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Collagen XIXa1 is crucial for motor axon navigation at intermediate targets

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SUMMARY
During development, motor axons navigate from the spinal cord to their muscle targets in the periphery using stereotyped pathways. These pathways are broken down into shorter segments by intermediate targets where axon growth cones are believed to coordinate guidance cues. In zebrafish *stumpy* mutants, embryonic development proceeds normally; however, as trunk motor axons stall at their intermediate targets, suggesting that Stumpy is needed specifically for motor axon growth cones to proceed past intermediate targets. Fine mapping and positional cloning revealed that *stumpy* was the zebrafish homolog of the atypical FACIT collagen *collagenXIXa1* (*colXIX*). *colXIX* expression was observed in a temporal and spatial pattern, consistent with a role in motor axon guidance at intermediate targets. Knocking down zebrafish ColXIX phenocopied the *stumpy* phenotype and this morpholino phenotype could be rescued by adding back either mouse or zebrafish *colXIX* RNA. The *stumpy* phenotype was also partially rescued in mutants by first knocking down zebrafish ColXIX and adding back *colXIX* RNA, suggesting that the mutation is acting as a dominant negative. Together, these results demonstrate a novel function for a FACIT collagen in guiding vertebrate motor axons through intermediate targets.

KEY WORDS: Zebrafish, Motor axons, Axon guidance, Collagen, Intermediate targets

INTRODUCTION
Proper neuromuscular connections are essential for establishing a functional motor system in vertebrates. For these connections to be formed, axons must pathfind to specific targets that may be relatively far away. It has been observed in numerous model organisms that axons consistently take stereotypical pathways to their targets and rarely make mistakes. This is due to growth cones at the tip of the axons that are able to navigate using cues present in the environment. This pathfinding is made simpler by their pathways being broken down into shorter segments that are delineated by endpoints known as intermediate targets or choice points (reviewed in Cook et al., 1998). At intermediate targets, growth cones are observed to pause and make extensive contacts with other cells, presumably coordinating cues that convey information and determine directional growth (O'Connor, 1999). The growth cone then leaves the intermediate target and progresses to the next intermediate target or to its final target. Intermediate targets may be defined by a specific group of specialized cells such as guidepost cells in the developing grasshopper limb bud (reviewed in O'Connor, 1999). Another well-characterized intermediate target is the floor plate or midline glial cells, which are crucial intermediate targets for commissural axons in vertebrates and invertebrates, respectively. Molecules with attractive, repulsive or adhesive properties function at this intermediate target to guide axons to this region and to enable them to then leave this region so that they can extend to their final targets (summarized in Kaprielian et al., 2001). By comparison, molecules that act at motor axon intermediate targets have not yet been identified.

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Zebrafish has proven to be an effective model organism for addressing issues in vertebrate axon pathfinding (reviewed in Beattie, 2000; Beattie et al., 2002) and several mutagenesis screens have been performed to uncover cues involved in early axon guidance (Beattie et al., 1999; Beattie 2000; Hutson and Chien 2002; Schneider and Granato, 2003). The axial primary motor axons in particular have been useful in identifying guidance cues owing to their visibility and accessibility. In each trunk hemisegment of the developing zebrafish embryo, there are three primary motoneurons that innervate the axial muscle between 18 and 24 hours post fertilization (hpf). These three motoneurons, CaP, MiP and RoP for caudal, middle and rostral primary motoneuron, send axons ventrally out of the spinal cord following the 'common path' to the first intermediate target: the horizontal myoseptum (Eisen et al., 1986). Each axon then takes a distinct pathway to its respective targets, pausing, branching or turning at places that are consistent with intermediate targets. Several axon guidance cues have been identified through mutational analysis that guide these primary motor axons, namely *diwanka/LH3* (*plod3* – Zebrafish Information Network) *unplugged/musk*, *topped* and *stumpy* (Zeller and Granato, 1999; Schneider and Granato, 2006; Beattie et al., 2000; Zhang and Granato, 2000; Rodino-Klapac and Beattie, 2004). While *diwanka* and *unplugged* function to guide axons along the common pathway and *topped* functions to guide CaP into the ventral muscle, *stumpy* appears to function at intermediate targets.

The *stumpy* mutant was isolated in a screen designed to elucidate molecules that guide primary motor axons and was identified by its dramatic short axon phenotype during the first day of development (Beattie et al., 2000). Phenotypic characterization revealed that CaP, and all other axial motor axons, stalled at their intermediate targets in *stumpy* mutants (Beattie et al., 2000). Moreover, the mutants also exhibited a neuromuscular junction (NMJ) defect (Panzer et al., 2005). These data indicated that Stumpy functions to enable motor axon growth cones to navigate past intermediate

targets in vivo, and that this directly or indirectly affects NMJ formation. Genetically, the *stumpy*^{b393} allele is homozygous viable and the heterozygotes exhibit a mild phenotype, suggesting that the mutation acts as a partial dominant (Beattie et al., 2000).

Here, we report the positional cloning of *stumpy* and show that it encodes a member of the fibril-associated collagens with interrupted triple helix (FACIT) family of collagens, CollagenXIXa1 (ColXIX). Taken together, our data reveal a novel role for ColXIX in motor axon outgrowth and neuromuscular development.

MATERIALS AND METHODS

Fish care and maintenance

AB* embryos, ABLF embryos and LF embryos were used for morpholino and RNA injections, and were maintained between 25.5 and 28.5°C. Embryos were staged by converting the number of somites to hours post-fertilization (hpf) (Kimmel et al., 1995). *stumpy*^{b393} homozygous mutants were generated by natural mating of homozygous mutant adults.

Genetic mapping and cloning of zebrafish ColXIX

A map cross was generated between *stumpy*^{b393} mutants on the AB* background and wild-type WIK. Haploids were generated by in vitro fertilization of eggs from heterozygous females with UV inactivated sperm (Streisinger et al., 1981) and the *stumpy* phenotype determined by znp1 antibody labeling (Beattie et al., 2000). Microsatellite Z-marker primers (http://zfin.org/cgi-bin/mapper_select.cgi) were used to place *stumpy* on chromosome 13. Those that did not show polymorphisms using agarose gels were then analyzed by single-strand conformational polymorphisms (SSCP) (Sentinelli et al., 2000). For fine mapping, a panel of 2542 haploid embryos was generated and more z-markers screened until two more closely linked markers AI476945 (fb55g08) (0.27 cM) and Fc22c08 (0.08 cM) were identified that flanked *stumpy*. Using these markers, BAC pools were screened (Danio Key 735, ImaGenes). AI476945 mapped to BX3241887 and Fc22c08 mapped to BX322620. By mapping BAC ends and using overlapping BACS, *stumpy* was placed between 8 and 90 kb on BX322620.

The 5′ end of the zebrafish mRNA was obtained by a tBLASTn search using the first 268 amino acids sequence of mouse ColXIX. Two Ab initio predicted RNA sequences hmm478284 and hmm476284 were identified. Primers were designed from these sequences and RT-PCR was performed. We were able to obtain exons 1-26 in one piece using the Takara One-Step RNA PCR Kit. To obtain full-length zebrafish *colXIX* mRNA, cDNAs were first generated using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and then Platinum Taq DNA Polymerase High Fidelity for PCR (Invitrogen, Carlsbad, CA, USA) was used to amplify *colXIX*. The PCR product was then cloned into PCR8GW TOPO-TA vector (Invitrogen, Carlsbad, CA, USA) and a correctly oriented 3.5 kb piece was cloned using LR clonase (Invitrogen, Carlsbad, CA, USA) into a PCSeGFP-DEST vector (generously provided by the Lawson Lab) to generate the 9299 bp PCSeGFP-DEST-zf colXIX fl vector generating a 3521 bp full-length zebrafish *colXIX* cDNA.

RNA in situ hybridization

Whole-mount RNA in situ hybridization was performed in wild-type and *stumpy*^{b393}, as described by Thisse et al. (Thisse et al., 1993). The probe used covers exons 1-26 of the *colXIX* cDNA, which was TA cloned into a PCRII plasmid using the Topo TA cloning Kit with PCRII vector (Invitrogen, Carlsbad, CA, USA). An antisense digoxigenin zebrafish *colXIX* riboprobe was synthesized from a plasmid linearized with *Bam*HI and transcribed with T7 RNA polymerase (Roche, Indianapolis, IN, USA). The control sense probe was synthesized from the same plasmid linearized with *Not*I and transcribed with SP6 RNA Polymerase (Roche, Indianapolis, IN, USA).

