locus such as *nrt* or *trio*, allowing them to survive to the pupal stage or to fertile adults (Gertler et al., 1990). Therefore *ena* acts opposite of HDA loci, as a suppressor of *abl* phenotypes. The *Drosophila* Ena homologues include the three mammalian family members VASP, Mena, and EVL (Ena-VASP-like). Ena/Vasp have a large number of biochemical functions arising from the various protein binding domains in its structure, including EVH1, EVH2, and polyproline region. EVH1 localizes Ena to focal adhesion by binding zyxin and vinculin and it localizes Ena to neurites by binding receptors such as Robo and Sema. It can also localize Ena elsewhere by binding Proline rich motifs on other proteins. The polyproline domain of Ena binds to the SH3

domains of Abl and Src tyrosine kinases and to profilin, while EVH2 motif contains G and F actin binding regions; in these ways directly linking Ena to the actin cytoskeleton. Currently, it is hypothesized that Ena serves three main biochemical functions. First by binding to F-actin barbed end it promotes actin polymerization. Second Ena helps to cluster the barbed ends to promote filament bundling for filopodia formation (Applewhite et al 2007). Indeed, Ena can be found at the tips of filopodia in migrating cells *in vivo* (Nowotarski et al 2014), but also at the tips of lamellipodia and filopodia in primary cultured neurons (Lanier et al 1999). Third, Ena antagonizes capping by actin Capping Protein *in vitro* and *in vivo* (Mejillano et al., 2004, Gates at al 2009). By promoting long, thin, bundled filaments, Ena at the leading edge of the cell increases cell speed at first, but ultimately the protrusions are withdrawn as the filaments are less stable. Reduction of Ena on the other hand promotes short branched filaments that protrude slower, but are more stable, ultimately having a stronger positive effect on cell motility (Bear et al 2002).

Ena is important for axon guidance and cell migration in both mice and *Drosophila*. FP4-mito is a construct containing poly-Proline motifs that bind to the EVH1 domain of Ena, sequester Ena to the mitochondria and essentially render the cells Ena-null like. In an experiment in which the developing neocortex was injected with an FP4-mito, pyramidal neurons did not migrate to the correct layers showing that Ena is essential for neuronal migration in vertebrates (Goh et al. 2002). Furthermore *mena* mouse mutants have axonal guidance defects in the formation of the corpus callosum, hippocampal commissure, and pontocerebellar fiber bundles (Lanier et al. 1999). Fruit fly *ena* mutant embryos display CNS defects including misrouting of all axon tracks and thinning of longitudinal tracks (Gertler et al 1995). Loss of *ena* causes a severe bypass defect, in which the ISNb PNS axon tracks follow the ISN nerve trajectory and

miss their muscle targets (Wills et al. 1999). In fruit flies, Ena, similarly to Abl, also participates in the development of many other events in embryogenesis, including the syncytial divisions, cellularization, dorsal closure, germband retraction, segmental groove retraction and head involution (Gates et al 2007).

What is the role of Ena in the Abl signaling pathway? Ena is a downstream antagonist of Abl, as suggested by *ena* acting as an *abl* mutant phenotype suppressor. Work by Grevengoed et al in 2003 suggests that Abl regulates Ena's subcellular localization, because during the syncytial divisions and cellularization stages of early embryogenesis, Ena is re-localized from the cell cytoplasm to the apical cortex in *abl* mutants. Peifer and co-workers argue that this localization of Ena is the key function of Abl, as the aberrant actin structures seen in *abl* mutants are formed precisely at the ectopic sites of Ena accumulation, and reduction of that ectopic Ena suppresses the actin defects of *abl* mutants. Furthermore, Abl phosphorylates Ena on six Tyrosine residues *in vitro* and *in vivo*. This phosphorylation is required for adult fly viability. Also Abl phosphorylation of Ena prevents binding of Ena to SH3 domains *in vitro*, thus this phosphorylation may be important for Ena's protein interactions (Comer et al 1998). Ena co-localizes with Abl in the axon tracts in Drosophila CNS (Gertler et al 1995). Our lab has shown that *ena* heterozygous mutations enhance the $Notch^{ts}$ bypass phenotype (Crowner et al 2003). Dab^{mz} maternal zygotic mutant alters Ena localization during cellularization (Song et al 2010) and in the eye disk in the larvae (Kannan et al 2014), thus showing that Dab is an upstream regulator of Ena, and reinforcing the downstream role of Ena in the Abl signaling pathway.

In summary the current model of the Abl signaling pathway in Drosophila axons and its regulation by Notch is as follows. Notch at the membrane antagonizes the Abl signaling module in the cytoplasm and binds to Dab and Trio which are two bifurcating pathways upstream of Abl

and Rac, respectively. Dab directly regulates Abl localization, while Trio is a GEF for Rac GTPase. Downstream, Abl negatively regulates Ena via excluding it from its locations in the cytoplasm, or via phosphorylation, or direct binding. Ena and Rac modify the actin cytoskeleton to promote either the formation of filopodia or lamellipodia respectively, thus creating a molecular switch that allows dynamic modulation of the actin cytoskeleton (**Figure 1**).

Section 1.2 Disabled

In this project our goal is to understand the role of Disabled (Dab) in *Drosophila* development. It is plausible to speculate that Dab is a scaffolding protein that acts as an adapter to link multiple receptors to downstream signaling events. As discussed below, Dab is important in neuronal development. In mammals it regulates neuronal migration and in *Drosophila* it is important for axon guidance, specifically in its role as signal transducer in the Abl kinase signaling module.

In *Drosophila*, Dab was originally found in a genetic screen for enhancers of *abl* mutant phenotypes. Heterozygous mutation of any of various Abl-interacting genes, such as *nrt* or *trio*, causes haploinsufficiency dependent on Abl (HDA), and overexpression of *dab* suppresses that HDA effect (Gertler et al 1989; Liebl et al 2003). Dab and Abl are expressed in the embryonic CNS during all stages of embryogenesis and flies that are mutant for both have increased breaks in their embryonic CNS axon tracts (Gertler et al 1993). Much evidence suggests Dab interacts with a number of proteins in the Abl signaling pathway. Dab has potential binding sites for Abl SH3 domains. Dab binds to Notch as assayed with pure protein, with *in vitro* translated protein

and by co-IP out of WT fly extracts (Giniger 1998; LeGall et al 2008). Moreover, deletion of the Dab binding site from a Notch transgene selectively impairs the ability of that protein to support Notch-dependent axon guidance without affecting Notch cell fate control. Conversely, a *dab* transgene expressed in neurons specifically enhances the expressivity of the bypass phenotype of N^{ts}. This evidence supports that Dab not only binds to but also interacts antagonistically with Notch (LeGall et al 2008).

Dab^{mz} (maternal and zygotic) mutant was made in the Giniger lab, discussed in Song et al 2010. Two deletion alleles were made by mobilizing different P elements that were inserted in the 5' region of the *Dab* gene and isolated by imprecise excisions. Both alleles have deletions that span 5' promoter region and the first exon encoding the translation start and lack detectable protein by Western blot in head lysates. Dab^{mz} line is a hetero-allelic combination of both alleles. While overall Dab^{mz} mutant is viable to adulthood, Dab still plays important roles in the development of many fly tissues, suggesting yet unexplored redundant pathways to compensate for the arising defects. *Dab* mutant phenotypes have been characterized most thoroughly in four contexts and we will consider them in turn. First, Dab regulates axon guidance in the peripheral nervous system. In ISNb and SNa nerve growth dab^{mz} mutants have the same "stalling" defects as do *abl* mutants, in which ISNb fails to make the innervation into muscle 12 and SNa falls short of its targets muscles 23 and 24 (Song et al., 2010). These defects are further exacerbated in the *dab* mutant with one deleted copy of *abl*, further supporting the genetic interactions between *abl* and *dab*. Second, Dab affects larval eye development. Dab phosphorylation regulates nuclear position in the photoreceptor neurons of the larval retinal neuroepithelium. As such, expression of phosphorylation-competent mouse Dab1 rescues a nuclear misplacement phenotype in photoreceptor cells that is produced by altered activity of the Dynactin subunit, Gl, restoring

nuclei to their normal position near the apical surface of photoreceptor cell bodies. However, the unphosphorylatable *dab-5F* (Phenylalanine) mutant could not rescue this nuclear migration defect and the nuclei were seen in the optic stalk (Pramatarova A et al., 2006). Interestingly, nucleokinesis has been previously shown to contribute to neuronal migration. Dab also regulates the localization of Abl in these eye disk cells. In WT, Abl is localized cortically, while in dab^{mz} Abl is much more diffuse throughout the cell body (Song et al 2010). On the other hand, *Abl* mutants have WT Dab localization, supporting *dab*'s position genetically upstream of *abl* (Song et al., 2010). Dab is also important in the eye disk because loss of Dab in dab^{mz} leads to increased number of Golgi cisternae which are redistributed from everywhere in the cell body to the most basal portion of the cell body (Kannan R et al., 2014). The third context in which Dab was found to play a role is mid cellularization, when the surface of the early embryo is homogeneous, with dividing nuclei that are being separated into single blastoderm cells by invaginating membrane furrows. At this stage, unlike WT, dab^{mz} embryos have patches of pseudocells that are missing nuclei. Within those patches peripheral actin levels are lower than WT, Abl levels are similar to WT, but Ena levels are significantly elevated. In normal looking cells with nuclei, outside of these patches, actin and Ena are at WT levels, but interestingly Abl levels at cell junctions are increased significantly. This supports the notion that Dab regulates Abl and downstream Ena levels in these cells. Finally, Dab is also important during dorsal closure in late embryonic development. Dab^{mz} mutants, like abl^{mz} mutants (Grevengoed et al., 2001), have defects in zippering of the dorsal epithelium including breaks in and a jagged leading edge, improper elongation of the leading edge cells and severely disrupted peripheral localization of Ena in amnioserosal cells (Song et al., 2010).

In mammals Dab has been shown to play a key role in the Reelin signaling pathway that regulates cortical glutamatergic neuronal migration and is important for organization of the neocortex into six distinct layers. Dab acts downstream of the secreted protein, Reelin, described below. Reelin mouse mutant, called *reeler*, exhibits developmental and behavioral defects which are studied as a model of human lissencephaly. Lissencephaly, caused by a number of genes, is a group of neuronal migration disorders that are characterized by the smooth appearance of the brain and improper neuronal positioning. Human *reelin* mutations for example result in autosomal recessive lissencephaly with cerebellar hypoplasia (Hong et al 2000). Mouse *reeler* mutants display disrupted neocortical development. During normal development at embryonic stages 10 through 18 the layers form in an inside-out pattern. Waves of newly born postmitotic cells travel past their predecessors, forming layers II-VI, with the youngest cells closest to the marginal zone and the subplate older cells ending up closest to the ventricular zone. In *reeler* mutants, the inside out pattern is disrupted. The cells are unable to travel past the earlier born layers. However, the migrations of cells occur during correct times in development. There are also defects in the cerebellum, the hippocampus and other brain structures. These mice are ataxic. Spontaneous *mdab1* mutations such as *scrambler* and *yotari* mice as well as targeted mutations in *mdab1* result in ataxia and the same neuroanatomical defects as *reeler* (Gonzalez et al 1997, Sheldon et al 1997, Howell et al 1997b).

The vertebrate and *Drosophila dab* genes have related structures and the N terminus is highly conserved in sequence. As in *Drosophila dab*, the mouse *mdab1* and *mdab2* genes express proteins with a highly conserved phosphotyrosine-binding (PTB) domain in their first 200 residues, and with diverging C termini that have small regions of similarity throughout. Unlike other PTB containing adaptor proteins, the PTB domain of Dab binds unphosphorylated ligands.

PTB binds to an internalization sequence, Asn.Pro.X.Tyr (NPXY), located in the intracellular domains of the LDLR receptor family, in particular VLDLR, ApoER2 and amyloid precursor protein family, and can simultaneously bind to lipid bilayer phosphoinositides (Homayouni et al 1999, Howell et al 1999b, Trommsdorff et al 1998). The tertiary structure of the PTB domain resembles the pleckstrin homology (PH) domain (Howell et al., 1999). The current signaling model suggests that Reelin is secreted by Cajal-Retzius cells to activate neuronal migration in the forming neocortex. Dab1 is expressed in the migrating neurons. Upon binding of Reelin to lipoprotein receptors VLDLR and ApoER2, Dab is phosphorylated on five Tyrosine residues that are clustered close to the PTB domain. This phosphorylation is important for cell migration, as a 5Y (Tyrosine) to 5F (Phenylalanine) mutant, that is not phosphorylatable, is ataxic and has *reeler*-like defects (Howell et al., 2000).

