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# Functional dissection of the *C. elegans* cell adhesion molecule SAX-7, a homologue of human L1

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**Cell adhesion molecules of the Immunoglobulin superfamily (IgCAMs) play important roles in neuronal development, homeostasis and disease. Here, we use an animal *in vivo* assay system to study the function of *sax-7*, the *Caenorhabditis elegans* homologue of the human L1 IgCAM, a homophilic adhesion molecule involved in several neurological diseases. We show that the 6 Ig/5 FnIII domain protein SAX-7 acts autonomously in the nervous system to maintain axon position in the ventral nerve cord of the nematode. As previously reported, *sax-7* is also required to maintain the relative positioning of neuronal cell bodies in several head ganglia. We use the loss of cellular adhesiveness in *sax-7* null mutants as an assay system to investigate the contribution of individual domains and sequence motifs to the function of SAX-7, utilizing transgenic rescue approaches. By shortening the hinge region between the Ig1+2 and Ig3+4 domains, we improve the adhesive function of SAX-7, thereby providing support for a previously proposed autoinhibitory "horseshoe" conformation of IgCAMs. However, we find that Ig3+4 are the only Ig domains required and sufficient for the adhesive function of SAX-7. Previous models of L1-type IgCAMs that invoke an important role of the first two Ig domains in controlling adhesion therefore do not appear to apply to SAX-7. Moreover, we find that neither the 5 FnIII domains, nor the protease cleavage site embedded in them, are required for the adhesive function of SAX-7. Lastly, we show that of the several protein binding motifs present in the intracellular region of SAX-7, only its ankyrin binding motif is required and also solely sufficient to confer the adhesive functions of SAX-7.**


*Keywords:* IgSF; IgCAM; L1; Cell adhesion; Nervous system maintenance; Axon position

## Introduction

Cell adhesion molecules of the immunoglobulin superfamily (IgCAMs) engage in a variety of interactions to affect tissue development and integrity (reviewed in Rougon and Hobert, 2003; Sonderegger, 1998). Based on their domain composition, IgCAMs can be subdivided into various distinct families, one of them the L1 family of IgCAMs. L1-type IgCAMs are transmembrane proteins composed of 6 Ig domains, 3 to 5 FnIII domains and a characteristic short intracellular domain typically containing an ankyrin binding motif (Fig. 1A). A common feature of almost all L1 family members is that they engage in homophilic interactions through their extracellular domains, resulting in the homophilic adhesion of cells expressing these molecules (reviewed in Brummendorf et al., 1998; Brummendorf and Rathjen, 1996; Haspel and Grumet, 2003; Hortsch, 2000). Interactions have also been observed between L1 family members and various other non-Ig domain containing proteins, such as extracellular matrix molecules, integrins or neuropilin (Brummendorf et al., 1998; Brummendorf and Rathjen, 1996; Haspel and Grumet, 2003; Hortsch, 2000; Rougon and Hobert, 2003).

Vertebrate and invertebrate L1 family members have been implicated in many aspects of nervous system function, development and maintenance (Brummendorf et al., 1998; Brummendorf and Rathjen, 1996; Chen et al., 2001; Godenschwege et al., 2006; Hortsch, 2000; Rougon and Hobert, 2003; Sasakura et al., 2005; Wang et al., 2005; Yamamoto et al., 2006). Mutations in human L1 result in a wide spectrum of neurological abnormalities, including X-linked hydrocephalus, MASA syndrome, X-linked complicated spastic paraplegia type 1 and X-linked agenesis of the corpus callosum (Fransen et al., 1997).

Vertebrate genomes typically contain four L1 family members (L1, CHL1, Neurofascin and NrCAM; Hortsch, 2000). The nematode *Caenorhabditis elegans* contains two homologs of the L1 family, the broadly expressed *sax-7* (Chen et al., 2001; Sasakura et al., 2005; Wang et al., 2005) and the restrictively expressed *lad-2/Y54F2A.25* (Aurelio et al., 2002) (Supplementary Fig. 1). Like its vertebrate homologs, SAX-7 can engage in homophilic interactions in *trans* as assessed by cell clustering assays (Sasakura et al., 2005). The intracellular domain of SAX-7 contains a functional ankyrin binding motif and is phosphorylated by the FGF receptor *egl-15* (Chen et al., 2001). While the function of *lad-2* is currently

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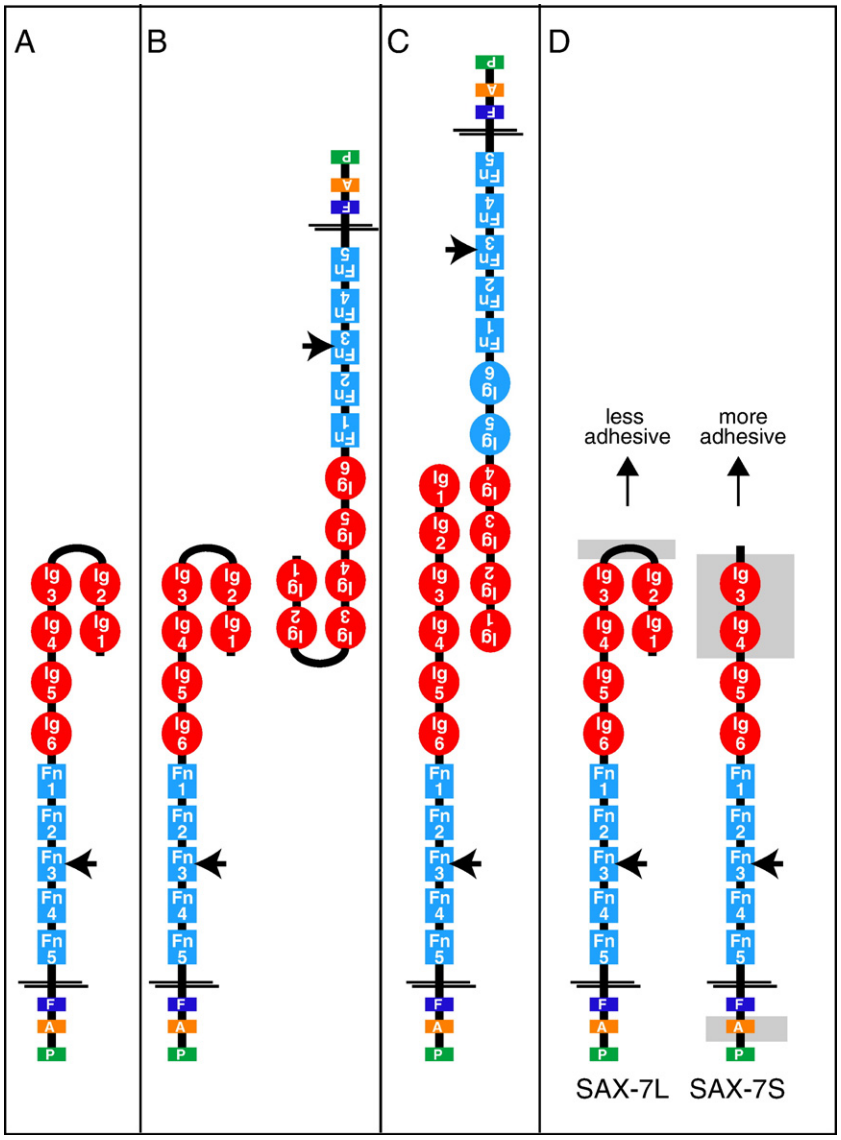
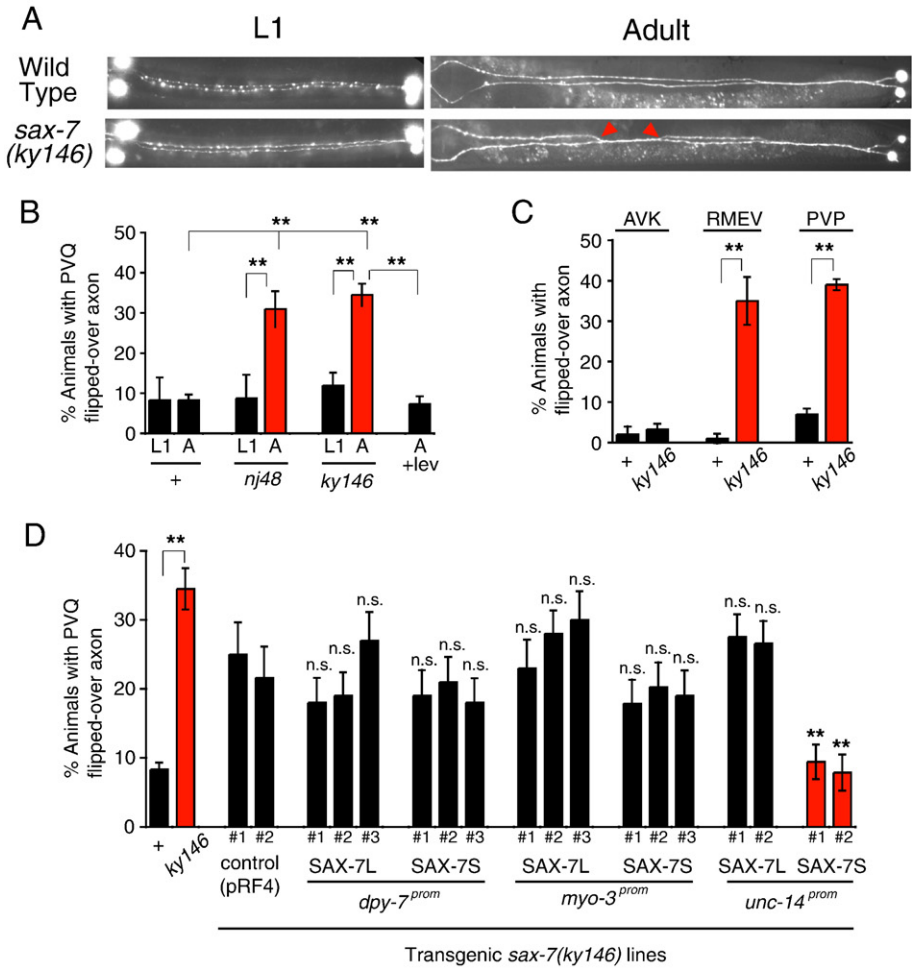



