Abelson kinase acts as a robust, multifunctional scaffold in regulating embryonic morphogenesis

Edward M. Rogers[a], Andrew J. Spracklen[b], Colleen G. Bilancia[a,†], Kaelyn D. Sumigray[b,†], S. Colby Allred[a], Stephanie H. Nowotarski[a], Kristina N. Schaefer[c], Benjamin J. Ritchie[a], and Mark Peifer[a,b,c,*]

[a]Department of Biology, [b]Lineberger Comprehensive Cancer Center, and [c]Curriculum in Genetics and Molecular Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

ABSTRACT **Abelson family kinases (Abls) are key regulators of cell behavior and the cytoskeleton during development and in leukemia. Abl's SH3, SH2, and tyrosine kinase domains are joined via a linker to an F-actin–binding domain (FABD). Research on Abl's roles in cell culture led to several hypotheses for its mechanism of action: 1) Abl phosphorylates other proteins, modulating their activity, 2) Abl directly regulates the cytoskeleton via its cytoskeletal interaction domains, and/or 3) Abl is a scaffold for a signaling complex. The importance of these roles during normal development remains untested. We tested these mechanistic hypotheses during *Drosophila* morphogenesis using a series of mutants to examine Abl's many cell biological roles. Strikingly, Abl lacking the FABD fully rescued morphogenesis, cell shape change, actin regulation, and viability, whereas kinase-dead Abl, although reduced in function, retained substantial rescuing ability in some but not all Abl functions. We also tested the function of four conserved motifs in the linker region, revealing a key role for a conserved PXXP motif known to bind Crk and Abi. We propose that Abl acts as a robust multidomain scaffold with different protein motifs and activities contributing differentially to diverse cellular behaviors.**

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INTRODUCTION

Biomedical science has twin goals: to define how cells and organisms work and to use this information to reveal what goes wrong in disease and develop better treatments. Collaboration between basic scientists and clinicians is key, with both sides informing the other. Few stories exemplify this better than that of Abelson kinase (Abl), one of the first identified human oncogenes. Chromosomal translocations activating Abl play a key role in chronic myelogenous leukemia (CML; Druker, 2008). The past 20 yr have seen parallel progress in understanding Abl's normal function in development and revealing how its activation contributes to disease, thus improving treatment (Khatri *et al.*, 2016).

Abl and its mammalian paralogue, Abl-related gene (Arg), are part of a kinase superfamily including Src that shares tandem N-terminal Src-homology domain 3 (SH3), Src-homology domain 2 (SH2), and tyrosine kinase domains (Figure 1A). In Abl family proteins, this is coupled to a C-terminal F-actin–binding domain (FABD; van Etten *et al.*, 1994) via a long, less well-conserved linker. This structure suggested the hypothesis that Abl links cell signaling and cytoskeletal regulation, which was abundantly confirmed in vivo (Bradley and Koleske, 2009). For example, in immune cells, Abl regulates cytokine responses, immune synapse assembly, and T-cell migration in response to chemotactic cues (Huang *et al.*, 2008). In the nervous system, Abl family members act downstream of axon guidance receptors to modulate growth cone guidance, synaptogenesis, and dendrite formation, regulating both actin and microtubules (Moresco and Koleske, 2003; Moresco *et al.*, 2005; Lee *et al.*, 2004; Li *et al.*, 2005; Lin *et al.*, 2009; O'Donnell and Bashaw, 2013). Finally, Abl family members modulate morphogenesis. The *abl arg* double-mutant mouse has neural tube closure defects (Koleske *et al.*, 1998). *Drosophila* Abl regulates diverse events ranging from coordinated apical constriction in the invaginating mesoderm to cell shape

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†These authors contributed equally.

*Address correspondence to: Mark Peifer (peifer@unc.edu).

Abbreviations used: Abl, Abelson kinase; AblΔFABD, Abl kinase lacking the conserved F-actin–binding domain; AblKD, Abl kinase dead; CR, conserved region; Ena, Enabled; LE, leading edge; SH2, Src-homology domain 2; SH3, Src-homology domain 3.

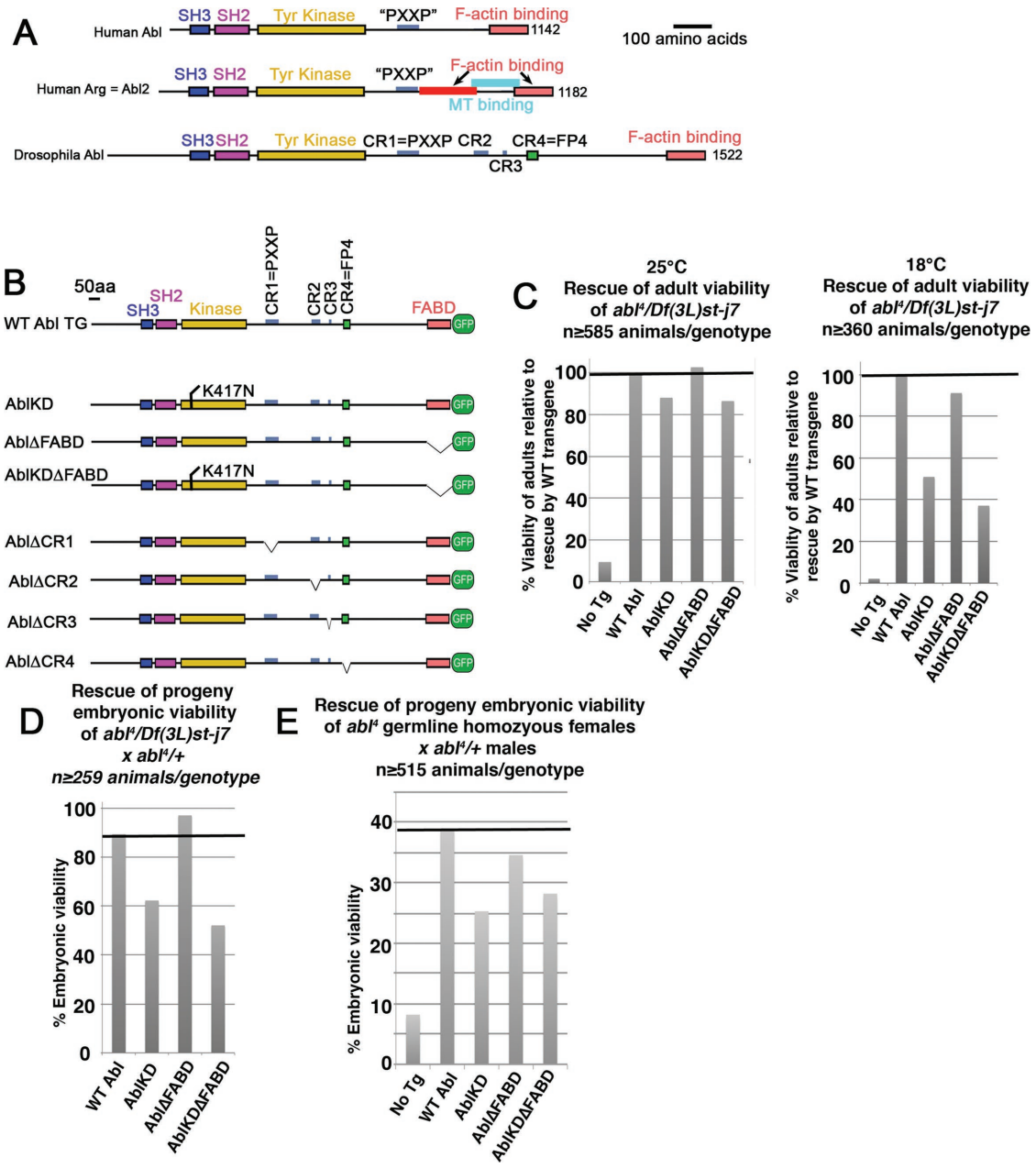


FIGURE 1: Wild-type human and *Drosophila* Abl proteins, mutants used, and rescue of viability. (A) Mammalian Abl and Arg and the single fly Abl share a core including highly conserved SH3, SH2, and tyrosine kinase domains and a C-terminal conserved region that binds actin in Abl and Arg (FABD). The linker is much less well conserved. In Arg, it contains a microtubule- and an additional actin-binding site, but the only shared motif is a PXXP SH3-binding site. (B) Mutants tested. All were C-terminally GFP-tagged, driven by the endogenous promoter (Fox and Peifer, 2007), and verified by Western blot to be expressed at levels similar to AblWT (Supplemental Figure S1). (C) Rescue of adult viability of abl^4 hemizygous zygotic mutants at indicated temperature normalized to rescue by AblWT. (D, E) Rescue of embryonic viability of *ablMZ* mutants from abl^4/ *Df(3L)st-j7* (D) or abl^4 germline homozygous mothers (E; 50% receive a wild-type *abl* gene paternally in both; crosses in Supplemental Figure S2). Full data for C–E are given in Table 1.

changes in epidermal cells undergoing convergent elongation, collective cell migration, or wound repair, stem cell maintenance in the germline, and Golgi localization in neurons (Grevengoed *et al.*, 2001, 2003; Fox and Peifer, 2007; Tamada *et al.*, 2012; Kannan *et al.*, 2014; Stine *et al.*, 2014; Zulueta-Coarasa *et al.*, 2014). Abl's roles in cell migration are also important in cancer: deregulated Abl kinases can drive invadopodia formation and invasive behavior (Smith-Pearson *et al.*, 2010; Mader *et al.*, 2011).

The importance of the Abl family in development and oncogenesis makes determining their mechanisms of action critical. Their structure and cell biological roles led to three models for function, which are not mutually exclusive. In one model, Abl/Arg, like other cytoplasmic kinases, phosphorylates protein targets that regulate cell behavior, thus altering their function. This model has substantial support. For example, Arg phosphorylates p190RhoGAP, regulating its subcellular localization and activity, with effects on cell contractility and

migration. Contractility defects can be rescued by wild-type but not kinase-dead Arg (Hernandez *et al.*, 2004; Bradley *et al.*, 2006; Peacock *et al.*, 2007). Arg also phosphorylates cortactin (Lapetina *et al.*, 2009), altering actin assembly and regulating ruffling. Surprisingly, Arg's effects on cell protrusions are largely rescued by kinase-dead Arg (Lapetina *et al.*, 2009); inhibitor studies suggest that Abl kinase may take over in phosphorylating cortactin. *Drosophila* Abl's best-characterized target is the actin regulator Enabled (Ena), which Abl negatively regulates (Gertler *et al.*, 1995). Consistent with the kinase model, Abl phosphorylates Ena in vitro and in vivo. However, in contrast to this model's simplest expectations, mutating all of the Abl phosphorylation sites on Ena does *not* hyperactivate Ena (Comer *et al.*, 1998). Thus, whereas Abl family proteins phosphorylate many targets and can alter their activities, kinase activity is not the whole story.

In the second model, Abl family proteins directly regulate the cytoskeleton via their C-terminal FABD as well as, in mammalian Arg, other actin- and microtubule-binding sites in the linker. For example, microtubule-actin cross-linking by Arg's C-terminal region is necessary and sufficient to modulate fibroblast lamellipodial dynamics (Miller *et al.*, 2004), whereas the actin-binding domain can modulate cortactin (Lin *et al.*, 2013). Intriguingly, Arg's second actin-binding domain, which is not conserved in Abl, can stabilize actin filaments and activate the Arp2/3 complex (Courtemanche *et al.*, 2015). The FABD also modulates kinase activity (Woodring *et al.*, 2001) and targets Abl to certain substrates (Mitra and Radha, 2010).

In the third model, Abl family kinases act as scaffolds for assembling a signaling complex. In addition to the kinase domain and FABD, the Abl family shares protein-binding sites for other partners that mediate Abl/Arg localization or activity, position substrates for phosphorylation, or assemble multiprotein complexes. Ligand engagement by the SH2 and SH3 domains helps activate Abl (Hantschel and Superti-Furga, 2004) and modulates partner/substrate docking; for example, Arg's SH3 domain mediates N-WASP interaction (Miller *et al.*, 2010). The SH3 domain can also negatively regulate kinase activity—surprisingly, its ability to bind ligands is not essential for Bcr-Abl disease induction (Smith *et al.*, 2003). Proline-rich motifs in the long, poorly conserved linker between the kinase domain and FABD bind other partners/substrates; for example, the most N-terminal PXXP motif binds the adaptors Crk and Nck (Feller, 2001) and the actin regulator Abi (Ibarra *et al.*, 2005). These motifs play important roles in cell spreading (Antoku *et al.*, 2008) and protrusive behavior (Lapetina *et al.*, 2009). The sequence surrounding this N-terminal PXXP motif is the only linker region conserved between mammalian Abl, Arg, and *Drosophila* Abl (Figure 1A; we refer to it as conserved region 1 [CR1]).

Most studies probed individual properties conferred by Abl or Arg in cultured cells. In intact animals, the rules may be different, with essential roles for either kinase activity or the FABD in a subset of biological events or with greater redundancy. There have only been a handful of tests in whole animals, which probed only a small subset of Abl functions. In mice, kinase activity is important in rescuing *abl*-mutant postnatal lethality (Hardin *et al.*, 1996), but most other murine Abl or Arg functions have not been similarly tested. Similarly, early experiments supported a role for the FABD in rescuing the partial lethality and immune defects of *abl*-null mice (Schwartzberg *et al.*, 1991), but this mutant was assessed for only a few other tissues and developmental times (Qiu *et al.*, 2010). Experiments in the 1990's from Hoffmann's lab began to test the importance of Abl kinase activity in *Drosophila*. Kinase-dead Abl restored adult viability of zygotic *abl* mutants, but tests in a sensitized genetic background suggested that it did not retain full function in this assay (Henkemeyer *et al.*, 1990). In contrast, a severely truncated Abl retaining kinase activity but lacking both the linker and FABD could not rescue adult viability. However, this work was done when the array of known Abl functions was very limited, and the approach has not been substantially extended since then to explore Abl's diverse roles in morphogenesis or the CNS. Similarly, although Abl family proteins clearly can act as scaffolds, the importance of the scaffolding role has been examined only in cultured cells.

