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Crk-associated substrate (Cas) signaling protein functions with integrins to specify axon guidance during development

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Members of the Cas family of Src homology 3 (SH3)-domain-containing cytosolic signaling proteins are crucial regulators of actin cytoskeletal dynamics in non-neuronal cells; however, their neuronal functions are poorly understood. Here, we identify a *Drosophila* Cas (DCas), find that Cas proteins are highly expressed in neurons and show that DCas is required for correct axon guidance during development. Functional analyses reveal that Cas specifies axon guidance by regulating the degree of fasciculation among axons. These guidance defects are similar to those observed in integrin mutants, and genetic analysis shows that integrins function together with Cas to facilitate axonal defasciculation. These results strongly support Cas proteins working together with integrins in vivo to direct axon guidance events.

KEY WORDS: p130Cas, Axon guidance, *Drosophila*, CasL

INTRODUCTION

The identification of molecular cues that guide neuronal processes to their targets in vertebrates and invertebrates reveals fundamental mechanisms underlying the development of neural networks (Huber et al., 2003; Tessier-Lavigne and Goodman, 1996). These guidance cues include both membrane-bound and secreted proteins that instruct the navigation of axons and dendrites through both attraction and repulsion. Cell surface receptors for these guidance cues initiate the signal transduction pathways essential for steering neuronal processes (Dickson, 2002). However, the signaling pathways initiated by guidance cue receptors, and the molecular mechanisms through which these signaling cascades alter axonal and dendritic cytoskeletal dynamics to direct attractive or repulsive steering events, are still poorly defined.

Some of the mechanisms that direct growing axons are similar to those that promote cell migration (Ridley et al., 2003). Proteins originally characterized as important for cell migration through their effects on cytoskeletal dynamics have more recently been shown to play roles in axon guidance during development. Integrins are a large family of receptors crucial for promoting cell migration, supporting cellular adhesion to extracellular matrix (ECM) components, and regulating intracellular actin filament dynamics (Hynes, 2002; Vicente-Manzanares et al., 2005). Integrins also participate in directing growing axons to their targets (Clegg et al., 2003; Nakamoto et al., 2004). Genetic analyses in *C. elegans*, *Drosophila* and mice reveal that axon extension in integrin mutants in vivo is not compromised during development; however, defects in axon guidance result from loss of integrin function (Baum and Garriga, 1997; Billuart et al., 2001; Hoang and Chiba, 1998; Pietri et al., 2004; Stevens and Jacobs, 2002). In combination with in vitro and in vivo observations addressing the effects integrins and their ligands exert on growing axons (Adams et al., 2005; Bonner and O'Connor, 2001; Garcia-Alonso et al., 1996; Gomez et al., 1996; Hopker et al., 1999; Kuhn et al., 1995), these studies define functions for integrin signaling in guiding growing axons, strongly suggesting that integrins are essential for axon guidance.

The molecular mechanisms by which integrins guide neuronal processes during development remain to be determined. Integrin receptors are heterodimers composed of a ligand-binding α-subunit and a β-subunit that together signal through their cytoplasmic domains (Hynes, 2002). Numerous molecules function downstream of integrin receptor activation to mediate cell migration, including tyrosine kinases such as focal adhesion kinase (FAK) and Src, Rho GTPases, focal adhesion proteins including Paxillin, and proteins in the Crk-associated substrate (Cas) family (Wiesner et al., 2005). Cas proteins are intriguing integrin signaling components because they are Src homology 3 (SH3)-domain-containing proteins that physically link integrins to kinases, phosphatases, guanine nucleotide exchange factors and proteins that nucleate actin filaments (Chodniewicz and Klemke, 2004; O'Neill et al., 2000). Furthermore, Cas proteins transduce intracellular signals that stimulate actin filament assembly (Bouton et al., 2001) and act as force sensors during cell motility (Sawada et al., 2006). Do Cas proteins provide a crucial link between integrin activation and actin dynamics essential for guiding growing axons to their targets in vivo? We show here that Cas proteins are highly expressed in the nervous system and together with integrins direct discrete axonal steering events during development.

MATERIALS AND METHODS

Molecular analysis and transformation

A *Drosophila Cas* cDNA (EST RE07188) was sequenced on both strands. ^{Myc}*DCas* was made using PCR to obtain a small *DCas* linker sequence that was placed, using restriction endonucleases, downstream of a 3×Myc tag and sequenced. *DCas* was then subcloned in-frame downstream of this linker. ^{Myc}*DCas* was then inserted into the pUAST vector for *Drosophila* transformation.

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In situ hybridization

Standard RNA in situ analysis of wild-type *Drosophila* embryos was carried out using sense and antisense *DCas* cRNA probes (Terman et al., 2002). No specific staining was observed in sense controls. In situ analyses on specific genotypes were conducted with the aid of a 'blue balancer' chromosome and staining embryos with a β-galactosidase antibody (1:3000, Cappell).

Western analysis and immunohistochemistry

Western analysis and *Drosophila* immunostaining were performed as described (Terman et al., 2002). Monoclonal antibodies against Fasciclin II (Fas2) (1D4), BP102, Myc (9E10), muscle myosin (FMM5), Even-skipped (3C10), β1 integrin betaPS (CF.6G11), α1 integrin alphaPS1 (DK.1A4), α2 integrin alphaPS2 (CF.2C7) were obtained from Developmental Studies Hybridoma Bank, University of Iowa. Embryos heterozygous for both *DCas* and β1, α1 or α2 were genotyped using a 'blue balancer' and the respective integrin antibodies to identify integrin heterozygotes. Cas antibodies [Phospho-p130Cas (Tyr410); #4011, Lot #1, Cell Signaling Technologies (Fonseca et al., 2004)] were used for western analysis (1:500) and *Drosophila* immunostaining (1:300).

Genetics and phenotypic characterization

Flies were obtained from Bloomington [*Df(3L)ED201*, $DCas^{Df(3L)Exel6083}$ and integrin mutants β1 (mys^{1}), α1 (mew^{M6}) and α2 (if^{k27E})] and the *Drosophila* Genetic Resource Center [$DCas^{P1}$ (NP4466)]. *Drosophila* genetics and scoring of axon guidance defects were performed using standard approaches (Terman et al., 2002). All images were captured using a Zeiss Axioimager upright microscope with an Axiocam HRc camera and Axiovision software. Brightness, contrast and color balance of images were adjusted using Adobe Photoshop.

RESULTS

Identification of a highly conserved Cas family member in *Drosophila*

Cas proteins play central roles in mediating cell migration and actin cytoskeletal reorganization, but their contributions to the establishment of nervous system connectivity remain to be determined. Therefore, we sought to understand how Cas proteins function during neural development using *Drosophila* as a model system. We identified several overlapping *Drosophila* EST sequences with a high degree of similarity to human *CAS* (*BCAR1* –Human Gene Nomenclature Database) and localized these ESTs to a single gene on the third chromosome that we named *Drosophila Cas* (*DCas*, *CG1212*; not to be confused with the *Drosophila* genes *CAS/CSE1 segregation protein* and *castor*) (Fig. 1A). The GenBank accession number for *DCas* is EF599121. The *DCas* locus covers ~13 kb of genomic sequence and includes at least four exons (Fig. 1A). The longest *DCas* cDNA encodes a protein of 793 amino acids (Fig. 1B). In addition, we found a smaller *DCas* EST that when translated is missing the DCas SH3 domain (data not shown; accession number AAF47336).