Morpholino analysis

A splice-blocking morpholino was designed to exons 17 and 18 (Gene tools, *colXIX MO*: GGCAAACCCTGCAAGCCAAAGGAG). Two doses, 9 and 4.5 ng, were injected into wild-type embryos at the one- to two-cell stage. A translation blocking MO was also designed (Gene tools, *colXIX* ATG MO2: TGCGGAGAAAGTTTATTATTCCAGC) and injected at 4 ng/embryo.

cDNA constructs, RNA synthesis and RNA injections

Mouse *ColXIX* cDNA clones were obtained from Imagenes (BC118970). The cDNA was cut from PCR-Blunt II-TOPO using *Xba*I and *Pvu*II, and ligated into the PCS2 vector, which was cut with *Eco*RI and blunted. The mutated RNA constructs were generated from this using Stratagene Site-Directed Mutagenesis kit. The following primers were used: KRsense, 5′-TCCTGGTCCCACTGGAGCAAGAGGTGACAAGGGTAGTGAGGG-3′ and KRantisense, 5′-CCCTCACTACCCTTGTCACCTCTTGC-TCCAGTGGGACCAGGA-3′ to generate the K791R mutation; sdm1, 5′-CCCTCACTACCCTTGTCACCTCTTGCTCCAGTGGGACCAGGA-3′ and sdm2, 5′-TCCTGGTCCCACTGGAGCAAGAGGTGACAAGGGT-AGTGAGGG-3′ to confirm the sequence; Rstopsense, 5′-CCAGGGGAGCAGGGTGAATGAGGACCTATTGGAGATACAG-3′ and Rstopantisense, 5′-CTGTATCTCCAATAGGTCCTCATTCACCCT-GCTCCCCTGGT-3′ to generate the R901stop mutation; and to confirm the sequence: sdmstop1, 5′-AATCCAGGGAGGGGTGAAT-3′; and sdmstop2, 5′-GAGGGCCTCTGTCTCCTG-3′.

The full-length zebrafish *colXIX* cDNA was cloned into a PCSDest-eGFP vector (kindly provided by Dr Nathan Lawson, University of Massachusetts Medical School) and capped poly-A mRNA generated mMessage mMachine (Ambion, Austin, TX). RNA doses are given for each experiment in the text. For RNA rescue experiments, the *colXIX* MO and the RNA were mixed before injecting. To ensure that this was not introducing any experimental error, we also performed experiments where we first injected the MO then injected half of the MO injected embryos with RNA (sequential injections). Both of these methods showed the same result. For example, we found that there was no statistically significant difference between sequential injections (58±2% long axons) and the mixed injections (63±5% long axons) P-value=0.0653 for rescuing the *colXIX* MO phenotype with mouse *ColXIX* RNA.

Whole-mount antibody labeling

Whole-mount antibody labeling was performed as described previously (Eisen et al., 1986; Beattie et al., 2000). The znp1 monoclonal antibody that recognizes primary and secondary motor axons (Trevarrow et al., 1990; Melancon et al., 1997) was detected using the Sternberger Clonal-PAP system with diaminobenzidine (DAB) as a substrate (Beattie and Eisen, 1997) or with the Goat-anti-mouse Oregon Green secondary antibody (Invitrogen). Znp1 recognizes synaptotagmin II (Fox and Sanes, 2007). Embryos were analyzed with a Zeiss axioplan microscope (Thornwood, NY, USA). CaP axons in segments 5-15 on both sides of the embryo were analyzed. The heparan sulfate proteoglycan antibody (10E4 epitope) was obtained from US Biological and used at 1:100. Anti-Laminin 1 (L 9393) obtained from Sigma (St Louis, MO, USA) was used at 1:100. Anti-chondroitin sulphate was (CS56) was obtained from Sigma (St Louis, MO, USA) and used at 1:100.

RESULTS

stumpy maps to the zebrafish homolog of CollagenXIXa1

To positionally clone the *stumpy* mutation, we generated a map cross between *stumpy*$^{-/-}$ (on the AB* line) and WIK. All mapping was carried out on haploid DNA obtained from *stumpy* heterozygous embryos from the map cross. A haploid panel of 2542 embryos was collected, the *stumpy* phenotype was determined by znp1 antibody labeling as previously described (Beattie et al., 2000), and DNA was extracted from each individual embryo. We determined that the two closest z markers flanking the mutation were z25253 (2.1 cM) and z44520 (19.3 cM) on chromosome 13. Using a combination of the meiotic map from the Talbot laboratory (Woods et al., 2005) and the Vega site on the Sanger Zebrafish sequencing project (http://vega.sanger.ac.uk/Danio_rerio/index.html), numerous markers were tested until we found two more closely linked markers

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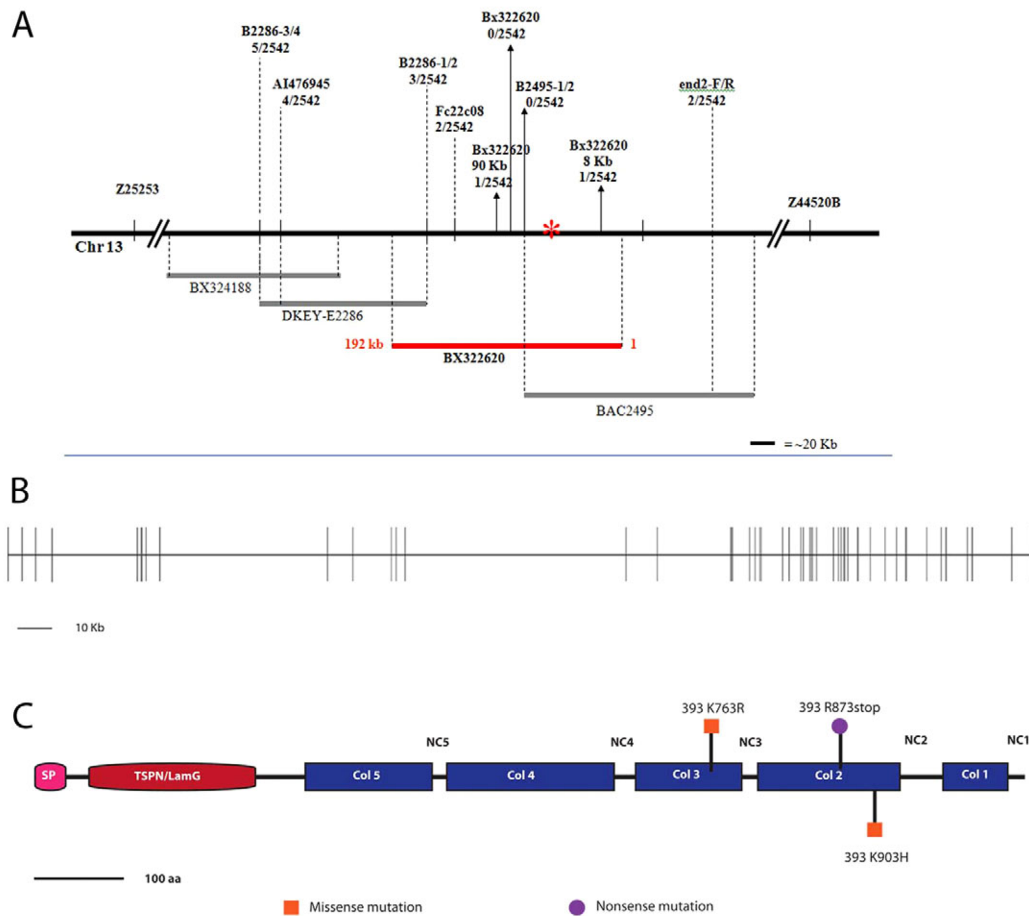

Fig. 1. Positional cloning of the *stumpy* gene. (**A**) The *stumpy*^{b393} mutation was mapped to chromosome 13 (Chr 13) and further mapped to BAC BX322620. The solid black line represents the relevant genomic region on Chr 13; lines beneath indicate individual BACs that were mapped to this region with the *stumpy* BAC in red; primer names and number of recombinants are listed above the line. The asterisk denotes the location of the *stumpy* gene coincident with the location of ColXIX. (**B**) Intron-exon structure of the ColXIX gene. The gene consists of 50 exons and spans ~300 kb. (**C**) ColXIX protein structure with *stumpy*^{b393} mutations indicated. ColXIX is composed of an N terminus head consisting of a signal peptide and a LamG/TSPN domain (red). The C-terminal tail has five collagenous domains (Col1-Col5) ranging from 72 amino acids to 186 amino acids in length interrupted by five non-collagenous domains (NC1-NC5) ranging from 18 amino acids to 158 amino acids in length.

AI476945 (4/1466, 0.27 cM) and Fc22c08 (2/2542, 0.08 cM) that flanked the *stumpy* mutation (Fig. 1A). By screening BAC pools (Danio Key BAC library 735, ImaGenes), we mapped AI476945 (fb55g08) on BX324188 and Fc22c08 on BX322620. By SSCP mapping of BAC ends and using overlapping BACs, *stumpy* was placed between 8 and 90 kb on BX322620.