Fig. 1. Schematic representation of L1 family members. (A) Schematic structure of L1 family members, including SAX-7. "Ig" indicates "immunoglobulin domain", "Fn" indicates "Fibronectin type III domain", "F" indicates "FERM domain binding motif", "A" indicates "ankyrin binding motif", "P" indicates "PDZ domain binding motif". The sequence of the latter 3 motifs is shown in Fig. 7A. The arrow indicates a phylogenetically conserved protease cleavage site. L1 IgCAMs and related molecules, such as TAG-1, are thought to exist in a horseshoe configuration in solution, in which the first two Ig domains directly interact with the 3rd and 4th Ig domain. (B, C) The prevalent models of homophilic interaction of L1-like IgCAMs (Haspel and Grumet, 2003). In the zipper model (B), the globular horseshoe domains interact with one another. The critically interacting motifs appear to be a loop protruding from Ig3 in a hole of the opposing horseshoe, making contacts with residues from Ig2 (Freigang et al., 2000). In the domain swapping model (C) (Su et al., 1998), the horseshoe configuration is not stable and the Ig1+2 domains swap their binding partners. (D) The two isoforms of SAX-7. The long isoform (SAX-7L) is less adhesive than the short isoform (SAX-7S) (Sasakura et al., 2005). Grey shading indicates regions that we have identified in this paper as critical for the respective activities. Shortening the hinge region between Ig2 and Ig3 promotes adhesiveness of SAX-7L. The Ig3+4 domains and ankyrin binding domains are regions critical for the adhesive activity.

unknown, mutant analysis of *sax-7* has shown that the gene is involved in maintaining the integrity of several tissue types (Axang et al., 2007; Chen et al., 2001; Sasakura et al., 2005; Wang et al., 2005; Zallen et al., 1999). This is particularly evident in the nervous system where *sax-7* is not required for initial neuronal cell body and axon development, but rather involved in maintaining the specific position of neuronal structures postembryonically. Three other Ig domain family members, the secreted Ig domain protein ZIG-4, the giant multidomain protein DIG-1 and the FGF receptor EGL-15 display similar functions in maintaining axon position in the ventral nerve cord (Aurelio et al., 2002; Benard et al., 2006; Bülow et al., 2004). Here, we expand the phenotypic characterization of *sax-7* mutants and show that SAX-7 also affects axon positioning along the midline in the ventral nerve cord.

The robustness of the *sax-7* mutant phenotype provides an unprecedented opportunity to dissect the requirement for individual sequence features of SAX-7 in an *in vivo* context. Through re-introduction of mutated versions of SAX-7 into *sax-7* null mutant animals, we investigate here which parts of this L1 family member are involved in its *in vivo* function. We use the *C. elegans* system to ask several specific questions about members of the L1 family of IgCAMs:

(1) Based on electron microscopical and X-ray crystallographic studies, L1-type proteins are thought to exist in a horseshoe conformation in solution (Freigang et al., 2000; Schurmann et al., 2001; Su et al., 1998). In this conformation, the first 2 Ig domains (from here on termed "Ig1+2") fold back onto Ig domains 3 and 4 (from here on termed "Ig3+4"), thereby creating a characteristic

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| Axon | zig-4 | egl-15 | sax-7 | dig-1 |
|---|---|---|---|---|
| PVQ | + | + | + | + |
| PVP | + | + | + | n.d. |
| AVK | + | - | - | + |
| RMEV | - | - | + | + |

Fig. 2. Axon positioning defects in the ventral nerve cord of *sax-7* mutants. (A) Maintenance defect of the two PVQ axons in *sax-7(ky146)* mutants. Note that first larval (L1) stage animals appear wild type, with both axons being separated by the ventral midline. In contrast, by the adult stage, axons have flipped over the midline (red arrowheads). (B) Quantification of PVQ midline flip-over defect (L1: $n=47–101$; A; $n=179–300$). "L1" are freshly hatched first larval stage animals. "A" are young adults that have just molted. PVQ flip-over defect of *ky146* mutants can be suppressed by paralysis, induced pharmacologically (50 μM levamisole; indicated as "+lev"). Note that *kyIs146* and *nj48* show similar severity of defects, as previously observed for head neuron defects (Sasakura et al., 2005). Error bars, standard error of the proportion. (C) Axonal midline flip-over phenotype of AVK, RMEV and PVP neurons. Animals were scored as young adults ($n=63–184$). (D) The axonal defects of *sax-7* mutants are rescued by pan-neuronal expression of the short isoform of *sax-7* (SAX-7S) under the control of the *unc-14* promoter, but not under control of a muscle (*myo-3*) or hypodermis-specific (*dpy-7*) promoter. All transgenic lines carry the coinjection marker *rol-6*. "#" refers to independent transgenic lines examined for a given construct. Error bars are standard error of the proportion. Statistical significance was assessed by comparing (*z*-test) each transgenic line in which a *sax-7* isoform is expressed under a heterologous promoter to each of the two control transgenic lines which carry solely the coinjection marker *rol-6*. $^{**}p<0.01$, n.s.=difference from control not statistically significant. Bars with significant difference to controls are shown in red. Sample size is 88–203, depending on strain/transgenic line. (E) Comparison of the spectrum of mutant phenotypes of known axon maintenance factors. "+" indicates that defects can be observed upon loss of the gene, "–" indicates that no or <10% penetrant defects are observed (Aurelio et al., 2002; Benard et al., 2006; Bülow et al., 2004) (this paper). n.d., not determined.

horseshoe shape (Fig. 1A). However, it is not clear whether this conformation remains stable in an adhesive complex. It has been proposed that a globular horseshoe domain can homophilically interact in its globular form with similar globular horseshoes *in trans* in a zipper-like manner (Freigang et al., 2000; Kunz et al., 2002) (Fig. 1B). Alternatively, a domain swapping mechanism has been proposed in which the horseshoe unfolds upon homophilic interaction, leading to an interaction between Ig1+2 of one molecule