The Cas family includes the mammalian p130Cas (Cas, Bcar1), Cas-L (Hef1, Nedd9) and Efs (Sin) proteins, all of which contain, along with several additional conserved motifs, a highly conserved SH3 domain that is important for Cas-dependent cell migration (Fig. 1B) (Cary et al., 1996; Cary et al., 1998; Garton and Tonks, 1999). DCas, like vertebrate Cas proteins, includes this N-terminal SH3 domain and is 70% identical at the amino acid level to the SH3 domain of human CAS (Fig. 1B,C). Cas proteins are also characterized by their 'substrate' and 'serine-rich (Ser)' regions, which constitute a major portion of the Cas protein and contain several conserved tyrosine, serine and threonine phosphorylation sites (Fig. 1B,C). Many of these amino acid residues are conserved between DCas and human CAS, suggesting they are important for Cas function (Fig. 1C). Among these conserved tyrosine residues are five YxxP motifs that, when phosphorylated, serve as binding sites for proteins with Src

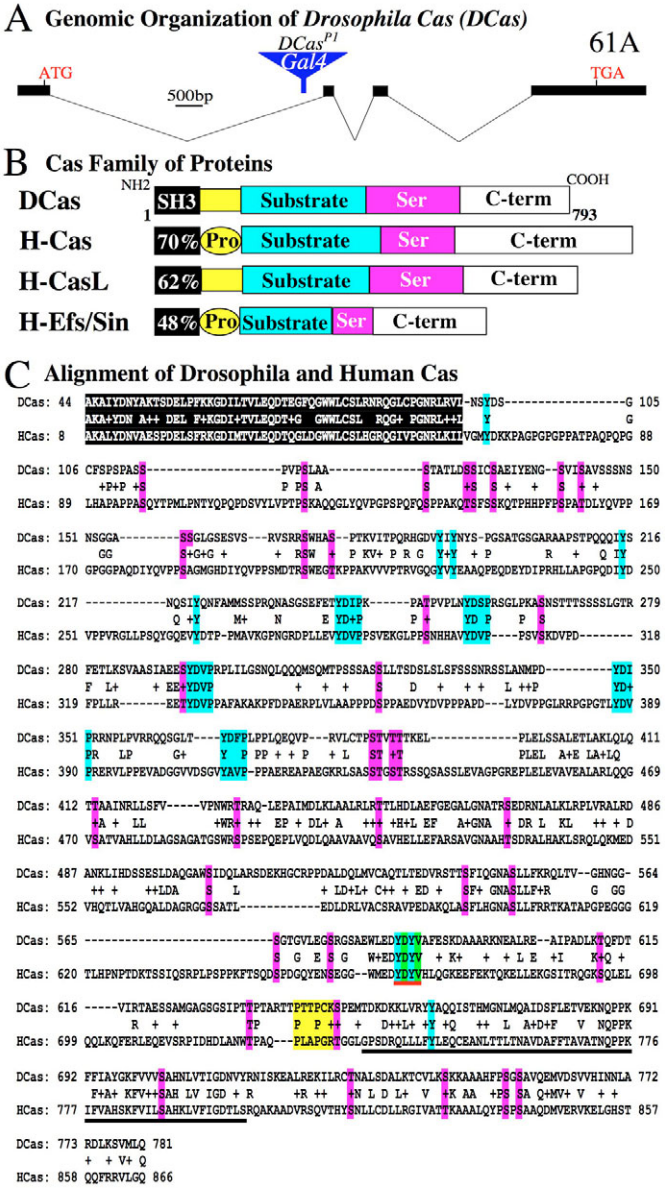

Fig. 1. Molecular characterization of *Drosophila* Cas. (**A**) Organization of the *DCas* locus ($DCas^{P1}$: site of *GAL4*-containing P-element insertion). (**B**) Domain organization of human (H) and *Drosophila* (D) Cas proteins, and percentage amino acid identity of human Cas-family SH3-domains compared with DCas. (**C**) Alignment of the *Drosophila* and human Cas proteins, with conserved residues highlighted. In addition to the SH3 domain (black), tyrosine residues (blue), including those residing in consensus Crk/Nck SH2-binding sites (YXXP, also blue), and serine/threonine residues (purple) are conserved. Additional conserved regions include an SH3-domain-binding motif (yellow), a dimerization motif (black underline) and a Src-binding site (pYDYV, red underline).

homology 2 (SH2) or phosphotyrosine-binding (PTB) domains. These proteins include the adaptor proteins Crk and Nck (Dock) and also the phosphatase Ship2 (Inppl1) (Fig. 1C) (Bouton et al., 2001). DCas also contains a stretch of conserved amino acids found in all vertebrate Cas proteins, YDYV, that serves as an Src tyrosine kinase-binding site (Fig. 1C) (Bouton et al., 2001). At its C-terminus, DCas includes residues, conserved in all mammalian Cas proteins, that are important for dimerization, suggesting that DCas might also exist as a dimer

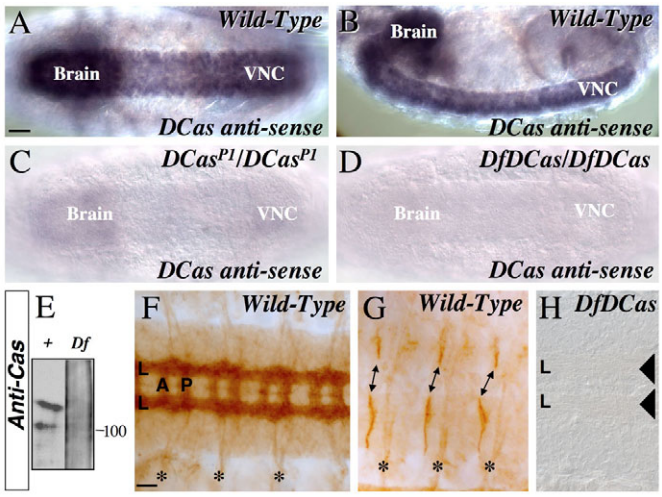

Fig. 2. *Drosophila* Cas is highly expressed in the embryonic nervous system. Localization of (**A-D**) *DCas* RNA and (**E-H**) protein reveals that *DCas* is highly expressed in the central and peripheral embryonic nervous system. (A,B) Stage 15 whole-mount *Drosophila* wild-type embryos viewed ventrally (A) and laterally (B) following in situ hybridization with antisense *DCas* RNA probes. Note the prominent *DCas* expression in the ventral nerve cord (VNC) and brain. Anterior, left. (C,D) A $DCas^{P1}/DCas^{P1}$ *Drosophila* embryo (C) subjected to in situ hybridization exhibits significantly reduced levels of *DCas* transcript, whereas an embryo deficient for the *DCas* gene (D) shows little to no *DCas* transcript (ventral views). (E) Western analysis of lysates obtained from late stage 16/17 embryos using an antibody generated against Cas (see text). The Cas antibody recognizes two prominent bands in wild-type embryo lysates (+) that are absent in *DCas* mutant lysates (Df). Molecular weight (100 kDa) is indicated. (F,G) A stage 15 wild-type embryo immunostained with Cas, showing Cas in axons within the anterior (A) and posterior (P) commissures, and longitudinal connectives (L) and motor nerves (asterisks). Cas immunostaining is also present at muscle attachment sites (arrows). (H) A stage 15 embryo homozygous for a *DCas* deficiency exhibits little to no Cas immunostaining (arrowheads, unstained longitudinal connective axons visualized using Nomarski optics). Scale bar: 30 μm in A-D; 10 μm in F-H.

(Fig. 1B,C) (Law et al., 1999). Human CAS and EFS, but not CAS-L, also contain a proline-rich (PxxP) region immediately C-terminal to their SH3 domains. However, although DCas contains conserved proline residues both within this region and C-terminal to the serine-rich region, it is most similar to CAS-L (Fig. 1B,C). Therefore, *Drosophila*, unlike mammals, has only one Cas family member, eliminating issues of functional redundancy in assessing Cas contributions to neural development.

Cas is highly expressed in the nervous system and in axons

We next analyzed DCas expression during embryonic development. In situ hybridization analysis using *DCas* antisense and sense probes showed that *DCas* mRNA is deposited maternally and is also highly expressed during embryonic development. During embryonic stages that include the period of axon pathfinding, we observed high levels of *DCas* in most, if not all, CNS neurons within the developing brain and nerve cord (Fig. 2A,B). *DCas* mRNA was also found at low levels in embryonic peripheral sensory neurons, including chordotonal organs, and in the developing musculature (data not shown).