Scientists also probed Abl's mechanisms of action in leukemia. Kinase activity is clearly important for oncogenesis, such that the kinase inhibitor imatinib revolutionized CML treatment (O'Hare *et al.*, 2005). Consistent with this, whereas Bcr-Abl induces a CML-like myeloproliferative disease in mice, kinase-dead Bcr-Abl does not (Zhang and Ren, 1998). However, although kinase activity is necessary for oncogenesis, it may not be sufficient. Roles for the FABD in cancer remain controversial, with the result differing, depending on the amount of Bcr sequence included in the Bcr-Abl fusion protein. p210Bcr-Abl does not require its FABD to induce leukemia (Wertheim *et al.*, 2003), but deleting the FABD attenuates leukemogenesis by p190Bcr-Abl (Heisterkamp *et al.*, 2000). Several effects of oncogenic Bcr-Abl or v-abl in cultured cells depend on the FABD, including the ability to transform fibroblasts, make lymphoblasts interleukin independent (McWhirter and Wang, 1993), modulate matrix adhesion (Wertheim *et al.*, 2003), and suppress apoptosis and confer drug resistance to hematopoietic progenitors (Underhill-Day *et al.*, 2006). Thus, although elegant experiments in cultured cells have revealed how kinase activity, cytoskeletal interactions, and scaffolding can influence individual cellular properties, the relative importance of these roles in whole animals is less clear. We thus tested the different hypotheses for Abl's mechanism of action in morphogenesis, evaluating the importance of kinase activity, the FABD, and conserved linker motifs.

RESULTS

The F-actin–binding domain is not essential for rescuing embryonic or adult viability or fertility of *abl*-null mutants, whereas loss of kinase activity impairs but does not eliminate Abl function

To test the importance of kinase activity or direct regulation of the actin cytoskeleton in Abl function in vivo, we generated two mutants (Figure 1B): Abl kinase-dead (AblKD = AblK417N) and AblΔFABD, which deletes the conserved FABD (van Etten *et al.*, 1994). Fly Lys-417 corresponds to Lys-271 of human Abl, which the crystal structure reveals is part of the ATP-binding pocket (Schindler *et al.*, 2000). This residue is highly conserved in tyrosine kinases, and its mutation eliminates their function. AblK417N eliminates kinase activity of fly Abl, as assessed by both kinase assays on Abl immunoprecipitated from *Drosophila* embryos (Henkemeyer *et al.*, 1990) and failure to elevate phosphotyrosine levels after overexpression in vivo, in contrast to wild-type Abl (Stevens *et al.*, 2007). Mutation of the analogous lysine in mouse Arg also eliminates kinase activity, and thus this mutant is the canonical kinase-dead mutant (e.g., Peacock *et al.*, 2007).

Both mutants, as well as other mutants described later, were C-terminally green fluorescent protein (GFP) tagged and driven by the endogenous *abl* promoter. All transgenes were integrated into the same chromosomal location to minimize position effects, and transgenics were verified by PCR and/or sequencing (Supplemental Figure S1, A–H). GFP tagging does not interfere with the ability of Abl to rescue, and the endogenous promoter drives expression levels equivalent to that of endogenous Abl (Fox and Peifer, 2007). We confirmed that all of our transgenes are equivalently expressed (Supplemental Figure S1I).

Construct	Residues changed or deleted	Percentage rescue of adult viability in *abl*^{4}/*Df(3L)st-j7* background (normalized to WT)		Percentage rescue of progeny, embryonic viability of *abl*^{4}/*Df(3L)st-j7* female × *abl*^{4}/+ male	Percentage rescue of progeny, embryonic viability of *abl*^{4} *maternal germline* female × *abl*^{4}/+ male
		25°C	18°C		
No transgene	N/A	9.4 (911)[a]	2.1 (733)	N/A	8.2 (527)
AblWT	N/A	100 (1502)[b]	100 (1335)[b]	89.4 (901)	38.9 (540)[b]
AblKD	K417N	88.0 (1225)[a,b]	50.9 (1009)[a,b]	62.3 (705)[a]	25.2 (726)[a,b]
AblΔFABD	Δ1418–1520	102.6 (1238)[b]	91.1 (1474)[a,b]	97.1 (362)	34.6 (515)[b]
AblKDΔFABD	K417N,Δ1418–1520	86.4 (585)[a,b]	37.1 (361)[a,b]	52.1 (259)[a]	28.2 (560)[a,b]
AblΔCR1	Δ734–789	69.6 (2149)[a,b]	7.7 (972)[a,b]	53.9 (267)[a]	12.4 (606)[a,c]
AblΔCR2	Δ930–965	103.8 (56)[b]	ND	ND	54.0 (303)[b]
AblΔCR3	Δ1003–1014	111.7 (110)[b]	ND	ND	68.7 (256)[b]
AblΔCR4	Δ1063–1095	120.4 (906)[b]	95.3 (797)[a,b]	87.1 (340)	52.0 (254)[b]

In evaluating differences between genotypes, it is important to note that "statistically significant" differences using the test chosen may not always reflect important biological differences. We were thus cautious not to overinterpret small differences. Our more qualitative assessments of relevance are presented in Supplemental Figure S3.

Numbers in columns 5 and 6 (rescue of embryonic viability) are fractions of viable progeny and are not normalized to rescue by AblWT. Numbers in columns 3 and 4 (adult viability rescued) are reported as normalized to WTAbl. This was done because the cross (*abl*^{4}*/TM3 Sb* × *Df(3L)st-j7/TM3Sb*) produced three classes of adults (*abl*^{4}*/TM3 Sb, Df(3L)st-j7/TM3Sb,* and the relevant class *abl*^{4}*/Df(3L)st-j7*). Because none of the genotypes is fully wild type, it would be problematic to compare progeny numbers to those expected from Mendelian segregation, as the mutations on the TM3 Balancer chromosome and other mutations on the Deficiency chromosome may also affect adult viability. We counted the number of adults of each genotype (distinguishable by linked markers *Sb* and *Ki*). For example, at 18°C, for AblWT, 488/1335 (36.6%) of the progeny were *abl*^{4}*/TM3 Sb,* 397/1335 (29.7%) were *Df(3L)st-j7/TM3Sb*, and 450/1335 (33.7%) were the relevant class, *abl*^{4}*/Df(3L)st-j7*. Thus, for other genotypes scored for adult viability at 18°C, the percentage of the progeny of the *abl*^{4}*/Df(3L)st-j7* genotype was divided by 33.7 to normalize to wild-type survival.

Numbers in parentheses are numbers of adults/embryos scored; N/A, not applicable; ND, not determined.

[a]Survival *worse* than rescue by AblWT at $p < 0.001$ (all others not significantly worse or actually better than AblWT).

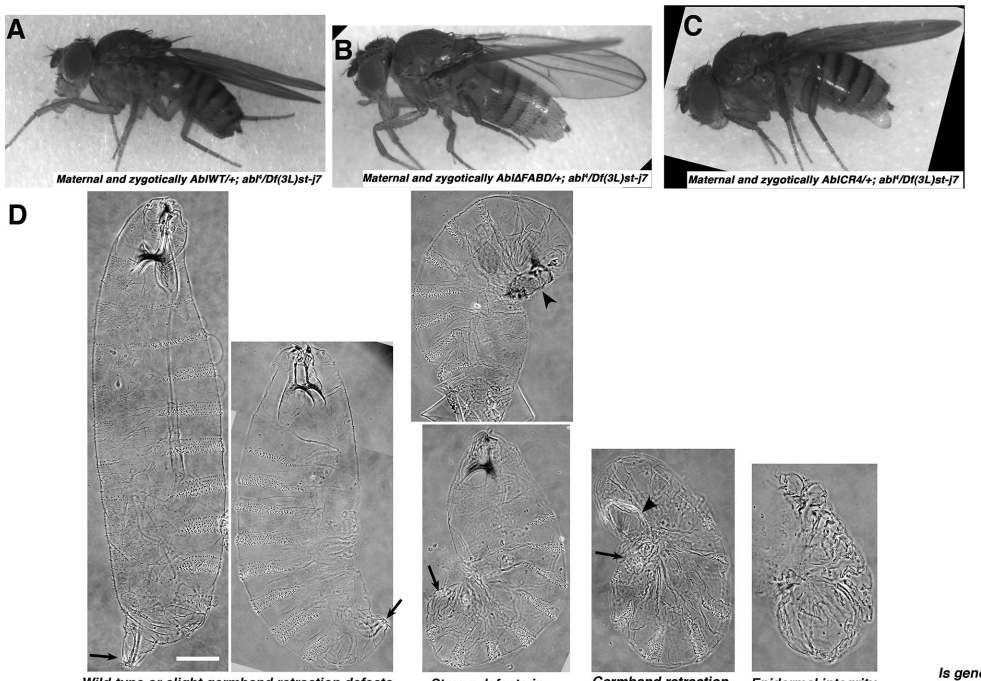
[b]Survival *better* than no transgene at $p < 0.01$.

[c]Survival *better* than no transgene at $p < 0.05$ (all others not significant).

TABLE 1: Rescue of adult or embryonic viability of *abl* mutants by mutant transgenes.

We initially hypothesized that *both* kinase activity and the FABD would be essential for *all* Abl functions in morphogenesis or that *each* would be required for a *subset* of Abl's functions. To test these hypotheses, we expressed each transgene in the absence of endogenous Abl and assessed their function. As a first functional test, we assessed whether they rescued the lethality caused by Abl loss. The *abl* zygotic mutants survive embryogenesis due to maternally contributed Abl, but most die as pupae (no transgene = no TG; Figure 1C, left, and Table 1). We first assessed whether our mutants rescued adult viability at 25°C. AblΔFABD rescued adult viability as well as our wild-type *abl* transgene (AblWT; Figure 1C, left; full data and statistical tests in Table 1; rescue was normalized to that of AblWT). AblKD retained substantial rescuing ability only slightly reduced from that of AblWT (88%; Figure 1C, left, and Table 1). We further challenged the mutants by testing them at 18°C, which increases phenotypic consequences of Abl loss, perhaps by impairing cytoskeletal dynamics (Grevengoed *et al*., 2003). At 18°C, *abl* zygotic mutants are almost fully pupal lethal, but AblΔFABD once again rescued as well or nearly as well as AblWT (91%; Figure 1C, right, and Table 1). In this assay, although AblKD retained substantial rescuing ability, it was significantly reduced from that of AblWT (51%) but did provide significant rescue over no transgene (2%; Figure 1C, right, and Table 1). We next tested whether AblKD and AblΔFABD rescued embryonic viability of *abl* maternal and zygotic mutants (*ablMZ*), which are fully embryonic lethal (Grevengoed *et al*., 2001). To do this, we crossed adult females that lack zygotic Abl expression and carry one copy of specific Abl transgenes (*abl*^{4}*/Df(3L)st-j7*; transgene/+ females; *abl*^{4} is a null allele (Fox and Peifer, 2007), and *Df(3L)st-j7*a is a chromosomal deletion removing *abl* and nearby genes) to *abl*^{4}/+ heterozygous males that were homozygous for the transgene (Supplemental Figure S2A). Thus 50% of the progeny were maternally and zygotically *abl* null. By using two different alleles in these crosses, we removed issues associated with effects of other mutations on the chromosome during oogenesis of the *abl*^{4}*/Df(3L)st-j7* mothers. Surprisingly, AblΔFABD fully rescued embryonic viability (97% viable; Figure 1D and Table 1); this is comparable to the viability of wild-type control flies and matches rescue by AblWT. AblKD once again was reduced in its function (embryonic viability of 62 vs. 89% for AblWT; Figure 1D and Table 1), but the level of rescue suggested that a subset of AblKD maternal/zygotic mutants survive embryogenesis.

Most striking, AblΔFABD, like AblWT, allowed *ablMZ* mutants, which lack any endogenous Abl from the start of development, not only to survive embryogenesis, but also to survive to become fertile adults without obvious defects (Figure 2, A and B) that produce viable progeny. To confirm this surprising result, we PCR-genotyped adult progeny, verifying rescue (Supplemental Figure S3). In contrast, the subset of *ablMZ* mutants whose embryonic viability was rescued by AblKD did not survive to adulthood. Thus the FABD of Abl is *not* essential for successful embryonic or postembryonic development. In contrast, Abl kinase activity is critical for full Abl function, but AblKD retains significant function in promoting embryonic and postembryonic viability.