Interestingly, Reelin regulates levels of Dab, because *reeler* brains have significantly overexpressed levels of Dab, although the level of phosphorylation on Tyrosines is lower (Rice et al 1998). Tyrosine-phosphorylated mDab1 associates with the SH2 domains of nonreceptor Tyrosine kinases Src, Fyn and Abl (Howell B.W., et al., 1997a) and binds to a number of downstream effectors including PI3Kinase and Notch (Bock HH, et al., 2003, Hashimoto-Torii K, et al., 2008).

The structure of Dab suggests that it is a scaffolding protein that acts as a link to connect multiple receptors to kinase cascades, to ultimately reshape the actin network. In this work we aim to understand how the structure of Dab affects its function and its interaction with Abl kinase signaling module specifically.

CHAPTER 2: *DISABLED* DOMAIN MUTANTS

Introduction

As described previously, Disabled (Dab) is an adaptor protein that acts upstream to regulate Abl and Ena localization in multiple *Drosophila* tissues during development. Furthermore, it is important for axon guidance of peripheral axon tracts in the embryo and interacts with Abl pathway genes in this context (Song et al 2010). Dab's structure is important for biochemical interactions with proteins in the pathway. Dab PTB domain pulled down purified Ram23 Notch domain *in vitro* (Giniger 1998). Furthermore Dab co-immunoprecipitates from embryo and head lysates with Notch and Trio (LeGall et al 2008). Also the Dab sequence has putative binding motifs for Abl SH3 domains. This evidence suggests that Dab may act as a scaffold in the Abl signaling pathway in *Drosophila* axon guidance. We asked the question of what domains of Dab are essential for these functions and for accumulation of Dab in axons of the embryonic CNS. For this purpose we designed Dab deletion DNA constructs based on an analysis of the protein domains and established fly lines that express various partially deleted derivatives of Dab. These lines were analyzed by genotyping and expression of Dab by anti-Dab immunostaining in the late stage embryos. Expression of two of the derivatives was confirmed by Western blot. Other constructs remain to be tested for expression. Future experiments will determine what domains of Dab are necessary and sufficient to rescue dab^{mz} axon and cellularization phenotypes. For this,

transgenes directing expression of Dab derivatives will be introduced into the *dab*^{mz} mutant background and assayed for levels of defects compared to the *dab*^{mz} mutant alone.

Section 2.1 Methods

DNA constructs: We utilized the Gateway system (Invitrogen) using PCR8 as entry vector and pAttB UASp GW Gateway destination vector (Josh Currie, Stephen Rogers lab). UASp promoter was replaced with endogenous promoter for endogenously driven Dab constructs. Endogenous promoter spans the sequence from the 3' end of *lasp* gene to the second start codon of *disabled* gene, with a deleted first start ATG. FlyC31 system (flyc31.org) was used to recombine *dab* cDNA from pAttB UASp GW vector into chromosome 2 site 51C (BestGene). Primers were 1. Full length: F: 5'-CACC GTC AAG TCC CTG GT-3' R:5-CTA CCA CAG CAC ATC ATA ATC GT-3', 2. PTB domain: F: 5'-CACC GTC AAG TCC CTG GT-3' R: '5-CTA GAT CTG CTG GCG CGC-3' 3. PTB+Center domain: F: 5'-GTC AAG TCC CTG GTG GC-3' R: 5'-CTA CCC ATC GTA GTC GTA GTC C-3' 4. Central domain+C terminus F: 5'-CAG GGT AAG TCC CTG CAC G-3' R: 5'-CTA CCA CAG CAC ATC ATA ATC G-3' 5. 5. PTB+ C terminus: We created ecoRI cut site after PTB domain and using ecoRI site after Center domain, cut out Center domain in full length construct. 6. C terminus F: 5'-GAA TTC GTT GCA CGC CGA G-3' R: 5'-CTA CCA CAG CAC ATC ATA ATC G-3'.

Genotyping: Used 10 males and 10 females ground up with a pestle. DNA was lysed using DNAzol reagent (Life Technologies). Sequencing is by ACGT, Inc.

Westerns: Overnight embryo lysates (~50ul) were separated on SDS polyacrylamide gels, transferred to nitrocellulose, probed with mouse anti-Dab P6E11 Ascites (Hybridoma Bank, Iowa, USA) 1:3 (Le Gall M., et al., 2008) and mouse anti-β tubulin (E7 Hybridoma Bank, Iowa, USA), and detected by peroxidase –coupled chemiluminescence (Lumigen).

Immunohistochemistry: Stage 17 (12-14hr at 25^{o}C) embryos (~30ul) were fixed with 4% Paraformaldehyde, stained with antibodies and visualized by immunofluorescence with appropriate secondary antibodies. Antibodies included polyclonal rabbit anti-Dab 1:1000, chicken anti-GFP 1:10000 (Aves Labs, Inc.), and mouse anti-FasII (1D4, Hybridoma Bank, Iowa, USA) was used at 1:50 as a control. *Z* stacks were collected using a Zeiss LSM510 Confocal microscope at 63x magnification.

Section 2.2 Results and Discussion

In order to understand how Dab structure affects its functions in ISNb growth and guidance, eye disk and epithelial development, we designed Dab deletion DNA constructs, which were incorporated into the fly genomes. The constructs are based on an analysis of the protein structure and previous analysis of Dab biochemistry, see below. The fly lines were confirmed for the presence of transgenes by genotyping and expression of Dab by anti-Dab immunostaining in the late stage embryos. We were also able to test two of the fly line domain mutants for protein expression by Western blot. These fly lines can also be used in the future to test what domains of Dab regulate Abl localization, activity and its interaction with downstream proteins.

In order to create the constructs we first did a phylogenetic and structure analysis of the Dab protein sequence. In *Drosophila*, there is one *dab* gene that has at least eight splice isoforms, currently annotated on Flybase (**Figure 2A**). There are four major splice regions accounting for the isoforms. Firstly the start site in isoforms C,D, and G is 5' to the start site in isoforms A,E,H, F, I. Second, the 5th exon is spliced out in isoforms C, H and E. Third, the 6th exon is longer on the 5' end in isoforms D, G, F, H, and A. Lastly, the 10th and last exon is longer on the 3' end in isoforms G and F (**Figure 2A**). We sequenced and analyzed the full length cDNA our lab received from the Gertler laboratory. Its exon structure appears to originate from isoform E (Dab-RE on Flybase.org) and corresponds to the cDNA MIP12186 submitted by the Berkeley Drosophila Genome Project .This cDNA isoform was previously shown to rescue dab^{mz} embryonic motonerve defects, and codes for a functional protein (Song et al., 2010). All of the isoforms code for proteins containing a PTB domain highly conserved with vertebrates (**Figure 2C**). Adjacent to the PTB domain the N terminal 2/3 of the protein is highly conserved with other insects. We refer to this domain as the Center domain. The Center domain has many polyproline sequences, nine of which are putative SH3 Abl binding sites that are located within scattered conserved motifs in this region of the protein (**Figure 2D**). The conserved motifs are embedded in short, conserved sequences that are predicted to form secondary structures such as a helices and β-sheets. The C terminus region is conserved only in *Drosopholids* and is not predicted to form extensive secondary structures. It contains three repeating 11-amino-acid motifs that consist of alternating Glutamic or Aspartic acid and Arginine residues (Gertler et al., 1993). The entire span of the protein contains 122 phosphorylatable Serines, 35 Threonines and 30 Tyrosines, including some in consensus Abl phosphorylation motifs (NetPhos 2.0 Server). In all isoforms, one putative Abl phosphorylation site is located in the PTB domain and one in the

Center domain. In isoforms G and F which have longer 3' ends, there is a third site in the longer 10th exon (http://kinasephos.mbc.nctu.edu.tw) (**Figure 2A, B, and C**).

Based on this sequence analysis, we decided to split Dab into three major domains: the PTB domain, the Center domain and the C terminus. Therefore the deletion derivatives included 1. PTB domain alone, 2. PTB+ Central domain, 3. PTB+ C terminus, and 4. Central domain+C terminus 5. C terminus and 6. Full length (**Figure 3 and Table 1**). We chose to truncate the Center domain at the same point as a peptide used to make anti-Dab antibodies (Le Gall M, et al., 2008).To be noted, recently available phylogenetic data (Blast NCBI) also suggest that the conserved domain may extend 300 codons beyond the endpoint of the Center domain in my initial set of constructs, which may be taken into consideration for any future set of deletion constructs (**Figure 2C**).

We expressed the transgenes under the Dab endogenous promoter to recapitulate WT Dab protein levels for rescue. Full length Dab was also expressed under the UAS promoter which can be driven via GAL4 in a variety of tissues. To prepare the constructs, we utilized the Gateway system (Invitrogen), which allows easy shuttling of transgenes into vectors with different tags and promoters. We used the Destination vector pAttB UASp GW, (Josh Currie) for transgene injection into embryos. Its features include the *K10* gene terminator, which allows protein expression in the germline, and N-terminal eGFP, for live imaging and immunohistochemical analysis. An N terminal GST-Dab fusion was shown to rescue motonerve defects to the same level as an untagged construct or a genomic duplication (Song et al., 2010). All transgenes were recombined via an attB sequence onto chromosome 2 site (51C) via PhiC31 integrase technology, which avoids possible differences in genomic positional effects between transgenes.

The transformants were subsequently bred into stable balanced viable lines and confirmed by genotyping.

The *dab* fly mutant lines were assessed for expression of Dab protein by immunostaining and western blot. We found that eGFP and Dab are co-localized with FASII in the embryonic CNS and PNS axon tract bundles at stage 17 in endogenously driven Full length, PTB+Center, Center+C terminus and Full length driven by elav-Gal4 expressing embryos (**Figure 4A**). This confirms previous findings that Dab is expressed in axons (Gertler et al 1989) and shows that the endogenous promoter works to localize the Dab protein domains in the expected time and place in development. We also confirmed protein expression of full length Dab and Dab PTB+Center domain driven by the endogenous promoter in embryonic lysates on Western blot (**Figure 4B**). The full length and endogenous proteins are indicated by 256kD band while the conserved PTB+Center domains peptide is 178kD. Future work, in particular Western blots, will have to resolve if expression of other domains mutants, such as the PTB domain and the C terminus, is at the same or similar level as Dab full length, in order to have an even level of comparison of rescue effects of dab^{mz} mutant phenotypes by different Dab domains. This protein expression analysis will have to be performed using antibodies to eGFP, instead of to Dab, because we previously found that the anti-Dab P6E11 antibody binds to an epitope in the Center domain.

In summary, we have constructed flies expressing different domains of Dab. We confirmed that these domains are expressed in the nervous system in the late stage embryo. The full length and conserved region of PTB+Center domains are expressed at detectable levels by Western blot from embryo lysates. Future work will use these fly mutants to analyze what domains of Dab are important for its function in the nervous system and in epithelial development.