We next assessed the distribution of endogenous DCas protein. Our attempts to generate a DCas antibody were unsuccessful. However, we tested several different antibodies generated against Cas proteins and found that an antibody against an epitope highly conserved between mammalian and *Drosophila* Cas (Fonseca et al., 2004) recognizes DCas (see Fig. S1 in the supplementary material). Western analysis using this polyclonal antibody revealed that this vertebrate Cas antibody recognizes bands at ~105 kDa and ~140 kDa in *Drosophila* embryos, and that these bands are absent in embryos harboring a deletion of the *DCas* gene (Fig. 2E). In *Drosophila* embryos, DCas immunostaining was observed during neural development in a pattern consistent with that of *DCas* mRNA and with the expression of a $^{Myc}DCas$ transgene under the control of a *GAL4* enhancer trap insertion in *DCas* ($DCas^{P1}$-*GAL4*; Fig. 1A; see Fig. S1D,E in the supplementary material). DCas protein first appeared in motor and CNS projections during initial stages of axon outgrowth. At later embryonic stages, DCas immunostaining was present in neuronal cell bodies and in axons that contribute to all motor axon pathways (Fig. 2F,G; data not shown). We also observed DCas immunostaining in the chordotonal sensory organs (not shown) and in muscle attachment sites (Fig. 2G). Importantly, no Cas immunostaining was observed in embryos harboring a small deficiency that includes the *DCas* locus (Fig. 2H), showing that this Cas antibody generated against vertebrate Cas specifically recognizes DCas.

To further address the role of Cas proteins in neurons, we asked whether any of the mammalian Cas proteins is also expressed in axons during development. *p130Cas* and *Cas-L* transcripts are found in the developing and postnatal brain (Huang et al., 2006; Merrill et al., 2004), and p130Cas localizes to axons in early development and into adulthood in the spinal cord, cerebellum and cerebral cortex (Huang et al., 2006; Liu et al., 2007; Nishio and Suzuki, 2002). We used several antibodies that specifically recognize Cas proteins and examined their patterns of immunostaining in the developing rat spinal cord. We observed that mammalian Cas family members are indeed highly expressed in neurons during development (see Fig. S2 in the supplementary material).

DCas is required for axon pathfinding during development

To determine whether Cas proteins serve axon guidance functions during development, we analyzed *DCas* mutant *Drosophila* embryos. A search of public *Drosophila* stock collections identified two potential *DCas* loss-of-function (LOF) mutants as well as a large deficiency that removes *DCas* (*Df(3L)ED201*). One of these potential *DCas* LOF mutants, $DCas^{P1}$, is the *GAL4*-containing P-element transposon situated in the intron downstream from the start of *DCas* translation (Fig. 1A). Another potential *DCas* LOF mutant is a small deficiency (*Df(3L)Exel6083*) that removes *DCas* and five putative adjacent genes. No *DCas* mRNA transcript or immunostaining were observed in embryos homozygous for the *DCas* deficiencies (Fig. 2D,H), and very little *DCas* transcript or immunostaining was present in homozygous $DCas^{P1}$ embryos (Fig. 2C, data not shown). These expression data, in combination with genetic experiments described below, show that *Df(3L)Exel6083* is a *DCas*-null allele and that $DCas^{P1}$ is a *DCas* hypomorphic LOF allele.

We assessed DCas function in the development of the stereotypic *Drosophila* embryonic neuromuscular connectivity pattern as this is an excellent system to study axon guidance events, including motor axon fasciculation and defasciculation and target recognition (Araujo and Tear, 2003). All motor axon trajectories can be easily observed in late stage 16 to early stage 17 embryos using the mAb 1D4 (anti-Fasciclin II), and individual motor axons can be followed to their targets (Van Vactor et al., 1993). All five motor axon

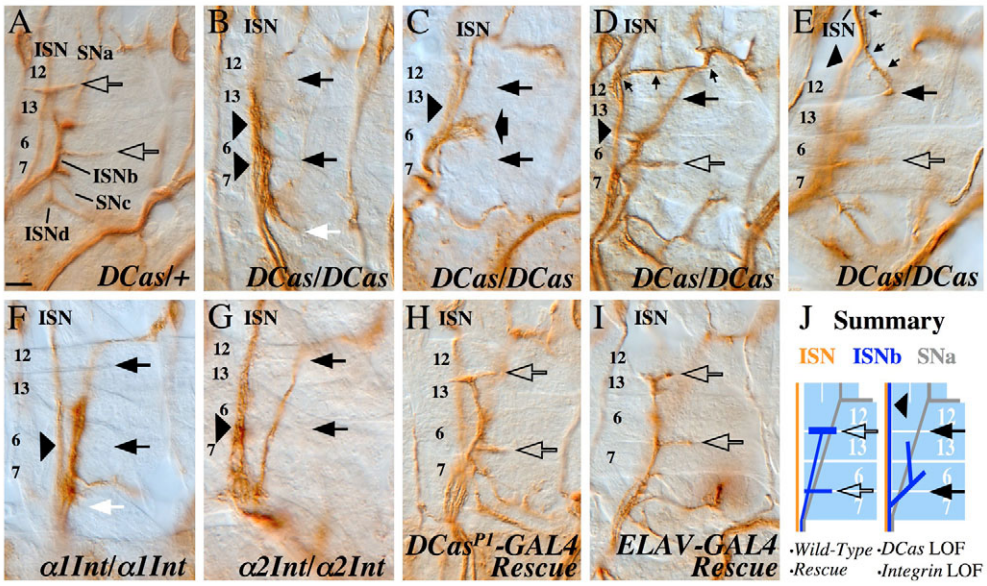

Fig. 3. *Drosophila Cas* and *integrin* mutants exhibit ISNb motor axon defasciculation defects. (**A-I**) Abdominal segments of filleted stage 16/17 *Drosophila* embryos immunostained with the monoclonal antibody 1D4 to visualize all motor axons (Anterior, left, dorsal, up). The intersegmental nerve (ISN), intersegmental nerve b (ISNb), intersegmental nerve d (ISNd), segmental nerve c (SNc), segmental nerve a (SNa) and ventral muscles 6, 7, 12 and 13 are indicated. (A) ISNb motor axons in $DCas^{Df(3L)Exel6083}$/+ embryos defasciculate normally from the ISN and grow dorsally to innervate muscles 6/7 and 12/13 (open arrows, used similarly in D,E,H,I). (B-E) *DCas* loss-of-function mutant embryos often fail to innervate their muscle targets (black arrows) and exhibit a range of ISNb guidance phenotypes indicative of an increase in axon-axon fasciculation. These defects include ISNb axons remaining abnormally fasciculated with the ISN (B,C, arrowheads), and ISNb axons abnormally bundled together (C, broad arrow). ISNd axons also do not defasciculate normally to their muscle targets (B, white arrow). (D) ISNb axons in *DCas* mutants are also abnormally fasciculated with the ISN (arrowhead), where they often separate at inappropriate locations and extend abnormally (small arrows). (E) ISNb axons in a *DCas* mutant are abnormally fasciculated such that they extend past their muscle targets (arrowhead, data not shown) and eventually take abnormal routes to reach their muscle targets (large black arrow). (F,G) Axons in *α1 integrin* (mew^{M6}) (F, *α1Int*) and *α2 integrin* (if^{K27E}) (G, *α2Int*) LOF mutants exhibit axon guidance defects resembling *DCas* LOF mutants, including abnormal fasciculation of ISNb axons with the ISN (arrowheads), abnormal fasciculation of ISNd axons with the ISN (white arrow, F) and failure of ISNb axons to innervate their muscle targets (black arrows). (H,I) Expression of DCas rescues ISNb defects observed in *DCas* LOF mutants. Expression of either ^{Myc}DCas under the $DCas^{P1}$*-GAL4* driver (H, *UAS-*^{Myc}*DCas/+; Df(3L)ED201/*$DCas^{P1}$) or ^{Myc}DCas in all neurons using the *ELAV-GAL4* driver (I, *ELAV-GAL4/UAS-*^{Myc}*DCas;* $DCas^{Df(3L)Exel6083}$/$DCas^{Df(3L)Exel6083}$) restores the normal ISNb innervation pattern in *DCas* mutants (open arrows). Scale bar: 15 μm. (**J**) Schematic summarizing ISNb motor axon guidance phenotypes.