Genotype		Wild-type or slight germband retraction defects	Strong defects in germband retraction or head involution	Germband retraction, head involution, and dorsal closure failure	Epidermal integrity defects	Is genotype significantly different than abl^{MZ}	Is genotype significantly different than abl^{MZ}; AblWT
abl^{MZ}	(n=450)	30%	27%	28%	15%	N.A.	p<0.0001
abl^{MZ}; AblWT	(n=252)	77%	9%	7%	5%	p<0.0001	N.A.
abl^{MZ}; AblKD	(n=605)	76%	15%	4%	4%	p<0.0001	N.S.
abl^{MZ}; AblΔFABD	(n=375)	79%	5%	11%	4%	p<0.0001	N.S.
abl^{MZ}; AblKDΔFABD	(n=170)	84%	8%	7%	1%	p<0.0001	N.S.
abl^{MZ}; AblΔCR1	(n=343)	55%	22%	12%	9%	p<0.0001	p<0.0001

FIGURE 2: Neither kinase activity nor the FABD is required to rescue adult viability and fertility of *abl*^{4} maternal/zygotic mutants or for many morphogenetic movements, as assessed by cuticle pattern. (A–C) Adults, anterior left, rescued by indicated transgene. All were otherwise maternally and zygotically *abl*-null mutant (*abl*^{4}*/Df(3L)st-j7* progeny of zygotically *abl*-null mutant mothers (maternal genotype *abl*^{4}*/Df(3L)st-j7;* Supplemental Figure S2A; the altered bristles are caused by the *Ki* mutation on the *Df(3L)st-j7* chromosome). (D) Rescue of embryonic morphogenesis, as assessed by larval cuticles, presented anterior up. Top, four classes of increasing severity. Arrows, correct germband retraction (left cuticle) or increasingly severe defects. Arrowheads indicate defects in head involution and dorsal closure. Scale bar, 50 μm. Bottom, fraction of cuticles in each category. All except AblΔCR1 provide essentially wild-type levels of rescue. In D, embryos are progeny of a cross in which mothers had germlines homozygous for the null allele *abl*^{4} and fathers were *abl*^{4}*/+*. Thus all embryos were maternally mutant for endogenous *abl*, and half were maternally and zygotically mutant.

Neither kinase activity nor the FABD is essential for cortical localization, localization to axons, or overall epidermal morphogenesis

One function of kinase activity or the FABD might be in ensuring correct subcellular localization. From gastrulation to germband retraction, Abl is found in a cytoplasmic pool and enriched at the cell cortex in cells of the ectoderm, a localization that is replicated by GFP-tagged AblWT (Fox and Peifer, 2007; Supplemental Figure S4A). AblKD and AblΔFABD both retained cortical enrichment (Supplemental Figure S4, B and C). Abl is also enriched in CNS axons (Henkemeyer *et al.*, 1990), as is GFP-tagged AblWT (Supplemental Figure S4G). AblKD and AblΔFABD show no alterations in axonal enrichment (Supplemental Figure S4, H and I), even in the absence of wild-type Abl (Supplemental Figure S4, H and J; as we document later, however, AblKD did not rescue CNS defects). Thus neither kinase activity nor the FABD is essential for these aspects of Abl localization.

The lethality of *abl* mutants reflects Abl's many roles in embryonic and adult morphogenesis and CNS development. The disadvantage of our *abl*^{4}*/Df(3L)st-j7* approach is that we could not directly compare embryos carrying our transgenes to comparable embryos without any transgene, since *abl*^{4}*/Df(3L)st-j7* females are essentially inviable (Figure 1C) and infertile. To circumvent this, we used the FLP/FRT/DFS approach (Chou and Perrimon, 1996) to generate females with germlines homozygous for *abl*^{4} either in the presence of a transgene or in the absence of any transgene as a control. We crossed them to males heterozygous for *abl*^{4}—thus half of the progeny have no maternal or zygotic Abl, whereas half express paternally contributed Abl (Supplemental Figure S2B). This allowed direct comparison of *ablMZ* mutants rescued with our mutant transgenes to control *ablMZ* mutants without any transgene; the disadvantage is that our wild-type *abl* transgene (AblWT) does not fully rescue viability in this context, presumably due to homozygosity of other loci on the *abl*^{4} chromosome that enhance effects of Abl reduction.

We thus used rescue by AblWT as our baseline. AblΔFABD also significantly rescued embryonic viability, although it might be slightly diminished relative to AblWT (35% embryonic viability vs. 39% viability for AblWT and 9% viability for no transgene; Figure 1E and Table 1). AblKD also significantly rescued embryonic viability but was less effective than AblWT (25% viability vs. 39% viability for AblWT and 9% viability for no transgene; Figure 1E and Table 1).

Abl's role in embryonic viability reflects important roles in cell behavior in many different tissues. To initially assess the requirement for kinase activity or the FABD in regulating morphogenetic movements, we examined cuticles of mutant embryos. The cuticle is the larva's external skeleton and is secreted by the epidermis, and its features allow one to assess completion of morphogenetic movements such as germband retraction, dorsal closure, and head involution, all of which are defective in *ablMZ* mutants (Figure 2D; Grevengoed *et al.*, 2001; half of the embryos receive a paternal wild-type *abl* gene and are partially rescued). Surprisingly, both AblKD and AblΔFABD substantially rescued morphogenesis defects at rates similar to AblWT (Figure 2D, bottom). These data suggest that neither kinase activity nor the FABD is essential for completion of many morphogenetic movements, at least as assessed by cuticle patterning.

AblΔFABD rescues cellularization, mesoderm invagination, and CNS development as effectively as AblWT, whereas AblKD has differential function in these events

These data were surprising, demonstrating that neither kinase activity nor the conserved FABD is absolutely essential for viability or some aspects of normal morphogenesis. Thus we tested an alternate mechanistic hypothesis: that kinase activity and/or the FABD play specific roles in a subset of processes requiring Abl. The robustness of the embryonic program means embryos can survive despite defects in some cell biological events of morphogenesis. We thus examined cell shapes and cell movements underlying morphogenetic events depending on Abl: cellularization, coordinated mesoderm invagination, germband retraction, dorsal closure, and axon outgrowth (Grevengoed *et al.*, 2001, 2003; Fox and Peifer, 2007). Abl loss during cellularization leads to excess Ena accumulation at the apical cortex, leading to aberrant actin accumulation, defects in actomyosin furrow ingression, and formation of multinucleate cells (Figure 3, A vs. B; Grevengoed *et al.*, 2003). *ablMZ* mutants also have defects in coordinated apical constriction during gastrulation (Figure 3F; Fox and Peifer, 2007) and thus mesodermal cell invagination into the ventral furrow, once again due to aberrant Ena accumulation and apical actin abnormalities. This leads to defects at the ventral midline (Figure 3, C vs. D). Strikingly, AblΔFABD rescued both cellularization and mesodermal invagination at rates similar to AblWT (Figure 3, C, E, and G; representative images of all genotypes are in Supplemental Figure S5). In contrast, AblKD provided only a subtle rescue of cellularization (Figure 3E and Supplemental Figure S5D; 32% cellularized normally vs. 21% for no transgene and 85% for AblWT). However, AblKD effectively rescued mesoderm invagination (Figure 3E and Supplemental Figure S5K).

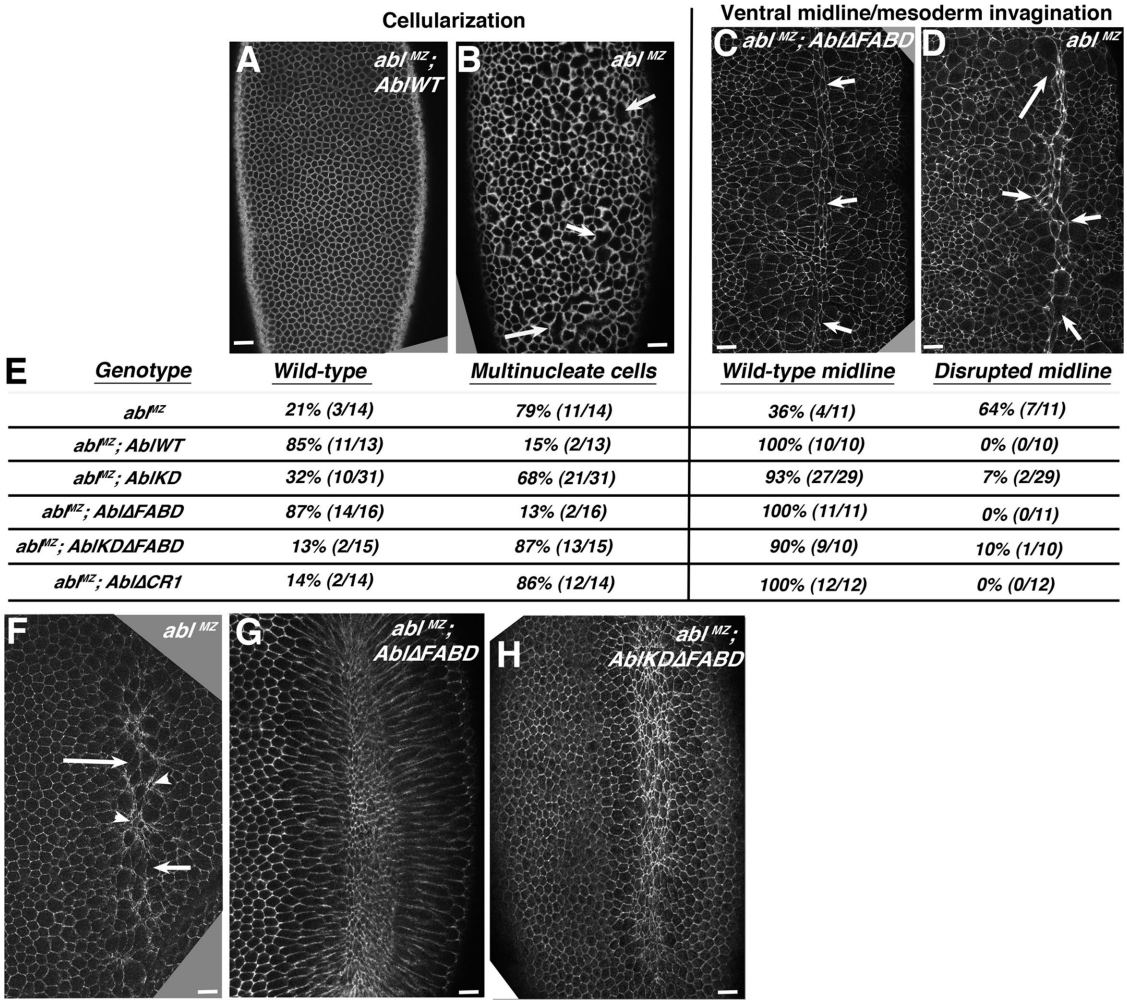
Abl also regulates CNS development via effects on axon path finding (Gertler *et al.*, 1993; Grevengoed *et al.*, 2001). Maternal/zygotic Abl loss leads to severe CNS defects—instead of the correct ladder of longitudinal and commissural axons, *ablMZ* mutants either have gaps in commissures (Figure 4, A vs. B and H vs. I, quantified in K) or more-severe axon disorganization (Figure 4, H vs. J, quantified in K). These defects are substantially but not fully rescued by AblWT—60% of embryos are fully rescued, whereas the others have commissural axon defects (Figure 4, C and K). Once again, AblΔFABD (Figure 4E) rescued CNS defects as well as AblWT (Figure 4K). In contrast, AblKD retained only partial function (Figure 4D), rescuing the severe axon disorganization phenotype of some *ablMZ* mutants but providing little or no rescue of the commissureless phenotype (Figure 4K). Thus the FABD is not essential for completion of *any* of these three morphogenetic events, whereas kinase activity is more important for regulating some events (cellularization and CNS axon outgrowth) and less important for others (mesoderm invagination).

The FABD is not essential for cell shape changes or actin regulation during dorsal closure, whereas kinase activity plays a modulatory role

Among the processes most drastically affected by Abl loss is dorsal closure, in which epidermal cell sheets move dorsally to meet at the midline to enclose the embryo (Grevengoed *et al.*, 2001). This involves coordinated cell shape change and migration in two tissues (Martin and Parkhurst, 2004) and thus offers an opportunity to look for subtle effects of loss of kinase activity or the FABD. The squamous amnioserosal cells cover the dorsal surface before closure (Figure 5, A and B, asterisks) and are attached laterally to the two epidermal epithelial sheets (Figure 5, A and B, arrowheads). Three forces drive closure, all of which require a well-organized actin cytoskeleton: 1) Epidermal leading edge (LE) cells assemble a contractile actomyosin cable, anchored cell to cell at cell junctions; this contracts, helping power closure. 2) Amnioserosal cells apically constrict, pulling the epidermal sheets dorsally. 3) Dorsal protrusions of LE cells guide zippering at the canthi as the sheets meet (Figure 5, A and B, arrows).

The *ablMZ* mutants have major defects in both dorsal closure and germband retraction, which precedes it (Figure 5, A and B vs. D and E, quantitated in M; Grevengoed *et al.*, 2001). Thus most *ablMZ* mutants have very abnormally shaped "dorsal openings" (Figure 5, A–C vs. D–F, and M). We tested whether kinase activity or the FABD are essential for cell shape changes or collective cell migration during dorsal closure. Consistent with our cuticle data, we saw striking rescue of overall dorsal closure by both AblKD and AblΔFABD (Figure 5, G–J, quantitated in M), with rescue by AblΔFABD equivalent to that provided by AblWT (Figure 5C) and rescue by AblKD nearly as effective. In almost all embryos, germband retraction was successfully completed, the LE was relatively straight (Figure 5, G–J, arrowheads), and zippering at the canthi occurred normally (Figure 5, G–J, arrows). This suggests that kinase-dead and FABD-deleted mutants retain substantial function in regulating morphogenesis.