CHAPTER 3: *DAB*^{MZ} MATERNAL ZYGOTIC MUTANT RE-ISOLATION AND CHARACTERIZATION

Introduction

Since we made *dab* domain mutants, we want to test what domains of Dab are important in guidance of axon tracts in the peripheral nervous system, as well as what domains are important in eye disk development, cellularization and dorsal closure. For these purposes Dab domain mutants have to be tested in the level of rescue of *dab*^{mz} phenotypes. We have to create lines bearing a Dab cDNA insertion on the 2nd chromosome and *dab*^{mz} deletion on the 3rd chromosome. Originally, *dab*^{mz} was made by a heterozygous combination of two deletion alleles, *dab1* and *dab2*, that were each created by imprecise excisions via mobilization of two P-elements P{EPgy2}Dab[EY10190] and P{XP}Dab[d11255] (Song et al 2010). *Dab*^{mz} embryos exhibit an ISNb nerve stalling defect in which the axon bundles fail to reach the cleft of muscles 13/12 in 46% of hemisegments. A similar expressivity is seen in *dab*^{1}/*dab*^{1} embryos from homozygous *dab*^{1} mothers. The stalling defect is further enhanced to 77% of hemisegments upon removal of one copy of *abl* using *Df(3L)std11* allele. Another phenotype characteristic of *dab*^{mz} is in the larval eye disk photoreceptors. Here Ena, a downstream effector of Dab and Abl, is re-localized from puncta everywhere in the cell body to basal accumulation in the cell body where it co-localizes with Cis-golgi marker (Kannan et al 2014). We tested whether *dab*^{mz} mutant still demonstrated the 46% ISNb stall phenotype in the late stage embryo, as shown by Song et al in

2010. We found that in the original *dab*^{mz} the stall defect was the same level as WT, <1%, even though the *dab* deletion is present, as assayed by PCR. We therefore suspect that a suppressor mutation has overtaken the stock over time resulting in inhibition of the stall phenotype. Interestingly, we also found by genotyping that the *dab*^{mz} that originally was *dab*^{1}/*dab*^{2} is now mostly *dab*^{1}/*dab*^{1}.

As a result of this finding we wanted to remake the stock in order to remove the suppressor mutation. We successfully remade the mutant *dab*^{mz} line and tested it for defects in ISNb outgrowth and eye disk localization of Ena.

Section 3.1 Methods

Genetics: *Dab*^{1}*/TM3* x *Sp/cyo actin lacZ; Dab*^{2}*/TM3*. We made lines: *dab*^{mz} line *dab*^{1}/*dab*^{2} and *dab*^{mz} line *Sp/cyo actin lacZ; dab*^{1}/*dab*$^{2.}$

DAB immunostaining and immunohistochemistry: For visualizing the nervous system in embryos, female *dab*^{mz} (line #2) x male *STD11/actin GFP* progeny embryos were collected for 12-14hr, fixed and stained by standard methods (Bodmer et al., 1987). Antibodies used were anti-FasII (1D4, Hybridoma Bank, Iowa, USA) 1:50 and chicken anti-GFP 1:5000 (Aves Labs, Inc.) as a marker of the balancer chromosome. Biotinylated secondary antibody (Jackson ImmunoResearch Laboratories) staining was amplified using Vectastain Elite tertiary reagent (Vector Labs) and visualized using 3,3-diaminobenzidine (DAB). Embryos were filleted in 90% glycerol in PBS and examined by Nomarski microscopy. For eye disc immunostaining, late third-instar larval eye disk and antennal discs were dissected with the two brain lobes and fixed

in 4% paraformaldehyde for 20 minutes at room temperature. The antibodies used were: mouse anti-Ena (1:50) (5G2, Hybridoma Bank, Iowa, USA) and rabbit anti-GM130 1:100. Z stacks were collected using a Zeiss LSM510 Confocal microscope at 63x magnification.

ISNb stall counting: Hemisegments A2-A7 were used for analyzing Intersegmental nerve b (ISNb) phenotypes. 204 hemisegments were analyzed.

Genotyping: Used 10 males and 10 females ground up with a pestle. DNA was lysed using DNAzol reagent (Life Technologies). Primers used were F: 5'-tgcgccgttattttcgag-3' and R: 5'-gcctgctcgttccattcg-3'. Sequencing is by ACGT, Inc.

Section 3.2 Results and Discussion

In order to remove the suppressor mutation that inhibits phenotypes previously shown in dab^{mz} animals, we re-made new dab^{mz} fly lines. We crossed balanced Dab^{1} ($Dab^{1}/TM3$) and Dab^{2} (*Sp/cyo actin lacZ;* $Dab^{2}/TM3$) alleles, isolated dab^{1}/dab^{2} males and females and crossed them to generate a stock. After a number of generations, however, genotyping shows that all lines created are now mostly dab^{1}/dab^{1}. For reasons we do not understand, the Dab^{2} allele seems be selected against in the mixed stock, though, genotyping does still show the presence of some dab^{2}. In order to determine whether the suppressor mutation does not occur in the new stock, we checked the stalling defect in line dab^{1}/dab^{2}. We crossed female dab^{1}/dab^{2} with male *STD11/actin GFP* with the expectation that 50% of the embryos will have WT looking hemisegments with ISNb nerves making innervations into the cleft of muscles 12 and 13, while 50% of embryos will have a stalling defect in approximately 70% of hemisegments. Unfortunately, the actin-GFP marker

on the balancer could not be scored reliably at stage 17, when the phenotype is manifest. Therefore, we counted all embryos, expecting to see a mixture of heterozygous over balancer and homozygous mutant embryos. We saw two populations of embryos. In 8 embryos, expressivity of stall defect was 89%, in line with dab^{mz}. In 10 embryos, expressivity of stall defect was 7%, in line with WT. Total number of hemisegments was 204. This is consistent with having homozygotes that are true dab^{mz} (**Figure 5A**).

To further examine the new dab^{mz} lines, we looked at the localization of Enabled and cis-golgi in larval eye disk photoreceptors. Previously it was shown that in WT photoreceptors, Ena is co-localized with the cis-golgi marker GM130 in puncta throughout the soma. In dab^{mz}, Ena and cis-golgi are re-localized to the basal region of the soma (Kannan et al 2014). In the analysis of the new *dab* mutant lines, we indeed see co-localization of anti-Ena marker with GM130 and re-localization of these markers to the basal region of the soma as compared to WT (**Figure 5B**). In summary, we have remade new dab^{mz} lines that recapitulate the phenotypes published in the lab previously.

CHAPTER 4: DAB REGULATION OF ABL LOCALIZATION AND ACTIVITY IN S2 CULTURED CELLS

Introduction

In our analysis of the role of the Dab protein in the Abl signaling module during development, previous experiments established that Dab regulates Abl localization (Song et al 2010).The absence of Dab protein in *dab*^{mz} maternal zygotic fruit fly embryos causes inhomogeneity in Abl localization during the midcellularization stage of early embryogenesis, as discussed in the Introduction (JK Song et al 2010). Disabled structure, such as its polyproline domains which are potential SH3 binding regions, and the PTB domain, suggests that Dab may act as a scaffold and can potentially recruit and localize Abl, by binding it directly or in a complex and may regulate its ability to bind to other proteins. We also wondered whether Dab regulates Abl kinase activity.

We decided to perform a structure-function analysis to determine what domains of Dab may regulate Abl localization. For this purpose we used the eGFP fusion Dab domain constructs we developed (**Figure 3 and Table 1)** to transiently transfect cells in culture and subsequently assay Abl localization by immunoflourescence. We use Schneider 2 (S2) cell line, a *Drosophila* cell line derived from a primary culture of late stage (20-24 hours old) embryos (Schneider, 1972). It appears to have a macrophage-like lineage. S2 cells grow in suspension at room temperature

without CO2. They can be induced to adhere weakly and spread on a surface coated with polylysine and concanavalin A. They are not motile.

To assay Abl activity, we took advantage of a FRET based Abl kinase reporter, previously made in the lab by Ram Kannan. It contains an Abl-phosphorylatable Tyrosine and the cognate SH2 and SH3 domains (from c-Crk II), linked to CFP and YFP (**Figure 6**). It is a UAS construct, modified from Ting et al., 2001, which can be driven in the cells by a GAL4 expressing construct. Seven lines of evidence suggest that this is a faithful reporter of Abl activity: it produces a FRET in WT cells in culture, the FRET signal goes up if Abl is overexpressed or activated, it goes down in *abl* mutant cells, it is reversibly inhibited by application of a specific chemical blocker of Abl kinase, a reporter derivative lacking the phosphorylatable Tyrosine fails to show FRET activity, photobleaching of the FRET acceptor chromophore enhances the fluorescence yield of the FRET donor chromophore, and it shows a FRET signal in embryos that is consistent with the known pattern of Abl activity (Kannan, et al., ms in preparation).

Section 4.1 Methods

S2 cells were cultured in SF900 II media (Life Technologies cat # 10902) with Antibiotic-Antimycotic (Life technologies cat # 15240-062) and split every 4-5 days from 10 million cells to 2 million cells. At 5-8million cells/ml the cells were transfected with DNA constructs by using DDAB reagent (Han Kyuhyung 1996) and consequently stored at RT. Dab Constructs include UAS-eGFP-Dab FL, UAS-FLAG-Dab FL, UAS-GST-Dab FL, UAS-eGFP- Dab PTB+Center, UAS-eGFP-Dab Center+C terminus, UAS-eGFP-Dab ΔCenter and UAS-eGFP-Dab C terminus.

We used Actin-Gal4 as a driver construct and UAS-Abl-FRET for FRET analysis. 72 hours post transfection, 50ul of cells were fixed with 4% PFA in PBS on 1:1 0.5mg/ml polylysine L-Concanavalin A coated coverslips. Subsequently they were stained and imaged. The antibodies used were rabbit anti-Dab 1:200, rabbit anti-Abl 1:100, mouse anti-Flag 1:500 (M2, Sigma), chicken anti-GFP1:5000 (Aves Labs, Inc**.),** WGA-Alexa Fluor 647 125ug/ml (Invitrogen), Dapi 1:1000, rabbit anti-GM130 1:100, syntaxin 16 1:50, rabbit anti-Rab5 1:100 (Abcam), LysoTracker Red DND-99 400nM (Invitrogen). Imaging was performed on Zeiss confocal microscope using 63x oil objective.

Ratiometric FRET imaging was performed by exciting donor CFP and collecting the emission from acceptor YFP as the FRET channel. In addition, we also independently excited donor CFP and acceptor YFP and collected emission from donor and acceptor using appropriate laser lines. Ratio FRET is calculated separately for each image plane by dividing the average intensity from FRET channel to CFP channel after subtracting the background values from the respective channels, and then averaged across the Z axis for all planes for a given cell. Nuclear signals often show artifactual signal in the FRET channel unrelated to Abl activity and were excluded from the analysis.

Section 4.2 Results and Discussion

Disabled and Abl protein localization in cultured cells

We first determined levels of endogenous proteins by immunostaining WT S2 cells in culture with Dab and Abl antibodies. Plating the cells on polylysine + conA causes them to spread,

causing a rounded appearance with some cells having protruding filopodia and lamellipodia. Dab is expressed throughout the cell cytoplasm. Dab immunoflourescent signals are weak compared to those observed in embryos or imaginal discs, suggesting that the expression level of the protein is rather lower than that in developing animals. Unlike Dab, we found that Abl is abundant throughout the cytoplasm and the localization pattern is variable throughout the cell. Moreover some cells have higher and some have lower levels of Abl expression. Abl can be found in puncta of different sizes, homogeneously throughout the cytoplasm and also at the cell cortex (**Figure 7A**).

S2 cells were co-transfected with UAS-eGFP-Dab + actin-Gal4, fixed, and visualized by double labeling with anti-GFP and anti-Dab. In another experiment we co-transfected UAS-Flag-Dab and actin-Gal4, fixed and visualized by double labeling with anti-Flag and anti-Dab. When Dab was expressed from UAS under control of a co-transfected actin-Gal4, the immunofluorescence levels were substantially higher, suggesting significant overexpression compared to endogenous Dab. We saw that overexpressed eGFP-Dab and Flag-Dab in these cases coalesces into puncta throughout the cytoplasm (**Figure 7B**). We tested if the Dab puncta co-localize with organelles in the cell. We tested co-localization with GM130, a marker of cis-Golgi, syntaxin 16, a marker of trans-Golgi, Rab5, an early endosome marker, and lysotracker, a stain for the Lysosome or late endosome. The puncta appear not to co-localize with any organelles tested so far (**Figure 7C**). To test for co-localization of Dab and Abl in S2 cells, cells were co-transfected with UAS-eGFP-Dab + actin-Gal4, fixed, and visualized by double labeling with anti-GFP and anti-Abl. We found that the puncta of endogenous Abl co-localize with the Dab puncta in cells overexpressing Dab (**Figure 8A**). Next we were interested in determining what domains of Dab are responsible for Dab punctal accumulations co-localizing with Abl

endogenous puncta in S2 cells. For this we used the structure-function approach, developing UAS-eGFP-Dab deletion constructs that can be overexpressed in S2 cells via a co-transfection of an actin-Gal4 driver construct. Interestingly, we found that domain constructs containing the Center domain of Dab, including UAS-eGFP-Dab FL, UAS-eGFP- Dab PTB+Center, and UAS-eGFP-Dab Center+C terminus, all accumulated in cytoplasmic puncta marked with anti-GFP staining which co-localized with the anti-Abl puncta (**Figure 8A and Table 2**). On the other hand, when the transfected Dab construct does not contain the Center domain, such as UAS-eGFP-Dab ΔCenter and UAS-eGFP-Dab C terminus, Dab localizes to fewer, one to five, puncta and sometimes to the cell cortex. Furthermore, these puncta do not co-localize with Abl endogenous puncta, which are excluded from the nucleus (**Figure 8A**). These Dab puncta appear to be inside the nuclear envelope, visualized by the wheat germ agglutinin staining (**Figure 8B**). Interestingly, using NetNES 1.1 Server online software we found that Dab contains a potential nuclear export sequence in its Center domain, as well as the PTB domain (**Figure 2B**). This suggests that by removing the Center domain from Dab, it gets sequestered in the nucleus of the S2 cell. Previously it has not been shown that Dab has nucleic functions or is trafficked into the nucleus in *Drosophila*, however it has been shown that Dab affects nuclear translocation in larval photoreceptors by regulation of the perinuclear actin cytoskeleton (Pramatarova A et al., 2006). When transfecting PTB domain alone, we were not able to get any positively transfected cells.