pathways, and also the transverse nerve, were found to be defective in *DCas* mutant embryos (Figs 3 and 4; Table 1; data not shown). The ISNb pathway in wild-type embryos, and also in *DCas* heterozygotes, is formed correctly by motor axons defasciculating from the ISN and extending dorsally through the ventral musculature to innervate muscles 6, 7, 12 and 13 (Fig. 3A,J; Table 1). In *DCas* mutants, ISNb axons exhibited highly penetrant axon guidance phenotypes, often failing to innervate muscles 6 and 7 and muscles 12 and 13 (Fig. 3B-E,J; Table 1). These guidance phenotypes include the failure of ISNb axons to defasciculate from the ISN (Fig. 3B), ISNb axons stalling within the ISNb following defasciculation from the ISN (Fig. 3C), ISNb axons bypassing their target muscles (Fig. 3D), and abnormal ISNb axonal trajectories to their targets (Fig. 3E). Characterization and quantification of these phenotypes reveal that these defects are indicative of increased motor axon fasciculation (Table 1), as has been observed in the absence of several different guidance-cue signaling cascades that regulate fasciculation of developing *Drosophila* motor projections (Araujo and Tear, 2003; Van Vactor, 1998). In *DCas* mutant embryos, SNa motor axons (Fig. 4; Table 1) and CNS axons (see Fig. S2 in the supplementary material; Table 1) also exhibit highly penetrant guidance defects consistent with increased axonal fasciculation. Phenotypes characteristic of decreased fasciculation are not observed to any appreciable extent in *DCas* LOF mutants (Table 1). Importantly, we observed no defects in muscle integrity or neuronal cell fate determination in *DCas* LOF mutants (see Fig. S1 in the supplementary material).

To confirm that *DCas* axon guidance phenotypes result from decreased *DCas* function, we restored *DCas* expression in homozygous *DCas* mutant embryos using the GAL4-UAS system (Brand and Perrimon, 1993). We utilized the $DCas^{P1}$ allele to express ^{Myc}DCas under control of what is likely to be the endogenous *DCas* promoter, as $DCas^{P1}$ is a *GAL4*-containing P-element insertion located downstream of the *DCas* ATG (Fig. 1A) and drives gene expression in a pattern similar to that detected with a Cas antibody (see Fig. S1D,E in the supplementary material). Indeed, expression of the ^{Myc}*DCas* transgene in a *DCas* mutant background that includes the $DCas^{P1}$ allele significantly rescued the embryonic ISNb (Fig. 3H), SNa (Fig. 4I) and CNS (see Fig. S2 in the supplementary material) axon guidance phenotypes (Table 1). To confirm that this rescue results from DCas expression in neurons, we placed *UAS-*^{Myc}*DCas* under the transcriptional control of the neuron-specific driver *ELAV-GAL4*, which drives robust expression of ^{Myc}DCas in motor and CNS neurons (see Fig. S1C in the supplementary material). Neuronal ^{Myc}*DCas* expression rescued ISNb (Fig. 3I), SNa (Fig. 4J) and CNS (see Fig. S2 in the supplementary material) *DCas* embryonic axon guidance defects (Table 1). Therefore, axon guidance phenotypes observed in *DCas*

Table 1. Axon guidance phenotypes (ISNb, SNa and CNS defects)

Genotype (hemisegments)	Abnormal ISNb pathway* (a; x)	Abnormal SNa pathway† (b; y)	Abnormal CNS pathway‡ (c; z)
Controls:			
+/+ (*n*=137)	2.9	8.0	2.9
$DCas^{Df(3L)Exel6083}$/+ (*n*=98)	2.9	5.9	3.1
$DCas^{P1}$/+ (*n*=90)	6.7	3.3	1.6
$\beta1(mys^{1})$/+ (*n*=97)	9.3	14.4	9.3
$\alpha1(mew^{M6})$/+ (*n*=84)	7.1	15.3	4.8
$\alpha2(if^{K27E})$/+ (*n*=84)	5.0	8.6	4.5
Loss of function			
$DCas^{Df(3L)Exel6083}/DCas^{Df(3L)Exel6083}$ (*n*=156)	73.9 (98.3; 1.7)	69.0 (90.7; 9.3)	58.9 (94.7; 5.3)
$DCas^{P1}$/*Df(3L)ED201* (*n*=84)	78.0 (100; 0)	69.6 (100; 0)	47.6 (100; 0)
$DCas^{P1}/DCas^{P1}$ (*n*=204)	56.0 (100; 0)	49.0 (98.8; 1.2)	37.7 (93.5; 6.5)
$\alpha1(mew^{M6})/\alpha1(mew^{M6})$ (*n*=103)	82.3 (98.8; 1.2)	71.8 (98.6; 1.4)	55.8 (88.4; 1.6)
$\alpha2(if^{K27E})/\alpha2(if^{K27E})$ (*n*=89)	80.0 (100; 0)	56.2 (100; 0)	36.4 (69.6; 30.4)
Rescue			
UAS^{Myc}*Cas;* $DCas^{Df(3L)Exel6083}$/*ELAV-GAL4;* $Cas^{Df(3L)Exel6083}$ (*n*=168)	11.3 (100; 0)	28.2 (93.5; 6.5)	16.7 (100; 0)
UAS^{Myc}*DCas;* $DCas^{P1}$/*Df(3L)ED201* (*n*=100)	26.7 (93.5; 6.25)	27.0 (100; 0)	6.4 (100; 0)
Gain of function			
(+) *ELAV-GAL4,* UAS^{Myc}*DCas*/+ (*n*=154)	44.3 (96.3; 3.7)	44.9 (95.8; 4.2)	42.2 (96.9; 3.1)
(+++) *ELAV-GAL4,* UAS^{Myc}*DCas/ELAV-GAL4,* UAS^{Myc}*DCas* (*n*=154)	82.1 (98.9; 1.1)	86.5 (100; 0)	92.2 (100; 0)
UAS^{Myc}*DCas;* $DCas^{P1}/UAS^{Myc}$*DCas;* $DCas^{P1}$ (*n*=112)	98.7 (100; 0)	90.8 (100; 0)	83.0 (100; 0)
Genetic interactions			
$\beta1(mys^{1})$/+; $DCas^{Df(3L)Exel6083}$/+ (*n*=140)	75.4 (100; 0)	41.8 (97.6; 2.4)	40.0 (98.2; 1.8)
$\alpha1(mew^{M6})$/+; $DCas^{Df(3L)Exel6083}$/+ (*n*=126)	73.6 (100; 0)	55.3 (100; 0)	40.5 (100; 0)
$\alpha2(if^{K27E})$/+; $DCas^{Df(3L)Exel6083}$/+ (*n*=126)	72.0 (100; 0)	53.7 (100; 0)	42.1 (94.3; 5.7)
ELAV-GAL4, UAS^{Myc}*DCas/+;* $DCas^{Df(3L)Exel6083}$/+ (*n*=84)	28.8 (100; 0)	26.7 (93.7; 6.3)	14.3 (58.3; 41.7)
$\beta1(mys^{1})$/+; *ELAV-GAL4,* UAS^{Myc}*DCas*/+ (*n*=168)	27.2 (97.3; 2.7)	27.2 (96.8; 3.2)	27.4 (63.0; 37.0)

Phenotypes characteristic of increased fasciculation (a, b and c indicate percentage of defects in abnormal ISNb, SNa and CNS pathways, respectively). (a) ISNb defects: stalling of motor axons exiting the CNS; or failure of ISNb axons to defasciculate from the ISN; or ISNb axons following the ISNd or SNc; or ISNb axons stalling; or ISNb axons remaining fasciculated with the ISN but wandering in the lateral muscle fields or projecting back to innervate ventral muscles; or ISNb axons bypassing targets; or absent or decreased 6/7 or 12/13 muscle innervation. (b) SNa defects: stalling of motor axons exiting the CNS; or SNa axons fasciculated with the ISN nerve; or failure of the dorsal branch of the SNa to separate from the lateral branch of the SNa; or failure of the lateral branch of the SNa to separate from the dorsal branch of the SNa; or SNa axons failing to make two turns between muscles 22 and 23 and muscles 23 and 24 (either stalling near muscle 12 or projecting long distances between muscles 22 and 23). (c) CNS axons: bundling of more than one longitudinal axonal connective; or absent or thin Fas2 CNS third longitudinals; or abnormally clumped axons; or stalling of axons; or axons exiting the CNS as large bundles.
Phenotypes characteristic of decreased fasciculation (x, y and z indicate percentage of defects in abnormal ISNb, SNa and CNS pathways, respectively). (x) ISNb defects: abnormal projections of ISNb axons; or projecting into abnormal target fields; or presence of single axons in abnormal areas; or premature innervation of abnormal targets. (y) SNa defects: premature branching of SNa axons; or abnormal defasciculation of SNa axons; or SNa axon termination at incorrect muscles; or SNa single axons projecting abnormally. (z) CNS axons: longitudinal connective axons defasciculated; or singularly extending away from the CNS.
α1, *α2* and *β1* refer to the *α1*, *α2* and *β1 integrin* genes.
*Percentage of hemisegments with a or x.
†Percentage of hemisegments with b or y.
‡Percentage of hemisegments with c or z.

mutant embryos result from a lack of neuronal DCas, demonstrating that DCas plays a key role in the regulation of motor axon defasciculation.

