Disabled is a bona fide component of the Abl signaling network

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SUMMARY
Abl is an essential regulator of cell migration and morphogenesis in both vertebrates and invertebrates. It has long been speculated that the adaptor protein Disabled (Dab), which is a key regulator of neuronal migration in the vertebrate brain, might be a component of this signaling pathway, but this idea has been controversial. We now demonstrate that null mutations of Drosophila Dab result in phenotypes that mimic Abl mutant phenotypes, both in axon guidance and epithelial morphogenesis. The Dab mutant interacts genetically with mutations in Abl, and with mutations in the Abl accessory factors trio and enabled (ena). Genetic epistasis tests show that Dab functions upstream of Abl and ena, and, consistent with this, we show that Dab is required for the subcellular localization of these two proteins. We therefore infer that Dab is a bona fide component of the core Abl signaling pathway in Drosophila.

KEY WORDS: Disabled, Abl tyrosine kinase, Axon guidance, Drosophila, Epithelial morphogenesis

INTRODUCTION
The Abelson non-receptor tyrosine kinase (Abl) family has been implicated in a broad range of biological processes, such as oncogenesis, cell growth, adhesion, migration, neurite extension and growth cone motility (for a review, see Pendergast, 2002). For example, in Drosophila, Abl mutants often display guidance defects in CNS and motor axons (Wills et al., 1999a; Wills et al., 2002), as well as epithelial morphogenesis defects during syncytial pseudo-cleavage, cellularization and dorsal closure (Grevengoed et al., 2003; Grevengoed et al., 2001; Liebl et al., 2003). In mice, knockout of the Abl family kinase genes Abl (Abl1 – Mouse Genome Informatics) and Abl-related gene (Arg; Abl2 – Mouse Genome Informatics) (Gitali et al., 2003) causes defects in neural tube closure with cytoskeletal abnormalities such as disruption of the apical latticework and enrichment of actin at the basolateral surface (Koleske et al., 1998; Moreasco et al., 2005). Abl acts at the center of the complex signaling network that executes neuronal morphogenesis (for a review, see Moreasco and Koleske, 2003). For example, Abl interacts with axon guidance receptors such as Dcc, Robo, Lar and Notch (Bashaw et al., 2000; Crouner et al., 2003; Forsthoefel et al., 2005; Wills et al., 1999a), suggesting that a variety of guidance signals converge on the Abl signaling pathway and are integrated.

Abl acts in concert with conserved signaling components. Genetic experiments in fly identified one antagonist, Enabled (Ena) (Gertler et al., 1990) and three cooperating factors: Neurotactin (Nrt) (Liebl et al., 2003), Trio (Liebl et al., 2000) and Failed axon connections (Fax) (Hill et al., 1995). Nrt is a single-pass transmembrane protein involved in cell adhesion through the binding of its ligand, Amalgam (Fremon et al., 2000). Trio contains tandemly encoded guanine exchange factor (GEF) domains and activates the small GTPases Rac and Rho (Bataimen et al., 2000; Briancon-Marjollet et al., 2008; Debant et al., 1996). Fax, a dominant enhancer of Abl (Hill et al., 1995), interacts in a dosage-sensitive manner with Trio (Liebl et al., 2000). Ena, a substrate of Abl (Gertler et al., 1995), facilitates actin polymerization at the barbed ends of actin filaments, in part by acting as an anti-capping protein (Barzik et al., 2005; Bear et al., 2002). Ena localization is disrupted by the absence of Abl, implying that Ena works downstream of Abl (Grevengoed et al., 2003). Recently, it has been shown that Abl interacting protein 1 (Abi1), first identified biochemically in mammalian cultured cells, acts as an antagonist of Abl signaling during axonogenesis through the modulation of F-actin distribution (Lin et al., 2009).