Dorsal closure is a robust process that compensates for loss of individual forces (Kiehart *et al.*, 2000). Thus we tested whether our overall analysis missed subtle defects in cell biological events caused by absence of kinase activity or the FABD. Constriction of the LE actomyosin cable is one important driving force (Hutson *et al.*, 2003). Normally, LE cells maintain even tension along the cable and thus have fairly similar widths at the leading edge (Figure 6, A–C, T-shapes). In *ablMZ* mutants, in contrast, some LE cells have splayed-open leading edges (Figure 6, D and E, arrows) whereas neighbors are hyperconstricted (Figure 6, D and E, arrowheads; Grevengoed *et al.*, 2001), suggesting that actin cable integrity is reduced, and, when it fails, cells splay open while neighbors constrict. In addition, a subset of *ablMZ* epidermal cells completely fail to elongate along their dorsal-ventral axes, for unknown reasons (Figure 6, D and E, brackets). We hypothesized that although AblKD and AblΔFABD rescued completion of closure, they might have defects in cell shape change. Strikingly, both



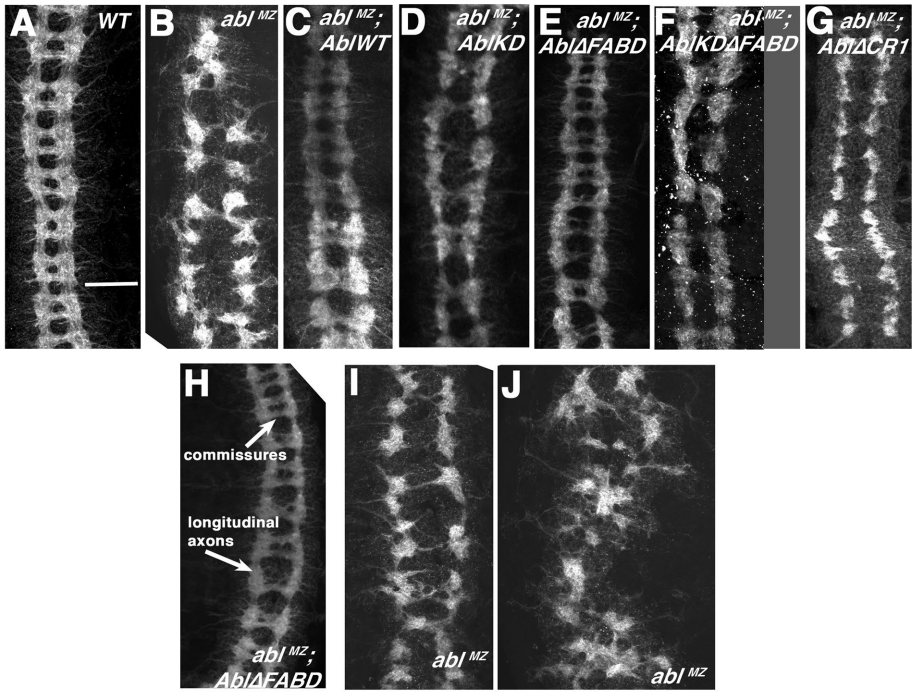
E

Genotype	Wild-type	Multinucleate cells	Wild-type midline	Disrupted midline
abl^{MZ}	21% (3/14)	79% (11/14)	36% (4/11)	64% (7/11)
abl^{MZ}; AblWT	85% (11/13)	15% (2/13)	100% (10/10)	0% (0/10)
abl^{MZ}; AblKD	32% (10/31)	68% (21/31)	93% (27/29)	7% (2/29)
abl^{MZ}; AblΔFABD	87% (14/16)	13% (2/16)	100% (11/11)	0% (0/11)
abl^{MZ}; AblKDΔFABD	13% (2/15)	87% (13/15)	90% (9/10)	10% (1/10)
abl^{MZ}; AblΔCR1	14% (2/14)	86% (12/14)	100% (12/12)	0% (0/12)

FIGURE 3: The FABD is not required for correct cellularization or mesoderm invagination, whereas AblKD and AblKDΔFABD display differential activity in these events. (A–D, F–H) Embryos, anterior at top. (A, B) abl^4 maternal mutants have multinucleate cells at cellularization (B, arrows), which is effectively rescued by AblWT (A). (C, D) Defects in coordinated mesoderm invagination in abl^4 maternal mutants lead to a very irregular ventral midline at stage 9 (D, arrows), which is rescued by AblΔFABD (C, arrows). (E) Quantitation of degree of rescue by the indicated mutants. Left, multinucleate cells. Right, mesoderm invagination. Representative images of embryos of each genotype are shown in Supplemental Figure S5. (F–H) Embryos during mesoderm invagination. (F) *ablMZ* mutant showing lack of coordinated apical constriction (arrows vs. arrowheads). (G, H) Fixed images suggest that coordinated apical constriction is largely restored by AblΔFABD or AblKDΔFABD. Scale bars, 15 μm. Embryos are progeny of a cross in which mothers had germlines homozygous for the null allele abl^4 and fathers were $abl^4/+$. Thus all embryos were maternally mutant for endogenous *abl*, and half were maternally and zygotically mutant.

AblKD (Figure 6, H and I, T-shapes) and AblΔFABD (Figure 6, J and K, T-shapes) largely restored wild-type LE cell shapes, reducing the splayed-open and hyperconstricted leading edges seen in *ablMZ* mutants (Figure 6, D and E, arrows; rescue quantitated in Table 2) and rescuing uniform dorsal-ventral cell elongation and AblWT (Figure 6, F and G, and Table 2).

The cell shape defects in *ablMZ* mutants reflect underlying changes in Ena-mediated actin regulation (Grevengoed *et al.*, 2001). Wild-type LE cells assemble a leading edge actomyosin cable (Figure 7, A–B″, arrowheads), whereas more-ventral epidermal cells accumulate actin all around their cortex, with subtle enrichment at tricellular junctions (Figure 7, A–B″, arrows). The actin regulator Ena is enriched at tricellular junctions of all epidermal cells during early dorsal closure (Figure 7, A–B″, arrows; Gates *et al.*, 2007); these coincide with and may promote the modest actin accumulation seen at tricellular junctions. During later dorsal closure, Ena accumulates in prominent "leading edge dots" at presumptive sites of cell–cell cable attachment (Figure 7, I–I″, arrows). In *ablMZ* mutants, actin becomes highly elevated at dorsal-ventral cell boundaries of many epidermal cells during early dorsal closure, including those not at the LE (Figure 7, C–D″, arrows). These "actin flares" overlap Ena's normal tricellular junction localization, consistent with the possibility that they result from Ena misregulation. In later dorsal closure, LE Ena dots become irregular in *ablMZ* mutants (Figure 7, J–J″, arrows), with some cells accumulating much less Ena. AblΔFABD restored both normal low-level actin accumulation at tricellular junctions during early dorsal closure (Figure 7, G–H″, arrows) and normal Ena LE dot localization later (Figure 7L″, arrows). AblKD restored normal low-level actin accumulation at tricellular junctions during early dorsal closure, (Figure 7, E–F″, arrows) but only partially restored Ena LE dot localization later (Figure 7, K′ and K″, arrows). Together these data strongly suggest that the FABD is not essential



K Genotype	Near wild-type	No commisures	Severely disrupted
abl^{MZ}	0% (0/9)	44% (4/9)	56% (5/9)
abl^{MZ}; AblWT	60% (6/10)	30% (3/10)	10% (1/10)
abl^{MZ}; AblKD	10% (2/21)	81% (17/21)	10% (2/21)
abl^{MZ}; AblΔFABD	69% (9/13)	31% (4/13)	0% (0/13)
abl^{MZ}; AblKDΔFABD	13% (1/8)	75% (6/8)	13% (1/8)
abl^{MZ}; AblΔCR1	22% (5/23)	70% (16/23)	9% (2/23)

FIGURE 4: AblΔFABD rescues the CNS defects of *ablMZ* mutants, whereas AblKD, AblKDΔFABD, and AblΔCR1 retain only partial activity. (A–G) Representative CNS pictures of wild type and indicated mutants, presented anterior up. (H–J) Examples of wild-type or near-wild-type CNS with normal longitudinal and commissural axons (H), a mutant lacking most commissures (I), and a mutant showing the severely disrupted phenotype (J). (K) Quantitation of defect frequency in the indicated mutants. Embryos are progeny of a cross in which mothers had germlines homozygous for the null allele abl^4 and fathers were abl^4/+. Thus all embryos were maternally mutant for endogenous *abl*, and half were maternally and zygotically mutant. However, for CNS scoring, mutants scored were selected to be maternally *and* zygotically mutant using a GFP-marked Balancer chromosome. Scale bar, 25 μm.

for Abl to correctly regulate the cytoskeleton or cell shape changes during dorsal closure, whereas kinase activity plays a modulatory but not essential role.

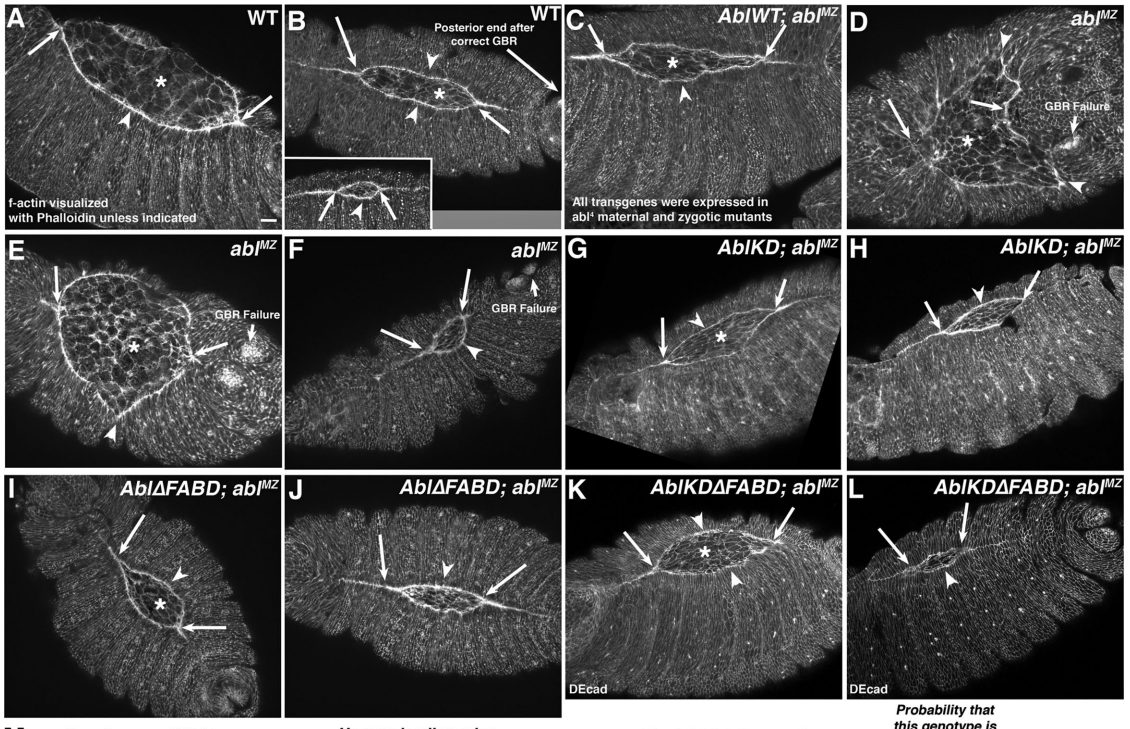
Deleting the FABD does not affect dorsal closure dynamics

Given the complete rescue of viability and morphogenesis by AblΔFABD, we hypothesized that its role might only be apparent in effects on the dynamics of morphogenesis rather than their completion. Dorsal closure is robust (Kiehart *et al.*, 2000), and some mutations, like that in the cadherin regulator p120catenin, do not affect completion of closure or accompanying cell shape changes but do slow the process (Fox *et al.*, 2005). We thus used live imaging to examine whether FABD loss significantly slowed dorsal closure. We first measured the overall closure rate, assessing the rate of decrease in amnioserosal area in the last 60 min of dorsal closure. The wild-type closure rate is quite variable but averaged 63 μm^2/min (Figure 8, A and E, and Supplemental Movie S1). The closure rate in *ablMZ* mutants (zygotically rescued embryos were eliminated using a GFP-marked Balancer chromosome) was significantly reduced (32 μm^2/min; $p = 0.03$; Figure 8, B and E, and Supplemental Movie S2). Strikingly, AblWT (Figure 8C and Supplemental Movie S3) and AblΔFABD (Figure 8D and Supplemental Movie S4) both rescued closure to rates statistically indistinguishable from wild type (Figure 8E). We also assessed the rate of zippering from the canthi, beginning when the opening was 120 μm—due to variability, even *ablMZ*-null mutants were not statistically distinguishable from wild type, but the trends were similar (Figure 8F)—with the slowest rate seen for *ablMZ*. Our quantitation of closure rates underestimates the rescue by AblWT and AblΔFABD because almost half of unrescued *ablMZ* mutants completely failed to close (Figure 2D), and even in those that did, closure was qualitatively quite abnormal, with zippering defects (Figure 8B4, red arrow), abnormal leading edges, and failure to retract deep segmental grooves (Figure 8B6, blue arrows). AblΔFABD also effectively rescued these qualitative aspects of closure. In summary, *none* of our assays revealed a significant diminution in the function of Abl lacking the FABD.

Removing *both* kinase activity and the FABD severely reduces but does not eliminate Abl function

We considered two hypotheses explaining why both AblKD and AblΔFABD retain substantial function: 1) *both* kinase activity and the FABD may be largely dispensable for some events, or 2) kinase activity and the FABD may function somewhat redundantly in modulating Abl's scaffolding role, so that removing one does not totally disable Abl. To distinguish between these hypotheses, we generated a double mutant that is both kinase dead and lacks the FABD (AblKDΔFABD; Figure 1B).