Overexpression of neuronal DCas results in motor and CNS axon guidance defects

To further characterize DCas neuronal functions, we asked whether neuronal overexpression of *DCas* affects axon pathfinding. Overexpression of ^{Myc}*DCas* in all neurons in a wild-type background led to highly penetrant motor axon guidance phenotypes (Fig. 5; Table 1). Interestingly, most of these *DCas* gain-of-function (GOF) axon guidance defects are similar to those we observed in *DCas* LOF mutants: reductions in motor axon defasciculation at characteristic choice points (Fig. 5; Table 1). For example, when one copy of the ^{Myc}*DCas* transgene was expressed in all neurons with one copy of the *ELAV-GAL4* driver in a wild-type background (designated '+'), ISNb axons often failed to innervate their muscle targets and instead remained fasciculated in the ISNb nerve (Fig. 5A; Table 1). Increasing the levels of neuronal DCas (++) resulted in more-severe motor axon pathfinding defects, including the inability of ISNb axons to defasciculate from the ISN nerve (Fig. 5B), and extensive stalling and aberrant bundling of ISNb axons along their trajectory (Fig. 5C). Increasing neuronal DCas levels still further (+++) produced dramatic motor axon stalling within their nerve roots, just after their exit from the ventral nerve cord, resulting in noticeably fewer motor axons extending into the periphery (Fig. 5G). SNa motor axon guidance phenotypes in *DCas* neuronal GOF mutants were also similar to those we observed in *DCas* LOF mutants and exhibited a similar dosage-dependent increase in the severity of abnormal fasciculation and stalling defects (Fig. 5D-G). We also found that *DCas* GOF dramatically affects CNS axonal trajectories, producing phenotypes reminiscent of those observed in the absence of the Slit receptor Roundabout (Robo) or by increased Netrin attractive signaling (see Fig. S2 in the supplementary material) (Bashaw and Goodman, 1999; Seeger et al., 1993). We also observed increased fasciculation and stalling of CNS axons (see Fig. S2 in the supplementary material). As was

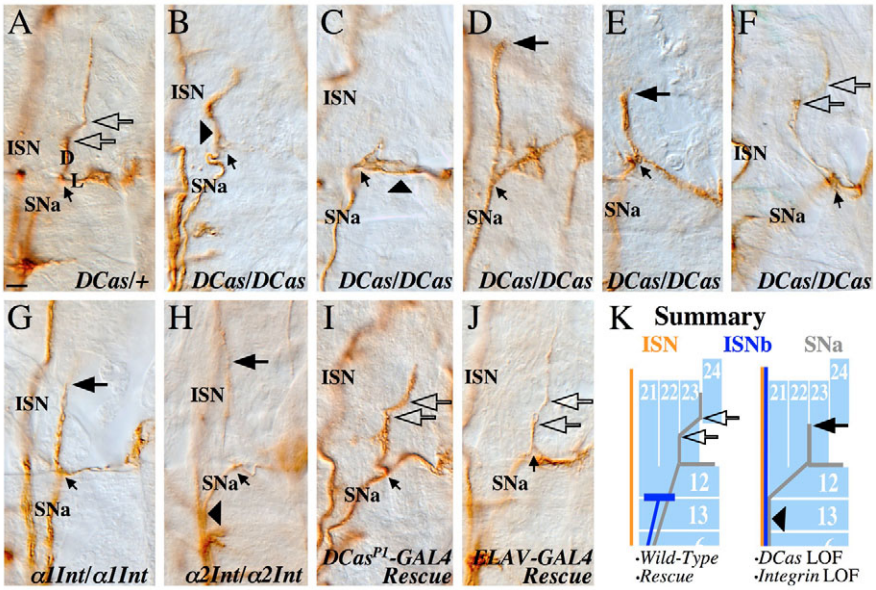

Fig. 4. *Drosophila Cas* and *integrin* mutants exhibit SNa motor axon defasciculation defects. (**A-J**) Immunostaining as in Fig. 3. The intersegmental nerve (ISN) and the dorsal (D) and lateral (L) branch of segmental nerve a (SNa) are indicated. (A) In $DCas^{Df(3L)Exel6083}$/+ embryos, axons within the SNa defasciculate normally from the SN and extend past the ventral musculature as a single tightly fasciculated bundle. Near the dorsal edge of muscle 12, SNa axons defasciculate (small arrow) to generate a dorsal (D) and a lateral (L) branch. Axons within the dorsal branch extend dorsally between muscles 22 and 23 and then make two characteristic turns (open arrows), continuing dorsally between muscles 23 and 24. (B-F) *DCas* loss-of-function mutant embryos often fail to reach their muscle targets and exhibit a range of SNa axon guidance phenotypes indicative of defects in axon-axon defasciculation. (B,C) SNa axons in *DCas* mutants exhibit abnormal fasciculation (arrowheads) of the lateral branch with the dorsal branch (B, arrow), and also of the dorsal branch with the lateral branch (C, arrow). (D,E) In *DCas* mutants, axons within the dorsal branch of the SNa often do not make their two characteristic turns, extending past their defasciculation choice point (arrow, D) or stalling in the vicinity of this choice point (arrow, E). (F) SNa axons in *DCas* mutants are also abnormally fasciculated, extending past their defasciculation point (arrow) but eventually taking an abnormal route to reach lateral muscle targets (open arrows). (G,H) SNa axon guidance defects seen in *α1 integrin* (mew^{M6}) (G, *α1Int*) and *α2 integrin* (if^{K27E}) (H, *α2Int*) LOF mutants resemble those observed in *DCas* LOF mutants, including: SNa stalling past the dorsal SNa choice point (large arrow, G), axons fused with the ISN (arrowhead, H), and SNa axons within the dorsal branch growing past their choice point (large arrow, H). (I,J) Expression of DCas rescues SNa defects observed in *DCas* LOF mutants. Expression of either ^{Myc}DCas under the $DCas^{P1}$-*GAL4* driver (I, *UAS-*^{Myc}*DCas/+; Df(3L)ED201/*$DCas^{P1}$) or ^{Myc}DCas in all neurons using the *ELAV-GAL4* driver (J, *ELAV-GAL4/UAS-*^{Myc}*DCas;* $DCas^{Df(3L)Exel6083}$/$DCas^{Df(3L)Exel6083}$) restores the normal SNa trajectory and innervation pattern in *DCas* mutants (open arrows). Scale bar: 15 μm. (**K**) Schematic summary of SNa motor axon guidance phenotypes.

the case for *DCas* LOF mutants, we observed no defects in neuronal cell fate in these neuronal *DCas* GOF mutants (see Fig. S1 in the supplementary material).

To better characterize mechanisms underlying these *DCas* GOF axon guidance defects, we used our $DCas^{P1}$-*GAL4* driver to overexpress DCas in its endogenous temporal and spatial pattern. DCas overexpression using the $DCas^{P1}$-*GAL4* driver resulted in axon guidance defects qualitatively and quantitatively similar to those observed using the *ELAV-GAL4* driver (Table 1; data not shown). These results show that overexpression of DCas results in phenotypes strikingly similar to those we observe in *DCas* LOF mutants: in both cases we observed highly penetrant reductions in the ability of motor axons to separate from their original trajectories, resulting in increased fasciculation of these axons. Taken together, these neuronal *DCas* LOF and GOF phenotypes show that although DCas is necessary for axon defasciculation, it is not sufficient, as DCas neuronal overexpression does not cause an increase in axonal defasciculation.