In addition to those molecules, Disabled (Dab) may also be a component of the Abl signaling pathway. Disabled is a tyrosine-phosphorylated adaptor protein (Gertler et al., 1993; Howell et al., 1997a; Le and Simon, 1998). It is a possible point of linkage of intracellular signaling to cell surface receptors. In Drosophila, it binds to the receptor Notch, and altering Dab levels modifies axon guidance phenotypes in Notch mutants (Le Gall et al., 2008; Le Gall et Giniger, 2004). In vertebrates, Dab is essential for neuronal migration and cortical layering during brain development (Howell et al., 1997b). Binding of the secreted protein reelin to its receptors, such as Vldlr and ApoE receptor 2, induces tyrosine phosphorylation of Dab1, a mouse homolog of Dab. Activated Dab1 then binds to Notch, which directly controls the radial migration of neurons to the appropriate layer of the developing neocortex (Hashimoto-Torii et al., 2008). When Disabled was first identified, it was thought to be a core component of the Abl signaling pathway (Hoffmann, 1991). In Drosophila, Dab is extensively co-expressed with Abl during development and modest overexpression of Dab suppresses the genetic interaction of Abl with Nrt and fax (Gertler et al., 1993; Hill et al., 1995; Liebl et al.,

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2003). However, all the mutations originally ascribed to Dab were later found to be mutations of the nearby gene Nrt, and, as a result, the notion that Dab is linked to the Abl signaling pathway has become quite controversial (Liebl et al., 2003). To date, no mutations have been identified in Drosophila Dab.

We now show that Dab is a bona fide component of the Abl signaling pathway in Drosophila. We generate two independent null alleles of Dab and show that they produce motor axon patterning defects that are very similar to those of Abl pathway mutations. The Dab mutations interact synergistically with mutations in Abl and trio, and antagonistically to ena, demonstrating that Dab is a positive component of the pathway, and that, by genetic criteria, Dab functions upstream of Abl and ena. The Dab mutants, moreover, mimic non-neuronal phenotypes of Abl in epithelial morphogenesis such as syncytial pseudo-cleavage furrow formation, cellularization and dorsal closure. Finally, we show that Dab is required for proper subcellular distribution of Abl in those epithelia and in the retinal neuroepithelium, confirming its role as an upstream component of the Abl signaling module.

MATERIALS AND METHODS

Genetics

Dab1 and Dab2 were generated by transposase-mediated imprecise excisions of DNA by mobilizing two independent P-elements, P[EPgy2]Dab[EY10190] and P[XP]Dab[d11255], respectively (obtained from the Bloomington Stock Center and the Exelixis Collection at Harvard, respectively) and screening by PCR. Dab1 and Dab2 have deletion mutations and lack 5' promoter and coding region of the Dab gene. We confirmed that both alleles are protein-null in the animal (Fig. 1). The hetero-allelic null mutant Dab1/Dab2 is viable and fertile. We used Dab1/Dab2 females for maternal zygote experiments.

Sources of fly stocks were as follows: Abl1, A. Goodman (PhyloTech, San Francisco, CA, USA); Abl2, UAS-Abl, enaGC5, enaGC6, UAS-Da吸纳, D. Van Vactor (Harvard University, MA, USA); elav-Gal4, Y. N. Jan (UCSF, CA, USA); Df(3L)st100.62, E. Liebl (Dennison University, OH, USA). trio1 and trio8 were obtained from the Bloomington Drosophila Stock Center. Balancer chromosomes containing β-galactosidase [β-gal (TM6B-T8-lacZ) and CyO-act-lacZ)] and green fluorescent protein [GFP (TM3-act-GFP and CyO-kruppel-GFP)] expression markers were used in all genetic experiments.

Abl antibody generation

A fragment of Drosophila Abl kinase (including the kinase domain) was cloned into pQE6X His vector (Qiagen). Expression was induced using 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in M15 competent cells. Induced Abl kinase protein was purified under denaturing conditions [8M urea, 25 mM Tris (pH 7.6), 100 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and protease and phosphatase inhibitors (Sigma)] using Ni-NTA agarose beads (Qiagen) in Poly-Prep chromatography columns (Bio-Rad) to pull down the His-tagged Abl fragment. Purified Abl protein was dialyzed (Slide-A-Lyzer, Pierce) and refolded in refolding Buffer (0.1 M Tris, 0.4 M L-Arginine, 1 mM EDTA and Phosphatase and protease inhibitors) supplemented with decreasing amounts of urea. Purified Abl protein was injected into two New Zealand White rabbits (Covance Research) and sera obtained from different bleed were tested for their specificity.