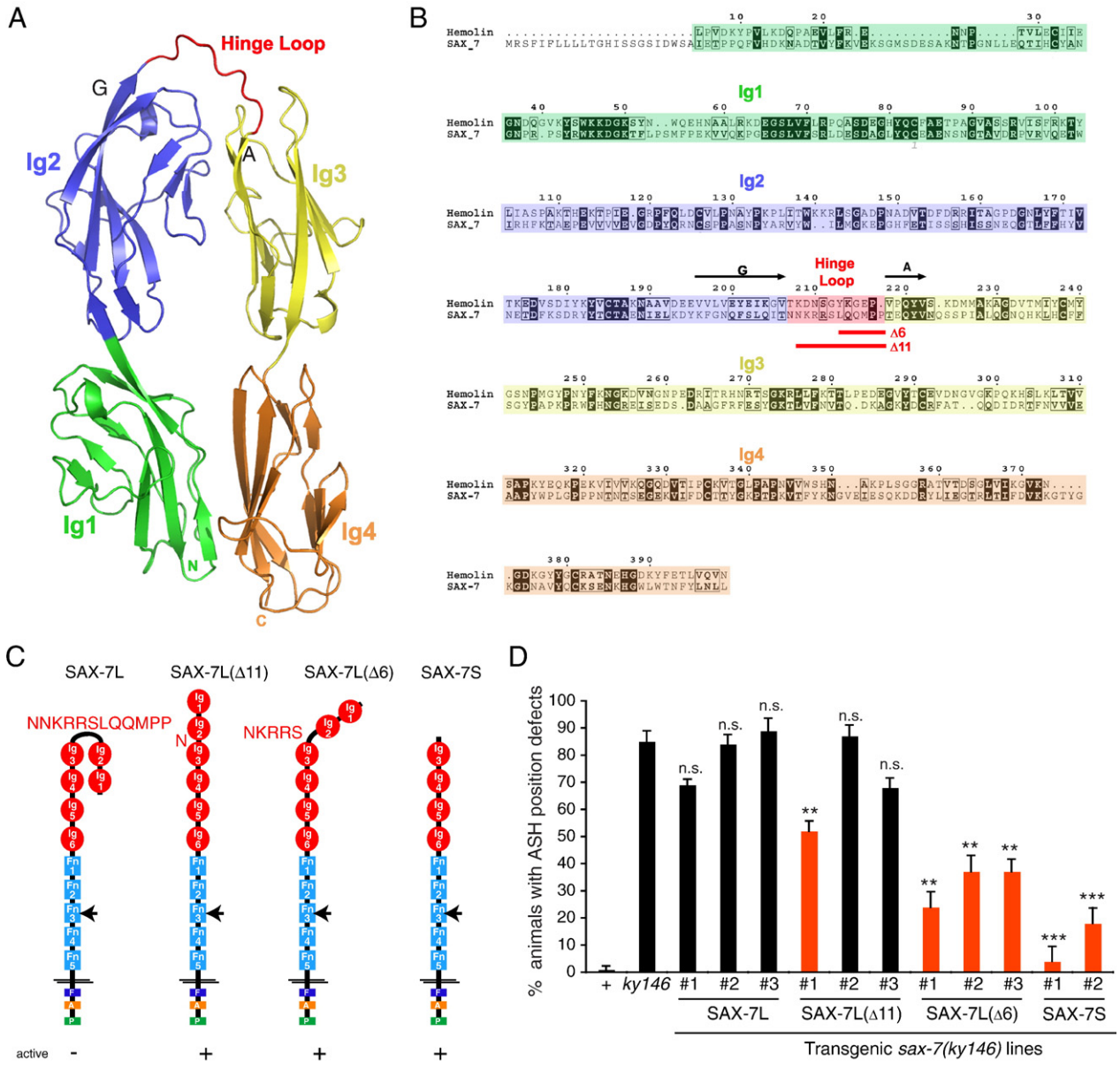



Fig. 3. Importance of the hinge region of SAX-7. (A) The horseshoe structure of hemolin defines a hinge loop region (in red) that connects the Ig1+2 and Ig3+4 modules. The structure is taken from Su et al. (1998). Note that a linker region only exists between Ig2 and Ig3 and not between other Ig domains. (B) ClustalW alignment of the first 440 amino acids of SAX-7L with the four Ig domains of hemolin defines a putative hinge loop region in SAX-7L. "G" and "A" denote the last β-strand of Ig2 and the first β-strand of Ig3 in the hemolin structure, respectively, which is also indicated in panel A. Black boxes indicate amino acid identity, open boxes similarity. (C) Schematic representation of constructs used. The sequences of the linker regions are indicated in red. (D) Rescue of the *sax-7 (ky146)* mutant phenotype by constructs indicated in panel C. "% animals with ASH position defects" indicates fraction of animals in which one or two ASH neuron cell bodies were mispositioned either on, or anterior to the nerve ring, scored with *oyIs14*. All transgenic lines described from here on carry the intestinal coinjection marker *elt-2*::*gfp*. "#" refers to independent lines examined for a given transgene. Error bars, standard error of the proportion. Statistical significance was assessed by comparing (*z*-test) each transgenic line with the parent null mutant strain *sax-7(ky146)* in which the DNA was injected. $*p<0.05$, $**p<0.01$, $***p<0.005$, n.s. = difference from mutant control not statistically significant. Bars with significant difference to mutant control are shown in red. Sample size is 69–118, depending on strain/transgenic line. Note that even though more efficient than the normal SAX-7L isoform, the 11 amino acid deletion construct SAX-7L(Δ11) is less effective in rescuing the mutant phenotype than the 6 amino acid deletion construct SAX-7L(Δ6). As inferred from the crystal structure of hemolin (Fig. 3A), the lack of any significant spacing between Ig2 and Ig3 in the Δ11 construct may bring the two domains in too close proximity and thereby interfere with the folding of Ig3.

with Ig3+4 of the other molecule and vice versa (Su et al., 1998) (Fig. 1C). Similar domain swapping mechanisms have been proposed for another class of cell adhesion molecules, the cadherins (Boggon et al., 2002; Chen et al., 2005). By testing the *in vivo* activity of an engineered, artificially extended version of SAX-7, we investigate here the importance of the globular vs. extended conformation of SAX-7.

(2) Mutational analysis has revealed that residues broadly distributed over all 6 Ig domains affect homophilic binding of vertebrate L1 in *in vitro* assays (De Angelis et al., 1999; Holm et al., 1995) (reviewed in Haspel and Grumet, 2003). Multiple Ig domains of vertebrate L1 were also shown to be required in neurite outgrowth and cell adhesion assays (Appel et al., 1993). In contrast, other studies mapped the homophilic L1 binding site exclusively to the