Two observations led us to wonder whether the apparent co-localization of Abl with expressed Dab was physiologically relevant. First, the puncta of expressed Dab were highly irregular in size, shape and number (**Figure 7B**). Furthermore, we were unable to document association with any characterized endomembranous compartment (**Figure 7C)**. We therefore performed a different test to see whether Dab full length recruits endogenous Abl protein. We

created a mito-RFP-Dab FL construct which recruits Dab to the mitochondria. It appears that this modified Dab does re-localize to the mitochondria, as it is now found in small, well-distributed puncta that co-localize significantly with mitotracker (**Figure 9A,B**). However, we do not observe recruitment of Abl to these mitochondrial Dab puncta. Abl puncta are still seen randomly throughout the cytoplasm (**Figure 9C**).

The observation that Dab puncta do not co-localize with any organelle markers lead us to suspect that Dab puncta may be aggregates of Dab that do not have a physiological function. This may explain why Dab binds to Abl puncta in this *in vitro* system. In this case Dab putative SH3 binding domains may interact with Abl simply because of their abundance and it remains to be determined if indeed Dab binds to Abl *in vivo*.

Disabled regulation of Abl kinase activity

We next tested whether Dab regulates Abl kinase activity. We expressed in S2 cells a UAS-FRET probe for Abl kinase activity under actin-Gal4 control, either by itself or in cells that also express UAS-GST Dab full length. These were plated on polylysine + ConA coverslips, as above, and average FRET activity was determined. FRET measurements were made only in the cytoplasm due to background signal in the nucleus that was unrelated to Abl activity. In this experiment we observed that the FRET probe accumulates in the Dab puncta with very little probe free in the cytoplasm (**Figure 10D**). Fret measurement in the Dab overexpressing puncta was higher compared to the FRET measurement in the cytoplasm in WT S2 cells which supports increased Abl kinase activity in these regions (**Figure 10A, C**). However, we were concerned

that analysis of FRET values in cells bearing varying amounts of aggregates might skew the results artifactually (Vogel et al 2006). For example, it could be that overexpressed Dab protein may be binding the SH2 or SH3 domains in the FRET probe, thus creating the observed accumulation of the FRET probe. Recruitment of the FRET probe at high level in Dab aggregates could lead to trans interactions of CFP and YFP from multiple FRET probe molecules and thus may be a non-physiological increase in Abl activity. We therefore repeated the experiment in another cell type where the aggregation phenotype was not observed. In experiments to be published elsewhere (Kannan et al, in prep), photoreceptor neurons expressing the Abl FRET probe were isolated from eye discs of WT, Dab-overexpressing and *dab*^{mz} 3rd instar larvae, dissociated, and plated, and Abl kinase activity was assayed by FRET (**Figure 10B**). WT cultured eye disks have mean FRET 1.52838 with SEM 0.09. Dab overexpressing photoreceptors showed a significant increase in FRET signal with 2.07 mean FRET with SEM 0.12. On the other hand *dab*^{mz} photoreceptors which lack Dab protein have significantly reduced FRET signal with 0.935 mean FRET with 0.18 SEM (**Figure 10C**). This *in vivo* experiment indeed shows that Dab does positively regulate the activity of Abl kinase.

FIGURES

Figure 1

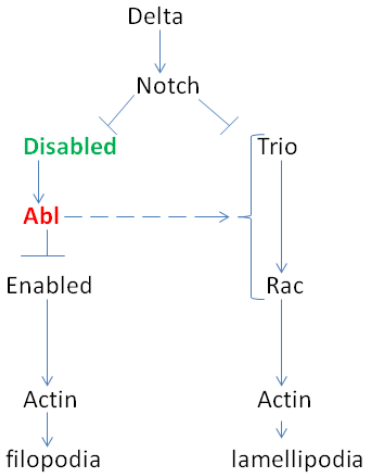
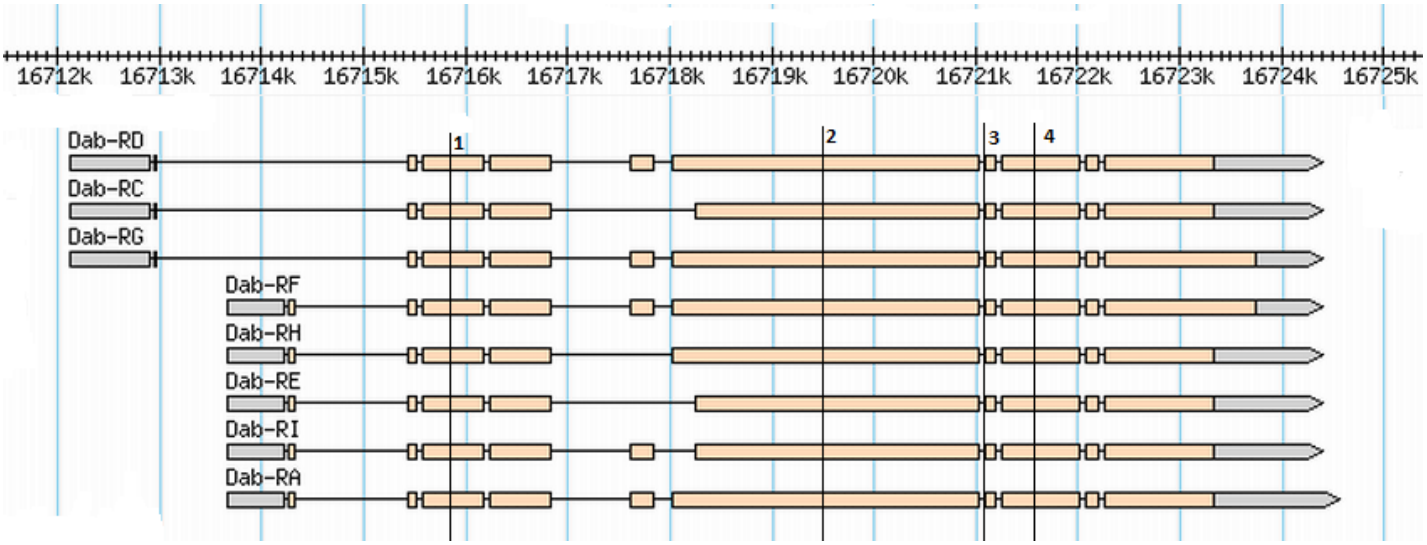


Figure 1: **Schematic of a model for the functional organization of the Abelson kinase signaling pathway in Drosophila axon guidance, and its regulation by Notch.**

Notch receptor is a negative regulator of the Abl signaling pathway. Notch binds to (Disabled) Dab adaptor protein and Trio GEF. Abl is downstream of Dab. Abl negatively regulates Enabled (Ena). Trio GEF regulates Rac GTPase. Preliminary experiments suggest an effect of Abl on Rac, dependent on Trio (Ram Kannan, in preparation). Ena promotes filopodia formation while Rac promotes lamellipodia formation. In other systems Rac was shown to work through Wave/Scar and Arp2/3. Our data is consistent with these findings (JK Song unpublished).

Figure 2

A.



B.

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MVKSLVAKLSTASSNLSLASTFGGGSGAAEETNYAKHRNDPGRFFGDGVQ    50 #1, #5
FKAKLIGILEVGEARGDRMCQEALQDLKMAIRAAGEHKQRITIHVTIDGL   100 #9, #9
RLRDEKTGDSLYHHPVHKISFIAQDMTDSRAFGYIFGSPDSGHRFFGIKT   150
DKAASQVVLAMRDLFQVVFELKKKEIEMARQQIQGKSLHDHSSQLASLSS   200
LKSSGLGGMGLGHSDLASGGISSGHALTLLGSSLSTTNGTSRLGVSLDVA   250
KASGSAAKEVSPESVADLVDLEQELTSLQRGISQMERITPNEPTTSSTGG   300
AGHPSLAKSASEDDPFGDSFIYVPSYSILPPPPDSGRNRHKPPNKTPDAV   350
TSLDAMLSPPPGTSSSHGSASAGLQAADNDDDNWLQELDQQNDVFDTSKV   400
VSSSGLGSVLAMAPLASSESTATPTQQLTEVAAGSGPLADLDIGLSTALG   450
NEEQTSTILSLEPPTLNSLENPHPPADPVLLPRDTDPFSPTRKKSDPDPF   500 #8
QESDLFAKLDAFEFEAPPAVPAPSIPNLATETKANVFNGPLQVQLPPEKE   550 #8
LQLQQPPSTVRNRPTASVSALPSGGALDVISSISNKKMPHLFGQARSFGK   600
SGSDIGSSVNMRRLQESDSLSETEAAPEPPPRPDSTPYSEPPPLPPKKQF   650 #8, #5
SDLVIRPSPANTTQPPTSGRYEYLNSNVTARRTASSVDAPPIPLPSRRVG   700 #8
RSDGCFPGPGRPRKPGHTEDDYLAPLGAPPPLLPPPSQGSSARARPQRQA   750 #8, #8
SLGRPQDIYENKAEILQAQAQAQAQAPEVAPSSNTLAPDITLTQLLTLGM   800
DDLAIKLNVPASKLSTMTLVQLTAYLSEYLSSEKSQVHSQERRSSPANTA   850 #9
PAPASTAAVFKVNFDQQTSFVAKFDDTFGEDEPVMPSGSSDSTFVANFAN   900
FNDAPTPVPTVSPVVATVPSADRYAVFREIIDQELQQQQQETDLMGDLTP   950
PPVDETQAKEISEGLEVNNVGAELPIDALDVKPAPKIDTKITEVVAQAKD  1000
RYAALRDIILVENLFDKPAIATDTQPEKEKDLLQDFPEFSDEFNEDHDLR  1050
QIMDHQNVQTHARDRHGLVDSRGFPTEPSSSALTVGDYDEDEDADAGGES  1100 #2
SLDSNEKDAEPVSGQDQYEKLSTSTQQLDAAAPALEDVQQLQQQSLPPKQ  1150
DQKFLSILTAPGGGTKDDIEIDELMHRAISNLSLDSRDRVSPATSSAAPS  1200
RGAPGLHTPSQFNDVSTSPIPLQKPGMGPSPVPSQLSAVSQLIDTATKQM  1250
MGDKDREKQSWATFDSPKAKGKARLTLPPPPPPASNTSQPDTVESPCSSD  1300 #8
PRDDGWSKQQRRWAKKERQQTSSSSRDLSPWDDETPEYLKRRQLAAAQMA  1350
```