Immunohistochemistry and immunoblotting

Embryos used for immunostaining were collected, fixed and stained by standard methods as described previously (Bodner et al., 1987). For phalloidin staining, fixed embryos were mechanically devitellinized by placing them under a coverslip and cracking them with gentle tapping. For axonal detection, anti-FasII (1D4, Hybridoma Bank, Iowa, USA) was used at 1:50. 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 filled in 90% glycerol in PBS. Embryos without the balanced chromosome were selected by anti-β-gal staining (1:1000; Cappel). Epithelial morphogenesis was analyzed using anti-Ena (1:100; 5G2, Hybridoma Bank, Iowa, USA), anti-phosphotyrosine (1:500; 4G10, Millipore), anti-β-tubulin (1:150; E7, Hybridoma Bank, Iowa, USA) and anti-Abl (1:300), fluorochrome-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) for the detection of primary antibodies, and rhodamine-phalloloid (Molecular Probes) and DAPI (Invitrogen) for the detection of F-actin and nuclei, respectively. For Dab protein detection, mouse monoclonal antibodies (P4D11, 1:2 and P6E11, Asctes 1:20 Hybridrida Bank, Iowa, USA) were used for immunoprecipitation-western experiments and a mouse polyclonal antibody (Le Gall et al., 2008) was used at 1:20 for immunohistochemistry. Anti-Ena (1:500), anti-Abl (1:1500) and anti-β-tubulin (1:750) were used for western blotting.

For eye disc immunostaining, late third-instar larval eye and antennal discs were dissected with the two brain lobes and fixed in 4% paraformaldehyde for 20 minutes at room temperature. The following antibodies were used: rabbit anti-Abl (1:300), rat anti-Elav 7E8A10 (1:20; Developmental Studies Hybridrida Bank) and rhodamine-phalloloid. Z-stacks were collected using a Zeiss LSM510 Confocal microscope at 63× magnification.

Quantification and statistical analysis.

For axonal analysis, hemisegments A2–A7 were used for analyzing intersegmental nerve b (ISNb) and segmental nerve a (SNa) defects. P-values were determined using the χ2 test. For epithelial analysis, images of embryos at mid-cellularization were taken by confocal microscopy and imported to the NIH image software, Image J. For quantification of the apical region, an area encompassing the most apical 5% to the surface was measured. Equivalent apical areas (30 μm2) were randomly selected for measurement. Lateral cortical accumulation was analyzed using profiles of fluorescence intensity from single planes. Ten cells from each of three embryos were examined per control and testing sample. Data were statistically analyzed using Student’s t-test.

RESULTS

Generation of Dab mutation

Two unrelated P-elements, P[EPgy2]Dab[EY10190] and P[XP]Dab[d11255], derived from different collections, were used as starting points for imprecise excision experiments, and deletion mutations were isolated and identified from each. Screening of genomic DNA by PCR yielded two independent alleles, Dab1 and Dab2. The alleles have deletions of 3070 bp and 1887 bp, respectively, in each case including the 5′ promoter region and the first exon encoding the translation start (Fig. 1A). We examined Dab protein expression from head lysates of each homozygous mutant and from the hetero-allelic combination. They all failed to show a band corresponding to Dab protein (~280 kDa) on western blots (Fig. 1B). In addition, CNS axon accumulation of Dab was not detectable in Dab mutant embryos derived from Dab mutant mothers (here called DabMS embryos) (Fig. 1C). Therefore, our molecular and biochemical evidence suggests that Dab1 and Dab2 are null alleles. Furthermore, Dab alleles complement mutations in the next upstream locus, Lasp, indicating that this gene is not affected. Homozygotes for both Dab alleles are viable and hetero-allelic Dab1/Dab2 flies are viable and fertile, allowing us to establish DabMS, a stock that allows recovery of embryos that lack all maternal and zygotic Dab (65–90% adult viability, depending on the alleles used).