To identify genes on this BAC region we used Genscan (http://genes.mit.edu/GENSCAN.html), which identified six potential open reading frames with one corresponding to exons 14-49 of zebrafish *colXIX*. RT-PCR with 3′ RACE was carried out to obtain the rest of the exons until a stop site was identified. The 5′end containing the start site was obtained using tBLASTn to find sequences in zebrafish chromosome 13 that were similar to the N terminus of mouse *ColXIX* (*Col19a1* – Mouse Genome Informatics). Primers were then designed to obtain these regions from cDNA. The entire zebrafish *colXIX* gene spans over 300 kb of chromosome 13 (Fig. 1B). By RT-PCR, we were able to sequence a 3521bp *colXIX* cDNA. Conceptual translation of this cDNA sequence yields a 1102 amino acid protein with a signal peptide sequence, a 185 amino acid N terminus non-collagenous domain with similarities to the Laminin G/Thrombospondin N domain and five collagenous (Col) domains of varying lengths separated by short non-collagenous (NC) domains (Fig. 1C). The amino acid sequence has 45 and 44% overall sequence identity with human and mouse ColXIX, respectively (see Fig. S1 in the supplementary material). There is especially high identity in the NC2-Col1-NC1 region at the C-terminal end of the protein with 62 and 61% identity in this region with human and mouse ColXIX, respectively.

colXIX is expressed at intermediate targets during pathfinding of primary motor axons

The horizontal myoseptum has long been considered an intermediate target for zebrafish motor axons as CaP growth cones pause at this location and all motor axons diverge here to extend into unique myotome regions (Eisen et al., 1986; Melancon et al., 1997). In *stumpy* mutants, all primary motor axons stall at this location (Beattie et al., 2000). In addition, *stumpy* CaP motor axons also stall at the ventral edge of the notochord, a position where wild-type CaP growth cones normally pause and/or form a small branch (Eisen et al., 1986). Stumpy CaP and MiP growth cones also stall at the dorsal- and ventral-most regions of the myotome,

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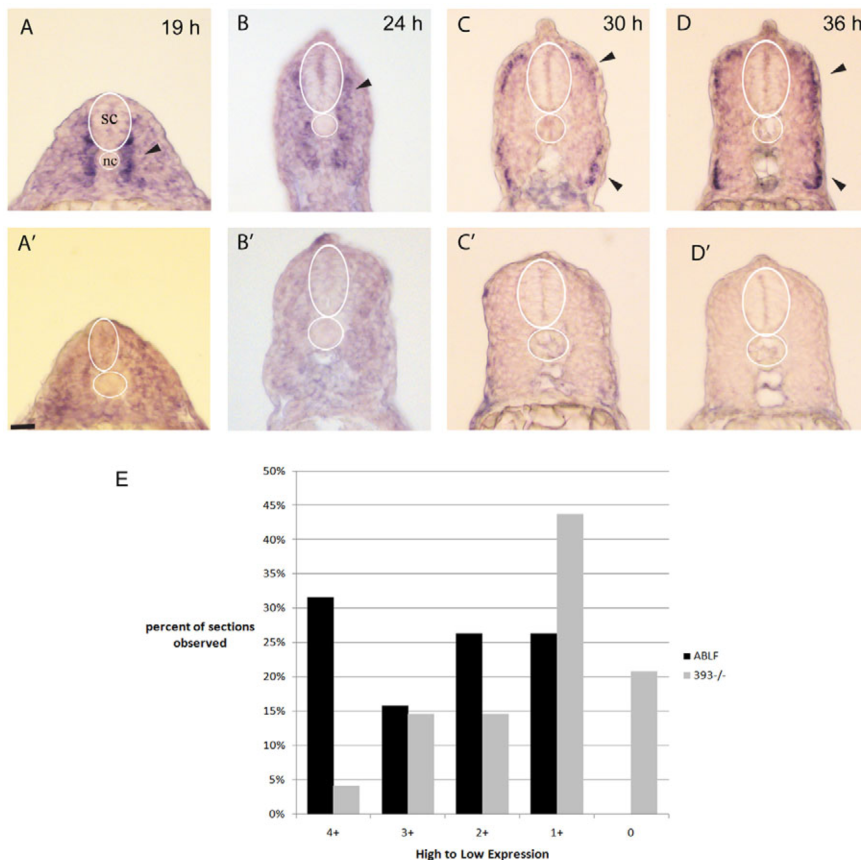

Fig. 2. *colXIX* RNA is dynamically expressed during motor axon outgrowth. (**A-D′**) RNA in situ hybridization using *colXIX* anti-sense (A-D) and sense (A′-D′) riboprobes that encompasses exons 1-27 of the zebrafish *colXIX* cDNA at (A,A′) 19 hpf, (B,B′) 24 hpf, (C,C′) 30 hpf and (D,D′) 36 hpf. Sections were from the mid-trunk region. (**E**) Histogram of *colXIX* RNA in situ hybridization intensity at 19 hpf for wild-type (*n*=8 embryos; ~40 sections) and *stumpy* mutant embryos (*n*=8 embryos; ~40 sections). The *x*-axis reflects the intensity of the *colXIX* expression. Spinal cord (sc) and notochord (nc) are indicated by white borders. Arrows show localization of *colXIX* transcripts in the myotome. Scale bar: 20 μm.

respectively, where they normally branch and turn, suggesting that these are also intermediate targets. If ColXIX protein were required for motor axon navigation at intermediate targets, we would expect expression of *colXIX* at these locations during the period of axon outgrowth. To test this, we generated two RNA in situ probes against *colXIX* transcripts, a N-terminal probe encompassing exons 1-26 and a C-terminal probe covering exons 17-21. The probe that covered the N terminus, a region of the gene with less homology to other collagens, gave a much clearer and specific signal, and was used to analyze expression in wild-type embryos. Focusing on the mid-trunk region, primary motor axons start to grow out of the spinal cord at ~18 hpf and most have reached their muscle targets by 24-26 hpf (Eisen et al., 1986). Secondary motoneurons start migrating to their muscle targets at ~26 hpf (Myers et al., 1986). If a gene is important for growth cone migration to their targets, it should be expressed at these time points. Examination of cross-sections from 19 hpf embryos revealed robust *colXIX* expression in the ventro-medial region of the myotome immediately adjacent to the notochord. This location corresponds to the horizontal myoseptum, the first intermediate target for all three primary motor axons (Fig. 2A,A′). By 24 hpf *colXIX* expression at the first intermediate target is reduced and expression is now observed in cells at dorsolateral and ventrolateral regions of the muscle (Fig. 2B,B′). The dorsolateral region of the dorsal muscle is where the MiP axon is observed to turn ventrally along the edge of the dorsal muscle and where MiP stalls in *stumpy* mutants. Expression then expands to lateral cells of the dorsal and ventral myotome towards the horizontal axis by 30-36 hpf (Fig. 2C,C′,D,D′); again, coincident with the location of the growing axons. These findings suggest that *colXIX* is expressed at the right place and time to influence motor axon growth cones as they navigate intermediate targets on their way to their final targets. We next investigated whether the *colXIX* transcript was altered in *stumpy*^{b393} mutants. We performed RNA in situ hybridization on 19 hpf *stumpy*$^{-/-}$ embryos and found that *colXIX* expression was decreased, but not absent, in *stumpy* mutants (Fig. 2E). This suggests that the mutant ColXIX is likely to be present in *stumpy* mutants.

Morpholino knockdown of ColXIX phenocopies stumpy

If Stumpy were indeed ColXIX, then we would expect that disrupting ColXIX would produce the same phenotype as *stumpy* mutants. Morpholinos are an effective way of knocking down gene expression in zebrafish (Nasevicius et al., 2000). We designed two morpholinos against zebrafish *colXIX*: a splice-site morpholino against the splice site junction of exons 17 and 18; and a translation blocking morpholino. The efficacy of the splice-site morpholino was confirmed by RT-PCR using exon-specific primers designed for exons 14 to 17 (Fig. 3A, MO). An incorrectly spliced band indicative of inclusion of the 109 bp intron was observed in morphants and not in wild-type uninjected embryos, which caused the remainder of the gene to be translated out of frame (Fig. 3A, WT). Injection of *colXIX* MO (4.5 ng) resulted in Stumpy-like CaP axon defects in 84.8±2% of axons examined (Fig. 4). The exact same Stumpy-like phenotype was observed with a translation-blocking (TB) MO (4 ng, 9 ng); however, owing to the lack of a zebrafish ColXIX antibody, the efficacy of the *colXIX* TB MO could not be confirmed. We therefore used the splice site MO for the rest of the experiments unless otherwise specified.