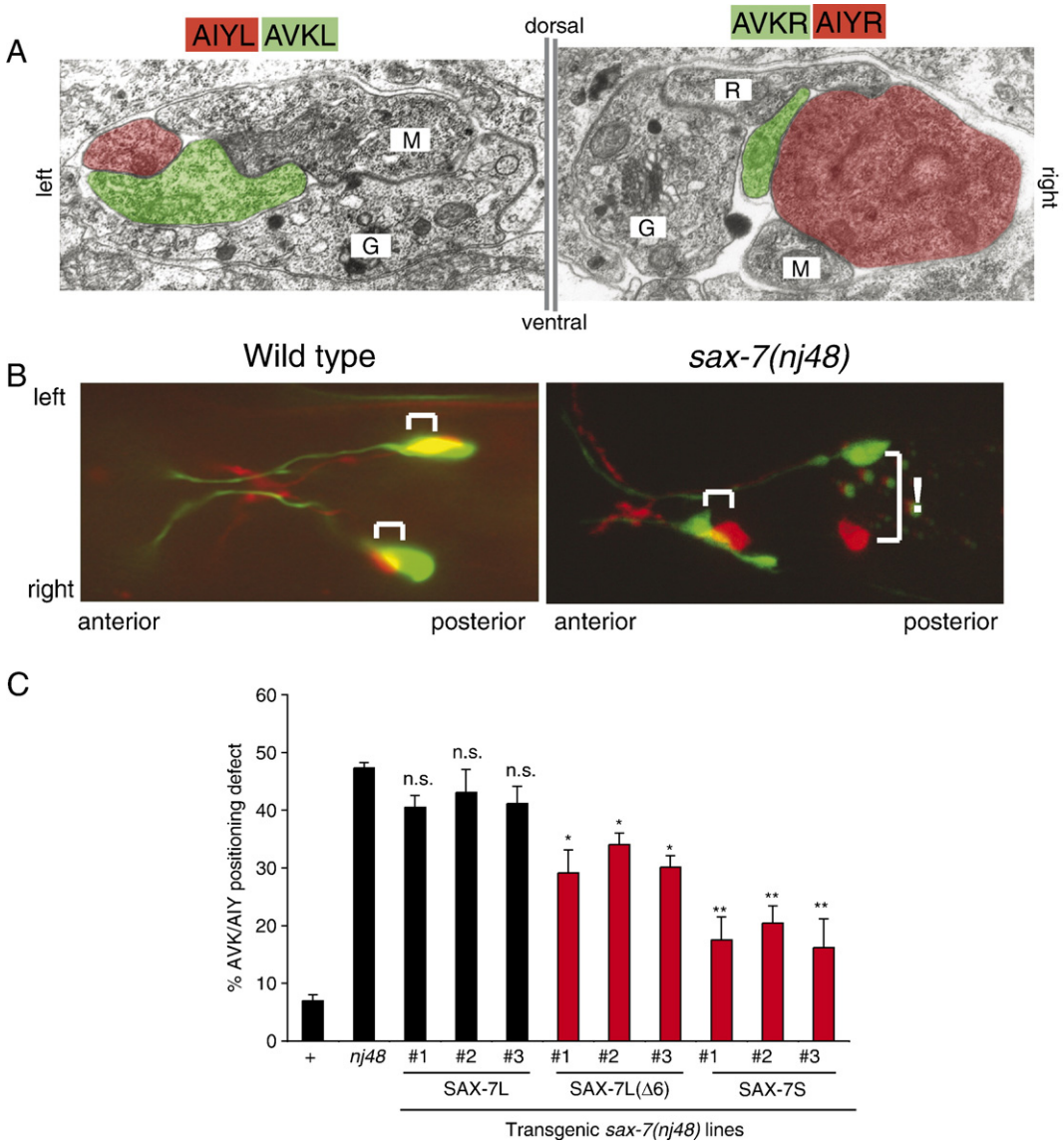



Fig. 4. Importance of the hinge region of SAX-7 determined by an *in vivo* adhesion assay (A) Electron micrographs collected in the course of the analysis by White et al. (1986) show that the left AIY and AVK neurons (AIYL and AVKL) and the right AIY and AVK neurons (AIYR and AVKR) normally adhere to one another. The two micrographs are from the same animal (N2U). The sections originate from the region of the lateral ganglion. Note that the cell diameter changes depending on the location of the section along the a/p axis. AIY neurons are pseudocolored in red, AVK neurons are pseudocolored in green. Magnification is 26,400. "M"=AIM, "R"=RIS (an unilateral neuron), "G" excretory gland. (B) Reporter genes monitor the adhesiveness of the AIYL and AVKL and AIYR and AVKR neurons (left panel; brackets). AIYL/R are visualized in a ventral view with dsRed2 (*otIs133*) and AVKL/R with GFP (*bwIs2)*. Yellow overlap is observed due to overlap of signals in different planes of focus. The right panel shows a representative example in *sax-7(nj48)* animals, in which the left cell pair becomes detached (marked with exclamation mark). See panel C for quantification of defects. (C) Rescue of the *sax-7(nj48)* mutant phenotype by the constructs indicated in Fig. 3C, expressed under control of the pan-neuronal *unc-14* promoter. Note that the deletion construct SAX-7L(D6) rescues the mutant phenotype more efficiently than the wild-type SAX-7L isoform. "#" indicates independent transgenic lines. "% AVK/AIY positioning defect" indicates fraction of cases in which any of the AIY and AVK pairs are detached from one another. Statistical significance was assessed by comparing (*z*-test) each transgenic line with the parent mutant strain *sax-7(nj48)* in which the DNA was injected. $*p<0.05$, $**p<0.01$, n.s.=difference from mutant control not statistically significant. Bars with significant difference to mutant control are shown in red. Sample size is 93–152, depending on strain/transgenic line.

second Ig domain (Kunz et al., 1998; Zhao and Siu, 1995; Zhao et al., 1998). Binding to heterophilic ligands, such as neurocan or neuropilin, was shown to be mediated by the first Ig domain (Castellani et al., 2002; Oleszewski et al., 1999). Through deletion analysis, we describe here which Ig domains are required and sufficient for SAX-7 function *in vivo*.

(3) Multiple functions have been assigned to the FnIII domains of L1 family members *in vitro*. L1 proteins have been shown to undergo ectodomain shedding that is mediated through protease cleavage sites within the 3rd and distal to the the 5th FnIII domains (Kalus et al., 2003; Maretzky et al., 2005; Moos et al., 1988; Naus et al., 2004; Nayeem et al., 1999). While tissue culture experiments have ascribed functional relevance to this shedding in vertebrates, it is less clear whether shedding is also required in an *in vivo* context. Apart from containing protease cleavage sites, the FnIII domains of L1 have also been implicated in homophilic binding, receptor multimerization, recruitment of integrins and promotion of neurite outgrowth (Appel et al., 1995; Koticha et al., 2005; Silletti et al., 2000). We test the relevance of the FnIII domains of SAX-7 by removing all its FnIII domains and asking whether the molecule retains its function.

(4) The intracellular domain of L1 family members contain several motifs that are thought to couple this transmembrane protein to various cytoskeletal proteins. These motifs include a FERM domain binding motif, an ankyrin binding motif and a PDZ binding motif. Interaction between these motifs of vertebrate L1 and their cognate binding proteins has been documented in cell culture (Davey et al., 2005; Davis and Bennett, 1994; Dirks et al., 2006; Falk et al., 2004; Gunn-Moore et al., 2006; Koroll et al., 2001), but the physiological relevance of these interactions is little understood. We test the importance of these binding motifs by mutagenizing individual motifs and asking whether this impedes the function of SAX-7 *in vivo*.

## Results and discussion

### *sax-7 functions in neurons to maintain axon position in the ventral nerve cord*

Our first goal was to compare the phenotypic spectrum of *sax-7* with that of other IgSF members that were previously implicated in maintaining nervous system architecture. Animals lacking the maintenance factors ZIG-4, a secreted 2 Ig domain protein, or the FGF receptor isoform EGL-15(5a), develop normally but fail to maintain the position of interneuronal axon tracts along the ventral midline of *C. elegans* (Aurelio et al., 2002; Bülow et al., 2004). Animals lacking the maintenance factor DIG-1, a large extracellular protein with various protein interaction domains, also develop normally but fail to maintain the position of large number of neurons in the head, ventral nerve cord and tail ganglia (Benard et al., 2006). *sax-7* mutants were previously shown to fail to maintain the position of sensory neuron cell bodies and their axons in various head ganglia, as well as the cell bodies of ventral cord motor neuron and their circumferential axonal commissures (Sasakura et al., 2005; Wang et al., 2005; Zallen et al., 1999). However, the positioning of longitudinal interneuronal axon tracts within the ventral nerve cord has not previously been examined in *sax-7* mutants.