Dab is required for proper growth and guidance of motor axons

Dab mutant embryos display abnormal patterning of motor axons. In the wild type, the eight axons of intersegmental nerve b (ISNb) innervate a field of ventrolateral body wall muscles (Fig. 2A,B). In
the Dab mutants, these axons stalled and failed to innervate their most distal target muscle, muscle 12 (defects are seen in 46% of total hemisegments in DabMZ/Dab1 versus 2% in Dab heterozygote embryos; Fig. 2C and Table 1). In addition, we found analogous defects in another motor nerve, segmental nerve a (SNas). Normally, SNas diverges into dorsal and lateral branches with dorsal branch axons innervating muscles 21, 22, 23 and 24 (Fig. 3A,B). However, DabMZ dorsal branch SNax axons often stopped short and failed to reach their muscle target (39% of hemisegments affected; Fig. 3C and Table 1). The same defects were observed in the zygotic Dab mutant but at much lower expressivity (9% in homozygous Dab null mutants nor in a hetero-allelic combination. The lower band at ~150 kDa is a non-specific band (asterisk). The western blot was probed with mAb D2, which recognizes an epitope that is common to both Dab isoforms (Gertler et al., 1993) and that is well beyond the region deleted by the mutations. Similar results were obtained in a western blot using a polyclonal antibody raised against the N-terminal two-thirds of the protein (data not shown). (C) Anti-Dab immunohistochemistry in late stage 16 embryos using the polyclonal serum. Dab is expressed in axons of the CNS, however, the signal is absent in DabMZ, an embryo that lacks both maternal and zygotic Dab.

We next asked whether Dab interacts genetically with Abl signaling components by using deficiency chromosomes to reduce the dosage of Abl and its interacting partners in the Dab mutant background. DabMZ axon phenotypes were dramatically enhanced in the presence of deficiency chromosomes (Table 1). The moderate expressivity of ISNb axon defects in DabMZ (46%) was increased by heterozygosity for deficiency chromosomes reducing gene copies of Abl and Nrt [77% with Df(3L)std11/+, or Abl and fax [65% with Df(3L)std100.62/+] . These interactions were observed for both Dab1 and Dab2. An analogous synergistic interaction was observed for SNas (Table 1). We found that Abl is the major gene in the deficiencies that contributes to this interaction, because a single copy of a wild-type Abl genomic transgene rescued the synergistic interaction, as assessed in ISNb using Df(3L)std11, back to the level of the Dab mutant alone (from 77% defective to 42%). We also examined interactions of two other mutants in the Abl pathway, trio and ena, in the Dab null environment. trio mutations similarly enhanced Dab ISNb axon defects (78% with trio1/+ and 80% with trio2/+, Table 1). Conversely, heterozygous loss-of-function mutations of the Abl antagonist ena suppressed the Dab phenotype in ISNb axons (from 46% to 26% with enaGC1/+, and to 36% with enaGC1/++; Table 1).

**Dab is genetically upstream of Abl and ena**

As DabMZ-null embryos have a well-formed nervous system, we were able to perform classical genetic epistasis to determine the order of the pathway. We found that pan-neuronal overexpression of Abl suppressed the DabMZ ISNb phenotypes (46% to 29%; Table 1), whereas overexpression of Dab failed to suppress the...
highly penetrant ISNb phenotype of Abl. Together, these data suggest that Abl acts downstream of Dab (Table 1). Overexpression of Dab with the same Gal4 driver does significantly modify a different phenotype of these same ISNb axons; the bypass phenotype of a Notchα mutant (Le Gall et al., 2008). This demonstrates that the level of Dab overexpression achieved in this experiment was sufficient to significantly modulate the growth of ISNb axons, and thus validates our interpretation of the ISNb defect of DabMZ (white asterisks).

Dab functions in Abl-dependent epithelial morphogenesis

In addition to its roles in axon patterning, Abl is required for several aspects of epithelial morphogenesis in the early embryo. Maternal-zygote Abl mutants display defects during syncytial pseudocleavage and cellularization (Grevengoed et al., 2003), ventral furrow formation (Fox and Peifer, 2007), and dorsal closure (Grevengoed et al., 2001). We therefore examined epithelial morphogenesis in DabMZ embryos.