AblKDΔFABD significantly rescued *abl* zygotic mutant adult viability at 25°C (Figure 1C, left;, and Table 1; 86% viable normalized to AblWT vs. 9% without a transgene). This rescuing activity was significantly reduced but not eliminated when we challenged AblKDΔFABD function further by assessing adult viability at 18°C (Figure 1C, right, and Table 1; 37% of the viability of AblWT vs. 2% without a transgene). Of interest, this was lower than that of both AblΔFABD (91%) and AblKD (51%), suggesting that removing both kinase activity and the FABD may have additive effects on Abl function. AblKDΔFABD also retained some ability to rescue *ablMZ* mutant embryonic viability, but this was substantially reduced from AblWT (Figure 1, D and E, and Table 1); the 52% viable progeny of AblKDΔFABD; abl^4/*Df(3L)st-j7* + mothers (Figure 1D) likely relates only to those receiving paternal wild-type *abl*. Once again, the double mutant appeared more impaired than either single mutant (AblΔFABD, 97% viability; AblKD, 62% viability). Finally, unlike AblWT or AblΔFABD,



M

Genotype	Wild-type or slightly wavy LE (C)	Uneven leading edge or slowed zippering (E above)	Total GBR failure and dorsal opening misshapen (D above)	Probability that this genotype is different than: abl^{MZ}	Probability that this genotype is different than: abl^{MZ}; AblWT
abl^{MZ}	5% (2/43)	60% (26/43)	35% (15/43)	N.A.	p=0.0001
abl^{MZ}; AblWT	94% (17/18)	6% (1/18)	0% (0/18)	p<0.0001	N.A.
abl^{MZ}; AblKD	72% (21/29)	17% (5/29)	10% (3/29)	p<0.0001	N.S.
abl^{MZ}; AblΔFABD	88% (46/52)	12% (6/52)	0% (0/52)	p<0.0001	N.S.
abl^{MZ}; AblKDΔFABD	94% (15/16)	6% (1/16)	0% (0/16)	p<0.0001	N.S.
abl^{MZ}; AblΔCR1	11% (2/18)	56% (10/18)	33% (6/18)	N.S.	p<0.0001

FIGURE 5: Abl proteins lacking kinase activity, the FABD, or both can restore a straight leading edge (LE) and effective zippering at the canthi during dorsal closure. Embryos, anterior left, dorsal toward viewer. Early (A, D), mid (B, C, E, G, I, K), or late dorsal closure (B, inset, F, H, J, L). Cell shapes and actin cable were visualized using phalloidin to detect F-actin. All mutants were selected to be maternally and zygotically mutant for endogenous Abl using a GFP-marked Balancer. (A, B) In wild type, amnioserosal contraction (asterisks) and the LE actin cable (arrowheads) drive closure, and epidermal sheets zip together at the canthi (arrows). Balanced forces leave the LE straight (arrowheads). Prior completion of germband retraction placed spiracles at the posterior end (beyond the right arrow in B). (C) AblWT rescues dorsal closure in abl^4 maternal/zygotic mutants. (D–F) Almost all *ablMZ* mutants fail to fully retract the germband, so the spiracles are not at the posterior (germband retraction [GBR] failure). Most have severe LE defects (D, E, arrowheads), and zippering is delayed (D, E, arrows). Occasional mutants have a less severe phenotype (F) but still fail to fully retract their germband. (G–L) AblKD (G, H), AblΔFABD (I, J), and AblKDΔFABD (K, L) each largely restore normal dorsal closure, with a relatively straight LE (arrowheads) and effective zippering (arrows). The mild LE waviness was also observed in embryos rescued with AblWT (C). Scale bar, 15 μm. (M) Quantitation of degree of rescue of dorsal closure in the different mutants. Embryos are progeny of a cross in which mothers had germlines homozygous for the null allele abl^4 and fathers were abl^4/+. Thus all embryos were maternally mutant for endogenous *abl*, and half were maternally and zygotically mutant. However, in this figure, mutants scored were selected to be maternally *and* zygotically mutant using a GFP-marked Balancer chromosome.

AblKDΔFABD did not rescue adult viability of *ablMZ* mutants. These data are consistent with the possibility that whereas loss of the FABD alone does not substantially impair Abl function, removing it further reduces function of AblKD. However, Abl lacking both kinase activity and the FABD retained significant residual function.

We next explored whether AblKDΔFABD differentially rescued Abl's embryonic roles. Of interest, morphogenesis (as assessed by cuticle patterning; Figure 2D) and coordinated mesoderm apical constriction (Figure 3, E, and F vs. H, and Supplemental Figure S5M) were rescued as well or nearly as well by AblKDΔFABD as by AblWT. Strikingly, however, Abl's regulation of cellularization was not effectively rescued by AblKDΔFABD (Figure 3E and Supplemental Figure S5F), nor did it rescue correct midline axon guidance (Figure 4, F and K)–these paralleled roles in which AblKD also had defects. Finally, we examined dorsal closure. Consistent with the cuticle phenotype, overall dorsal closure was substantially rescued by AblKDΔFABD (Figure 5, K and L, quantitated in M), with an even LE (Figure 5, K and L, arrowheads) and sheet zippering (Figure 5, K and L, arrows) largely restored. AblKDΔFABD also largely rescued uniform LE cell shape (Figure 6, L and M, T-shapes, and Table 2), although we occasionally saw epidermal cell elongation failure and an uneven LE (Figure 6M, brackets and arrowhead). These data suggest that kinase activity and the FABD both contribute to full Abl function, but Abl lacking both still rescues many aspects of morphogenesis. These deficits in function are not solely due to a complete failure of cortical enrichment of AblKDΔFABD, as it

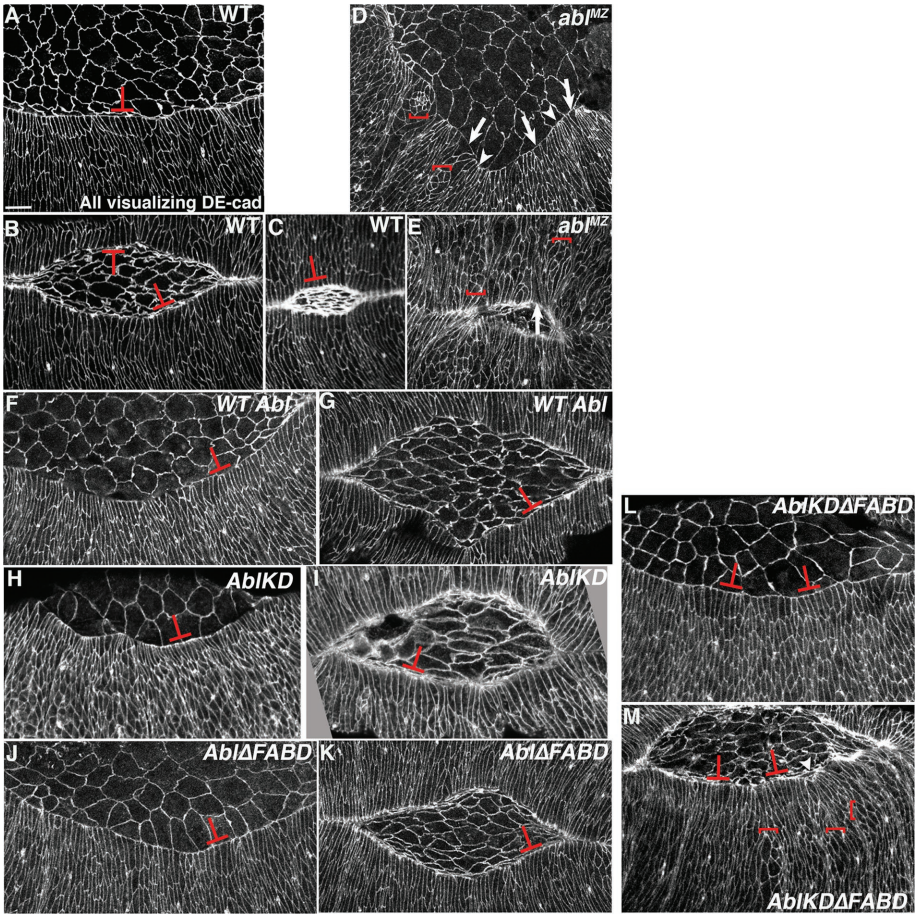


FIGURE 6: AblKD, AblΔFABD, and AblKDΔFABD each rescue the cell shapes of leading edge (LE) cells during dorsal closure. Embryos, anterior left and dorsal facing viewer, during early (A, D, F, H, J, L), mid (B, G, I, K, M), or late (C, E) dorsal closure, with cell shapes visualized using DE-cadherin. (A–C) Wild type. Even cable tension maintains relatively even-length LEs (T-shapes). (D, E) *ablMZ*. Some LE cells have splayed open LEs (arrows) while neighbors are hyperconstricted (arrowheads). In addition, some epidermal cells do not elongate (brackets). (F–M) AblWT (F, G), AblKD (H, I), AblΔFABD (J, K), and AblKDΔFABD (L, M) each restores relatively even LE shapes (T-shapes; Table 2) and uniform epidermal cell elongation, although in AblKDΔFABD (L, M) occasional epidermal cells fail to elongate (M, brackets). Scale bar, 10 μm. Embryos are progeny of a cross in which mothers had germlines homozygous for the null allele abl^4 and fathers were $abl^4/+$. Thus all embryos were maternally mutant for endogenous *abl*, and half were maternally and zygotically mutant. However, in this figure, mutants scored were selected to be maternally *and* zygotically mutant using a GFP-marked Balancer chromosome.

consensus binding site for the actin regulator Ena. To identify potential functional regions, we compared linkers of insect Abls, defining four short conserved regions of 12–56 amino acids, which we refer to as CR1–CR4 (Supplemental Figure S6). CR2 and CR3 do not contain any recognizable motifs. CR4 carries a perfect match to the "FP4 motif" that binds Ena's EVH1 domain, as well as a second, less perfect match (LPPPP; Supplemental Figure S6). CR1 is the only motif with recognizable similarity to human Abl and Arg linkers—it includes the PXXP motif known to bind the adapters Crk and Nck and the actin regulator Abi (Figure 1A and Supplemental Figure S6; Feller, 2001; Ibarra *et al.*, 2005).

To test whether these motifs contribute to Abl's mechanism of action in development, we generated mutants deleting each motif individually. We first tested rescue of adult viability of *abl* zygotic mutants or embryonic viability of *ablMZ* mutants. AblΔCR2 and AblΔCR3 rescued both as well as AblWT (Figure 9, A and C, left, and Table 1). This suggests that CR2 and CR3 are not essential for Abl function in morphogenesis, and we did not analyze these mutants further. Owing to the presence of the FP4 motif, we examined AblΔCR4 in more depth. Strikingly, AblΔCR4 was as effective as AblWT at rescuing embryonic viability of *ablMZ* mutants (Figure 9, A and B; full data and statistical tests in Table 1), adult viability of abl^4 zygotic mutants at 25°C (Figure 9C, left, and Table 1), and zygotic viability in our sensitized assay, which stresses cytoskeletal regulation by raising animals at 18°C (Figure 9C, right, and Table 1). Further, AblΔCR4 rescued *ablMZ* mutants to adulthood (Figure 2C). Finally, AblΔCR4 remained cortically enriched in vivo (Supplemental Figure S4F). Thus the putative Ena-binding motifs in CR4 are dispensable for Abl function, at least in

remains cortically enriched (Supplemental Figure S4D). The differential rescuing ability of AblKDΔFABD could mean that different roles require quantitatively different thresholds of Abl activity or differ qualitatively, requiring particular combinations of Abl protein domains.

A conserved region in the Abl linker encoding a PXXP motif is critical for Abl function in vivo

The significant residual function of AblKDΔFABD suggested the hypothesis that other regions of Abl are critical for function. In addition to the SH3/SH2/kinase module and FABD, all Abl proteins contain a long linker, but its sequence is not well conserved from fly to mammal or even between mammalian Abl and Arg (Figure 1A). In Arg, the linker carries another actin-binding domain and a microtubule-binding domain (Miller *et al.*, 2004), whereas fly Abl has a

Genotype	Number of "splayed open" leading edge cells per leading edge	Number of leading edges scored	Number of embryos scored
Wild type	1.5	33	28
abl^4 MZ	6.4	32	29
abl^4; AblWT	2.3	26	18
abl^4; AblKD	2.6	28	26
abl^4; AblΔFABD	2	51	34
abl^4; AblKDΔFABD	1.9	16	12
abl^4; AblΔCR1	4.8	18	12

TABLE 2: Rescue of leading edge cell shape.