To ensure that the phenotype observed was specifically caused by ColXIX knockdown, we examined whether the *colXIX* RNA could rescue the morphant phenotype. For these experiments, we

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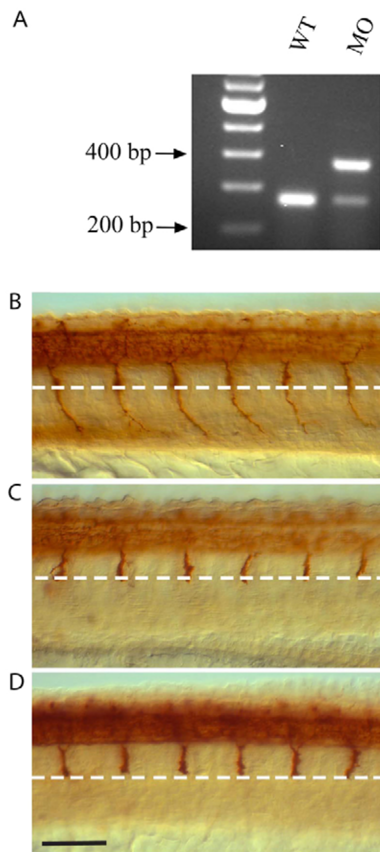

Fig. 3. ColXIX knock down phenocopies the *stumpy* mutation. (**A**) RT-PCR shows inclusion of a 109 bp fragment in splice-blocking *colXIX* MO injected (MO) compared to wild-type (wt) embryos. (**B-D**) CaP axon phenotypes as visualized using znp-1 antibody in (B) uninjected wild-type embryos, (C) wild-type embryos injected with 9 ng *colXIX* MO and (D) *stumpy*$^{b393-/-}$ mutant embryos. White dashed line indicates the horizontal myoseptum. Scale bar: 70 μm.

generated *ColXIX* RNA from full-length mouse *ColXIX* cDNA obtained from Imagenes. We co-injected *colXIX* MO (4.5 ng) with full-length mouse *ColXIX* RNA (250 pg) at the one-cell stage. Only 41.8±2% of observed CaP axons had the Stumpy-like axon phenotype versus the 84.8±2% seen in morphants (P<0.0001, Fig. 4). We subsequently cloned the zebrafish *colXIX* cDNA and generated capped mRNA to use for morphant rescue. We observed a much higher rate of rescue using zebrafish *colXIX* RNA with only 7±1% of CaP axons having the Stumpy-like phenotype (P<0.0001 compared with morphants, Fig. 4). This indicates that mouse ColXIX is functional in zebrafish and both mouse and zebrafish ColXIX significantly rescue the phenotype caused by ColXIX knockdown.

Identifying mutations in ColXIX in stumpy mutants

If *colXIX* were *stumpy*, then we would expect to find DNA changes in the *colXIX* gene in *stumpy* mutants. RT-PCR was performed using total RNA extracted from *stumpy*^{b393} mutant and wild-type 26 hpf embryos. Sequencing the 3.5 kb *colXIX* cDNA revealed three mutations in *stumpy*^{b393}. This relatively high number of mutations was unexpected, but could be due to the large size of the *colXIX* gene. The mutations identified were A2410G (L763R), C2739T (R873stop) and T2826C (L903H)) (Table 1). The A2410G and C2739T mutations were confirmed to not be naturally occurring polymorphism by sequencing the regions containing the mutations from 20 and 17 AB* individuals, respectively, confirming that these base pairs are conserved and the base changes in *stumpy* mutants are not polymorphisms (data not shown). Owing to the R873stop mutation, the mutant protein would lack the last collagen domain, Col1, and the last two non-collagenous domains NC1 and NC2 (see Fig. 1B) as well as a L903H mutation in the collagen domain Col 2. These lysine and arginine residues in zebrafish are both conserved in mouse and human ColXIX (see Fig. S1 in the supplementary material), suggesting that these amino acids are relevant to protein function.

Mutant ColXIX does not rescue *colXIX* morphants

To determine whether the mutations found in *stumpy*^{b393} mutants affected ColXIX function, we generated mouse *ColXIX* cDNA with these base changes and investigated whether the resulting RNA could rescue *colXIX* morphants. The two *stumpy*^{b393} mutated residues K763 and R873 are conserved in mouse and correspond to K792 and R901, so we generated these mutations (K792R and R901stop) both together and separately in mouse *ColXIX* cDNA and examined whether they could rescue the CaP axon defect in *colXIX* morphants. The double-mutant RNA, which is the form found in *stumpy*^{b393} mutants, did not rescue and, in fact, resulted in a worse phenotype than MO alone (P value=6.26×10^{-6}, Fig. 4). By analyzing the two mutations separately, we sought to understand their contribution to the mutant phenotype. Injection of RNA with the K792R mutation alone and *colXIX* MO resulted in a small but statistically significant rescue (P<0.001, Fig. 4) compared with morphants. This rescue, however, was much less robust compared with rescue by wild-type mouse RNA (P<0.001 Fig. 4). Surprisingly, the R901stop mutant RNA (250 pg) was able to rescue to a similar degree to the wild-type full-length protein (P=0.91, Fig. 4). These data suggest that the ColXIX protein containing the *stumpy*^{b393} mutations has lost its ability to function in guiding CaP axons growth cone past the intermediate target and both mutations are needed in combination to cause the *stumpy* phenotype.

Mouse ColXIX mRNA with *stumpy*^{b393} mutations acts in a dominant-negative manner to induce *stumpy* phenotype

The finding that adding ColXIX containing the *stumpy*^{b393} mutations made the morphant phenotype worse, suggests that these mutations make the protein a dominant negative. This is consistent

Table 1. Amino acid mutations found in *stumpy*$^{393-/-}$

Allele	Amino acid position (zebrafish)	Amino acid position (mouse)	Wild type	Mutant	Region
393	763	792	Lys	Arg	Col3
393	873	901	Arg	Stop	Col2
393	903	943	Lys	His	Col2

All three mutations are found in the 393 allele of *stumpy* and all are conserved between human, mouse and zebrafish (see Fig. S1 in the supplementary material). Column 3 indicates the equivalent amino acid position in the mouse *ColXIX* sequence.

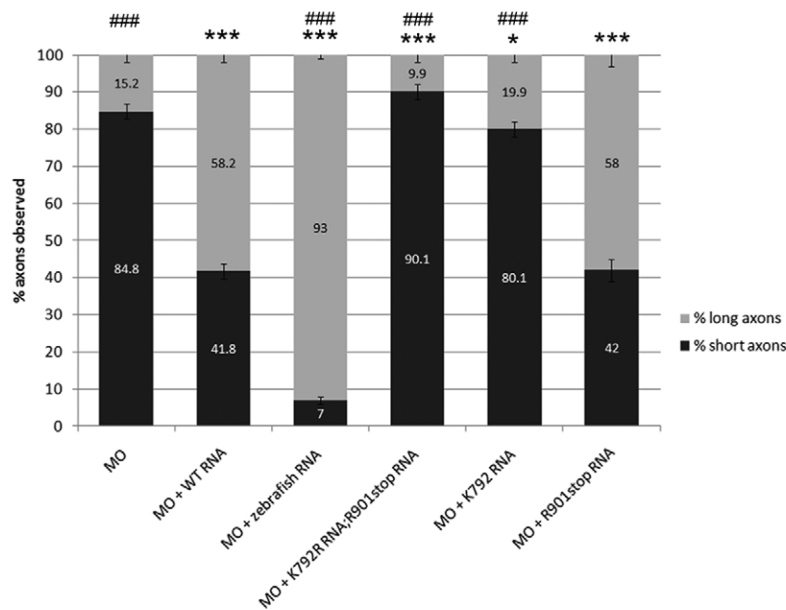

Fig. 4. *ColXIX* rescues the morphant phenotype. Wild-type mouse *ColXIX* RNA (WT RNA, 250 pg), wild-type zebrafish *colXIX* RNA (350 pg) and mouse *ColXIX* RNA (250 pg) double or single mutations were co-injected with 4.5 ng *colXIX* MO. *P*-values were calculated for % short axons observed versus MO-injected with significance indicated by a black asterisk (*), and against MO + WT mouse RNA (#): * or $^{\#}P<0.05$, ** or $^{\#\#}P<0.001$, *** or $^{\#\#\#}P<0.0001$.

with the *stumpy*^{b393} phenotype being partially dominant in that the heterozygotes show a mild phenotype (Beattie et al., 2000). To explore this in more detail, we injected mouse *ColXIX* mRNA with the *stumpy*^{b393} mutations into wild-type embryos. If the mutant protein is indeed acting as a dominant negative, we should observe the Stumpy CaP axon phenotype. We tested the two *stumpy*^{b393} mutations together and separately. We injected 300 pg each of the K792R:R901stop, K792R and R901stop mouse *ColXIX* RNA into wild-type embryos (Table 2). When we injected the mouse K792R:R901stop mutant *ColXIX* RNA, we observed Stumpy-like axons in 46±9% (*n*=123 embryos, Table 3) but in only 4±1% (*n*=2460 axons, Table 3) of CaP axons. Injection of K792R RNA did not cause any motor axon defects; however, embryos injected with the R901stop mouse RNA exhibited the Stumpy-like CaP axon phenotype in 38±8% (*n*=152 embryos, Table 3) of embryos observed but in only 3±1% of axons (*n*=3060 axons, Table 3). These values are less than the double mutant RNA and suggest that the combination of the mutations found in *stumpy*^{b393} causes ColXIX to function in a dominant-negative manner to inhibit extension of growth cones beyond intermediate targets. This finding is consistent with the fact that the *stumpy*^{b393} mutation genetically acts as a partial dominant (Beattie et al., 2000).