Cas signals integrin-dependent axon guidance during development

We next sought to better define the signaling pathway through which Cas regulates axon fasciculation. In mammals, Cas proteins function downstream of several different receptors in non-neuronal cells, including growth factor receptors, G-protein-coupled receptors, T-cell receptors, B-cell receptors and integrins (Defilippi et al., 2006). Interestingly, integrin receptor subunit mutations in *Drosophila* give rise to CNS and motor axon guidance defects that are strikingly similar to those we observe in *DCas* mutants (Hoang and Chiba, 1998; Stevens and Jacobs, 2002), suggesting that Cas might function together with integrin receptors to guide axons.

Drosophila integrins, like vertebrate integrins, are composed of an α-subunit and a β-subunit. In *Drosophila*, there is one gene encoding a typical laminin-binding-type α-subunit (α1, called *mew*), one encoding an RGD-binding-type α-subunit (α2, called *if*), and a single β-subunit gene (β1, called *mys*) very similar to the prototype vertebrate β1 receptor (Bokel and Brown, 2002; Brower, 2003). To investigate the connection between integrin and Cas signaling, we revisited the role of integrin receptor function in embryonic motor axon pathfinding and found that integrin-null mutant embryos exhibit defects that are qualitatively and quantitatively similar to *DCas* mutants (Figs 3, 4, and see Fig. S2 in the supplementary material; Table 1) (see also Hoang and Chiba, 1998). Embryos harboring null alleles for either *α1* (mew^{M6}) or *α2* (if^{K27E}) *integrin* genes exhibited ISNb and SNa axon guidance defects very similar to those observed in *DCas* mutants, including increased fasciculation resulting in the absence of muscle innervation (Fig. 3F,G; Fig. 4G,H; Table 1) (see also Hoang and Chiba, 1998). We also observed CNS axon guidance defects in both *α1* and *α2 integrin* mutants that were similar to those we observed in *DCas* mutants (see Fig. S2 in the supplementary material; Table 1) (see also Hoang and Chiba, 1998; Stevens and Jacobs, 2002).

To further address DCas involvement in integrin-mediated axon guidance, we looked for dominant genetic interactions between *DCas* and integrin subunit LOF mutations. Such transheterozygous interactions provide genetic support for two proteins functioning together in the same signaling pathway. We asked whether removal of a single copy of *DCas* dominantly enhances heterozygosity at the *α1*, *α2* or *β1 integrin* loci. We found that in *α1*, *α2*, *β1* or *DCas* heterozygotes, motor axon trajectories were not significantly different from wild type (Fig. 3A, Fig. 6A,E; Table 1). However, removal of a single copy of *DCas* together with a single copy of *α1*,

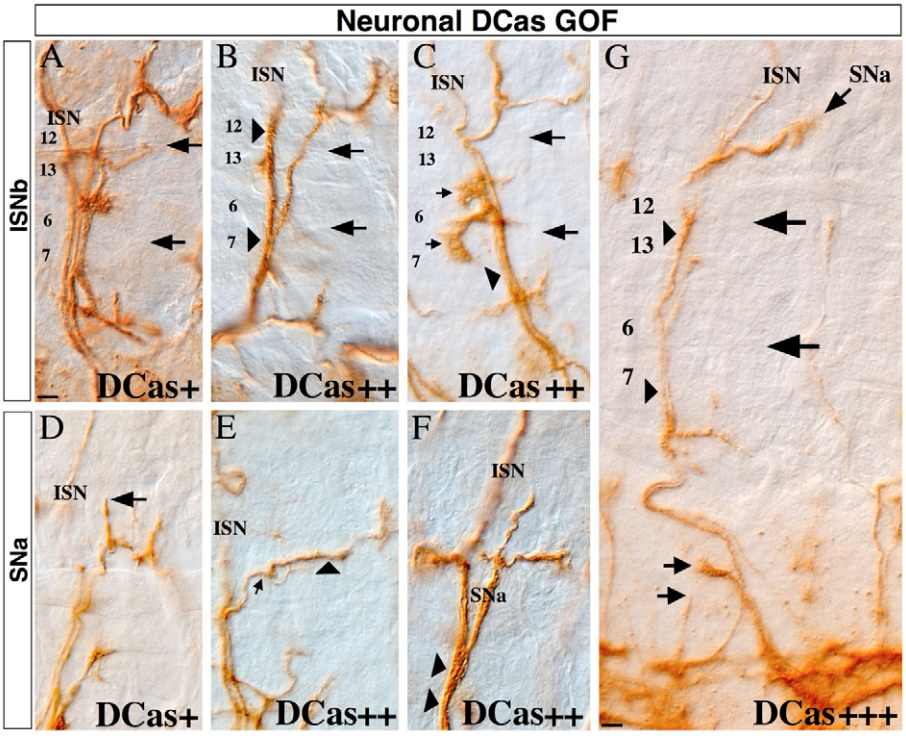

Fig. 5. Neuronal DCas overexpression results in axon guidance defects similar to those seen in *DCas* loss-of-function mutants. Overexpression of ^{Myc}DCas in all neurons in a wild-type background using the *ELAV-GAL4* driver results in ISNb (A-C,G) and SNa (D-G) guidance defects characterized by increased fasciculation of axons and decreased target innervation (A-G, large arrows). (**A**) Overexpression using one copy (+) each of both the ^{Myc}*DCas* reporter and the *ELAV-GAL4* driver in all neurons results in a reduction of ISNb muscle target innervation (arrows). (**B**,**C**) Overexpression using two copies (++) of both ^{Myc}*DCas* and *ELAV-GAL4* in all neurons results in ISNb axons remaining fasciculated with the ISN (arrowheads), and sometimes exiting the ISN in abnormal locations and stalling in large clumps of fasciculated axons (C, small arrows). (**D**) Overexpression of one copy (+) of both ^{Myc}*DCas* and *ELAV-GAL4* in all neurons results in a reduction of SNa axon target innervation; SNa axons often stalled at the choice point in the dorsal branch of the SNa (D, arrow). (**E**,**F**) Overexpression using two copies (++) of both ^{Myc}*DCas* and *ELAV-GAL4* in all neurons results in SNa axons remaining abnormally fasciculated with the lateral branch of the SNa (E, arrowhead) at the choice point (E, small arrow) and remaining abnormally fasciculated with the ISN (F, arrowheads). (**G**) Following temperature (29°C)-induced elevated expression using two copies of both *UAS-*^{Myc}*DCas* and *ELAV-GAL4* (+++), many motor axons stalled in the motor roots following exit from the CNS (small arrows), and those ISNb and SNa axons that did extend into the muscle fields often remained fasciculated with the ISN (arrowheads), failing to innervate muscle targets (large arrows). Scale bar: 15 μm in A-F; 7 μm in G.

α2 or *β1 integrin* resulted in highly penetrant axon guidance defects, suggesting that these three genes function in the same signaling pathway (Fig. 6B-E; Table 1). Importantly, the phenotypes resulting from dominant enhancement by *DCas* are indicative of increased fasciculation, similar to those observed in *DCas*, *α1* or *α2 integrin* LOF embryos (Table 1).

To further assess the role integrins play in DCas-mediated axon guidance, we asked whether *β1 integrin* LOF mutants dominantly suppress *DCas* GOF motor axon guidance phenotypes. The *Drosophila* β1 integrin (encoded by *mys*^{1}) is the predominant neuronal β1 integrin and therefore likely to mediate most, if not all, nervous system integrin signaling (Devenport and Brown, 2004; Yee and Hynes, 1993). If integrins are indeed necessary for activating DCas signaling, removing a single copy of *β1 integrin* should suppress *DCas* GOF phenotypes. When low levels of DCas were expressed in all neurons in an otherwise wild-type background, moderate guidance defects were observed involving axons of the ISNb, SNa and CNS third longitudinal (Fig. 5A,D, Fig. 6F,H and see Fig. S2 in the supplementary material; Table 1). Removing a single copy of the *β1 integrin* gene in this same neuronal *DCas* GOF genetic background significantly rescued axon guidance phenotypes resulting from *DCas* GOF (Fig. 6G,H; Table 1). Importantly, these results also show that neuronal overexpression of DCas does not simply function in a dominant-negative fashion to block integrin, or other, signaling pathways.