```
HPHQPPMQAPPQHTDRHGYYMRHARRMNSCDEDYDYDGEFVARRDQPQHQ  1400 #3
QQQRKFKHGLSRSRDNFELESPSWYHHPAHHTWSPQEIEQVRVRSFDRTA  1450 #7
YERSSYGPPPPIYDKRGQLRGKYRGDHRDRERERDRDRERYRDYARPSYDF  1500 #8, #6
DYENVYEERGGRSPLAYKPGRGGGDYLYDRERDRDRERDRKSFDRESLES  1550 #6
YESATRRRRSFGSGNDVYGSLDSRDDYRGDRERDRERDREQMKTRSLRKP  1600 #6
TTTSGKLRISGDIDYEQDSEQDFQQRSGVRSLQRPNQLGGDVVLPSNAVV  1650
GPQRLRKSSGSSPWDGEEPALPGQKSWKRPASAAETERRLAESRRAVALG  1700 #8
QTPSDGEKERRFRKKTRARSAKDLATVGAPSASTSAPSRSSYGRGIRDNY  1750 #4
DYICPGQRNDDDDDDDEDYVDDEPPTDEDKFERLNRRRHEMHQRMLESER  1800
RQMERHQPPSLAKLPGQNRTRGVVANSDYGFVDSYEQTPTPTPRSNASST  1850
GPGGLMMSGGESSAGVTSSKFNFDDGFESDFNQSSPPPAPAGTASSCNST  1900
PAGPVSANANNGGSKSLFRFSNDFSDREKREQFEMDTPPTSTPPITQKLR  1950
FDDNVKVSQFDDAAFEDDFAKASFDFEKEQAGSATAGAGGSGAMSRKQNM  2000
RTSKLQQRQELIKKSESVNIFAKKQEDPFEDDEFFKSPDQEQAMDQHNDD  2050
TEGGKFQWSEDANFAKFDENM-FDQLFAKASGQRPDPDIDNHNYAEIDVV  2099 STOP
NDTDFSFLANLNHYYQQQRLHSLRQSLNRHVYCNLPVQQLQEQVNQPMKK  2149
SIATSTSPWHEPKVKSKPLQQLLRKPKKWKLKRTLDDFLKCLIIASSEHV  2199 #9, #9
YDYDVLW-                                             2206 #5 STOP
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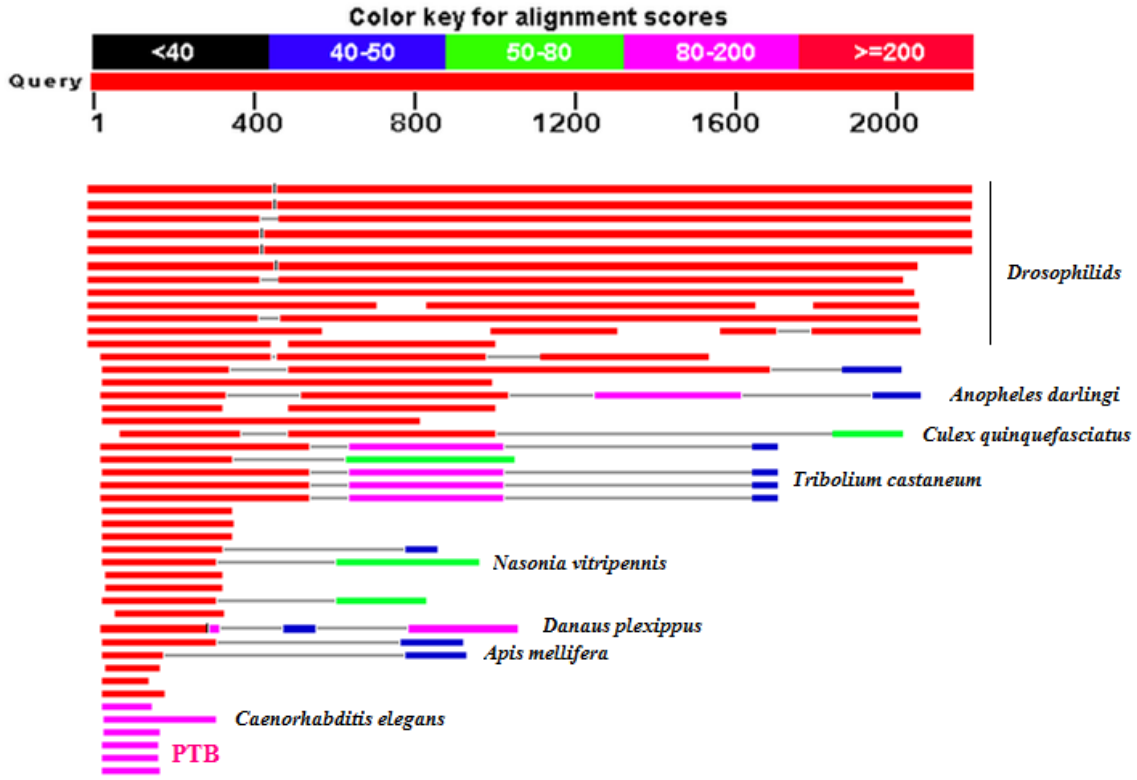
C.



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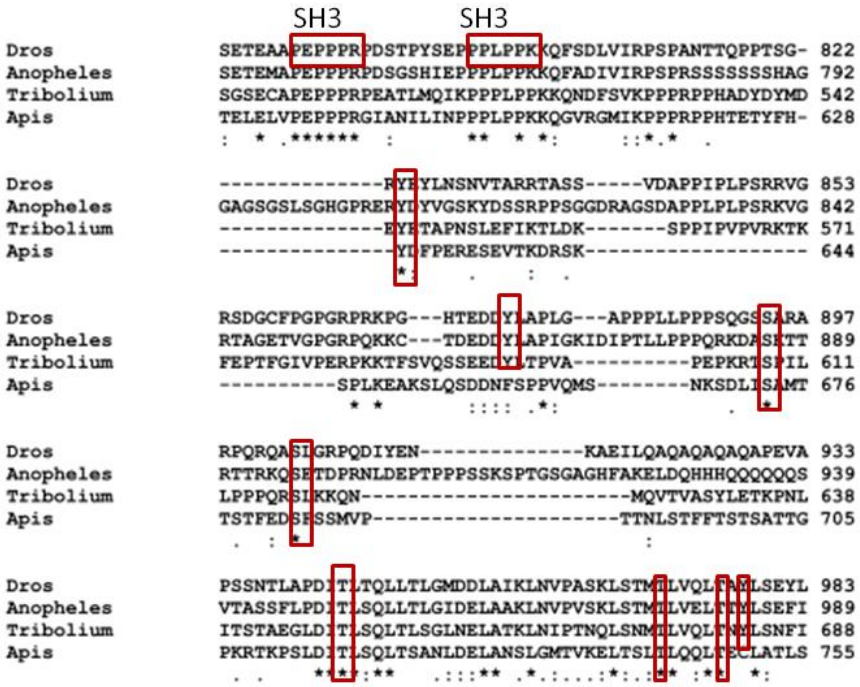


Figure 2: **Disabled: Genomic organization and predicted protein structure**
A. Dab DNA isoforms (Flybase.org). Boxes represent exons, gray represents untranslated regions; tan are predicted to be protein coding regions. **B.** Dab-RE isoform that was used as the starting point for transgenes in this study. **C.** Dab conservation (Pubmed Blast) with *Drosopholids*, other insects and vertebrates. **D.** Polyproline motifs and phosphorylatable Serines, Threonines and Tyrosines in conserved regions of the Dab Center domain.
Legend:

1. PTB domain, conserved with vertebrates, PTB K36-I174 (based on Giniger 1998)
2. Conserved with all insects, see *Danaus plexippus*
3. The Center domain ends (based on Le Gall et al 2008)
4. New conservation with some insects, see *Tribolium castaneum*
5. Predicted Abl kinase phosphorylatable Tyrosines (Y) (http://kinasephos.mbc.nctu.edu.tw/)
6. Acidic/basic repeats (Gertler et al 1993)
7. The C terminus
8. Predicted polyproline sequences that are potential Abl binding sites (http://cbm.bio.uniroma2.it/SH3-Hunter/)
9. Predicted nuclear export sequences (www.cbs.dtu.dk/services/NetNES/)

Figure 3

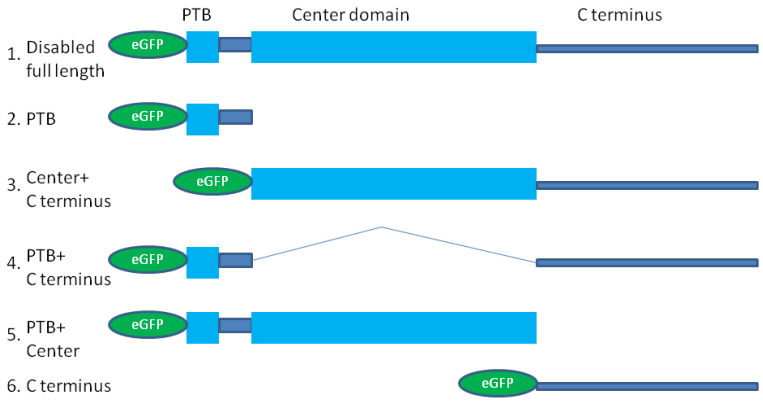
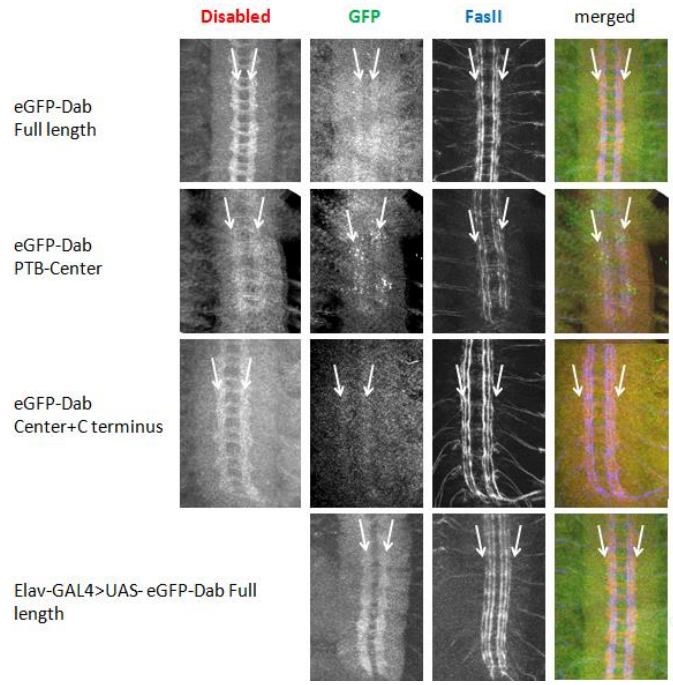


Figure 3: **Block Diagram of Dab deletion derivatives.**
All constructs have an N terminal eGFP fused to 1. Full length 2. PTB 3.Center domain+C terminus 4. PTB+ C terminus 5. PTB+ Center domain 6. C terminus of Disabled.

Figure 4

A.



B.