We first investigated the syncytial pseudocleavage and cellularization of DabMZ embryos and found that they mimicked Abl phenotypes (Grevengoed et al., 2003) in this context. As observed for Abl mutants, absence of Dab caused abnormal compartmentalization, such as anucleated pseudocells (Fig. 4A, short white arrows), incomplete pseudocleavage furrows and multinucleated cells (Fig. 4A, long yellow arrows) during cellularization (92% of embryos display defective cells; n=51). However, the microtubule baskets surrounding each nucleus in multinucleated cells looked normal (Fig. 4A). Previously, it has been shown that F-actin in Abl maternal-zygote mutants was apically accumulated (Grevengoed et al., 2003). Similarly, DabMZ mutants exhibited abnormal apical actin accumulation throughout cellularization (Fig. 4B). At mid-cellularization, for example, overall apical F-actin in DabMZ mutants was increased by ~ 49% (Fig. 4C).

Ena and Abl localization are disrupted in DabMZ mutant

In addition to the apical accumulation of actin in cellularizing DabMZ mutant embryos, we observed correlated alterations of actin, phosphotyrosine and Enabled protein localization at a mid-lateral level in this epithelium. The mid-lateral localization of actin was extremely variegated, with patches of contiguous pseudocells showing a disrupted actin pattern. Wild-type embryos, by contrast, displayed uniform lateral actin localization (Fig. 5A). The fluctuations in actin localization were strongly correlated with fluctuations in phosphotyrosine accumulation (Fig. 5B). The subcellular distribution of Enabled protein was also highly variegated at a mid-lateral level in DabMZ, but in a pattern that was complementary to that of actin and phosphotyrosine. Thus, in places along the mid-lateral cortex of the pseudocells where F-actin was reduced, Ena was selectively enriched (Fig. 6B). In such...
Disabled is required for Abl signaling

<table>
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| **ISNb bypass** |          |                                               |       | Dab1/+                                  |
| enaGC5/ enaGCS | +/+      | 99                                            | 180   | Dab1/+                                  |
| Dab1/+         | 99       | 240                                          |       | Dab1/+                                  |

| **SNa stop short** |          |                                               |       | Dab1/+                                  |
| DabMZ/ Dab1     | +/+      | 39                                            | 150   | DabMZ/+                                 |
| Df(3L)std11/+   | 69       | 238                                          |       | Df(3L)std11/+                           |
| Df(3L)std11, P[Abll]/+ | 39 | 132                                           |       | P[Abll]/+                              |
| Df(3L)std100.62/+ | 57 | 168                                           |       | Df(3L)std100.62/+                       |
| trio1/+        | 80       | 168                                          |       | trio1/+                                 |
| DabMZ/ Dab2    | +/+      | 33                                            | 132   | DabMZ/+                                 |
| Df(3L)std11/+   | 62       | 180                                          |       | Df(3L)std11/+                           |
| Dab1/ Dab1     | +/+      | 9                                            | 1136  | Dab1/+                                  |
| Df(3L)std11/+   | 52       | 204                                          |       | Df(3L)std11/+                           |
| trio1/+        | 35       | 240                                          |       | trio1/+                                 |

Early stage 17 embryos were fixed, stained with anti-FasII (1D4) and dissected. Hemisegments A2–A7 were analyzed for the scoring of ISNb (stall or bypass) and SNa defects (stop short of dorsal branch in Fig. 3). ‘ISNb bypass’ is a completely different defect from ‘ISNb stall’. ISNb axons do not enter the ventral longitudinal muscles, rather they bypass along the ISN root. P-values were determined by χ² test. All comparisons are statistically significant (P<0.001). Unless otherwise indicated, the comparison is between the homozygote mutant with and without the indicated modifier.

*In the case of DabMZ/ Df(3L)std11, P[Abl], the comparison is with DabMZ/Df(3L)std11 stall.
†In addition to the stall phenotype, 25% of total hemisegments display bypass phenotypes. In the DabMZ/ Dab1 background, the expressivity of ISNb bypass is increased to 33%.
‡In some cases, the same control genotype is the appropriate comparison for more than one experimental genotype. In these cases, data for the control genotype were pooled and that value is presented for all applicable table entries.