Taken together, our analysis of integrin LOF mutations and our observation of robust genetic interactions between *DCas* and *α1*, *α2* and *β1 integrin* mutants strongly support DCas serving a crucial role in transducing in vivo integrin-mediated signaling events essential for directing axon guidance. Furthermore, our results show that integrin/Cas signaling is necessary for axonal defasciculation events during neural development.

DISCUSSION

A rich diversity of neuronal guidance cues and their receptors modifies cytoskeletal components in growth cones, ultimately defining the trajectories of the growing neuronal processes. However, the manner in which distinct signaling cascades influence axonal or dendritic pathfinding events during neural development is poorly understood. We show here that a member of the Cas family of cytosolic SH3-domain-containing proteins is required for directing specific axon guidance events during embryonic development. We find that Cas proteins are highly expressed in neurons, and that Cas is required for CNS and motor axon

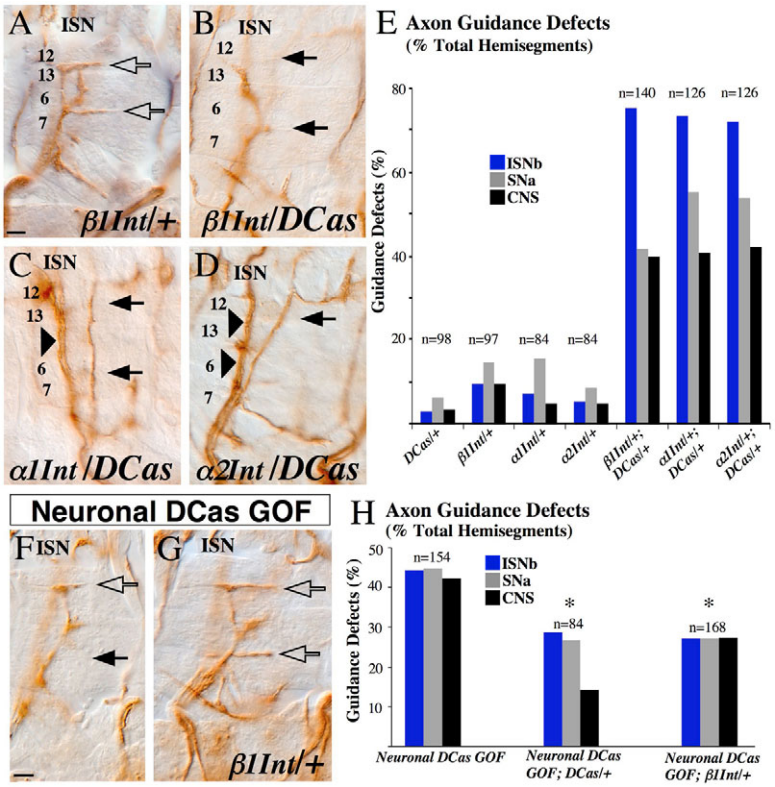

Fig. 6. DCas functions with integrins to regulate axon fasciculation during development. (**A-E**) Dominant (transheterozygous) genetic LOF interactions between *DCas* and the *β1*, *α1* or *α2 integrin* genes (*β1Int*, *α1Int* or *α2Int*). (A) ISNb axons in *β1 integrin* (*mys*^{1}) heterozygous embryos (*β1Int/+*) innervate muscles 6/7 and 12/13 (open arrows). (B-D) ISNb axons in embryos heterozygous for both *β1 integrin* (*mys*^{1}) (B), or *α1 integrin* (*mew*^{M6}) (C), or *α2 integrin* (*if*^{K27E}) (D) and *DCas*$^{Df(3L)Exel6083}$ fail to innervate their muscle targets (arrows), exhibiting increased axonal fasciculation (arrowheads). (E) The percentage of defective ISNb, SNa and CNS pathways in embryos heterozygous for either *DCas*, *β1Int*, *α1Int* or *α2Int*, or in embryos heterozygous for both *DCas* and *β1Int*, *α1Int* or *α2Int*. (**F-H**) Expression of one copy of ^{Myc}*DCas* and one copy of the *ELAV-GAL4* pan-neuronal driver results in axon guidance phenotypes that are suppressed by heterozygosity at the *β1 integrin* locus. (F) ISNb axon innervation of muscles 6/7 (black arrow) is absent in embryos expressing one copy of ^{Myc}*DCas* in all neurons in a wild-type background. (G) Normal ISNb axon innervation of muscles 6/7 and 12/13 (open arrows) in an embryo in which one copy of ^{Myc}*DCas* is expressed in all neurons in combination with heterozygosity at *β1 integrin*. (H) The percentage of defective ISNb, SNa and CNS pathways in embryos expressing low levels of neuronal DCas (Neuronal DCas GOF: *UAS-*^{Myc}*DCas/+, ELAV-GAL4/+*), in embryos expressing low levels of neuronal DCas that are also heterozygous for *DCas* LOF (Neuronal DCas GOF; *DCas/+*: *UAS-*^{Myc}*DCas, ELAV-GAL4/+; DCas*$^{Df(3L)Exel6083}$*/+*), and in embryos expressing low levels of neuronal DCas that are also heterozygous for *β1 integrin* LOF (Neuronal DCas GOF; *β1Int/+*: *mys*^{1}*/+; UAS-*^{Myc}*DCas, ELAV-GAL4/+*). The differences in the percentage of defective guidance phenotypes observed between Neuronal DCas GOF; *DCas/+* or Neuronal DCas GOF; *β1Int/+* and Neuronal DCas GOF for each axon pathway are statistically significant (*, $P<0.05$; χ^2 test). Scale bar: 15 μm in A-D; 15 μm in F,G.

pathfinding during development. Our results also strongly suggest that Cas and integrin receptors function together in this process. These observations strongly support a role for integrin/Cas signaling in mediating specific guidance events essential for establishing complex axonal trajectories during embryonic development.

Our genetic analyses suggest that DCas functions with integrin receptors in vivo to mediate correct motor and CNS axon guidance, providing mechanistic insight into how integrins direct axon guidance events during development. In vitro studies define roles for Cas proteins in promoting cell migration (Chodniewicz and Klemke, 2004), and Cas proteins have also been implicated as mediators of axon outgrowth and turning (Huang et al., 2006; Liu et al., 2007; Yang et al., 2002). Integrin effects on axon outgrowth have been studied extensively in vitro, but certain integrin functions suggested by these experiments have thus far not been reflected in the integrin mutant phenotypes observed in vivo (reviewed by Clegg et al., 2003). Examination of integrin mutant mice, flies and worms does not reveal a decrease in axon outgrowth during development (Baum and Garriga, 1997; Billuart et al., 2001; Hoang and Chiba, 1998; Pietri et al., 2004; Poinat et al., 2002; Stevens and Jacobs, 2002) (this study). Indeed, our in vivo functional analysis shows that one role of integrin/Cas signaling in neurons during development is to regulate the degree of fasciculation, or adhesion, among axons. *Drosophila* embryonic motor axons have proven useful for modeling these types of fasciculation defects and identifying the genes that govern them (Araujo and Tear, 2003; Van Vactor, 1998). Loss of DCas or integrin receptors from *Drosophila* motor neurons results in axons remaining abnormally fasciculated, growing past their normal defasciculation choice points, or sometimes stalling at these choice points. DCas neuronal overexpression produces similar hyperfasciculation defects, dependent upon integrins, including abnormal bundling and stalling of motor axons. Although other interpretations are possible, these results suggest a model in which integrin/Cas signaling normally serves to slow growing axons at choice points in vivo: experimentally decreasing integrin/Cas signaling attenuates adhesive functions that serve to slow growing axons, whereas

increasing integrin/Cas signaling results in excessive adhesion among axons, or between axons and other substrates upon which they extend (Fig. 7).