We examined the ventral nerve cord axons of the PVQ, PVP, AVK interneurons and the RMEV motoneuron in *sax-7* mutants (Fig. 2). We find that compared to the other known maintenance factors, an overlapping but partly distinct subset of axons is affected (Fig. 2). That is, the positioning of the PVQ, PVP and RMEV axons fails to be maintained along the midline in *sax-7* mutants, while the AVK axons are unaffected. This maintenance role is evidenced by an absence of positioning defects in the first

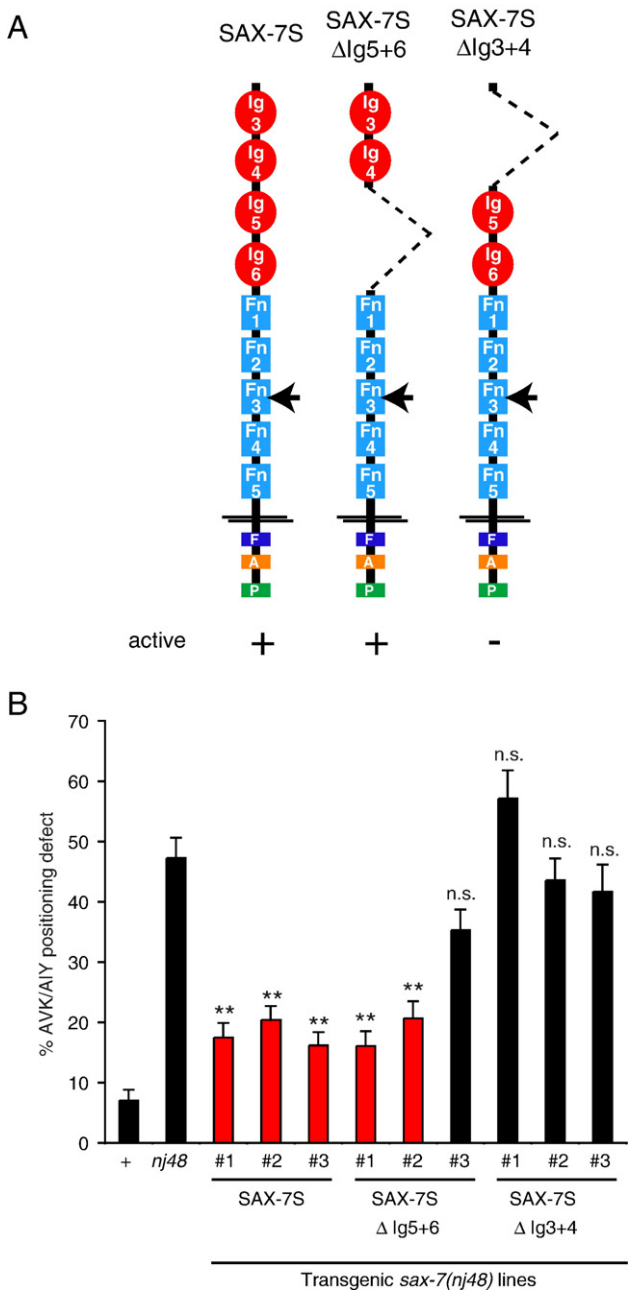



Fig. 5. Defining the Ig domains required for SAX-7 function. (A) Schematic representation of constructs used. (B) Rescue of the *sax-7(nj48)* mutant phenotype by constructs indicated in panel A, expressed under control of the pan-neuronal *unc-14* promoter. "#" indicates independent transgenic lines. "% AVK/AIY positioning defect" indicates fraction of cases in which any of the AIY and AVK pairs are detached from one another. Statistical significance was assessed by comparing (*z*-test) each transgenic line with the parent mutant strain *sax-7(nj48)* in which the DNA was injected. $^{**}p<0.01$, n.s. = difference from mutant control not statistically significant. Bars with significant difference to mutant control are shown in red. Sample size is 55–152, depending on strain/transgenic line. Data on wild-type and *sax-7(nj48)* null mutants is the same as shown in Fig. 4C and are shown for comparison only.

larval stage when most of the nervous system has already been generated and the presence of defects in the adult stage (Fig. 2B). Similar to the *zig-4*, *egl-15* and *dig-1* mutant phenotype (Aurelio et al., 2002; Benard et al., 2006; Bülow et al., 2004), the *sax-7* mutant phenotype is induced by mechanical stress exerted on the axons through the locomotory movement of worms, as the mutant phenotype can be suppressed by pharmacologic immobilization of worms (Fig. 2B).

*zig-4* is an Ig domain protein secreted from the PVT neuron, *egl-15* acts in the underlying hypodermis and *dig-1* functions in muscles to affect axon maintenance (Aurelio et al., 2002; Benard et al., 2006; Bülow et al., 2004). In contrast to all these non-autonomously acting precedents, we find that *sax-7* acts autonomously within neurons to affect axon maintenance along the ventral midline since a *sax-7* cDNA can rescue the *sax-7* mutant phenotype when expressed under control of a heterologous neuronal promoter (Fig. 2D). No rescue is