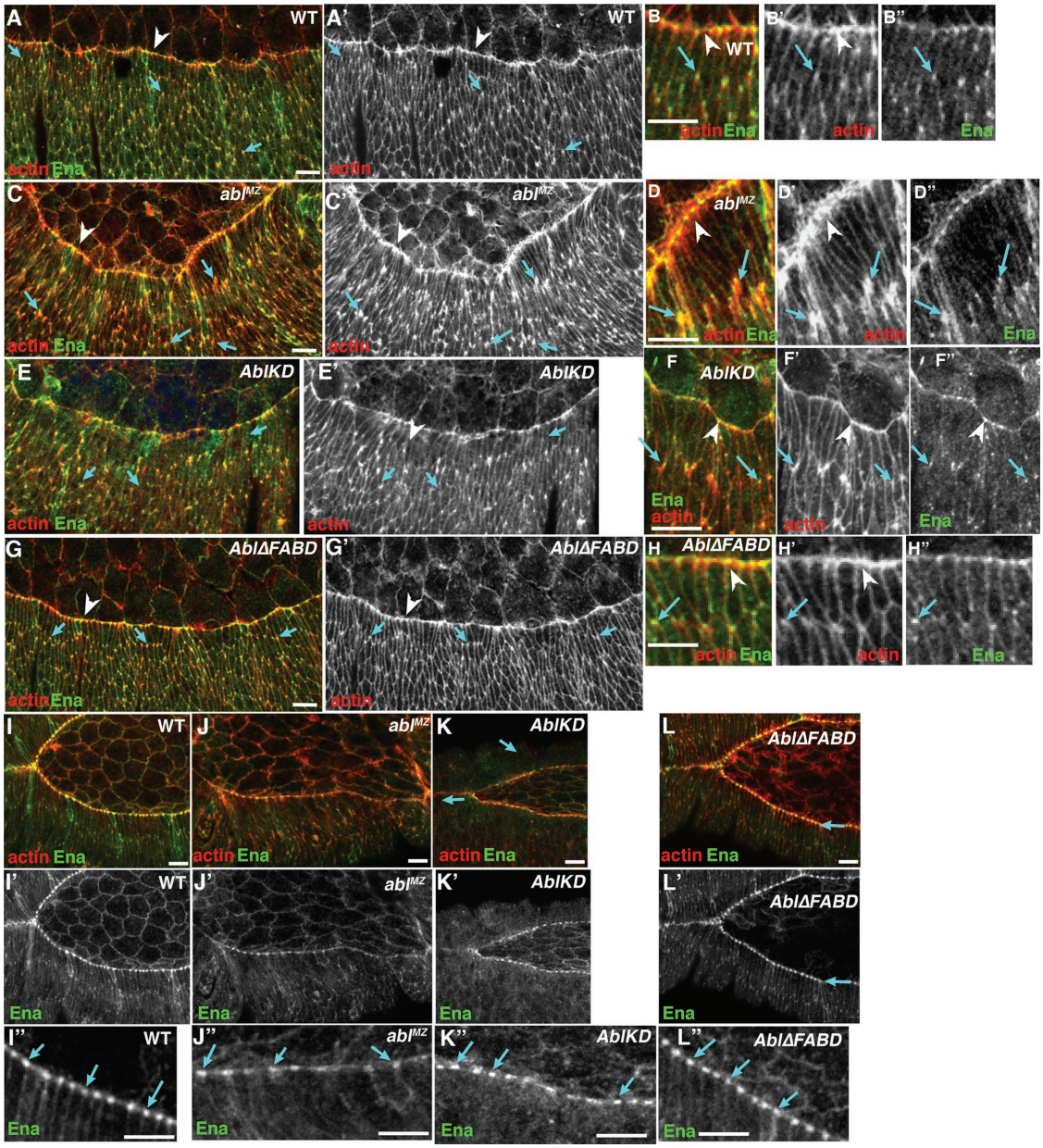


FIGURE 7: Abl regulation of actin levels and Ena localization does not require kinase activity or the FABD. (A–H) Lateral views of early stage 13 embryos, anterior to left. (B, D, F, H) Close-ups. (A, B) Wild type. The actin cable has formed at the LE (arrowheads), and Ena and actin also localize to tricellular junctions of more-ventral epidermal cells (arrows). (C, D) *ablMZ*. Elevated levels of actin accumulate both at LE (arrowheads) and tricellular junctions (arrows). (E–H) Defects in actin localization are rescued by both AblKD (E, F) and AblΔFABD (G, H). (I–L) Stage 14. (I′′–L′′) Close-ups. (I) Wild type. Ena localizes to LE "dots" located near ends of the actin cable within each cell (arrows). (J) *ablMZ*. Ena localization to LE dots is reduced overall and much less regular (arrows). (K–L) AblKD (K) and AblΔFABD (L) both rescue Ena localization. Scale bar, 10 μm. Embryos are progeny of a cross in which mothers had germlines homozygous for the null allele abl^4 and fathers were $abl^4/+$. Thus all embryos were maternally mutant for endogenous *abl*, and half were maternally and zygotically mutant. However, in this figure, mutants scored were selected to be maternally *and* zygotically mutant using a GFP-marked Balancer chromosome.

our assays. Of course, Ena can also associate with Abl via its SH3 domain (Ahern-Djamali *et al*., 1999), so the linker FP4 motif may be redundant.

In contrast, CR1 is critical for full Abl activity, although AblΔCR1 retained residual function. AblΔCR1 significantly rescued adult viability at 25°C (Figure 9C, left; full data and statistical tests in Table 1), although not as well as AblWT. However, at 18°C, AblΔCR1's ability to rescue adult viability was almost eliminated, although it did retain a small amount of residual activity (Figure 9C, right, and Table 1). AblΔCR1 also exhibited substantially reduced rescue of embryonic viability of *ablMZ* mutants (Figure 9, A and B, and Table 1) and could not rescue *ablMZ* mutants to adulthood. Thus AblΔCR1 was less functional than AblKD or AblΔFABD and, in some assays, also appeared less functional than AblKDΔFABD (Figure 9A and Supplemental Figure S7).

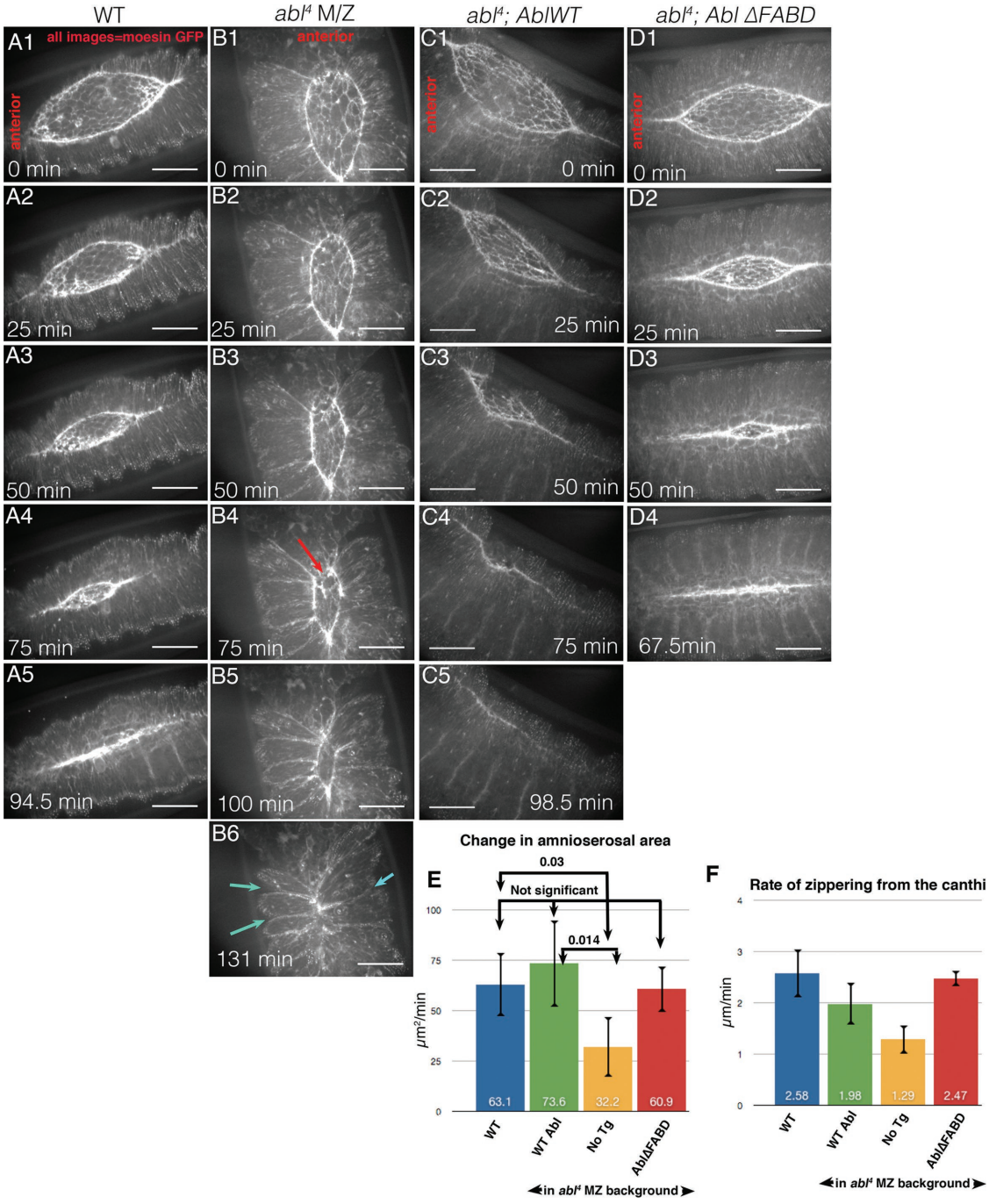


FIGURE 8: Loss of the FABD does not impair the rate of dorsal closure. (A–D) Representative movie stills of embryos expressing moesin-GFP in the indicated mutant, dorsal up, anterior, as indicated (A, Supplemental Movie S1; B, Supplemental Movie S2; C, Supplemental Movie S3; D, Supplemental Movie S4). (E) Quantitation of rate of closure ± SE. Mean rate of change in amnioserosal area from 60 to 30 min and from 30 to 0 min before closure; $n = 5–7$ embryos. (F) Quantitation of mean rate of zippering ± SE. Rate of change in intercanthi distance beginning at 120 μm apart; $n = 4–9$ embryos. Scale bars, 50 μm. Embryos are progeny of a cross in which mothers had germlines homozygous for the null allele *abl*^{4} and fathers were *abl*^{4}/+. Thus all embryos were maternally mutant for endogenous *abl*, and zygotically rescued mutants were eliminated using a GFP-marked Balancer chromosome.

AblΔCR1 has substantially reduced ability to regulate morphogenesis, cell shape changes, and localization of the cytoskeletal regulator Ena

These data suggest that the CR1 region is critical for full Abl function. This could be due to a role in a single process critical for viability (e.g., CNS development) or reflect broader reduction in Abl's diverse cell biological roles. To distinguish these hypotheses, we tested whether AblΔCR1 rescued different embryonic events. We first used cuticles to assess completion of key morphogenetic movements. AblΔCR1 failed to rescue two critical events—germband retraction and head involution (Figure 2D). This contrasted with all other mutants we tested, including

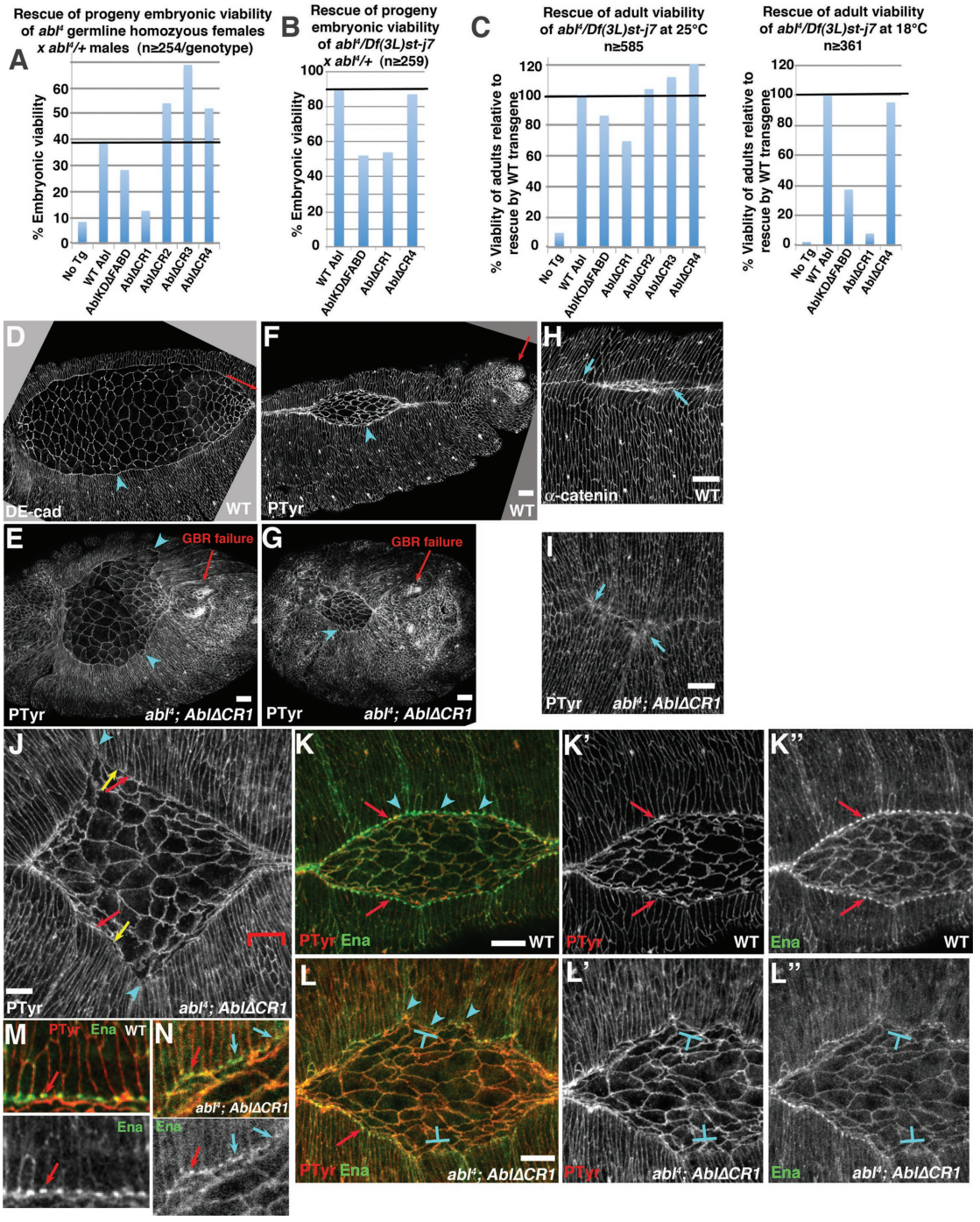


FIGURE 9: CR1 with the PXXP motif is critical for Abl function in morphogenesis. (A, B) Rescue of embryonic viability of *ablMZ* mutants from *abl*^{4} germline homozygous (A) or *abl*^{4}*/Df(3L)st-j7* (B) mothers (50% of progeny are paternally rescued). (C) Rescue of adult viability of *abl*^{4} zygotic mutants at indicated temperatures, normalized to AblWT rescue. Full data for A–C are given in Table 1. (D–N) Stage 13–14 embryos, anterior left, dorsal up. (D–I) Embryos stained to visualize cell–cell junctions. Relative to wild type (D, F, H), AblΔCR1 (E, G, I) fails to fully restore germband retraction (E, G, arrows) or a straight LE (D, F vs. E, G, arrowheads). (H, I) Even AblΔCR1 mutants that do close have severe dorsal midline discontinuities (I, arrows) relative to the straight midline in wild type (H, arrows). (J) AblΔCR1 mutants have the same large-scale defects at the LE (arrowheads), splayed open (red arrows; quantitated in Table 2) or hyperconstricted (yellow arrows) LE cells and failure of epidermal cells to elongate (brackets) seen in *ablMZ* mutants (Figure 6). (K–N) AblΔCR1 fails to rescue LE Ena localization. (K, M) Close-ups. Wild type. The LE is straight (arrowheads), and Ena localizes uniformly to LE "dots" (arrows). (L, N) Close-ups. AblΔCR1. The LE is wavy (arrowheads), and although Ena localizes to LE dots in occasional cells (red arrows), its localization is reduced and less well confined to the LE dots in most cells (T-shapes, blue arrows). Scale bars, 10 μm. In D–N, embryos are progeny of a cross in which mothers had germlines homozygous for the null allele *abl*^{4} and fathers were *abl*^{4}*/+*. Thus all embryos were maternally mutant for endogenous *abl*, and half were maternally and zygotically mutant. However, in this figure, mutants scored were selected to be maternally *and* zygotically mutant using a GFP-marked Balancer chromosome.