**Domains, cortical Ena was increased by 66-230% (Fig. 6D). Some cells with high levels of Ena protein demonstrated a constricted morphology with the nucleus either displaced or completely absent (Fig. 6C, dotted rectangle). Western blot analysis did not reveal any difference in overall levels of Ena protein between wild-type and DabMZ cellular blastoderm stage embryos (Fig. 6E), suggesting that Dab regulates the subcellular localization of Ena rather than its expression level.**

The hypothesis that Dab specifically controls Ena localization is further supported by analysis of Dab function during a different example of epithelial morphogenesis: dorsal closure. Like AblMZ mutants (Grevengoed et al., 2001), DabMZ mutants had disrupted dorsal closure with occasional breaks of the leading edge, failure of cell elongation (Fig. 7B,D) and disturbances in the ‘zippering’ of the dorsal epithelia (Fig. 7F). Visualization of Ena protein in the associated amnioserosa cells revealed that, here too, the subcellular localization of Ena was severely disturbed in DabMZ mutants (Fig. 7H,J). Taken together with the data on cellularization, the disrupted subcellular localization of Ena in DabMZ mutants implies that Ena is downstream of Dab in the genetic pathway.

We also examined the subcellular localization of Abl in DabMZ mutants. In the wild type, Abl was present homogenously across the embryo at the cellularization stage with only slight accumulation in mid-lateral membranes (Fig. 8A). In DabMZ mutants, Abl localization was substantially increased at cell junctions (Fig. 8B). At this apicobasal position, where correlated fluctuations were seen in actin and phosphotyrosine signals, the level of Abl protein was often seen to vary in parallel (Fig. 8B). However, there are also sites where increased Abl concentration is seen even though the actin signal remained normal (Fig. 8C). This suggests that Disabled may directly regulate the subcellular localization of Abl protein. This was confirmed by examining Abl localization in the retinal neuroepithelium in developing eye discs. In wild type animals, Abl was uniformly enriched along the membrane of developing photoreceptors (Fig. 9A). In DabMZ mice, cortical Abl was increased by 66-230% (Fig. 6D). Some cells with high levels of Abl showed a constricted morphology with the nucleus either displaced or completely absent (Fig. 6C, dotted rectangle). Western blot analysis did not reveal any difference in overall levels of Abl protein between wild-type and DabMZ cellular blastoderm stage embryos (Fig. 6E), suggesting that Dab regulates the subcellular localization of Abl rather than its expression level. The hypothesis that Dab specifically controls Abl localization is further supported by analysis of Dab function during a different example of epithelial morphogenesis: dorsal closure. Like AblMZ mutants (Grevengoed et al., 2001), DabMZ mutants had disrupted dorsal closure with occasional breaks of the leading edge, failure of cell elongation (Fig. 7B,D) and disturbances in the ‘zippering’ of the dorsal epithelia (Fig. 7F). Visualization of Abl protein in the associated amnioserosa cells revealed that, here too, the subcellular localization of Abl was severely disturbed in DabMZ mutants (Fig. 7H,J). Taken together with the data on cellularization, the disrupted subcellular localization of Abl in DabMZ mutants implies that Abl is downstream of Dab in the genetic pathway. We also examined the subcellular localization of Disabled in DabMZ mutants. In the wild type, Disabled was present homogenously across the embryo at the cellularization stage with only slight accumulation in mid-lateral membranes (Fig. 8A). In DabMZ mutants, Disabled localization was substantially increased at cell junctions (Fig. 8B). At this apicobasal position, where correlated fluctuations were seen in actin and phosphotyrosine signals, the level of Disabled protein was often seen to vary in parallel (Fig. 8B). However, there are also sites where increased Disabled concentration is seen even though the actin signal remained normal (Fig. 8C). This suggests that Disabled may directly regulate the subcellular localization of Disabled protein. This was confirmed by examining Disabled localization in the retinal neuroepithelium in developing eye discs. In wild type animals, Disabled was uniformly enriched along the membrane of developing photoreceptors (Fig. 9A).
mutants, conversely, there is significant disruption of Abl localization, with the cortical accumulation disturbed or lost in large patches of photoreceptor clusters (Fig. 9B). Unlike the blastoderm, however, overall F-actin organization was largely unaffected by Dab in the eye disc, with only scattered, relatively minor effects on actin structure (Fig. 9D). This supports the hypothesis that altered subcellular localization of Abl in the Dab mutant eye disc is a direct consequence of the absence of Dab function and not secondary to generalized disruption of actin patterning. Thus, altered Abl distribution in the absence of Dab suggests that Abl protein localization is downstream of Dab, consistent with the genetic data presented above. Together, these data strongly support the hypothesis that Dab acts upstream of Abl and Ena.