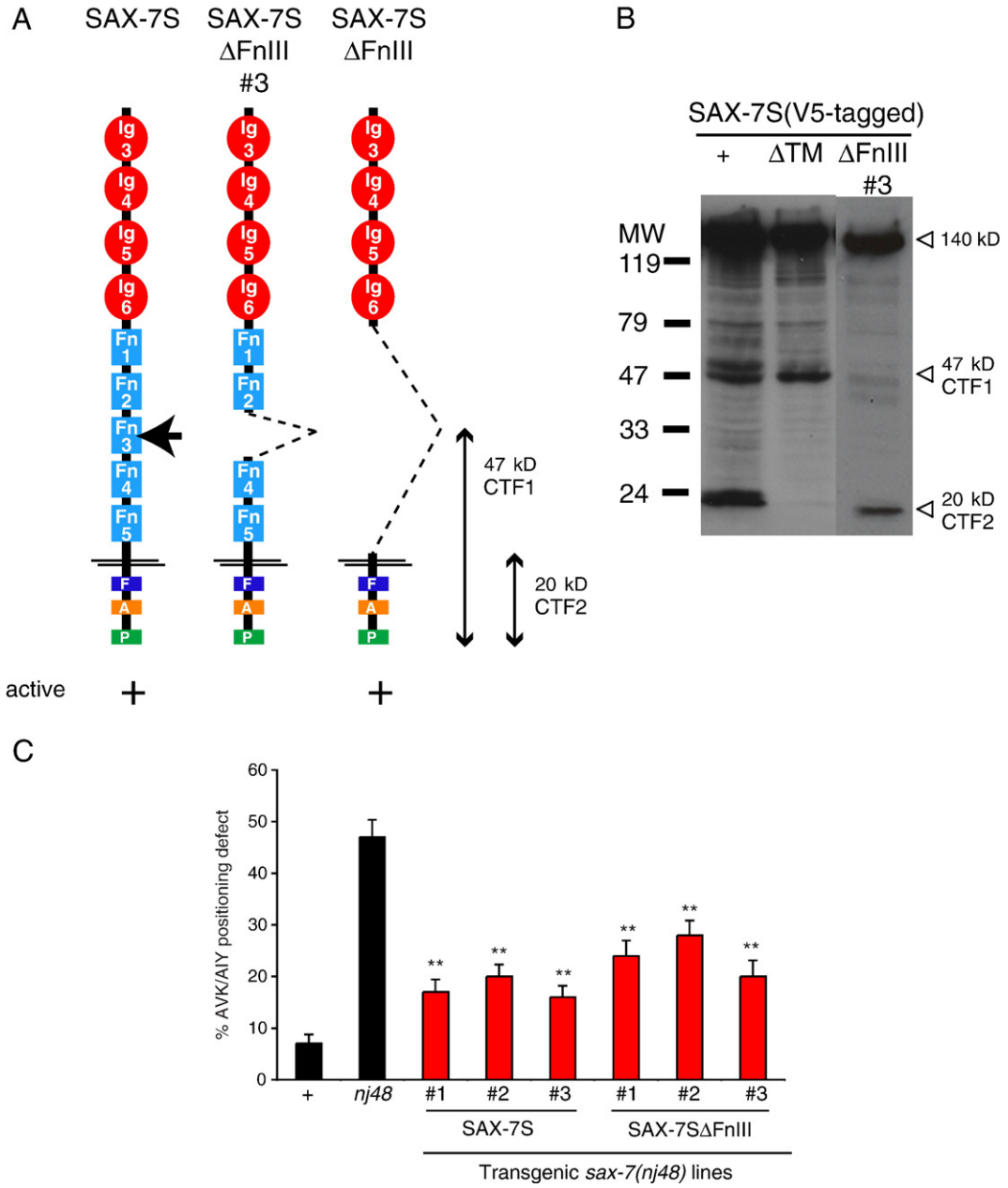



Fig. 6. FnIII domains are not required for SAX-7 function. (A) Schematic representation of constructs used. (B) Anti-V5 Western blot of lysates from COS cells transfected with SAX-7S, or mutated versions of SAX-7S in which either the transmembrane domain or the third FnIII domain are deleted. Each deletion eliminates specific cleavage events that in wild-type animals lead to the production of two distinct C-terminal fragments (CTFs; see panel A). (C) Rescue of the *sax-7(nj48)* mutant phenotype with a SAX-7S construct in which all FnIII domains are deleted, expressed under control of the pan-neuronal *unc-14* promoter. "#" indicates independent transgenic lines. "% AVK/AIY positioning defect" indicates fraction of cases in which any of the AIY and AVK pairs are detached from one another. Statistical significance was assessed by comparing (*z*-test) each transgenic line with the parent mutant strain *sax-7(nj48)* in which the DNA was injected. $^{**}p<0.01$. Bars with significant difference to mutant control are shown in red. Sample size is 43–152, depending on strain/transgenic line. Data on wild-type and *sax-7(nj48)* null mutants are the same as shown in Fig. 4C and are shown for comparison only.

observed when *sax-7* is expressed under control of a hypodermis or muscle-specific promoter (Fig. 2D). The neuronal focus of action mirrors the cell-autonomous focus of action of *sax-7* function in maintaining sensory neuron position in head ganglia (Sasakura et al., 2005).

Taken together, we conclude that each of the known axon maintenance factors affects a different, though overlapping spectrum of axons in the ventral nerve cord of *C. elegans* (Fig. 2E).

### *Altering the hinge region activates the long isoform of SAX-7*

The *sax-7* locus produces at least two isoforms, SAX-7L (for *L*ong) and SAX-7S (for *S*hort), which are distinguished by the presence or absence of the first two of a total of six Ig domains (Sasakura et al., 2005; Wang et al., 2005) (Fig. 1D). Previous work has shown that the short isoform (a) has a more potent ability to rescue adhesive defects of *sax-7* mutants and (b) is more adhesive in cell-culture-based interaction assays (Sasakura et al., 2005). We established several additional lines of independent evidence for SAX-7S being a more adhesive protein than SAX-7L. First, SAX-7S, but not SAX-7L, rescues the PVQ axon position defect at the ventral midline (Fig. 2D). Second, we examined the cell body of the ASH sensory neuron, visualized with a chromosomally integrated *sra-6*::*gfp* array, *oyIs14*. In wild-type animals, the ASH cell body is situated posteriorly to the nerve ring, while in *sax-7* mutants, a significant fraction of animals have their ASH neurons either located on or anterior to the nerve ring (Fig. 3). This phenotype can be efficiently rescued by pan-neuronal expression of SAX-7S, but less efficiently by SAX-7L (Fig. 3). The limited efficiency of rescue with the long isoform cannot be improved by a 10-fold increase in amount of injected DNA (data not shown).

In order to more precisely define mutant phenotypes, we generated a novel transgenic reporter strain in which we could specifically visualize the mutual adhesiveness of two pairs of interneurons, the AIY and AVK interneurons, whose cell bodies are located in the lateral head ganglion. EM analysis shows that the cellular membranes are indeed in direct contact (White et al., 1986) (Fig. 4A). We used single-cell-type-specific promoters (*ttx-3* and *flp-1*) to label AIY with *rfp* and AVK with *gfp*, respectively. In wild-type animals, AIY and AVK are located directly adjacent to one another (Fig. 4B). In *sax-7* mutants, however, AIY and AVK lose their direct cell contact (Figs. 4B, C). The differential ability of the long and short isoform of SAX-7 in rescuing this mutant phenotype is even more apparent in this assay; several transgenic lines expressing the long isoform show no statistically significant rescue while all transgenic lines expressing the short isoform do (Fig. 4C).

All these findings are supportive of a model in which the first 2 Ig domains have an inhibitory activity. According to the structure-based horse-shoe model of IgCAMs (Su et al., 1998) (Fig. 3A), a hinge loop between the 2nd and 3rd Ig domain may allow the first two Ig domains (Ig1+2) to fold back onto Ig domains 3 and 4 (Ig3+4), thereby inhibiting the homophilic binding activity of two SAX-7 molecules present on opposing cell surfaces. However, other possible explanations for this observation include a different overall protein stability or differences in protein trafficking of the long vs. short isoform. To seek more experimental support for the notion that Ig3+4 are indeed the major adhesive domains whose activity is inhibited by Ig1+2, we pursued two approaches. First, we removed residues in the hinge region between Ig1+2 and Ig3+4 that are predicted to prevent the hinging of this region. If the Ig1+2 / Ig3+4 interaction is indeed inhibitory, we would expect that the long isoform would thereby be transformed into a more adhesive molecule. Second, we directly tested the involvement of Ig domains 3 and 4 in the adhesive activity of SAX-7 by domain deletion analysis.

To test the importance of the hinge domain, we first defined the hinge region of SAX-7L as a 12 amino acid long region through aligning its primary sequence with that of hemolin (Fig. 3B). Hemolin is an insect IgSF member whose three dimensional structure was the first to point to the existence of a horseshoe conformation (Fig. 3A). We then shortened the hinge loop by deleting either 6 or 11 amino acids (Fig. 3C), reasoning that this may prevent the Ig1+2 module from folding back onto the Ig3+4 module, resulting in an extended conformation of the Ig1+2+3+4 domains. We indeed find that shortening in the hinge region significantly improves the ability of the long isoform of SAX-7 to rescue the mutant phenotype, assayed by either analyzing the position of the ASH sensory neuron or the relative position of the AIY and AVK interneurons (Figs. 3D, 4C).

The hinge loop deletion experiments suggest that the Ig1+2 module normally inhibits the function of Ig3+4 and that therefore the Ig3+4 module is the key determinant of the adhesive activity of SAX-7. To test this notion more directly, we asked whether it is indeed the Ig3+4 module rather than, for example, the Ig5+6 module that provides adhesive activity. We therefore deleted Ig3+4 or Ig5+6 from the short SAX-7 isoform. We find that a construct that solely contains Ig3+4 (in addition to the FnIII, transmembrane and intracellular domains) is capable of efficiently rescuing the *sax-7* mutant phenotype, that is, deleting Ig5+6 has no effect (Fig. 5). In contrast, deleting Ig3+4 abolishes the ability of SAX-7 to rescue the mutant phenotype. Taken together, unlike in vertebrate L1 family members, where important functions have been assigned to most of the Ig domains (De Angelis et al., 1999) and to the second Ig domain in particular (Freigang et al., 2000; Zhao and Siu, 1995; Zhao et al., 1998), of all Ig domains, only the 3rd and 4th Ig domains of SAX-7 are important for its adhesive function.

At this point it is not clear whether the adhesive SAX-7 activity that our assay measures is reflective of a homophilic interaction of SAX-7 molecules on opposing cellular surfaces or whether SAX-7 engages in an interaction with other proteins. Sasakura et al. (2005) showed that expression of SAX-7 in a single head sensory neuron of an otherwise *sax-7* mutant animal rescues the adhesive defects of this neuron, thereby suggesting heterophilic interactions. However, we fail to see single-cell-specific rescue when expressing SAX-7 exclusively in either the AIY or AVK interneurons (data not shown). At this point, it is therefore not conclusively proven or ruled out that SAX-7 homophilically interacts in *trans*. However, we consider this the most parsimonious model for SAX-7 function since (a) SAX-7 can homophilically interact in cell culture and upon ectopic expression in adjacent, normally non-interacting neurons (Sasakura et al., 2005), (b) SAX-7 is broadly expressed in the nervous system (Chen et al., 2001) and (c) the AIY and AVK cell bodies, which we assay here, are normally in direct contact with one another (Figs. 4A, B) and lose their contact in *sax-7* mutants (Figs. 4B, C).