Overexpression of mouse ColXIX induces CaP axon defects

Since *colXIX* has a very restricted expression pattern, we next examined whether overexpression/misexpression of wild-type ColXIX by itself could cause a phenotype. When we injected wild-type mouse *ColXIX* RNA (300 pg) into wild-type embryos, we observed short Stumpy-like CaP axons with 2±1% of axons observed being short, with this phenotype observed in 23±8% of the embryos (Table 2). In addition, we also observed branching in these over expressed embryos with 8% of CaP axons branched with this phenotype observed in 50±9% of embryos. Injection of zebrafish *colXIX* RNA (350 pg) into wild-type also induced Stumpy-like phenotypes with 4±1% of axons observed being short; this phenotype was seen in 43±15% of embryos scored (*n*=44, Table 3). However, only 1±1% of motor axons were branched in 18±11% of the embryos. As the most consistent defect seen with over expression of zebrafish or mouse *colXIX* is the short CaP axons, it suggest that the levels of ColXIX are crucial and overexpression can cause a similar defect to lower expression and/or expression of a mutant form of ColXIX.

Mouse and zebrafish ColXIX full-length RNA rescues *stumpy*^{b393} only with knockdown of mutant ColXIX

As we have determined that mouse ColXIX is functional in zebrafish, we next tested whether it could rescue the CaP axon phenotype in *stumpy* mutants. We injected increasing doses (250, 500 and 1000 pg) of mouse *colXIX* mRNA into *stumpy*^{b393} mutants and were unable to observe any rescue (data not shown). Because we had previously shown that the mutant protein acts as a dominant negative, we reasoned that perhaps just adding back wild-type ColXIX would not rescue the phenotype because of the presence of the mutant protein. We therefore co-injected *stumpy*^{b393} mutants with *colXIX* MO to knock down the mutant protein and simultaneously added back mouse *ColXIX* RNA. We did not observe any rescue with the splice-blocking MO co-injection (data not shown). This could be due to the possible maternal loading of pre-spliced *colXIX* mRNA, which would be impervious to the effect of the splice-blocking MO. However, with the translation-

Table 2. Overexpression of mutant *ColXIX* mouse RNA

Injection	Percentage of fish with short axons	Total fish	% axons affected	Total axons
K792R; R901stop RNA	46±9	123	4±1	2460
K792R RNA	0	150	0	3000
R901stop RNA	38±8	152	3±1	3060

Mutant RNA (250 pg) was injected into one- to two-cell stage embryos and motor axons were analyzed at 26 hpf.

Table 3. Overexpression of wild-type *colXIX*

Injected	Percentage of short axons	Percentage of embryos with short axons	Percentage of branched axons	Percentage of embryos with branched axons	Number of axons	Number of fish
Mouse *ColXIX* RNA	2±1	23±8	8±1	50±9	2220	111
Zebrafish *colXIX* RNA	4±1	43±15	1±1	18±11	880	44
Wild-type uninjected	0	0	0	0	1640	89

Mouse (300 pg) and zebrafish (350 pg) *colXIX* RNA were injected into one- to two-cell stage wild-type embryos and motor axons were analyzed at 26 hpf.

blocking *colXIX* MO (4.5 ng) in combination with 1000 pg wild-type mouse *ColXIX* mRNA, we observed CaP axon rescue in *stumpy*^{b393} mutants (Fig. 6). We observed rescued CaP axons, defined as those that progressed beyond the horizontal myoseptum, in 78±11% of the co-injected mutant embryos (n=55 embryos) with 12±2% (n=1100 axons) of CaP axons scored as rescued. In uninjected *stumpy*^{b393} mutants, we observed no axons extending beyond the horizontal myoseptum (n=600 axons); thus, this rescue was highly significant. Because this rescue was not observed when using a lower dose of mouse *ColXIX* mRNA and 1000 pg is a high dose of mouse RNA, we asked whether the zebrafish *colXIX* RNA could rescue at a lower dose. We co-injected 350 pg of zebrafish *colXIX* RNA with 4.5 pg *colXIX* ATG MO2 into *stumpy*^{b393} mutants. We found that 48±11% of embryos had some rescued CaP axons (n=79 embryos), with 4±1% of observed CaP axons rescued (n=1580 axons). These results indicate that the *stumpy* gene is indeed *colXIX* and that the mutant zebrafish ColXIX protein is acting as a dominant negative. These data also indicate that zebrafish *colXIX* RNA is more efficient than mouse *colXIX* at rescuing both the mutant and morpholino phenotypes.

DISCUSSION

The collagens are a family of extracellular matrix molecules whose role as structural proteins has been well studied. Collagens are characterized by Gly-X-Y repeats and form triple helices comprising homo- or heterogeneous polypeptide alpha (α) chains. At least 27 types of collagens have been identified in vertebrates, with 42 distinct α chains (Myllyharju and Kivirikko, 2004). Fibril-forming collagens are known mainly to provide structural support that maintains tissue integrity (reviewed in Kadler et al., 2007). Non-fibril-forming collagens, however, appear to have various non-structural functions. Their roles in the developing vertebrate nervous system have only recently been appreciated (reviewed in Fox, 2008). In studying the *stumpy* mutant, we have revealed a novel role for a FACIT collagen, ColXIX, in the formation of the neuromuscular network during early development.

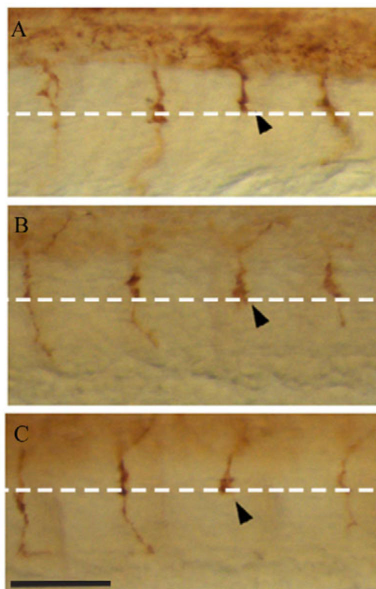

Fig. 5. Mutant ColXIX acts as a dominant negative. (**A-C**) Examples of CaP axon defects observed in embryos injected with mutant *ColXIX* RNA. White dashed line indicates the horizontal myoseptum. Arrowhead denotes Stumpy-like CaP axons.

Collagen XIX during development

ColXIX remains a poorly characterized collagen, especially in terms of its function. Moreover, it has limited homology to other FACITs, which makes it difficult to extrapolate any functional roles. Expression studies during early development in mice show that *ColXIX* transcripts are restricted in the developing muscle at embryonic day 11.5 and decrease by embryonic day 16.5

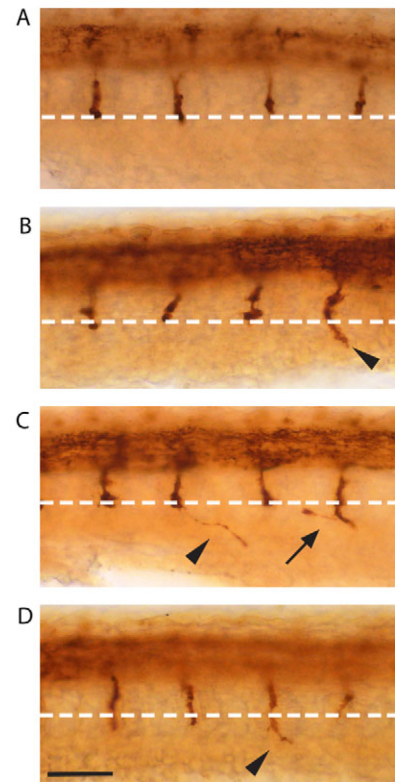

Fig. 6. Rescue of *stumpy*$^{b393-/-}$ mutants with mouse *ColXIX* RNA. (**A**) *stumpy*$^{b393-/-}$ embryo. (**B-D**) Representative images of *stumpy*$^{b393-/-}$ embryos co-injected with translation blocking *colXIX* MO and full-length mouse *ColXIX* RNA. Arrowheads indicate rescued CaP axons. An abnormal axon is indicated by the arrow. White dashed line indicates the horizontal myoseptum. Scale bar: 70 μm.