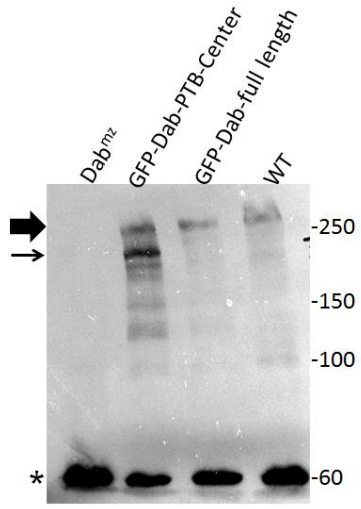
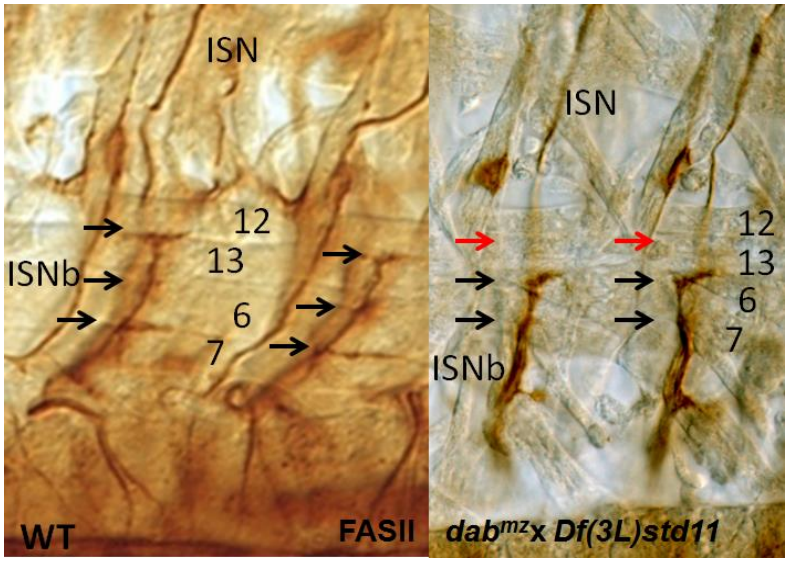


Figure 4: **EGFP-Dab transgenes are expressed under the endogenous Dab promoter in *Drosophila* embryos.**
A. Micrographs of embryos to visualize eGFP-Dab transgene expression in axon tracts in the Ventral Nerve Cord (arrows). Stage 17 embryos were fixed, stained with the indicated antibodies, dissected, Z stacks were taken with a confocal microscope, and max intensity projection was generated. Anti-Dab immunoreactivity is in red, eGFP is in green, FasII to identify axon tracts is in blue. **B.** Western blot of overnight embryos as in part A, boiled, ran on an SDS PAGE gel and probed with anti-Dab P6 antibody. Thick arrow points to position of full length Dab. Thin arrow points to truncated Dab (PTB+Center domains). Full length transgene product co-migrates with WT at the same position on the blot. Lane 1: Dab^{mz} embryos do not express Dab protein. Lane 2: Embryos expressing eGFP-Dab PTB+Center transgene and endogenous WT Dab. Lane 3: Embryos expressing eGFP-Dab full length transgene and endogenous Dab. Lane 4: WT embryos expressing endogenous Dab. 6% SDS PAGE. β-tubulin is loading control (asterisk).

Figure 5

A.



B.

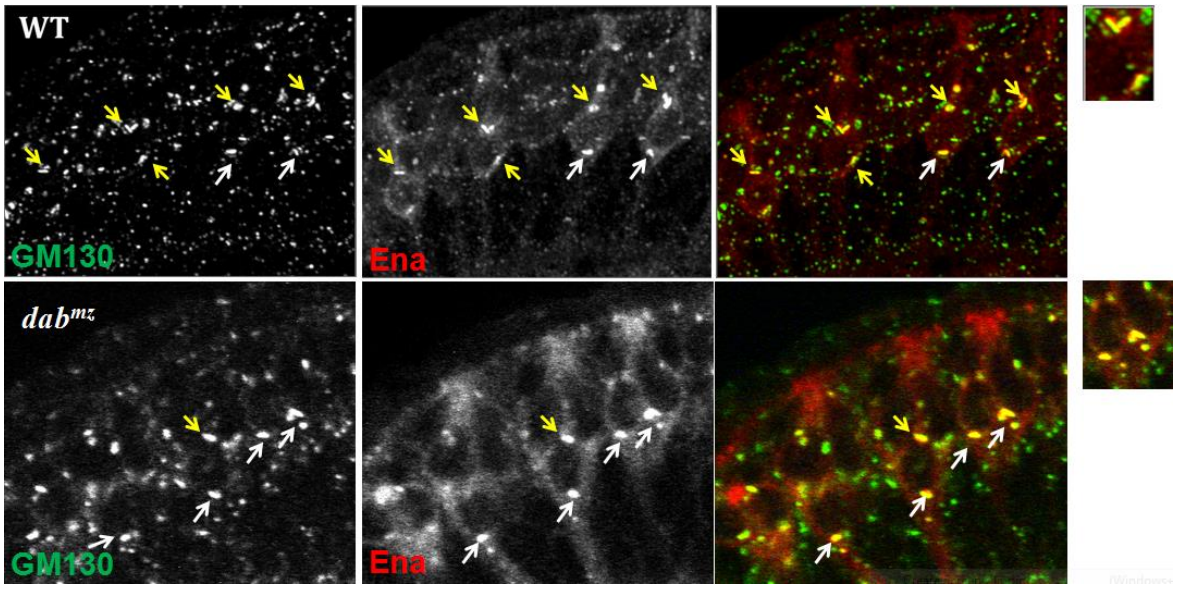


Figure 5: **Re-isolated *dab*^{mz} maternal zygotic mutant recapitulates published phenotypes.** **A.** 12-14 hr stage 17 embryos were collected, stained with anti-fasciclin II (1D4), visualized with DAB reagent, dissected and mounted in 90% glycerol. Hemisegments A2-A7 were scored. Wild-type (WT) ISNb always displays three major synaptic innervations to ventral longitudinal muscles (VLM), whereas mutation in *Dab* results in failure of ISNb to target muscle 12/13 cleft. Black arrows indicate NMJs, and red arrows indicate missing synaptic specializations. **B.** 3rd instar larval eye disk was dissected with brain, fixed, stained with anti cis-golgi (GM130, green)

and anti Ena (red), and imaged with a fluorescent microscope. In WT photoreceptors golgi is distributed throughout the soma, both apically and cortically (yellow arrows) and basally (white arrows). Ena co-localizes with cis-golgi. *Dab*^{mz} mutant cis-golgi accumulates in large aggregates that are more frequently localized toward the basal region of the soma (white arrows), than other areas of the cell. WT image from R. Kannan.

Figure 6

A.

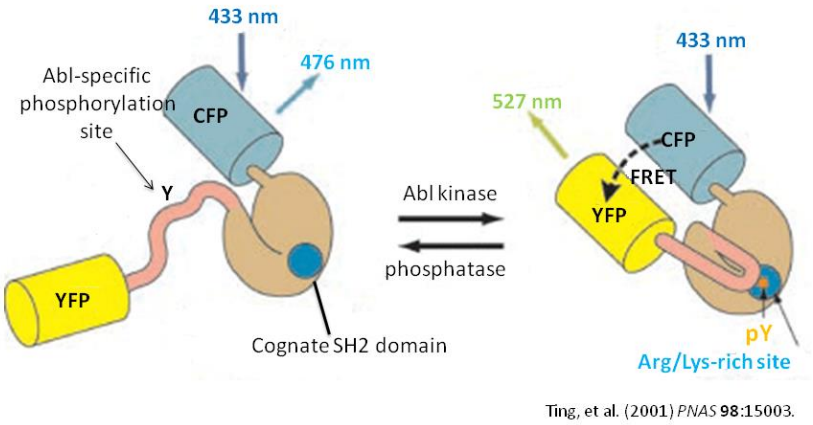


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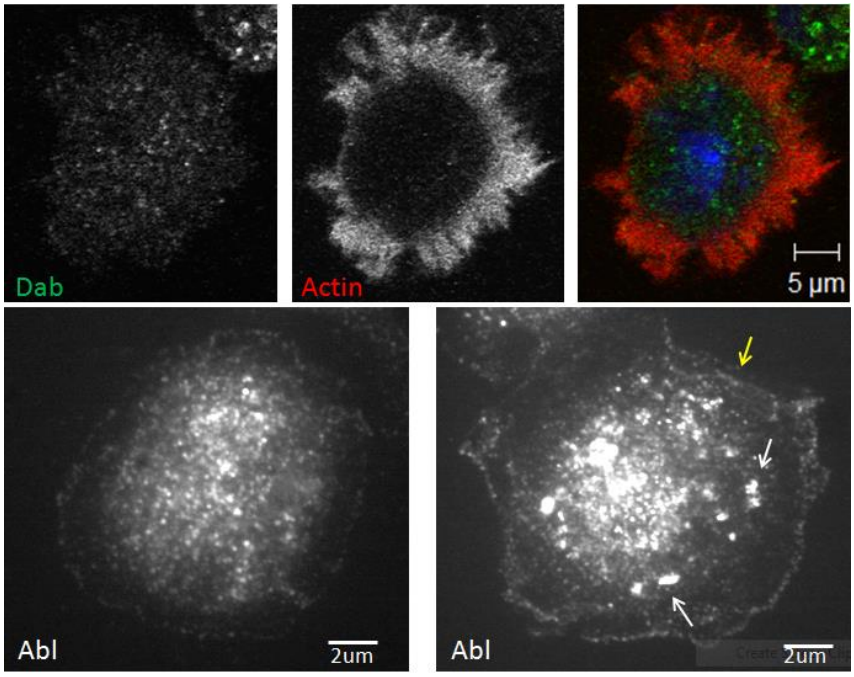
Figure 6: **Illustration of the FRET based Abl kinase reporter.**
UAS construct, modified from Ting et al., 2001, which can be driven in the cells by a GAL4 expressing construct. It contains an Abl-phosphorylatable Tyrosine and the cognate SH2 and SH3 domains (from c-Crk II), linked to CFP and YFP. The Abl FRET reporter displays FRET activity in cultured cells, and that activity is increased by co-expression of additional Abl kinase. FRET activity is also decreased in *abl* mutant cells or upon treatment with a specific Abl inhibitor gleevec (Kannan R. in prep.) Figure modified from Ting et al 2001.

Figure 7

A.



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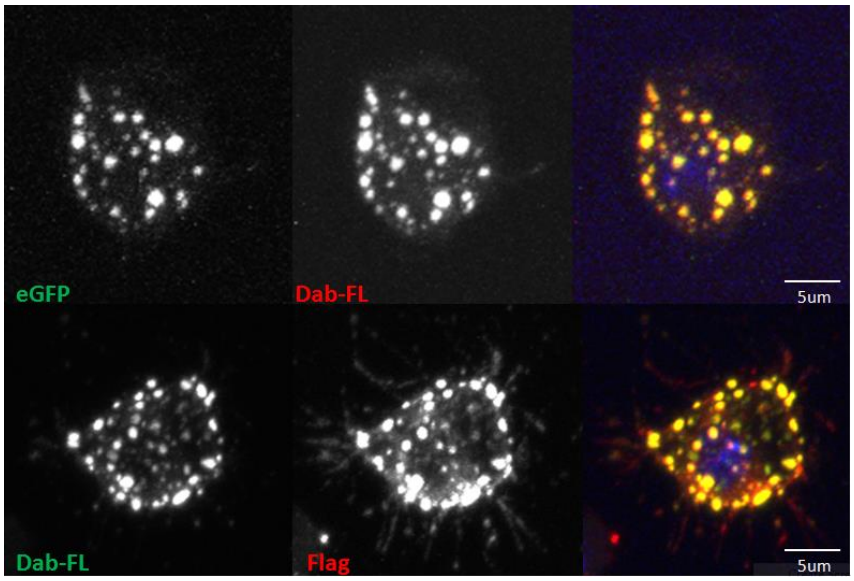


C.