AblKDΔFABD. However, AblΔCR1 did provide some rescue of epithelial integrity relative to *ablMZ* mutants lacking any transgene, as fewer embryos had the most-severe epidermal integrity defects (Figure 2D).

To define the cell biological events in which AblΔCR1 retained or lacked function, we examined Abl-regulated events in embryogenesis. AblΔCR1 retained essentially wild-type activity in promoting mesoderm invagination (Figure 3E and Supplemental Figure S5N). In contrast, however, AblΔCR1 failed to rescue cellularization (Figure 3E and Supplemental Figure S5G), and although AblΔCR1 rescued the severe CNS disruption seen in some *ablMZ* mutants, it did not restore correct midline guidance (Figure 4, G and K). These data suggest a possible differential loss of function in AblΔCR1. To further define the mechanistic functions retained or lost by AblΔCR1, we examined dorsal closure, exploring whether AblΔCR1 promoted correct cell shape change or regulated Ena localization. Consistent with cuticle analysis, most AblΔCR1 mutants failed to fully retract their germbands (Figure 9, E and G vs. D and F, red arrows; 12 of 15 embryos scored failed). They also had the extremely uneven LE (Figure 9, E and J vs. D and F, arrowheads; quantitated in Figure 5M) seen in unrescued *ablMZ* mutants (Figure 5K). Even the subset that closed had significant discontinuities at the dorsal midline (Figure 9, H vs. I). AblΔCR1 only partially rescued *ablMZ* mutant cell shape defects—LE cells had splayed open (Figure 9J, red arrows; quantitated in Table 2) and hyperconstricted LEs (Figure 9J, yellow arrows), suggesting defects in actin cable anchoring. Finally, AblΔCR1 failed to restore localization of the actin regulator Ena—whereas Ena is highly and uniformly enriched at LE dots in wild type (Figure 9, K and M, arrows), in AblΔCR1 mutants, Ena localization to LE dots was often reduced overall and less uniform (Figure 9, L, T-shapes, L″, and N, blue arrows), as in *ablMZ* mutants. Thus AblΔCR1 retains little if any function in dorsal closure. Together these data suggest that the CR1 motif plays a surprisingly important but not absolutely essential role in Abl function, with its loss differentially affecting some Abl-regulated events, like dorsal closure or CNS development, while not disrupting others, like mesoderm invagination. Once again, these deficits in function were not solely due to a failure of cortical enrichment, as AblΔCR1 remained cortically enriched (Supplemental Figure S4E), although the degree of enrichment may be reduced from wild type and thus contribute to the deficit in function.

DISCUSSION

As a key regulator of cell behavior in development and an iconic example of targeted cancer therapy, Abl has attracted attention from scientists in many fields. Their studies defined the pathways that Abl regulates and revealed its capabilities as a kinase and a direct cytoskeletal regulator in cultured cells, but the relative importance of each role in morphogenesis was largely untested. Here we directly tested a series of mechanistic hypotheses about how kinase activity, the FABD, and other conserved protein-docking sites contribute to Abl's diverse functions during morphogenesis in vivo.

The FABD is not essential for Abl's diverse roles in morphogenesis, whereas kinase activity plays differential roles in different events

Two mechanistic models for Abl function in development and disease suggest that 1) Abl phosphorylates target proteins to modulate their activity or 2) Abl directly regulates cytoskeletal dynamics via its cytoskeletal binding sites. We tested these hypotheses using mutants lacking either kinase activity or the only predicted cytoskeletal-binding site in fly Abl, the FABD (Supplemental Figure S7). To our surprise, AblΔFABD retained full activity in our assays—in the most dramatic demonstration, it rescued flies completely lacking endogenous maternal *and* zygotic Abl to fertile adults without morphological defects. Because some morphogenetic events regulated by Abl are robust, we looked carefully for cell biological defects that might not block viability, but even in those assays, AblΔFABD rescued as well as AblWT. Thus, despite its sequence conservation, the FABD is not essential for many of Abl's functions during normal development. It is interesting to note that O'Donnell and Bashaw (2013) carried out a detailed analysis of different roles played by Abl in the CNS, and their data suggest that although the FABD is dispensable for motor axon outgrowth, deleting it does lead to failure to rescue midline crossing of EW neurons, suggesting that AblΔFABD is subtly impaired. We note that although the FABDs of mammalian Arg and Abl bind actin, this has not been experimentally demonstrated for fly Abl but is only predicted based on sequence conservation. It will be important to determine whether and how fly Abl interacts with actin. In contrast, Abl kinase activity is critical for full Abl function. In some events, such as cellularization, AblKD was similar to the *abl*-null mutant; previous data also suggest key roles for phosphorylation of β-catenin during convergent extension (Tamada *et al.*, 2012) and Abl kinase activity in germline stem cells (Stine *et al.*, 2014). In other events, such as rescue of zygotic adult or maternal/zygotic embryonic viability, AblKD retained partial function but was significantly less functional than wild-type Abl. Perhaps most surprising, in some events, including mesoderm invagination and completion of dorsal closure and germband retraction, it retained full or nearly full function. Consistent with this, analysis of Abl's roles in axon outgrowth suggested both kinase-dependent and kinase-independent roles (O'Donnell and Bashaw, 2013). Thus kinase activity, although important for full Abl function, is not essential for all of its roles.

The conserved linker PXXP motif plays a surprisingly critical role in morphogenesis

In addition to the SH3:SH2:kinase module and FABD, all Abl proteins contain a long linker with a sequence that is divergent among mammalian paralogues or insect orthologues. Mammalian Arg has additional actin- and microtubule-binding sites in the linker, suggesting that family members may have distinct linker motifs contributing to function. Insect Abls share four short conserved motifs that may be partner-binding sites. Two, CR2 and CR3, do not contain known motifs, and neither proved critical for function. CR4 carries one perfect and one imperfect match for binding sites for Abl's key regulatory target, Ena. Surprisingly, AblΔCR4 was fully functional in all our assays. We hypothesize that this reflects redundancy/robustness of the Abl scaffold, as Abl's SH3 domain can also bind Ena (Ahern-Djamali *et al.*, 1999). Similarly, the second actin- and the microtubule-binding domains of Arg in the linker provide it with unique functions (e.g., Courtemanche *et al.*, 2015). Perhaps variant Abl linkers provide raw material to create new binding motifs that modulate but are not critical for Abl function.

Although CR2–4 were dispensable, CR1, the only linker motif shared by fly and mammalian Abl, proved critical for Abl function. AblΔCR1 failed to rescue embryonic viability of *ablMZ* mutants, and its activity in regulating individual morphogenetic events was severely impaired, although, like AblKD, AblΔCR1 retained residual function in a subset of Abl's roles. Thus CR1 is a critical and surprisingly less redundant part of Abl's mechanism of action (Supplemental Figure S7). Several partners bind human Abl CR1, including Crk, Nck, and Abi (Hossain *et al.*, 2012). These partners play strikingly different cell biological roles and can compete with one another for binding to Abl. Crk and Nck are signaling adapters, linking signals

from receptor and nonreceptor tyrosine kinases, integrins, or cadherins to diverse outputs (Zandy and Pendergast, 2008; Bell and Park, 2012), whereas Abi is a multifunctional regulator of distinct actin polymerization machines (e.g., Ryu *et al.*, 2009). It will be exciting to explore which contributes to different Abl functions in vivo. Existing mutational analysis may provide clues. *Drosophila* Abi is an excellent candidate, as it shares with Abl roles in cultured cell- protrusive behavior (Kunda *et al.*, 2003) and embryonic axon guidance (Lin *et al.*, 2009) and exhibits dose-sensitive genetic interactions with Abl (Lin *et al.*, 2009). Nck is also a reasonable candidate, as maternal and zygotic Nck mutants (= *Drosophila* Dock) share significant embryonic CNS defects with *abl*, although these are not as severe as those of *ablMZ* mutants and in fact exhibit increased rather than decreased midline crossing (Desai *et al.*, 1999)—effects on morphogenesis were not reported. Genetic analysis of *Drosophila* Crk was slowed by its location on the fourth chromosome and the lack of null alleles, but a cell-based RNA interference (RNAi) screen implicated both Abl and Crk in internalization of the pathogen *Pseudomonas aeruginosa* (Pielage *et al.*, 2008), and analysis of both RNAi and a hypomorphic allele support Crk roles in pupal thoracic closure downstream of the receptor tyrosine kinase Pvr (Ishimaru *et al.*, 2004). Of course, all three proteins likely have Abl-independent roles as well, complicating interpretation.

A model of Abl kinases as robust scaffolds with multiple semiredundant binding sites

Our understanding of receptor tyrosine kinases underwent a significant change 20 yr ago when it was realized that they are scaffolds that assemble signaling complexes, with kinase activity largely serving to create additional partner binding sites (Margolis and Skolnik, 1994). The surprising dispensability of the FABD and the significant function retained by both kinase-dead Abl and AblΔCR1 in some events support a similar speculative model for Abl in which Abl assembles a multiprotein signaling/actin regulatory complex, with individual partners recruited by interactions with several proteins (Supplemental Figure S7). Thus, for example, a particular protein partner might bind both a tyrosine residue phosphorylated by Abl's kinase and an actin-binding protein brought into the complex by Abl's actin interaction or an SH3 domain protein that binds CR1. Eliminating one recruitment mechanism might only reduce but not eliminate inclusion of this partner from the complex, while eliminating both potential recruitment mechanisms would be more deleterious. Consistent with this, a double mutant lacking both kinase activity and the FABD (AblKDΔFABD) has, in some cases, phenotypes quantitatively more severe than AblKD or AblΔFABD (Figure 9, A and C). However, AblKDΔFABD is not biologically dead—in fact, in some assays, it retained wild-type or nearly wild-type function, whereas in other events, it had residual activity. These data are consistent with a scaffolding model in which additional interaction sites exist, allowing AblKDΔFABD to retain residual scaffolding function by binding a subset of its partners. The SH2 and SH3 domains provide obvious examples. It will be important to explore their function in morphogenesis. In addition, in the absence of Abl kinase activity, other kinases may phosphorylate proteins in the complex, recreating SH2 binding sites normally created by Abl phosphorylation. Consistent with this, Abl is regulated by Src kinases in development and oncogenesis, with dual Abl/Src inhibitors emerging as effective treatments in CML resistant to the Abl-inhibitor Gleevec. Strikingly, overexpressing the Src-family kinase Hck confers Gleevec resistance; this requires Src kinase activity and correlates with Abl phosphorylation (Pene-Dumitrescu and Smithgall, 2010). Our data are reminiscent of work with platelet-derived growth factor receptors, for which mutating individual SH2-docking sites had complex, overlapping. or tissue-specific effects (Klinghoffer *et al.*, 2002; Tallquist *et al.*, 2003). This and recent work on phosphodependent protein complexes in yeast Hippo signaling (Rock *et al.*, 2013) suggest that robust and complex scaffold assembly may be a broad feature of signaling.

As we further test our scaffolding model, it will be important to determine whether the differential loss of function of AblKDΔFABD or AblΔCR1 in distinct morphogenetic events occurs because different roles require *quantitatively* different thresholds of Abl activity or because some roles require particular protein–protein interactions. Because deleting CR1 leads to defects in Ena localization, although Ena is not believed to bind this site, loss of particular interactions may have complex consequences.