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(Sumiyoshi et al., 2001). Expression was also observed in smooth muscle cells in the stomach and around the jaw (Sumiyoshi et al., 2001). ColXIX-null mice are normal at birth but, ~95% of pups die within the first 3 weeks, presumably caused by their inability to feed. Defects in muscle development in the lower esophageal sphincter probably caused this phenotype and demonstrated a role for ColXIX in the development of skeletal muscle transdifferentiation in the mouse esophagus (Sumiyoshi et al., 2004). A secondary phenotype has also been observed in hippocampal neurons in mice lacking ColXIX. Neuronal morphology is normal; however, some subtypes of hippocampal synapses were malformed, demonstrating a role for ColXIX in the nervous system (Su et al., 2010). These mouse studies suggest that ColXIX may be playing multiple functions during development.

Two other collagens have been found to function in axon guidance in the developing zebrafish. Knock down of ColXVIII results in CaP axon stalling soon after exiting the spinal cord, a phenotype similar to the *diwanka* mutant that has a mutation in the gene for the multifunctional enzyme LH3 (Schneider and Granato, 2006). This indicates that proper glycosyltransferase modification of ColXVIII is required for primary motor neuron growth cones to pioneer into the periphery (Schneider and Granato, 2006). A type IV collagen, Col4a5, has been shown to play a role in proper retinal ganglia cell axon targeting to the correct lamina of the tectal neuropil (Xiao and Baier, 2007). Col4a5 functions by anchoring heparan sulphate proteoglycans (HSPGs) and possibly other secreted factors onto the basement membrane, which then guide axons to their proper targets (Xiao and Baier, 2007). These data indicate that collagens can function with other ECM molecules to affect axon guidance.

Mutant ColXIX

The finding that both knock down of wild-type ColXIX and overexpression of the mutant cause a similar defect, is consistent with this mutant form of ColXIX acting as a dominant negative. This was confirmed by our inability to rescue the mutant phenotype by adding wild-type ColXIX. For any rescue to be observed, we first had to knock-down mutant Stumpy with a translation blocking MO, while adding back wild-type *ColXIX* RNA at the same time. Thus, the mutant ColXIX containing both the K792R and R901stop mutations is not only non-functional, as demonstrated by it inability to rescue *colXIX* morphants, but also acts to make any wild-type ColXIX non-functional. The *stumpy*^{b393} mutation acts partially dominant and the finding that mutant Stumpy acts as a dominant negative could cause this phenotype. However, we also show that *colXIX* expression is decreased in *stumpy*^{b393} mutants (Fig. 2E) and that knocking down ColXIX also causes the Stumpy CaP axon phenotype. Therefore, it is possible that haploinsufficiency also contributes to the partially dominant phenotype.

The double mutant ColXIX found in *stumpy*^{b393} mutants lacks the last two non-collagenous domains NC1 and NC2 thought to be crucial for chain selection and initiation of triple helix formation of ColXIX chains (Boudko et al., 2008). Our examination of the single mutant R901stop, which lacks NC1 and NC2, showed that this form acted as a dominant negative but could also rescue *colXIX* morphants. One explanation for these seemingly conflicting results is that in the presence of wild-type ColXIX, the truncated form causes a dominant-negative effect. However, when the wild-type form is knocked down, the truncated form can function at intermediate targets. When this mutant form is present along with the point mutation (k792R; R901stop) it acts as a stronger dominant negative and also fails to rescue *colXIX* morphants. These data indicate that the levels and constitution of the ColXIX complexes are crucial for their function at intermediate targets.

ColXIX at intermediate targets

The finding that *colXIX* is expressed at intermediate targets lends insight into ColXIX function. We had previously hypothesized that Stumpy could be acting as an attractant to facilitate axons to grow past their intermediate targets to their final, more distal targets (Beattie et al., 2000). However, *colXIX* transcripts are expressed specifically at identified motor axon intermediate targets in a temporally regulated manner, as shown by the dynamic expression pattern between 19 and 36 hpf. Signal was detectable at the horizontal myoseptum, an intermediate target for all three primary motor axons, the dorso-lateral edge of the dorsal muscle, which is an intermediate target for MiP axons, and the ventrolateral edge of the ventral muscle, which is an intermediate target for CaP (Myers et al., 1986; Beattie et al., 2000). As *colXIX* is expressed at intermediate targets and this expression pattern changes during development as motor axon extend along the myotome, it supports an alternative hypothesis that ColXIX is needed at intermediate targets for motor axons to proceed past these regions. It also appears that the levels and or localized expression of ColXIX is also important because over expression of wild-type *colXIX* also resulted in the Stumpy CaP phenotype. Thus, both genetics and gene expression indicate that ColXIX plays an important role at intermediate targets.

From what we know of its domains, we can make some inferences on the mechanism of ColXIX function at intermediate targets. Via its NC1 domain, ColXIX could be involved in interactions with fibrillar collagens that may be important for anchoring ColXIX to basement membranes at intermediate targets. The LamG/TSPN makes it possible that ColXIX localized to the intermediate targets could anchor HSPGs or other heparin-containing cues to the intermediate target that would then signal the growth cone to proceed past this region. Alternatively, the LamG/TSPN domain of ColXIX could be recognized directly by receptors expressed by the growth cone. Integrins are known to recognize both laminins and thrombospondin domains (DeFreitas et al., 1995; Hughes, 2001). In fact, Laminin-α1 has previously been shown to function in guidance of multiple axons in zebrafish (Paulus and Halloran, 2006).

One possibility for how ColXIX is functioning at intermediate targets is by anchoring cues needed for motor axons to move past their intermediate targets. To address this, we asked whether *stumpy* mutants exhibited changes in the expression of ECM components. We analyzed expression of chondroitin sulphate proteoglycans (CSPG), heparan sulphate proteoglycan (HSPG) and laminin. Compared with wild-type embryos, we did not observe any obvious differences in expression or levels of expression using any of these antibodies (data not shown). In another study, Schweitzer et al. (Schweitzer et al., 2005) also looked at expression of Tenascin-C in *stumpy* mutants and found no difference in expression level and pattern compared with wild types. Once specific cues are identified that guide motor axons, it will be important to test whether their expression is altered in *stumpy* mutants.

Cell-autonomy of ColXIX function

We had previously tested the cell autonomy of Stumpy function by single-motoneuron transplants and concluded that Stumpy function was needed both in motoneurons and in the environment (Beattie

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et al., 2000). However, this result was not unequivocal because when *stumpy*$^{b393-/-}$ CaP motoneurons were transplanted into wild-type hosts, 67% (6/9) were short and 33% had a wild-type phenotype (Beattie et al., 2000). This is in contrast to when we transplanted wild-type CaPs into *stumpy*$^{b393-/-}$ hosts, where 6/7 (89%) of motor axons were short. As shown above, *colXIX* is expressed in the myotome, which supports the non-cell autonomous role for ColXIX. We did not, however, see *colXIX* expression in the spinal cord. This may be due to the limitations of RNA in situ hybridization and/or transient low levels of expression. Therefore, at this time we cannot definitively say that ColXIX is not functioning in motoneurons but our data are more consistent with ColXIX functioning non-cell autonomously with respect to motoneurons.