### *Proteolytic cleavage and FnIII domains of SAX-7 are not required for in vivo function*

We expressed the epitope-tagged, short form of SAX-7 in COS cells and found the protein to be processed to release two C-terminal fragments of ~47 kDa and ~20 kDa (Fig. 6). We infer from the existence of these cleavage products that SAX-7, like other L1

family members (see Introduction), is processed in a membrane proximal region (releasing the ~20-kDa intracellular region) and within its third FnIII domain (releasing the ~47-kDa fragment). The third FnIII domain contains a phylogenetically conserved, predicted cleavage site for Furin and PC-type proprotein proteases (RWKR), which has previously been implicated in L1 processing (Hortsch, 2000; Kalus et al., 2003; Seidah and Chretien, 1999). We confirmed that each of these regions is indeed required for proteolytic cleavage by deleting either the transmembrane domain or the third FnIII domain, each of which leads to the disappearance of the respective cleavage product (Fig. 6B).

We assessed the physiological relevance of cleavage within the third FnIII domain. To abolish this cleavage event, we simply deleted all FnIII domains and asked whether the remaining Ig domains (plus transmembrane and cytoplasmic domains) are able to confer function, as measured by the mutual adhesiveness of the AIY and AVK interneurons. We find that the removal of all FnIII domains does not affect the ability of SAX-7S to rescue the adhesive defects of *sax-7* mutants (Fig. 6). We therefore conclude that ectodomain cleavage within any FnIII domain is not essential for SAX-7 function, at least in the cellular context assayed. Other functions previously associated with the FnIII domains of L1 family members (homophilic binding, receptor multimerization, recruitment of integrins; Appel et al., 1995; Koticha et al., 2005; Silletti et al., 2000) therefore also appear to have no relevance for SAX-7 function.

### *The ankyrin binding motif is the only functionally relevant intracellular motif of SAX-7*

The intracellular region of SAX-7 contains three phylogenetically conserved motifs that can couple to intracellular adaptor motifs, a FERM domain binding motif, an ankyrin binding motif and a C-terminal PDZ binding motif (Figs. 7A, B). In vertebrates, each of these motifs has been found to engage in physical contacts with FERM proteins, ankyrin and PDZ domain proteins (Davey et al., 2005; Davis and Bennett, 1994; Dirks et al., 2006; Falk et al., 2004; Gunn-Moore et al., 2006; Koroll et al., 2001). Moreover, L1 has been shown to be phosphorylated by casein kinase II (Wong et al., 1996) and the phosphorylation prediction program PredPhospho (Kim et al., 2004) identifies four CKII phosphorylation sites in SAX-7 (data not shown). However, the physiological relevance of protein–protein interactions and phosphorylation events has not yet been tested in a whole animal context.

We find that replacement of the complete intracellular region of SAX-7 with a heterologous protein (red fluorescent protein, RFP) abolishes the ability of the protein to rescue its mutant phenotype (Fig. 7C). As assessed by red fluorescence, this protein is correctly expressed and membrane localized (data not shown). The intracellular domain is therefore not required for membrane localization but is required for protein function. These results contrast previous in vitro findings which demonstrated that intracellular domain-deleted L1 supports homophilic adhesion of transfected cells (Wong et al., 1995).

To map which features of the SAX-7 intracellular region are required for its function in neuronal adhesion, we deleted individual protein binding motifs in the intracellular region. We find that deletions of the FERM domain binding motif or the PDZ domain binding motif do not abolish the ability to rescue the defects in AIY/AVK adhesiveness (Fig. 7C). However, deletion of the ankyrin binding motif completely abolishes the ability to rescue the mutant phenotype. This apparent importance of the ankyrin binding motif prompted us to test whether the ankyrin binding domain is perhaps the sole critical feature of the intracellular domain. To test this possibility, we appended the five amino acid long ankyrin binding motif (FIGQY) to the SAX-7 construct mentioned above, in which the entire intracellular domain is replaced with RFP (Fig. 7B). This construct is able to rescue the *sax-7* mutant phenotype (Fig. 7C). The ankyrin binding domain is therefore the sole intracellular functional determinant of SAX-7 in the context of neuronal adhesion in *C. elegans*.

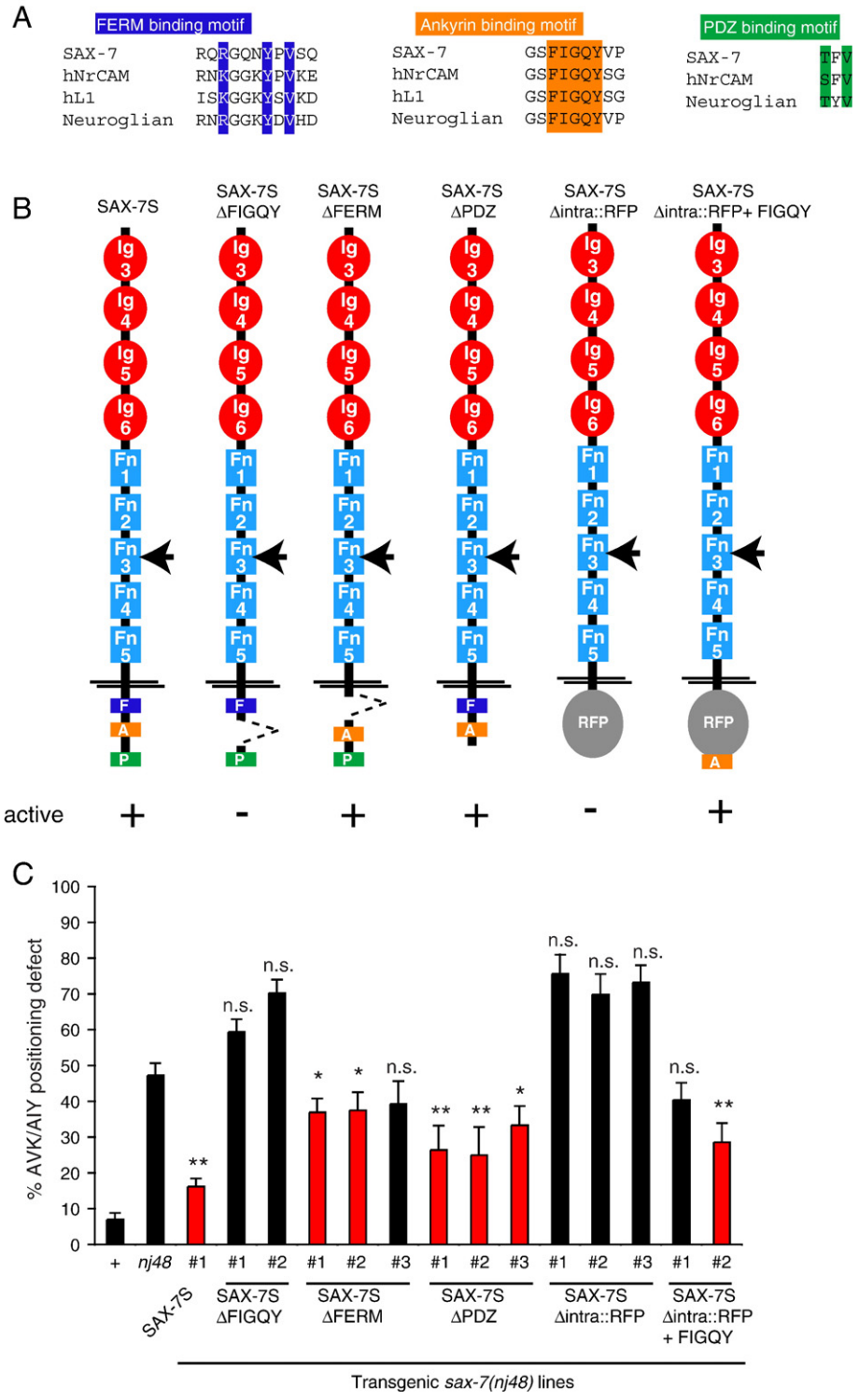
## Conclusions

We can draw three major conclusions from our work. First, we have identified a fourth IgSF member involved in maintaining the position of axons along the ventral midline. All 4 IgSF proteins affect a distinct, though largely overlapping spectrum of axons and are supplied by distinct cell types. ZIG-4 is secreted from the PVT interneuron to affect the maintenance of several sets of axons (Aurelio et al., 2002), EGL-15 acts in the hypodermis on which the axons are positioned (Bülow et al., 2004), DIG-1 is secreted from muscle (Benard et al., 2006) and, as we have shown here, SAX-7 acts autonomously within neurons to affect axon position. Future experiments will show whether these proteins interact with one another directly.