DISCUSSION

Data presented here demonstrate that Disabled is a bona fide component of the Abl signaling pathway. DabMZ embryos showed Abl-like phenotypes in different contexts: axon patterning, dorsal closure and cellularization. Dab interacts genetically with Abl and its genetic co-factors trio and ena during motor axon guidance, and is a positive regulator of Abl pathway signaling, acting upstream of Abl and Ena. Although the Dab mutant phenotypes mimicked Abl phenotypes, they were consistently milder. For example, the expressivity of the ISNb and SNa phenotypes of DabMZ was rather less than those of Abl. Similarly, the extent of Ena and actin accumulation in the apical region of blastoderm cells of DabMZ (up to 82% increase; data not shown) was rather less than the three-to fivefold increase reported previously for AblMZ mutants.
Disabled is required for Abl signaling

Fig. 6. Subcellular localization of Ena is altered in Dab \textsuperscript{MZ}.

(A–C) Embryos were labeled with phalloidin (F-actin; red), anti-Ena (green) and DAPI (blue). Mid-lateral views of mid-cellularization in wild type (A) and Dab \textsuperscript{MZ} (B,C). (A) Ena is slightly enriched at the cell periphery in the wild type. (B) Ena is highly accumulated at the cortical regions of some cells in Dab \textsuperscript{MZ}, corresponding to those places where the actin level is significantly reduced (white dotted circles, for example). (C) Ena accumulates at very high levels in pseudocells missing their nuclei, which also have reduced actin level at this focal plane (white dotted rectangles). Scale bars: 10 μm. (D) Quantification of cortical Ena localization. Confocal images were imported to Image J software and a line profile of fluorescence intensity was determined in the position of the dotted line (examples shown). Scale bar: 1 μm. Raw values of integrated Ena signal intensity in wild type and Dab \textsuperscript{MZ} were normalized to a scale from zero to 100. Ten cells in three embryos of each type (wild type, basal in Dab \textsuperscript{MZ} and enriched in Dab \textsuperscript{MZ}) were measured. * Statistical significance was determined by Student’s t-test (P<0.0001). Data presented in histogram are mean ± s.e.m. (E) Immunoblots of lysates from wild-type (WT) and Dab \textsuperscript{MZ} cellularizing embryos. β-tubulin was used as a loading control.

(Grevengoed et al., 2003). This suggests that although Dab contributes significantly to Abl pathway activity, it is not absolutely essential.

Dominant genetic interactions have been used to identify components of the Abl pathway (Hill et al., 1995), but it has not been possible to determine their order. Here, because we could examine the true null phenotypes of Dab, we have been able to perform classical epistasis and find that Dab acts genetically upstream of Abl and ena: altering Abl or Ena levels modified the Dab phenotype, but changing Dab could not alter an Abl or ena phenotype. This was confirmed by functional experiments as Abl and Ena localization required Dab during the cellularization of the blastoderm. Strikingly, Abl and Ena did not simply colocalize with the altered actin distribution in Dab \textsuperscript{MZ}. Abl was disrupted even in places where actin was not, and Ena disruptions were largely

Fig. 7. Epithelial morphogenetic defects in Dab \textsuperscript{MZ} during dorsal closure.