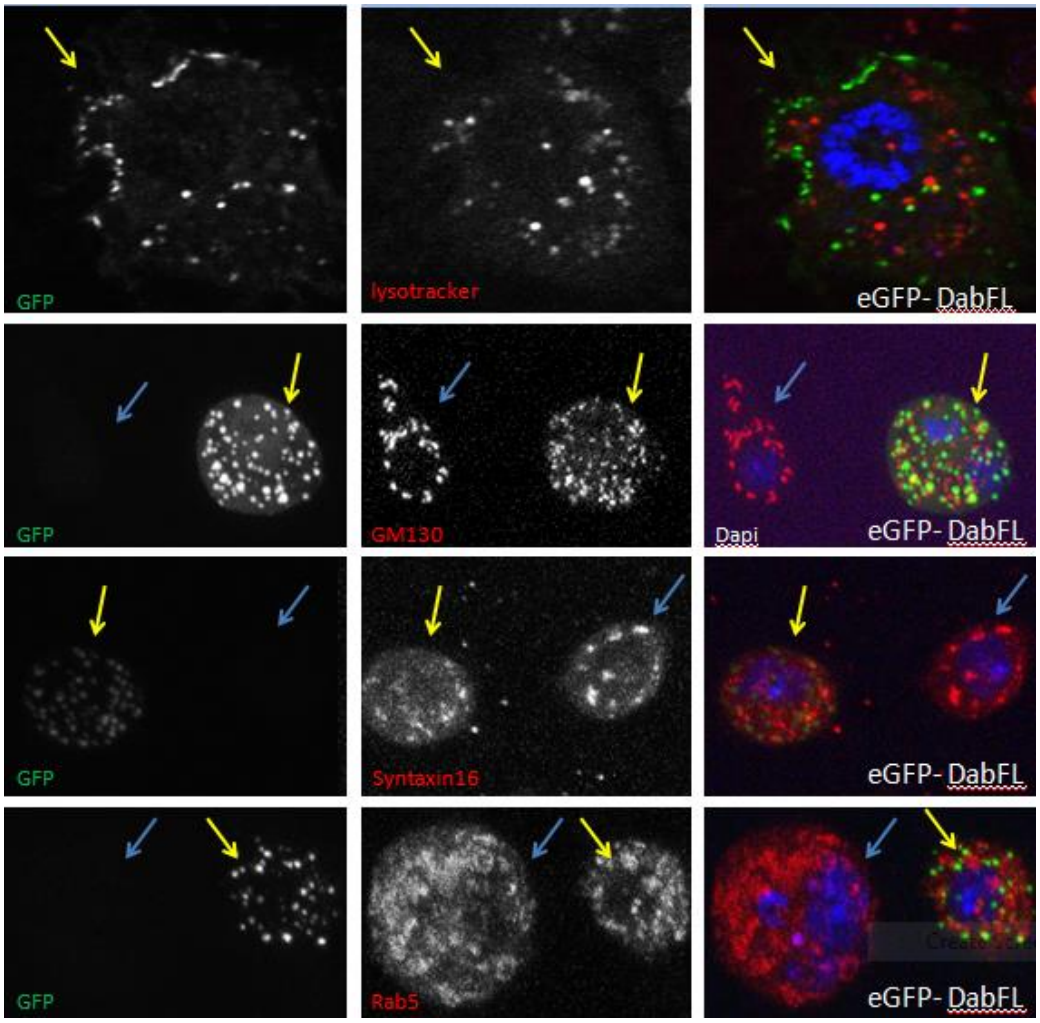
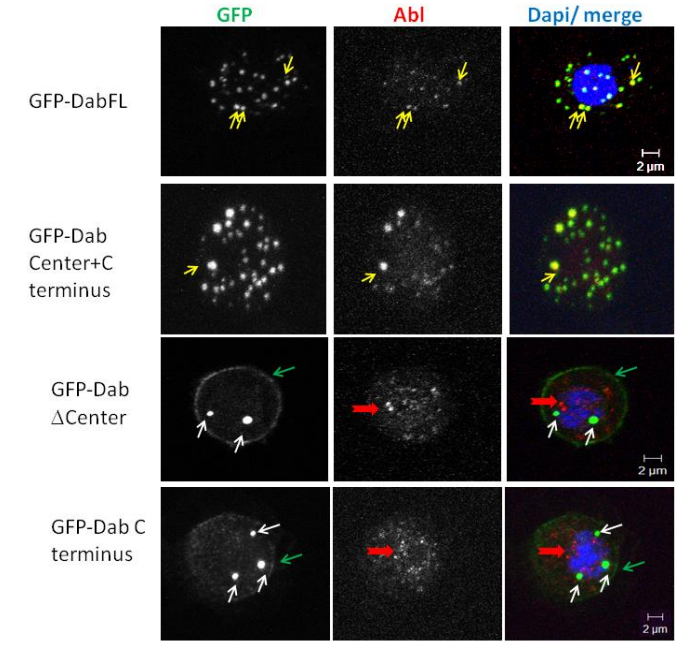


Figure 7: **Expression of Dab and Abl in *Drosophila* S2 cultured cells.**
A. S2 cells were plated on polylysine and concanavalin A 1:1 mix, fixed, stained and examined by fluorescence microscopy. Top panel: Endogenous Dab localization is at low levels and is diffuse throughout the cytoplasm. Anti-Dab is in green, actin is in red (phalloidin). Bottom panel: Abl is localized throughout the cytoplasm, in puncta of various sizes (white arrows) and at the cell cortex (yellow arrow). **B.** Top panel: eGFP-Dab full length localizes to puncta of varying sizes in the cytoplasm. EGFP is in green and Dab is in red. Bottom panel: Flag Dab full length is similarly localized in puncta throughout the cytoplasm..Anti-Dab is in green and anti-Flag is in red. **C.** S2 cells expressing eGFP-Dab full length transgene were plated and co-stained for GFP in green and various organelle markers in red. GFP puncta do not co-localize with lysotracker/lysosome (panel 1), GM130/Cis-golgi (panel 2), Syntaxin16/trans-golgi (panel 3) and Rab5/early endosome (panel 4).

Figure 8

A.



B.

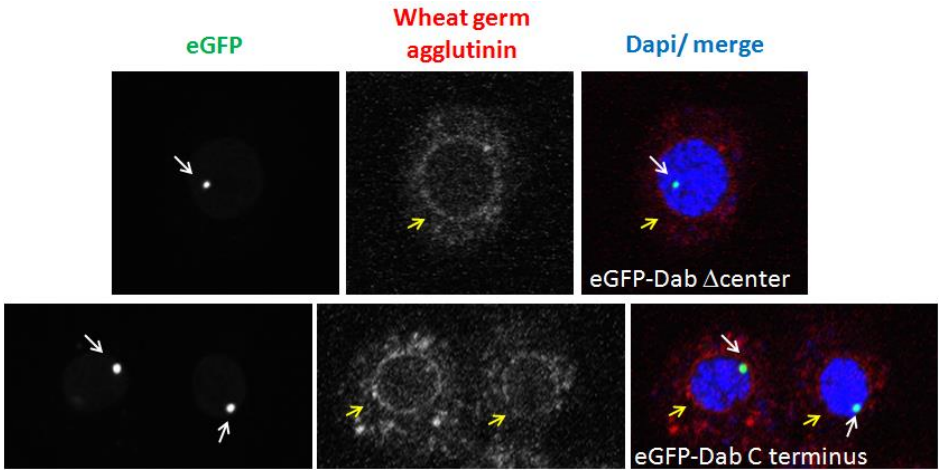


Figure 8: **Expression of eGFP-Dab deletion constructs in *Drosophila* S2 cultured cells.** **A.** S2 cells expressing eGFP-Dab deletion constructs were plated on polylysine and concanavalin A 1:1 mix, fixed, stained and examined by fluorescence microscopy. GFP is in green, Abl is in red and Dapi is in blue. Panels 1 and 2: eGFP-Dab full length and eGFP-Dab Center+C terminus are expressed in puncta in the cytoplasm. These puncta co-localize with Abl endogenous puncta (yellow arrows). EGFP-Dab PTB+Center domain had the same result, not shown. Panels 3 and 4: eGFP-Dab ΔCenter and eGFP-Dab C terminus have few puncta (white arrows) and tend to be

localized to the cell cortex (green arrows). These puncta do not co-localize with Abl puncta (red arrows). **B.** S2 cells expressing eGFP-Dab ΔCenter and eGFP-Dab C terminus transgenes were similarly plated, stained and imaged. GFP is in green, wheat germ agglutinin, a nuclear envelope marker, is in red, Dapi is in blue. These Dab constructs missing the Center domain are expressed in puncta (white arrows) inside the nuclear envelope (yellow arrows).

Figure 9

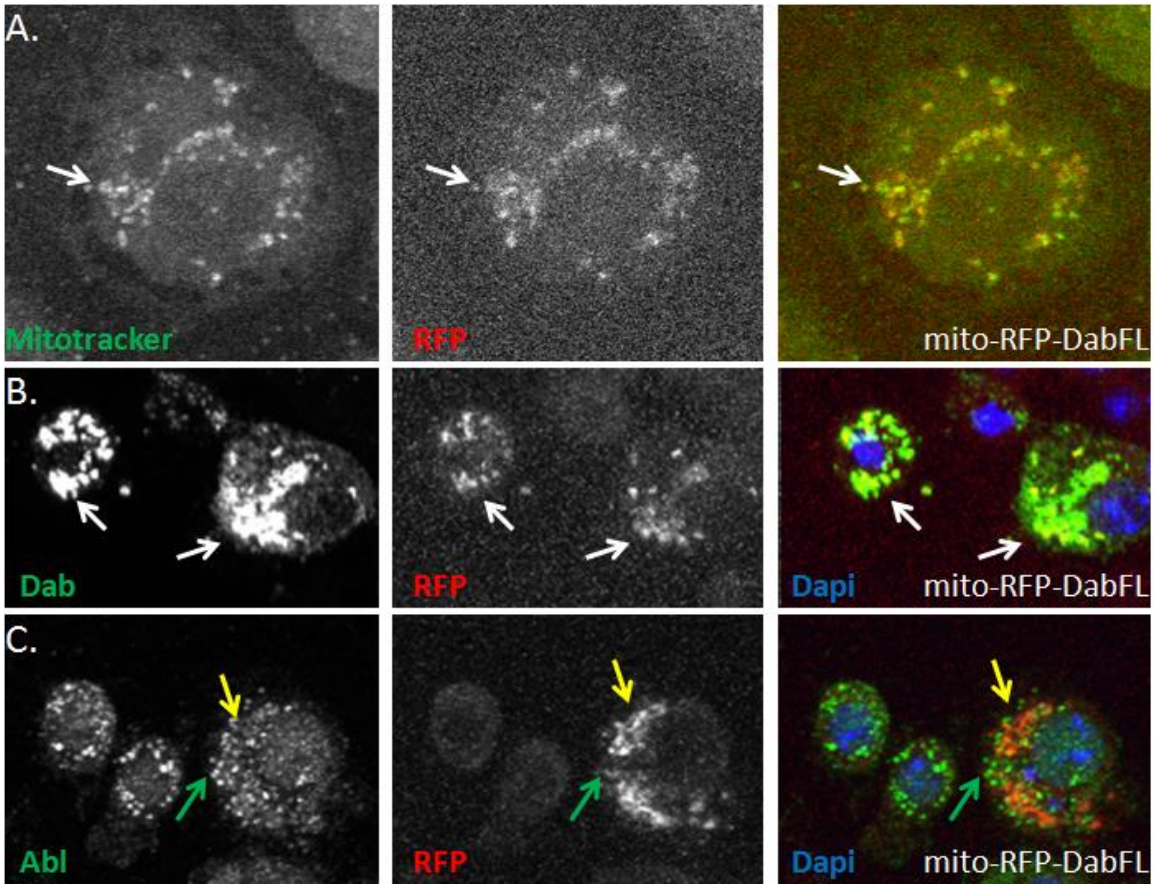
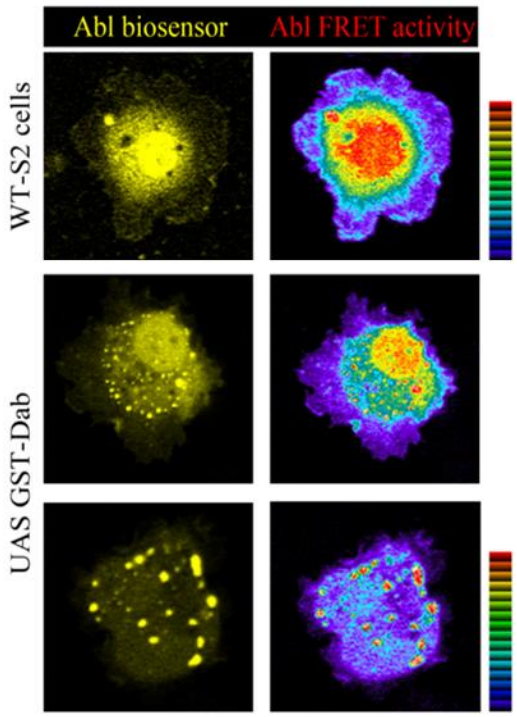