Our second conclusion relates to an open question in the field of IgCAM proteins. Structural studies have revealed the existence of horseshoe-type configurations of the first 4 Ig domains of several Ig domain proteins (Freigang et al., 2000; Schurmann et al., 2001; Su et al., 1998). Previous work (Sasakura et al., 2005) in conjunction with the work described here firmly establishes that the Ig1+2 module is indeed a negative regulator of SAX-7 function. As we describe here that the Ig3+4 module is the major adhesive component of SAX-7, it is reasonable to suggest that Ig1+2 inhibits SAX-7 function by binding Ig3+4 in a horseshoe configuration. However, conflicting models have been proposed in the literature for the arrangement of these domains in the context

Fig. 7. Assessing the importance of individual sequence motifs in the intracellular domain of SAX-7. (A) Sequence motifs in the intracellular domain of SAX-7. In contrast to other vertebrate L1 family members, L1 itself does not contain a canonical PDZ domain binding motif but nevertheless binds PDZ domain proteins (Dirks et al., 2006). FERM domain binding motifs are found in a large number of IgCAMs (Hamada et al., 2003). (B) Schematic representation of constructs used. "F" indicates "FERM domain binding motif", "A" indicates "ankyrin binding motif", "P" indicates "PDZ domain binding motif". (C) Rescue of the *sax-7(nj48)* mutant phenotype by constructs indicated in panel B, expressed under control of the pan-neuronal *unc-14* promoter. "#" indicates independent transgenic lines. "% AVK/AIY positioning defect" indicates fraction of cases in which any of the AIY and AVK pairs are detached from one another. Statistical significance was assessed by comparing (*z*-test) each transgenic line with the parent mutant strain *sax-7(nj48)* in which the DNA was injected. $*p<0.05$, $**p<0.01$, n.s. = difference from mutant control not statistically significant. Bars with significant difference to mutant control are shown in red. We also did a statistical comparison between the extent of rescue seen with the wild-type SAX-7S and SAX-7ΔFERM and SAX-7ΔPDZ constructs. Both SAX-7ΔFERM transgenic lines do not rescue quite as well as the wild-type construct ($p<0.05$) but 2/3 SAX-7ΔPDZ constructs do not differ from the wild-type construct in their ability to rescue the *sax-7* mutant phenotype ($p>0.05$). Sample size is 56–152, depending on strain/transgenic line. Data on wild-type and *sax-7(nj48)* null mutants are the same as shown in Fig. 4C and are shown for comparison only.

of an adhesive complex. In the domain swapping model (Fig. 1C), the first 2 Ig domains of two opposing, homophilically interacting IgCAMs swap their binding partners; the Ig1+2 module of one protein interacts with the Ig3+4 module of the other protein. In the zipper model, the horseshoe configuration remains intact and individual globular Ig1+2+3+4 horseshoes arrange in an alternating zipper pattern (Fig. 1B) (Freigang et al., 2000). Previous work (Sasakura et al., 2005) in conjunction with the work described here does not argue in favor of a domain swapping model for SAX-7 as the first 2 Ig domains are not required for the adhesive function of SAX-7, neither in a cell-based adhesion assay (Sasakura et al., 2005) nor in an organismal context (Sasakura et al., 2005) (this work). Rather, the Ig3+4 domains are sufficient to confer the adhesive function of SAX-7. The zipper model is also not supported by our findings. In the zipper model, the integrity of the globular module of the first 4 Ig domains is essential for binding activity and the Ig2



domain engages in critical contacts between the Ig1+2+3+4 modules (Freigang et al., 2000). However, in the case of SAX-7, the first 2 Ig domains are dispensable for activity. Moreover, we find that a shortening of the hinge region, which is predicted to disrupt the globular horseshoe structure, does not disrupt the adhesive activity of SAX-7, but rather promotes it. Taken together, the homophilic binding activity of SAX-7 may involve interactions of Ig domains in a previously non-anticipated manner. Structural analysis will eventually resolve this binding conformation.

The third conclusion is that the Ig3+4 module and the ankyrin binding motif appear to be the only essential determinants of SAX-7 protein function. The first and last two Ig domains, the FnIII domains with their embedded protease cleavage sites, intracellular binding sites for various adaptor proteins and predicted phosphorylation sites are dispensable for SAX-7 function in the neuronal adhesion context that we examined here. The sufficiency of the ankyrin binding motif also makes it unlikely that, at least in the context of neuronal adhesion, SAX-7 triggers intracellular, MAPK-mediated signal transduction events, a feature of vertebrate L1 that is mediated by a membrane proximal region (Cheng et al., 2005).

It is possible that the domain requirements that we describe here are specific for SAX-7 function in the organismal and cellular context that we have examined. For example, in *C. elegans*, SAX-7 may have a more static function solely in promoting neuronal adhesion, while in other systems, L1 protein function may be more dynamically controlled through protein processing (ectodomain shedding) and integration into cellular signaling events (e.g., through coupling to MAPK signaling). However, as our analysis is the first transgenic analysis of an L1 family member in an organismal context, one should not completely disregard the possibility that similar *in vivo* approaches in vertebrates may support a role for L1 more akin to its invertebrate homolog.

### Experimental methods

#### *Strains*

Mutant strains: *sax-7(ky146)*, *sax-7(nj48)*. Both alleles show similar severity of mutant phenotypes (this paper) (Sasakura et al., 2005) and we use them here interchangeably. Based on its molecular nature, a deletion at the beginning of the gene, *nj48* is a likely molecular null allele (Sasakura et al., 2005). Transgenic reporter strains that allow visualization of neuroanatomy are as follows. PVQ neurons: *oyIs14 Is[sra-6*::*gfp; lin-15(+)]*; PVP neurons: *hdIs26[sra-6*::*DsRed2; odr-1*::*gfp]*; RMEV neuron: *oxIs12 [unc47*::*gfp; lin-15(+)]*; AVK neurons: *bwIs2 Is[flp-1*::*gfp]*; AIY neurons: *otIs133 [ttx-3*::*dsRed2, unc-4(+)]*. All animals were grown and scored at 20 °C.

#### *DNA constructs and transgenic lines*

Information on DNA constructs and transgenic lines can be found in the Supplementary methods. Briefly, for *sax-7* rescuing constructs *sax-7* cDNA constructs were subcloned and expressed under control of the *myo-3* (Okkema et al., 1993), *dpy-7* (Gilleard et al., 1997) or *unc-14* promoter (Ogura et al., 1997). Constructs were injected at 5 ng/μL with either pRF4 *(rol-6(d))* at 100 ng/μL or *elt-2*::*gfp* at 20 ng/μL as injection marker.

#### *Cell culture expression of sax-7*

The *sax-7* cDNA was subcloned into the pcDNA 3.1D/V5-His TOPO (Invitrogen) expression vector, with a V5 epitope tag at its C-terminus. COS-7 cells were cultured in DMEM with 10% fetal bovine serum. Cells were transiently transfected using Lipofectamine (Invitrogen), collected after 36 h, and lysed in ice-cold IP buffer [5 mM Tris–HCl (pH 8), 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% Triton X-100 and protease inhibitor cocktail (Sigma)]. Lysates were resolved on a 7.5% SDS-PAGE gel, blotted and probed with peroxidase-conjugated antibodies and analyzed with the ECL plus Western Blotting Detection System.

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### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mcn.2007.08.014.

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