Supporting this model for how integrins and Cas regulate axonal fasciculation and pathfinding is extensive work on neuronal integrin functions in vitro. Growing axons and migrating cells preferentially elongate on surfaces to which they adhere most strongly, including integrin ligands (Bentley and Caudy, 1983; Bozyczko and Horwitz, 1986; Hammarback et al., 1988; Letourneau, 1975a; Raper et al., 1983). How might adhesive interactions influence axonal guidance decisions in vivo? Neuronal growth cones tend to form extensive lamellae, which are indicative of strong adhesive interactions, when cultured on highly adhesive substrata containing integrin ligands (Hammarback and Letourneau, 1986; Letourneau, 1975b; Letourneau, 1979). These adhesive interactions stabilize elongating nerve fibers by promoting filopodial extension and expansion of growth cone surfaces (Burden-Gulley et al., 1995; Gomez et al., 1996; Hammarback et al., 1988; Letourneau, 1979). Disruption of axon-substrate attachment in vitro with integrin function-blocking antibodies encourages axon-axon adhesive interactions (fasciculation) in place of axon-substrate adhesion (Bozyczko and Horwitz, 1986). Furthermore, contact with integrin ligands can slow axon elongation, as axons encountering an increasing gradient of laminin peptide exhibit reduced velocity, but growth cone velocity returns to previous rates when axons turn down the gradient (Adams et al., 2005). This in vitro observation resembles in vivo situations in which growth cones slow at a choice point, exhibit increased morphological complexity and then extend along distinct pathways (Godement et al., 1994; Gomez and Spitzer, 1999; Tosney and Landmesser, 1985). *Drosophila* motor axon growth cones also exhibit similar changes in morphological complexity upon contacting different substrates in vivo, suggesting that similar processes function to generate motor axon trajectories (Broadie et al., 1993; Johansen et al., 1989; Sink and Whitington, 1991). Different combinations of integrin ligands might be responsible for these effects. When vertebrate growth cones in vitro contact either the α1 or α2 integrin ligands, laminin and fibronectin respectively, they decelerate, pause and exhibit short-term growth arrest (Gomez et al., 1996; Kuhn et al., 1995). Interestingly, our in vivo observations show that DCas functions with both α1 (laminin-binding) and α2 [RGD (e.g. Tiggrin)-binding] integrins to mediate correct axon navigation by regulating motor axon fasciculation at choice points, suggesting that integrin/Cas-mediated spatial regulation of growth cone extension underlies correct navigation at these choice points.

The molecular mechanisms underlying integrin-mediated axon guidance remain to be completely defined. However, results derived from analysis of integrin/Cas signaling on cell migration shed light on how Cas and integrins might specify axonal defasciculation events in vivo. During cell migration, Cas proteins serve to establish linkage between migrating cells and the ECM (Defilippi et al., 2006). Cas plays an important role in regulating cytoskeletal organization, cell adhesion and force sensing, and fibroblasts isolated from *p130Cas*-null mutant mouse embryos exhibit disorganized and short actin filaments and decreased cell migration (Cho and Klemke, 2000; Honda et al., 1999; Honda et al., 1998). In non-neuronal cells, Cas becomes phosphorylated in response to integrin engagement by many ECM components, including fibronectin and laminin (Defilippi et al., 2006). FAK and Src family kinases have been implicated in integrin-dependent phosphorylation of Cas. Interestingly, recent in vitro observations reveal that FAK signaling at sites of integrin-mediated adhesion controls axon pathfinding (Ivankovic-Dikic et al., 2000; Rico et al., 2004; Robles and Gomez, 2006). Furthermore, pharmacological inhibitors of Src family kinases decrease the level of neuronal phosphorylated Cas in vitro (Huang et al., 2006; Liu et al., 2007), supporting a role for Src kinases in regulating Cas proteins in neurons. Finally, the activity of Rho-family small GTPases is also regulated by Cas interactions with the guanine nucleotide exchange factor Dock180 (Dock1) (Defilippi et al., 2006). Taken together, these links between Cas signaling components and cytoskeletal reorganization suggest that some of these signaling proteins might also influence axon guidance in vivo during development.

Our results demonstrate that integrin/Cas-mediated signaling is necessary but not sufficient for axonal defasciculation, revealing that integrin/Cas-mediated axon guidance must be integrated with other axon guidance signaling cascades to regulate axon defasciculation events during development (Fig. 7). The identity of these other axon guidance pathways is not known. The attractive/permissive guidance

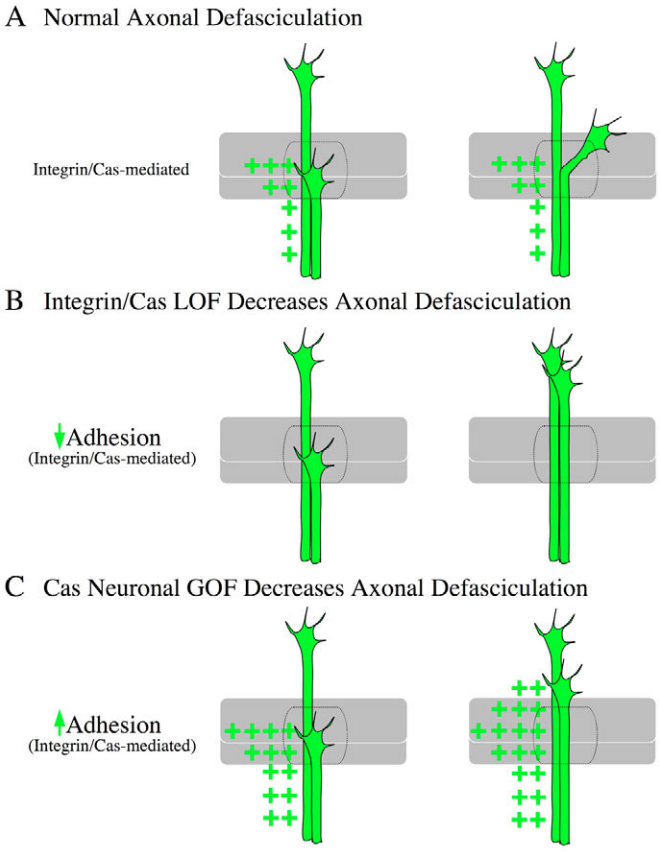

Fig. 7. Model for integrin/Cas-mediated regulation of axonal fasciculation. (**A**) During axon pathfinding, integrin/Cas signaling (+) functions to regulate axon-axon fasciculation. In this model, integrin/Cas-mediated adhesive interactions are strongest at choice points, slowing axons so that they can navigate specific defasciculation events. (**B**) Removal of integrin or Cas adhesive signaling in a loss-of-function (LOF) mutant background prevents axons from defasciculating at their choice points, maintaining fasciculation with the main axon bundle. (**C**) Neuronal overexpression (gain-of-function, GOF) of Cas increases integrin/Cas adhesive signaling and also prevents axons from defasciculating at their choice points. This model suggests that integrin/Cas adhesive signaling is necessary for axonal defasciculation but is not sufficient, such that other guidance cues, including axonal repellents, coordinately work together with integrin/Cas signaling to direct specific axon defasciculation.

cue Netrin binds to integrins, and functions with integrins in non-neuronal cells (Yebra et al., 2003). The Netrin receptor Deleted in colorectal cancer (DCC) has been found to utilize the integrin effector FAK and recently p130Cas, to mediate Netrin-dependent attractive growth cone steering (Li et al., 2004; Liu et al., 2004; Liu et al., 2007; Ren et al., 2004). Ephrins, best known for their role as repulsive axon guidance cues, also induce cell adhesion and actin cytoskeletal changes in fibroblasts in a p130Cas-dependent manner (Carter et al., 2002). Repulsive axon guidance cues may also regulate integrin/Cas-dependent axon guidance during development. The axonal repellent Slit genetically interacts with integrins and their ligands to guide commissural axons in *Drosophila* (Stevens and Jacobs, 2002). Semaphorin and Ephrin-mediated repulsive effects on non-neuronal cells also appear to involve inhibition of integrin signaling events (Miao et al., 2000; Serini et al., 2003). Interestingly, a crucial component of semaphorin-dependent repulsive axon guidance, a member of the molecule interacting with Cas-L (MICAL) family, physically associates with Cas-L (Suzuki et al., 2002; Terman et al., 2002) and our preliminary data suggest that these interactions are functionally important for axon guidance (A.L.K. and J.R.T., unpublished). Our observation that Cas functions with integrins to mediate axon guidance during development suggests new directions to better understand how integrin/Cas signaling modulates neuronal guidance through interactions with distinct axon guidance signaling pathways.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/12/2337/DC1

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