Identification of the *stumpy* gene as ColXIX has identified a novel role for a FACIT collagen in navigation of intermediate targets by motor axons in early development. Our data suggest that the levels of ColXIX at intermediate targets are crucial for normal motor axon outgrowth. Low levels, high levels and the presence of an aberrant form of ColXIX all cause motor axon stalling. This study further demonstrates that collagens are not merely structural molecules that lend stability to tissues and structures as was once thought, but that these molecules are also playing active roles in cellular processes, such as wiring the nervous system.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.051730/-/DC1

References

Beattie, C. E. (2000). Control of motor axon guidance in the zebrafish embryo. *Brain Res. Bull.* **53**, 489-500.
Beattie, C. E. and Eisen, J. S. (1997). Notochord alters the permissiveness of myotome for pathfinding by an identified motoneuron in embryonic zebrafish. *Development* **124**, 713-720.
Beattie, C. E., Raible, D. W., Henion, P. D. and Eisen, J. S. (1999). Early pressure screens. *Methods Cell Biol.* **60**, 71-86.
Beattie, C. E., Melancon, E. and Eisen, J. S. (2000). Mutations in the stumpy gene reveal intermediate targets for zebrafish motor axons. *Development* **127**, 2653-2662.
Beattie, C. E., Granato, M. and Kuwada, J. Y. (2002). Cellular, genetic, and molecular mechanisms of axonal guidance in the zebrafish. *Results Probl. Cell Differ.* **40**, 252-269.
Boudko, S. P., Engel, J. and Bachinger, H. P. (2008). Trimerization and triple helix stabilization of the collagen XIX NC2 domain. *J. Biol. Chem.* **283**, 34345-34351.
Cook, G., Tannahill, D. and Keynes, R. (1998). Axon guidance to and from choice points. *Curr. Opin. Neurobiol.* **8**, 64-72.
DeFreitas, M. F., Yoshida, C. K., Frazier, W. A., Mendrick, D. L., Kypta, R. M. and Reichardt, L. F. (1995). Identification of integrin alpha 3 beta 1 as a neuronal thrombospondin receptor mediating neurite outgrowth. *Neuron* **15**, 333-343.
Eisen, J. S., Myers, P. Z. and Westerfield, M. (1986). Pathway selection by growth cones of identified motoneurones in live zebrafish embryos. *Nature* **320**, 269-271.
Fox, M. A. (2008). Novel roles for collagens in wiring the vertebrate nervous system. *Curr. Opin. Cell Biol.* **20**, 508-513.
Fox, M. A. and Sanes, J. R. (2007). Synaptotagmin I and II are present in distinct subsets of central synapses. *J. Comp. Neurol.* **503**, 280-296.
Hughes, A. L. (2001). Evolution of the integrin α and β protein families. *J. Mol. Evol.* **52**, 0063-0072.
Hutson, L. D. and Chien, C. B. (2002). Wiring the zebrafish: axon guidance and synaptogenesis. *Curr. Opin. Neurobiol.* **12**, 87-92.
Kadler, K. E., Baldock, C., Bella, J. and Boot-Handford, R. P. (2007). Collagens at a glance. *J. Cell Sci.* **120**, 1955-1958.
Kaprielian, Z., Runko, E. and Imondi, R. (2001). Axon guidance at the midline choice point. *Dev. Dyn.* **221**, 154-181.
Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.
Melancon, E., Liu, D. W., Westerfield, M. and Eisen, J. S. (1997). Pathfinding by identified zebrafish motoneurons in the absence of muscle pioneers. *J. Neurosci.* **17**, 7796-7804.
Myers, P. Z., Eisen, J. S. and Westerfield, M. (1986). Development and axonal outgrowth of identified motoneurons in the zebrafish. *J. Neurosci.* **6**, 2278-2289.
Myllyharju, J. and Kivirikko, K. I. (2004). Collagens, modifying enzymes and their mutations in humans, flies and worms. *Trends Genet.* **20**, 33-43.
Nasevicius, A., Larson, J. and Ekker, S. C. (2000). Distinct requirements for zebrafish angiogenesis revealed by a VEGF-A morphant. *Yeast* **17**, 294-301.
O'Connor, T. P. (1999). Intermediate targets and segmental pathfinding. *CMLS* **55**, 1358-1364.
Panzer, J. A., Gibbs, S. M., Dosch, R., Wagner, D., Mullins, M. C., Granato, M. and Balice-Gordon, R. J. (2005). Neuromuscular synaptogenesis in wild-type and mutant zebrafish. *Dev. Biol.* **285**, 340-357.
Paulus, J. D. and Halloran, M. C. (2006). Zebrafish bashful/laminin-alpha 1 mutants exhibit multiple axon guidance defects. *Dev. Dyn.* **235**, 213-224.
Rodino-Klapac, L. R. and Beattie, C. E. (2004). Zebrafish topped is required for ventral motor axon guidance. *Dev. Biol.* **273**, 308-320.
Schneider, V. A. and Granato, M. (2006). The myotomal diwanka (lh3) glycosyltransferase and type xviii collagen are critical for motor growth cone migration. *Neuron* **50**, 683-695.
Schweitzer, J., Becker, T., Lefebvre, J., Granato, M., Schachner, M. and Becker, C. G. (2005). Tenascin-C is involved in motor axon outgrowth in the trunk of developing zebrafish. *Dev. Dyn.* **234**, 550-566.
Sentinelli, F., Lovari, S., Vitale, M., Giorgi, G., Di Mario, U. and Baroni, M. G. (2000). A simple method for non-radioactive pcr-sscp using mde gel solution and a midi gel format: application for the detection of variants in the GLUT1 and CTLA-4 genes. *J. Biotechnol.* **78**, 201-204.
Streisinger, G., Walker, C., Dower, N., Knauber, D. and Singer, F. (1981). Production of clones of homozygous diploid zebra fish (*Brachydanio rerio*). *Nature* **291**, 293-296.
Su, J., Gorse, K., Ramirez, F. and Fox, M. A. (2010). Collagen XIX is expressed by interneurons and contributes to the formation of hippocampal synapses. *J. Comp. Neurol.* **518**, 229-253.
Sumiyoshi, H., Laub, F., Yoshioka, H. and Ramirez, F. (2001). Embryonic expression of type XIX collagen is transient and confined to muscle cells. *Dev. Dyn.* **220**, 155-162.
Sumiyoshi, H., Mor, N., Lee, S. Y., Doty, S., Henderson, S., Tanaka, S., Yoshioka, H., Rattan, S. and Ramirez, F. (2004). Esophageal muscle physiology and morphogenesis require assembly of a collagen XIX-rich basement membrane zone. *J. Cell Biol.* **166**, 591-600.
Thisse, C., Thisse, B., Schilling, T. F. and Postlethwait, J. H. (1993). Structure of the zebrafish snail1 gene and its expression in wild-type, spadetail and no tail mutant embryos. *Development* **119**, 1203-1215.
Trevarrow, B., Marks, D. L. and Kimmel, C. B. (1990). Organization of hindbrain segments in the zebrafish embryo. *Neuron* **4**, 669-679.
Woods, I. G., Wilson, C., Friedlander, B., Chang, P., Reyes, D. K., Nix, R., Kelly, P. D., Chu, F., Postlethwait, J. H. and Talbot, W. S. (2005). The Zebrafish gene map defines ancestral vertebrate chromosomes. *Genome Res.* **15**, 1307-1314.
Xiao, T. and Baier, H. (2007). Lamina-specific axonal projections in the zebrafish tectum require the type IV collagen dragnet. *Nat. Neurosci.* **10**, 1529-1537.
Zeller, J. and Granato, M. (1999). The zebrafish diwanka gene controls an early step of motor growth cone migration. *Development* **126**, 3461-3472.
Zhang, J. and Granato, M. (2000). The zebrafish unplugged gene controls motor axon pathway selection. *Development* **127**, 2099-2111.

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Align human vs mouse vs zf colxix protein
CLUSTAL W (1.83) multiple sequence alignment


human           -------------MRLTGPWKLWLWMSIFLLPASTSVTVRDKTEESCPILRIEGHQLTYD 47
mouse           -------------MRHTGSWKLWTWVTTFLLPACTCLTVRDKPETTCPTLRTERYQ---D 44
zf              MFSRGPFSCAKDDMIHYLRWTVFLWIVN-SIPFASGMAVNERIDHTCPPLKLEDKWHTNV 59
                             *     *.:: *:    :* .: ::*.:: : :** *: *

human           NINKLEVSGFDLGDSFSLRR-AFCESDKTCFKLGSALLIRDTIKIFPKGLPEEYSVAAMF 106
mouse           DRNKSELSGFDLGESFALRH-AFCEGDKTCFKLGSVLLIRDTVKIFPKGLPEEYAIAVMF 103
zf              NLHR-EFTGFDLAEKFLLRKGTVTDSDTPLFRLGSKPLFKPTESVFPNGLXHEYSIVATF 118
                : :: *.:****.:.* **: :. :.*.. *:***  *:: * .:**:** .**::.. *

human           RVRRNAKKERWFLWQVLNQQNIPQISIVVDGGKKVVEFMFQATEGDVLNYIFRNRELRPL 166
mouse           RVRRSTKKERWFLWKILNQQNMAQISVVIDGTKKVVEFMFRGAEGDLLNYVFKNRELRPL 163
zf              RIRKTTKKDRWFVXQIFDKGGTSQVSLIVDGAKKSVEFLALGFLKNSXLYVFKNRDLHAL 178
                *:*:.:**:***: ::::: . .*:*:::** ** ***:  .   :   *:*:**:*:.*

human           FDRQWHKLGISIQSQVISLYMDCNLIARRQTDEKDTVDFHGRTVIATRASDGKPVDIELH 226
mouse           FDRQWHKLGIGVQSRVLSLYMDCNLIASRHTEEKNSVDFQGRTIIAARASDGKPVDIELH 223
zf              FDRQFHKLGVSVESNAVSIYLDCELIERQVTAERSGIDVSGRTFITTRLEDGKPVDVELQ 238
                ****:****:.::*..:*:*:**:**  : * *:. :*. ***.*::* .******:**:

human           QLKIYCSANLIAQETCCEISDTKCPEQDGFGNIASSWVTAHASKMSSYLPAKQELKDQCQ 286
mouse           QLRIYCNANFLAEESCCNLSPTKCPEQDDFGSTTSSWGTSNTGKMSSYLPGKQELKDTCQ 283
zf              EILVFCDSRIADLDRCCDSPGAMCEPTVTHNPTAIPLVTGYLQKMLSMP--AQLPTDRCH 296
                :: ::*.:.:   : **: . : *     ..  : .  *.   ** *     *  .* *:


human           CIPNKGEAGLPGAPGSPGQKGHKGEPGENGLHGAPGFPGQKGEQGFEGSKGETGEKGEQG 346
mouse           CIPNKEEAGLPGTLRSIGHKGDKGEPGEHGLDGTPGLPGQKGEQGLEGIKGEIGEKGEPG 343
zf              CPALKGLKGDPGPQGLPGLKGDKGDPGP---PGPGTLSVEKQAQ-----KGDQGPSGTPG 348
                * . *   * **.    * **.**:**     *.  :. :*  *     **: * .*  *

human           EKGDPALAGLNGENGLKGDLGPHGPPGPKGEKGDTGPPGPPALPGSLGIQGPQGPPGKEG 406
mouse           AKGDSGLDGLNGQDGLKGDSGPQGPPGPKGDKGDMGPPGPPALTGSIGIQGPQGPPGKEG 403
zf              EKGDVGPPGQPGAPGKEGKRGRRGKTGEPGTPGLQGPPGTCDAETVKGMKGDQGVAGERG 408
                 *** .  *  *  * :*. * :* .*  *  *  ****.       *::* ** .*:.*


human           QRGRRGKTGPPGKPGPPGPPGPPGIQGIHQTLGGYYNKDNKGNDEHEAGGLKGDKGETGL 466
mouse           QRGRRGKTGPPGNPGPPGPPGPPGLQGLQQPFGGYFN---KGTGEHGASGPKGEKGDTGL 460
zf              QKGDRGDSGLPGLEG--------------------------------AGGMKGQKGEEGP 436
                *:* **.:* **  *                                *.* **:**: *

human           PGFPGSVGPKGQKGEPGEPFTKGEKGDRGEPGVIGSQGVKGEPGDPGPPGLIGSPGLKGQ 526
mouse           PGFPGSVGPKGHKGEPGEPLTKGEKGDRGEPGLLGPQGIKGEPGDPGPPGLLGSPGLKGQ 520
zf              RGPPGPVMTAHGLRLSGPEGEGSEKGQKGEKGDQGEKGAEGSQGLQGPAGLTGPPGLEGK 496
                 * **.* .      .*     .***::** *  * :* :*. *  **.** *.***:*:

human           QGSAGSMGPRGPPGDVGLPGEHGIPGKQGIKGEKGDPGGIIGPPGLPGPKGEAGPPGKSL 586
mouse           QGPAGSMGPRGPPGDVGLPGEHGIPGKQGVKGEKGDPGGRLGPPGLPGLKGDAGPPGISL 580
zf              VGPPGPVGPRGLQGDPGSAGLIGLPGKDGMKGEKGDTGGAPGPAGPPGLKGEPGEP-CSV 555
                 *..*.:****  ** * .*  *:***:*:******.**  **.* ** **:.* *  *:

human           PGEPGLDGNPGAPGPRGPKGERGLPGVHGSPGDIGPQGIGIPGRTGAQGPAGEPGIQGPR 646
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mouse           PGKPGLDGNPGSPGPRGPKGERGLPGLHGSPGDTGPPGVGIPGRTGSQGPAGEPGIQGPR 640
zf              GLCGGEEVASGLRGLRGPKGERGVPGVPGDAGEKGEPGFGITGPPGLTGPKGEPGSQGPL 615
                    * :  .*  *  ********:**: *..*: *  *.**.* .*  ** **** ***

human           GLPGLPGTPGTPGNDGVPGRDGKPGLPGPPGDPIALPLLGDIGALLKNFCGNCQASVPGL 706
mouse           GLPGLPGTPGMPGNDGAPGKDGKPGLPGPPGDPIALPLLGDIGALLKNFCGNCQANVPGL 700
zf              GPQ---GPIGVPGIEGPPGPQGRPGLPGPPGEPTALPMVGDMGTLMKNACSVCQTRVPGL 672
                *     *. * ** :* ** :*:********:* ***::**:*:*:** *. **: ****

human           KSNKGEEGGAGEPGKYDSMARKGDIGPRGPPGIPGREGPKGSKGERGYPGIPGEKGDEGL 766
mouse           KSIKGDDGSTGEPGKYDPAARKGDVGPRGPPGFPGREGPKGSKGERGYPGIHGEKGDEGL 760
zf              PGQKGEKGSPGASGIPGMDGEKGDQGLRGPAGNPGKEGPKGVKGERGFPGPMGDKGDEGS 732
                 . **:.*..* .*  .  ..*** * ***.* **:***** *****:**  *:*****

human           QGIPGIPGAPGPTGPPGLMGRTGHPGPTGAKGEKGSDGPPGKPGPPGPPGIPFNERNGMS 826
mouse           QGIPGLSGAPGPTGPPGLTGRTGHPGPTGAKGDKGSEGPPGKPGPPGPPGVPLNEGNGMS 820
zf              PGVPGLPGSTGRTGSQGMNGRPGAVGPQGQKGDRGSEGPPGQPGPPGPPGAPYSEGNGMS 792
                 *:**:.*:.* **. *: **.*  ** * **::**:****:******** * .* ****

human           SLYKIKGGVNVPSYPGPPGPPGPKGDPGPVGEPGAMGLPGLEGFPGVKGDRGPAGPPGIA 886
mouse           SLYKIQGGVNVPGYPGPPGPPGPKGDPGPVGEPGAMGLPGLEGFPGVKGDRGPAGPPGIA 880
zf              SIYKLQNGAANGGQPGPPGPPGPKGDEGRMGEPGLMGLPGLEGLTGAKGDPGLPGPPGLN 852
                *:**::.*.    . *********** * :**** ********:.*.*** * .****:

human           GMSGKPGAPGPPGVPGEPGERGPVGDIGFPGPEGPSGKPGINGKDGIPGAQGIMGKPGDR 946
mouse           GISGKPGAPGPPGVPGEQGERGPIGDTGFPGPEGPSGKPGINGKDGLPGAQGIMGKPGDR 940
zf              GPVGKPGPRGETGIPGEPGERGPVGETGFPGPEGPPGAPGRPGKDGVPGYEGATGRPGDR 912
                *  ****. * .*:*** *****:*: ********.* **  ****:** :*  *:****

human           GPKGERGDQGIPGDRGSQGERGKPGLTGMKGAIGPMGPPGNKGSMGSPGHQGPPGSPGIP 1006
mouse           GPKGERGDQGIPGDRGPQGERGKPGLTGMKGAIGPVGPAGSKGSTGPPGHQGPPGNPGIP 1000
zf              GTKGERGDPGIPGERGVQGERGK---TGDKGTIGPQGPPGQKGEPGPPGSLTSPGS---V 966
                *.****** ****:** ******   ** **:*** **.*.**. *.**   .**.

human           GIPADAVSFEEIKKYINQEVLRIFEERMAVFLSQLK-LPAAMLAAQAY-GRPGPPGKDGL 1064
mouse           GTPADAVSFEEIKHYINQEVLRIFEERMAVFLSQLK-LPAAMLSAQAH-GRPGPPGKDGL 1058
zf              KLLSDTAALEEIKTFIRNEVLRVFEEKFSDSQTLLQKTPAAILAAQGRQGPPGPPGNDGS 1026
                   :*:.::****  :*.:****:***:::   : *:  ***:*:**.  * *****:**

human           PGPPGDPGP---QGYRGQKGERGEPGIGLPGSPGLPGTSALGLPGSPGAPGPQGPPGPSG 1121
mouse           PGPPGDPGP---QGYRGQKGERGEPGIGLPGSPGLPGSSAVGLPGSPGAPGPQGPPGPSG 1115
zf              PGPPGEPGPPGSQGYRGQKGERGQMGLGLPGAPGPAGPQGPVGLGPQGPSGPPGPPGPHG 1086
                *****:***   ***********: *:****:** .*...     *. *..** ***** *

human           RCNPEDCLYPVSHAHQRTGGN 1142
mouse           RCNPEDCLYPAPPPHQQAGGK 1136
zf              RCNPSDCFHPYG---RRDG-- 1102
                ****.**::*     :: *
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