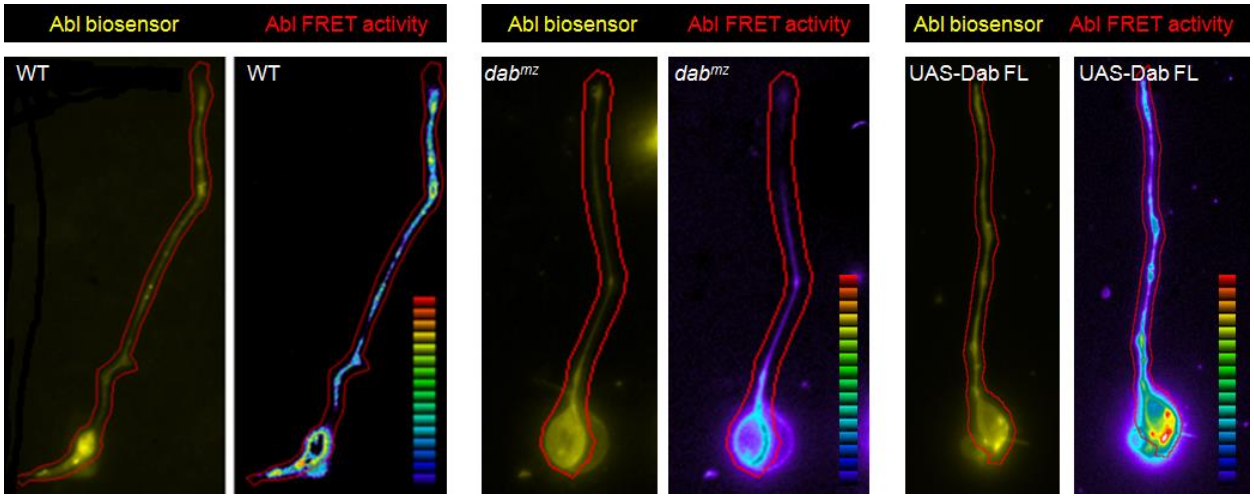
Figure 9: **Dab localized to the mitochondria in S2 cultured cells does not recruit endogenous Abl puncta.**
A. S2 cells expressing Mito-RFP-Dab full length were plated on polylysine and concanavalin A 1:1 mix, fixed, stained and examined by fluorescence microscopy. Mito-RFP-Dab co-localizes with mitotracker in S2 cells (white arrows). Mitotracker is in green, RFP is in red. **B.** S2 cells expressing Mito-RFP-Dab full length were similarly plated, stained and imaged. Anti-Dab antibody co-localizes with anti-RFP antibody (white arrows), as expected for the fusion protein. Dab is in green, RFP is in red and Dapi is in blue. **C.** S2 cells expressing Mito-RFP-Dab full length were similarly plated, stained and imaged. Mito-RFP-Dab, marked by anti-RFP antibody, does not recruit Abl to the mitochondria. Abl puncta (yellow arrow) do not co-localize with RFP-Dab puncta (green arrow). Abl is in green, RFP is in red and Dapi is in blue.

Figure 10.

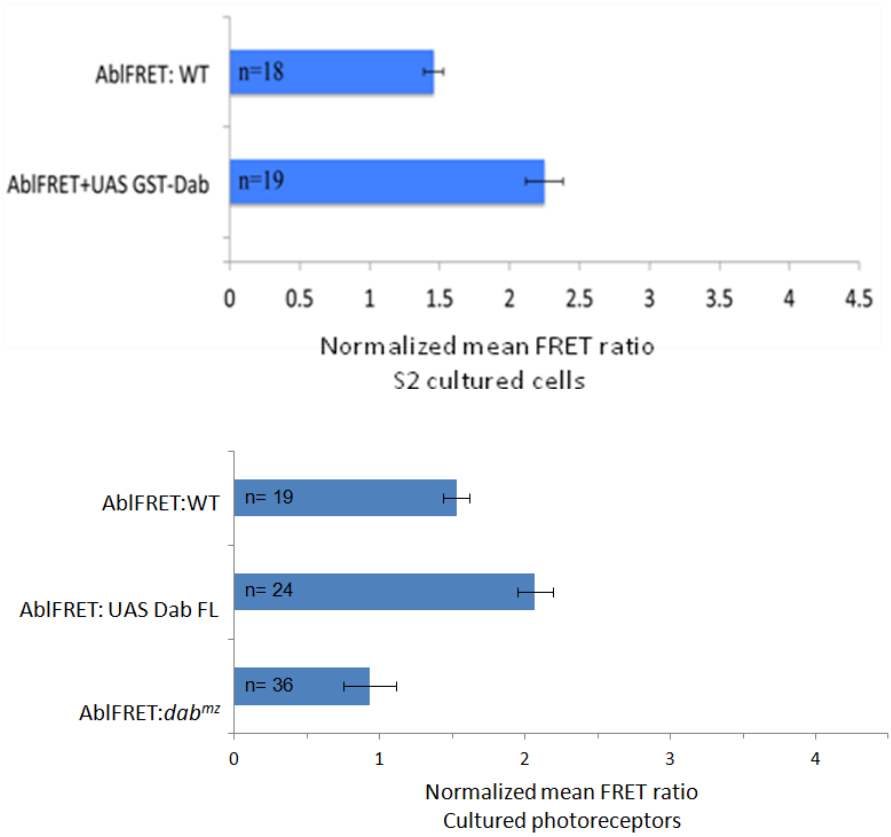
A.


Abl biosensor
Abl FRET activity
WT-S2 cells
UAS GST-Dab

B.


Abl biosensor
Abl FRET activity
WT
WT
dabmz
dabmz
UAS-Dab FL
UAS-Dab FL

C.


AblFRET: WT
n=18
AblFRET+UAS GST-Dab
n=19
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
Normalized mean FRET ratio
S2 cultured cells
AblFRET:WT
n= 19
AblFRET: UAS Dab FL
n= 24
AblFRET:dab^mz
n= 36
0
1
2
3
4
Normalized mean FRET ratio
Cultured photoreceptors

D.

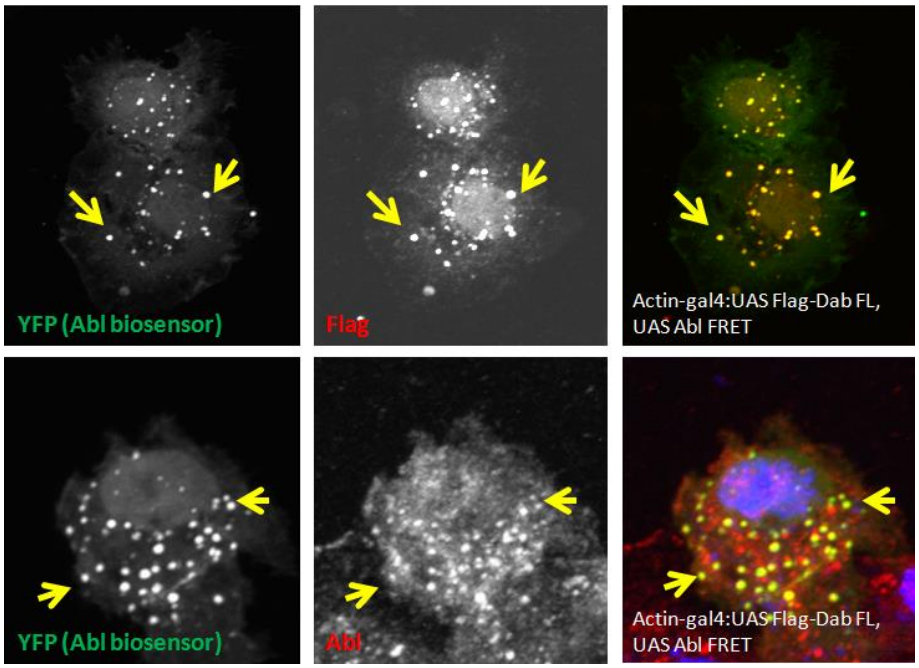
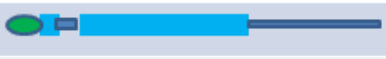

YFP (Abl biosensor)
Flag
Actin-gal4:UAS Flag-Dab FL,
UAS Abl FRET
YFP (Abl biosensor)
Abl
Actin-gal4:UAS Flag-Dab FL,
UAS Abl FRET

Figure 10: **Disabled regulates Abelson kinase activity.**
A. S2 cells expressing UAS Abl FRET Abl biosensor were plated on polylysine and concanavalin A 1:1 mix, fixed, stained and examined by Ratiometric FRET imaging. WT cells, top, have a baseline level of biosensor expression (YFP channel, left column) and FRET activity (FRET channel, right column). UAS GST-Dab expressing cells have increased Abl biosensor accumulation to puncta in the cytoplasm. These puncta have a higher FRET then the rest of the cell.**B.** 3rd instar eye disk photoreceptors expressing Abl FRET were dissected, placed in culture medium and imaged by Ram Kannan. WT cells, left, have a baseline level of biosensor expression (YFP channel, left column) and FRET activity (FRET channel, right column). *Dab*^{mz} photoreceptors (middle) show decreased Abl FRET activity compared to WT (ratio of red to blue). Photoreceptors overexpressing Dab full length (right) show increased FRET activity compared to WT (ratio of red to blue). **C.** Graphic summary of FRET measurement results for S2 cells (top) and photoreceptors (bottom). Y axis is normalized mean FRET ratio calculated by dividing the average intensity from FRET channel to CFP channel after subtracting the background values from the respective channels, and then averaged across the Z axis for all planes for a given cell. Nuclear signals were excluded from the analysis. Ns are indicated on graph. **D.** S2 cells expressing UAS Flag Dab full length and UAS Abl FRET were plated on polylysine and concanavalin A 1:1 mix, fixed, stained and examined by fluorescence microscopy. Anti YFP is in green and anti-Flag is in red (top). Anti Abl is in red (bottom). Abl biosensor accumulates in puncta, visualized by JL8 anti-YFP antibody (arrows). These puncta co-localize with Dab puncta, visualized by anti Flag antibody (top). Abl biosensor puncta also co-localize with Abl endogenous puncta (bottom).

TABLES

Table 1

Promoter	Tag	cDNA	
Endogenous	eGFP	Dab FL	
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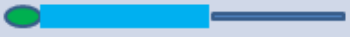
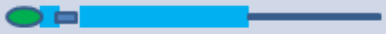
Endogenous	eGFP	DabPTB-Center	
Endogenous	eGFP	DabPTB-C terminus	
Endogenous	eGFP	DabCenter-C terminus	
Endogenous	eGFP	DabCterminus	
UASp	eGFP	Dab FL	
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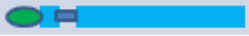
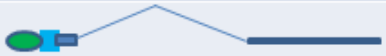
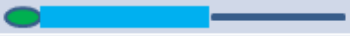
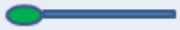
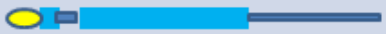
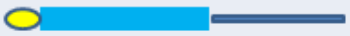
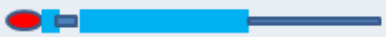
UASp	eGFP	DabPTB-Center	
UASp	eGFP	DabPTB-C terminus	
UASp	eGFP	DabCenter-C terminus	
UASp	eGFP	DabCterminus	
UASp	Flag	Dab FL	
UASp	Flag	DabPTB	
UASp	Flag	DabPTB-C terminus	
UASp	Flag	DabCenter-C term	
UASp	Flag	DabCterminus	
Actin	Mito RFP	Dab FL	

Table 1: **Tagged Dab deletion constructs.**
Constructs include endogenously driven eGFP tagged Dab deletion constructs, UASp driven eGFP tagged Dab deletion constructs, UASp driven Flag tagged Dab deletion constructs, Actin promoter driven RFP Dab full length that is recruited to the mitochondria.

Table 2

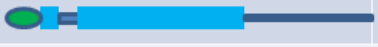
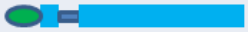
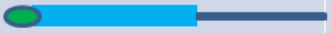
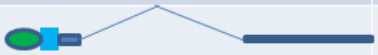
Domains	Cytoplasmic localization/ many puncta	Co-localization with Abl	Membrane localization
Full length 	+	+	-
PTB+Center 	+	+	-
Center+C terminus 	+	+	-
ΔCenter 	-	-	+
C terminus 	-	-	+

Table 2: **Summary of Dab deletion constructs localization in S2 cells.**
EGFP-Dab FL, eGFP-Dab PTB+Center, and eGFP-Dab Center+C terminus accumulate in cytoplasmic puncta which co-localizes with the anti-Abl puncta. In eGFP-Dab ΔCenter and eGFP-Dab C terminus expressing cells, Dab localizes to fewer, one to five, puncta which appear to be localized in the nucleus and sometimes to the cell cortex, and do not co-localize with anti-Abl puncta.

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