Abl in mammalian development and oncogenesis

Mammalian Abl and Arg play important roles in events ranging from neural tube closure to immune system function to synaptogenesis. Cell-based studies identified cellular events regulated by Abl kinase activity or cytoskeletal interactions, respectively. Given our data, it will be exciting to explore whether mouse Abl is as robust a scaffold, by introducing mutants analogous to ours into *abl* and *arg* single- or double-mutant mice and analyzing the diverse Abl/Arg functions. Our results may also have implications for Bcr-Abl's oncogenic role. Gleevec's effectiveness in CML and the strong selection for mutations restoring kinase activity in relapsing Gleevec-treated patients clearly demonstrate that kinase activity is *necessary* for oncogenesis. How can we reconcile these results with ours? First, Bcr-Abl's unregulated kinase likely phosphorylates targets outside Abl's normal repertoire, some of which may drive oncogenesis. Second, the data do not demonstrate that kinase activity is *sufficient* for oncogenesis but instead that suggest kinase activity and direct cytoskeletal regulation cooperate in cell transformation. Experiments defining which aspects of Abl function are required for oncogenesis may now be warranted, using inducible Bcr-Abl mutants altering single "functions" and testing the robustness of this oncogenic kinase to perturbation (Huettner *et al.*, 2000).

MATERIALS AND METHODS

Transgenic fly lines

The *Xba*/*Not*I fragment from pUASg-Abl::GFP (Fox and Peifer, 2007) containing 2 kb of upstream endogenous Abl promoter sequence and the Abl coding region was cloned into *Xba*/*Not*I sites of pUAStattP. Both missense and deletion mutants were made by PCR stitching of overlapping PCR products with mutations generated by primers in the overlapping segments. The forward mutagenic oligonucleotide primers for the Abl constructs are as follows:

AblΔCR1: 5′**CCAGCAGCAGGCCAGCACGCCCATG**CCAGCCAACGCCAGATGCAATTTCATCG-3′

AblΔCR2: 5′-**GGTGACCAGTGCTCATCCCATCACT**GAGGCTGCTCCTGCTCTTCCGGCAACTGC-3′

AblΔCR3: 5′**CGAAGGCCAGCCCCATTCCGCCACAG**ATGCAAAACAATGCGGCTGCCAGC-3′

AblΔCR4: 5′**GGAGTGCCTCGGGAGTGGCTTCAGGA**GCGCCGGAGAGCGCTGTGCAGGCC-3′

AblΔFABD: 5′**AGCTCCGCCAGCTCCACACAGATATCA**GGACTAGTGATTGGAGCTAGCATGGTG-3′

AblKD: 5′-TGGC AATACGGTGGCTGTTAACACGCTCAAGGA GGACACCAT-3′

The end of the boldface type in the primer sequences for the Abl deletions indicates the location of the start of the deletion. The underlined base indicates the location of the point mutation. The reverse primer for the respective constructs was the reverse complement of the forward primer. All constructs were sequenced to verify the desired mutational change and ensure that other changes had not been introduced. Transgenes were targeted to the left arm of the second chromosome by phiC31 integrase–mediated transgenesis (Bischof *et al.*, 2007). Injections of transgenic constructs were performed by BestGene (Chino Hills, CA) into PBacyellow[+]-attP-3BVK00037 (cytogenetic map position, 22A3).

Genotyping assays

Fly genomic DNA was prepared as follows: one to five flies were frozen overnight at –20°C and then crushed with a barrier tip in 50 µl of 1× squishing buffer (10 mM Tris, pH 8.2, 1 mM EDTA, 25 mM NaCl) plus 1 µl of Proteinase K (10 mg/ml), incubated at 37°C for 30–60 min, and heat inactivated for 1–2 min at 95°C. Genomic DNA was used as a template for PCR. In Supplemental Figure S1, A and B, we verify the presence of the Abl kinase-dead mutation. Single flies were crushed as described. PCR was used to amplify a 238–base pair region around the kinase-dead mutation. Primer sequences used were, for KD forward, GTCTTTCCGCTGAGTCCCGAGCCG, and for KD reverse, CACCAATGAGCTGCACCAGATTAGG.

The PCR product was sequenced using standard Sanger sequencing (University of North Carolina at Chapel Hill Genome Analysis Facility) using as primer KD-Seq, ACATCATGATGAAGCACAAGC. Note that the PCR primers used do not distinguish between the endogenous and the transgenic *abl* locus. Thus sequencing of this PCR product yields two peaks (an A for the endogenous locus and a C for the transgene) at the position of the point mutation, leading to an amino acid change that ablates Abl kinase activity.

In Supplemental Figure S1, D–H, we use PCR to verify the presence of the *abl* deletions in ΔFABD, ΔCR1, ΔCR2, ΔCR3, and ΔCR4. A 4-µl amount of each genomic DNA preparation was used for PCRs, following manufacturer's instructions (Phusion polymerase; Thermo Scientific, Waltham, MA). Then 10–20 µl of the PCR was run on a 1.3–1.5% gel at 100 V. Primers sequences used were as follows:

ΔFABD-F: GCACAAGCCAACAAAGCTAA

ΔFABD-R: GAACTTCAGGGTCAGCTTGC

ΔCR1-F: GGCGGTCAGGCCCTCACGCCGAACGCCCACCACAACGATCCGCACCAGCAGCAG

ΔCR1-R: CCATTCGTGCTGAGGTCGTCGATGAAATTGCATCTGGCG

ΔCR2-F: ACTTATCGCGAGGAGGATCC

ΔCR2-R: GGCCTTCGGATTTAGTCTGG

ΔCR3-F: GAGGCTGCTCCTGCTCTTC

ΔCR3-R: GGAGGAGAACGTCATCATGG

ΔCR4-F: CCACTACCGAAGGCACCATG

ΔCR4-R: GTTGCCATTGTTTGGCAGCTG

For Supplemental Figures S1C and S3, PCR was done using two primers that flank the first intron of *Drosophila abl* (forward, 5′-CCTGGTCCGTGAAAGTGAAA-3′, and reverse, 5′-GGATCCTCTGAGATGCGGTA-3′). PCR products were resolved on a 3.5% NuSieve GTG agarose (Lonza, Basel, Switzerland) gel in 1× TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0). Wild-type endogenous *abl* yields a 166–base pair fragment, the abl^4 mutant yields a 133–base pair fragment, and the *abl* transgenes yield a 93–base pair fragment due to the absence of an intron.

Fly stocks, viability, and phenotypic analysis of *abl* mutants and statistical tests

We used both *y* w and histone-GFP–expressing flies (Clarkson and Saint, 1999) as wild type. Zygotic *abl* mutants were generated by crossing *Df(3L)st-7 Ki/TM3 Sb* females to *abl* transgene *(Tn[Abl])/Tn[Abl]; abl^4/TM3 Sb* males and selecting for *Ki* and against *Sb* (*abl^4/Df(3L)st-7 Ki*). The fraction of progeny with this genotype seen when using the wild-type *abl* transgene (AblWT; 30.9% at 25°C and 33.7% at 18°C) was set as 100%, and other genotypes were normalized to this. Adult viabilities were compared by Fisher's exact test (GraphPad, La Jolla, CA). Embryos and flies maternally and zygotically *abl* mutant (*ablMZ*) were generated in two ways: 1) using the dominant female sterile method (Chou and Perrimon, 1996) to make abl^4 clones in the female germline and 2) using a deficiency spanning the *abl* locus transheterozygous to abl^4 (cross schemes are shown in Supplemental Figure S2). Briefly, to generate *abl* germline clones, *hs::Flp;;FRT 79 D-F ovoD/TM3* males were crossed with w;*Tn[Abl]/Tn[Abl];FRT 79 D-F abl^4/TM3* females. The 48- to 72-h-old progeny were heat shocked for 3 h at 37°C and allowed to develop to adulthood. Resulting virgin female flies of the genotype *hs::FLP/+;Tn[Abl]/+; FRT 79 D-F abl4/ FRT 79 D-F ovoD* were crossed with *w;Tn[Abl]/Tn[Abl];FRT 79 D-F abl^4/TM3, twi-GAL4,UAS-EGFP* males and put into cups with apple juice/agar plates and yeast paste for embryo collection. The presence of a GFP-expressing Balancer chromosome allowed identification of paternally rescued embryos after germband extension. To generate embryos and flies maternally and zygotically mutant for *abl* using a deficiency, we used *Df(3L) st-j7, Ki/TM6b* (Bloomington *Drosophila* Stock Center #5416, Deletes73A2-73B2). For both generation of *ablMZ* mutants and assessment of rescue to adult viability by the *abl* transgenes, *w; Df(3L) st-j7, Ki/ TM3, twi-GAL4,UAS-EGFP* females were crossed with *w;Tn[Abl]/Tn[Abl];FRT 79 D-F abl^4/TM3* males. If the resulting w;*Tn[Abl]/+;FRT 79 D-F abl^4/Df(3L) st-j7, Ki* females were viable, they were crossed to w;*Tn[Abl]/Tn[Abl];FRT 79 D-F abl^4/TM3, twi-GAL4,UAS-EGFP* males and put into collection cups. Assessment of embryonic lethality and preparation of embryonic cuticles were done as in Wieschaus and Nüsslein-Volhard (1986). To compare cuticle phenotypes of abl^4 mutants and embryos expressing different Abl transgenes in the abl^4 mutant background, we used Fisher's exact test (GraphPad). For each genotype, the numbers of cuticles falling into our three defective classes (strong defects in germband retraction, dorsal closure failure, and epidermal integrity defect) were grouped into a single defective category and compared with the number of cuticles in the wild-type category. Similarly, cuticle scores of each mutant transgene in the abl^4 mutant background were compared with the wild-type transgene (AblWT) using the same approach. Fisher's exact test was also used to compare the degree of rescue of dorsal closure in Figure 5M. To assess the completion of cellularization, we examined fixed embryos at cellularization, with cell outlines revealed by staining with phalloidin to mark either F-actin or DE-cadherin at a magnification that included ~70% of the embryo surface. Embryos were scored as mutant if these images showed any cells in which cellularization failed, as assessed by counting cells with apical ends two or more times larger than the typical cell in the field (Figure 3B, arrows). All scored as mutant had >10 cells in which cellularization failed. To assess successful completion of ventral furrow invagination, we

assessed fixed embryos after completion of germband extension. Successful completion of ventral furrow invagination was scored as embryos in which the ventral midline was straight and cells at the midline had relatively equal-sized apical ends (Figure 3C, arrows). In contrast, more than half of *abl*^{4} mutants had ventral midlines that were extremely irregular and in which many cells had failed to constrict apically (Figure 3D, arrows).

Embryo live imaging

Moesin-GFP–expressing embryos (Edwards *et al.*, 1997) were dechorionated in 50% bleach and mounted in halocarbon oil (series 700; Halocarbon Products, River Edge, NJ) between a coverslip and a gas-permeable membrane (Petriperm; Sartorius, Edgewood, NJ). Imaging was performed with a PerkinElmer (Waltham, MA) UltraView spinning-disk confocal ORCA-ER camera (Hamamatsu, Hamamatsu City, Japan), Nikon (Melville, NY) 40× Plan Apo numerical aperture 1.3 objective, and MetaMorph (Molecular Devices, Sunnyvale, CA) software. The 0.5-µm *z*-stacks were maximum intensity projected to visualize the amnioserosa and leading edge, using the ImageJ 5-D plugin (National Institutes of Health, Bethesda, MD). Dorsal closure rates based on area were calculated by measuring the dorsal opening area at 60 and 30 min before closure, and the final rate was determined by averaging the closure rates from 60 to 30 min and from 30 min to completion. Data were analyzed using a Mann–Whitney *U* test in Matlab (MathWorks, Natick, MA).

Immunohistochemistry and immunoblotting

Flies were allowed to lay eggs for the appropriate time (1–24 h) on apple juice/agar plates with yeast paste. Embryos were dechorionated in 50% bleach, washed in 0.1% Triton-X, and fixed at room temperature in 1:1 heptane/3.7% formaldehyde diluted in phosphate-buffered saline (PBS) for 20 min. Embryos were devitillenized either by shaking in 1:1 heptane/methanol or by hand for phalloidin staining. Embryos were incubated in blocking solution (PBS/0.1% Triton-X and 1% normal goat serum) for ≥30 min, incubated overnight at 4°C in primary antibody diluted in blocking solution, and washed three times in blocking solution. Embryos were incubated for 2 h at room temperature in secondary antibody in blocking solution and washed three times in blocking solution. Embryos were mounted on glass slides in Aquapolymount (Polysciences, Warrington, PA). Imaging was done on a Zeiss (Oberkochen, Germany) LSM-5 Pascal or a Zeiss 710 scanning confocal microscope. Primary and secondary antibodies used were anti–D-cadherin (1:100), anti-Ena (1:500), anti-BP102 (1:200; all from the Developmental Studies Hybridoma Bank, Iowa City, IA), and anti-mouse and anti-rat immunoglobulin G (IgG) Alexa Fluor 568 and 647 (Molecular Probes, Waltham MA); some secondary antibodies were preabsorbed with fixed *y w* embryos. For F-actin staining, tetramethylrhodamine isothiocyanate–labeled phalloidin (Sigma-Aldrich, St. Louis, MO) was used at a dilution of 1:500–1:1000.

For immunoblotting, equal volumes of embryos were homogenized with a pestle in a microfuge tube in SDS–PAGE sample buffer, boiled for 5 min, run on a 7.5% SDS–PAGE gel, and transferred to a nitrocellulose membrane. For detection of the transgenic protein, anti-GFP (JL-8, 1:500 or 1:1000; Clontech, Mountain View, CA) was used. Anti–α-tubulin (1:10,000; Sigma-Aldrich), anti-γ–tubulin (clone GTU-88; 1:2500; Sigma-Aldrich), and anti-Pnut (1:30; Developmental Studies Hybridoma Bank) were loading controls. Secondary antibody was horseradish peroxidase–conjugated anti-mouse IgG (1:50,000; Pierce, Waltham, MA), and ECL Plus substrate kit (Pierce) was used for detection.

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