(A–F) Embryos were stained with anti-phosphotyrosine. (A,B) In the wild type (A) at stage 13, the leading edge of dorsally extending epithelial cells produces a smooth, continuous line, whereas in Dab \textsuperscript{MZ} (B) the leading edge is jagged and irregular with occasional discontinuities (white curved arrow in inset). (C,D) At stage 14, wild-type (C) leading cells elongate dramatically. Leading cells of Dab \textsuperscript{MZ} (D) also elongate, but irregularly (curved arrow), with some cells failing to elongate (asterisks). (E,F) In wild type (E), apposed leading edges ‘zip’ together smoothly from the embryo termini towards the eye-shaped central region. In Dab \textsuperscript{MZ}, the final ‘zipping’ of the leading edges is not as tight as in the wild type, leading to abnormal shape of amnioserosa and loose association of the apposed epithelia (white bracket). Broad arrows indicate the direction of epithelial migration (A–F). (G–J) Ena subcellular localization in amnioserosa cells. Embryos were stained with anti-Ena at stage 14. (G) At the apical level in the wild type (G), Ena is tightly localized to the cortical membrane (white bracket) but in the Dab \textsuperscript{MZ} mutant (H), Ena is greatly decreased and loosely localized to the membrane with some cytosolic distribution (white dotted circle, for example). The basal view (I,J) shows Ena mostly localized to the cortical region (white bracket) in the wild type (I) but an overall increase in basal Ena that is homogenously distributed across the cell in the mutant (J; white dotted circle). Scale bar: 10 μm.
complementary to those of actin. Moreover, Abl localization was disrupted in developing larval eye discs in Dab mutants, even though actin structure was largely normal. This suggests that the effect of Dab on Abl and Ena localization is specific, not just secondary to disruption of F-actin.

Analysis of the Dab phenotypes in epithelial morphogenesis was particularly informative for illuminating the role of this protein in actin organization and its physical relationship to other components of the Abl pathway. In syncytial-stage Dab-null embryos, for example, in the mid-lateral region of the developing epithelium, we found that sites with high accumulation of Abl protein also had high phosphotyrosine content and a distribution of F-actin either equivalent to that in wild type or slightly elevated. By contrast, nearby regions without elevated Abl generally had reduced phosphotyrosine and lacked distinct F-actin staining, and these domains had elevated and delocalized Enabled. These data are consistent with the hypothesis of Peifer and co-workers (Gates et al., 2007; Stevens et al., 2008) that a key role of Abl is to exclude Enabled, thereby controlling actin distribution. Our data further suggest that Dab controls actin structure in this portion of the epithelium, in part, by controlling the distribution and activity of Abl, and therefore of Ena. One plausible hypothesis is that formation of proper actin structure requires a threshold level of Abl activity, and that, in the absence of Dab, high concentrations of Abl are required to achieve this threshold, whereas with the assistance of Dab, low homogeneous levels of Abl suffice. It is also noteworthy that the correlations we observe in localization of the various proteins at the blastoderm stage were selective for the mid-lateral level of the epithelium. Evidently, different molecular mechanisms are limiting for actin organization at different positions along the apico-basal axis.

The modest Abl-like phenotype of DabMZ in blastoderm stage cells manifests as a stochastic mixture of states: one close to (but distinguishable from) wild type and the other clearly aberrant. The switch between the two states is not strictly cell autonomous but heavily influenced by intercellular communication in this syncytial epithelium. Thus, strongly affected cells are almost never found in isolation but rather as strings or domains of contacting cells and cell membranes. Indeed, different cell-cell interfaces of a single cell can have clearly different patterns of Ena accumulation. Evidently, cell-cell contacts strongly influence the local structure of the cytoskeleton in these cells. The observation that intercellular communication coordinates cytoskeletal structure in the morphogenesis of this epithelium is reminiscent of observations of other epithelia, including planar cell polarity (Chen et al., 2008), coordination of ventral furrow involution (Parks and Wieschaus, 1991) and the function of the actin purse-string in dorsal closure (Kiehart et al., 2000). It is possible that such intercellular coordination of cell morphogenesis is a common feature of epithelial development.

In mammals, Dab is required for reelin-dependent localization of cortical neurons. This is mediated by interaction with Notch (Hashimoto-Torii et al., 2008; Sibbe et al., 2009). Our previous studies first identified a crucial Notch-Dab interaction in the context of embryonic axon patterning in the fly, based on studies
from biochemistry and gain-of-function experiments (Le Gall et al., 2008), and we have now verified this with loss of function mutations (J.K.S. and E.G., unpublished). The finding that Dab is a core element of Abl signaling raises the issue of whether Abl may also be involved in the Dab-dependent regulation of mammalian cortical neurons, acting in parallel or together with Notch to control their position in response to reelin.

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Competing interests statement
The authors declare no competing financial interests.

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