Evolution of *lbx* spinal cord expression and function

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Funding Information: NSF IOS 1755354, NIH NINDS R01 NS077947

Running Title: Evolution of *lbx* spinal expression

Keywords: Lbx1a, Lbx1b, Lbx2, Tlx3, dI4, dI5, dI6, dogfish, shark, *Xenopus tropicalis*, *Scyliorhinus canicula*

Abstract

Ladybird homeobox (Lbx) transcription factors have crucial functions in muscle and nervous system development in many different animals. Amniotes have two *Lbx* genes, *Lbx1* and *Lbx2*, but only *Lbx1* is expressed in the spinal cord. In contrast, teleosts have three *lbx* genes, *lbx1a*, *lbx1b* and *lbx2*. In this study, we characterize the spinal cord expression of zebrafish *lbx1a*, *lbx1b* and *lbx2* and show that each of these genes is expressed by distinct cell types. Our data suggest that *lbx1a* is expressed by dI4, dI5 and dI6 spinal interneurons, whereas *lbx1b* and *lbx2* are primarily expressed in different spinal cord progenitor domains. We investigated the evolution of *Lbx* spinal cord expression patterns by examining *Lbx1* and *Lbx2* expression in the lesser spotted dogfish, *Scyliorhinus canicula* and *Lbx1* expression in the tetrapod, *Xenopus tropicalis*. Our results suggest that zebrafish *lbx1a* spinal cord expression is conserved with that of *Lbx1* in other vertebrates, whereas *lbx1b* spinal cord expression probably evolved in teleosts after the duplication of *lbx1* into *lbx1a* and *lbx1b*. *lbx2* spinal expression was probably acquired somewhere in the ray-finned lineage, as this gene is not expressed in the spinal cords of either amniotes or *S. canicula*. Consistent with its conserved spinal cord expression pattern, we also show that the spinal cord function of zebrafish *lbx1a* is conserved with mouse Lbx1. In zebrafish *lbx1a* mutants, there is a reduction of inhibitory spinal neurons and an increase in excitatory neurons, similar to the phenotype of mouse *Lbx1* mutants. Interestingly, we also see a reduction of inhibitory spinal neurons in *lbx1b* mutants, although in this case there is not a corresponding increase in the number of excitatory neurons and *lbx1a;lbx1b* double mutants do not have a more severe spinal cord phenotype than *lbx1a* single mutants, suggesting that *lbx1a* and *lbx1b* do not act redundantly in spinal neuron development. This suggests that *lbx1b* and *lbx1a* may be required in succession for correct specification of inhibitory dI4 and dI6 interneurons, although only *lbx1a* is required for suppression of excitatory fates in these cells.

Introduction

The spinal cord is a crucial part of the central nervous system of all vertebrates as its neuronal circuitry controls movements and receives sensory inputs from the trunk and limbs. All of the data so far, suggest that the common ancestor of ray-finned and lobe-finned vertebrates had distinct classes of spinal neurons with particular functions, that were specified during embryonic development by different transcription factors and that critically, spinal cord patterning and neuronal circuitry are still highly conserved in extant vertebrates (e.g. Goulding and Pfaff, 2005; Higashijima et al., 2004a; Lewis, 2006; Li et al., 2004; Roberts, 2000), although in amniotes, which have evolved a more complex repertoire of movements, some classes of spinal neurons have diversified into more specialized sub-classes of highly-related neurons (e.g. Alvarez et al., 2005; Boije and Kullander, 2018; D'Elia and Dasen, 2018; Gosgnach et al., 2006; Goulding and Pfaff, 2005; Griener et al., 2015; Higashijima et al., 2004a; Kimura et al., 2006; Lewis and Eisen, 2001; Sapir et al., 2004). For example, analyses by ourselves and others have identified several important transcription factors that are expressed either by progenitor or post-mitotic cells at conserved dorsal-ventral positions in both amniote and zebrafish spinal cord, although the expression domains are usually larger in amniotes, corresponding to the larger and more complicated spinal cords in these vertebrates (e.g. Batista et al., 2008; Batista and Lewis, 2008; Briscoe et al., 2000; Juárez-Morales et al., 2016; Moran-Rivard et al., 2001; Pillai et al., 2007). Consistent with these conserved expression patterns, the types of neurons found in the spinal cord, and the functions of particular transcription factors in specifying these neuronal subtypes has also been highly conserved throughout vertebrate evolution (e.g. England et al., 2014; Gosgnach et al., 2006; Goulding and Pfaff, 2005; Juarez-Morales et al., 2017; Lewis, 2006; Li et al., 2004)}. However, most of these comparative analyses have so far been limited to ventral spinal cord and it is still unclear whether dorsal spinal cord neurons are as highly conserved.

The ventral spinal cord primarily contains neurons that are involved in controlling movements and relaying information about trunk and limb position. In contrast, the dorsal spinal cord primarily contains neurons that are crucial for processing and relaying sensory information. We already know that there is at least one major difference between the neurons in amniote and anamniote dorsal spinal cords, as anamniote embryos have large sensory neurons, called Rohon-Beard cells, that form in the most dorsal part of the spinal cord, whereas amniote embryos do not ((Lewis and Eisen, 2003; Roberts, 2000) although see (Reyes et al., 2004) and references therein for reports of RB-like cells in amniotes including mammals). In contrast, both amniote and anamniote embryos have dorsal spinal cord interneurons, although as mentioned above, it is not yet known the extent to which the specification and/or functions of these interneurons are conserved in different vertebrates. This is an

interesting question from both an evolutionary perspective and also for evaluating the efficacy of different animals as model systems for human spinal cord.

Ladybird homeobox (Lbx) transcription factors have crucial functions in muscle development in many different animals (Brohmann et al., 2000; Gross et al., 2000; Jagla et al., 1998; Kanamoto et al., 2006; Lou et al., 2012; Neyt et al., 2000; Schafer and Braun, 1999; Watanabe et al., 2007). In addition, they are required for specification of particular cells in the Drosophila nervous system and mammalian spinal cord (Cheng et al., 2005; De Graeve et al., 2004; Gross et al., 2002; Kruger et al., 2002; Müller et al., 2002). In mouse spinal cord, Lbx1 is expressed in three early-forming (dI4, dI5 & dI6) and two later-forming (DIL_A & DIL_B) classes of post-mitotic dorsal interneurons and it is essential for correct specification of these neurons (Cheng et al., 2005; Gross et al., 2002; Kruger et al., 2002; Müller et al., 2002). In chick, Lbx1 is expressed in a very similar spinal cord domain (Schubert et al., 2001). There is also an additional *Lbx* gene, *Lbx2*, in amniotes, but this is not expressed in the spinal cord (Chen et al., 2001; Chen et al., 1999; Kanamoto et al., 2006). In contrast, we have shown previously that zebrafish and other teleosts have 3 *lbx* genes (Wotton et al., 2008; Wotton et al., 2010) and our preliminary data and data from other labs suggested that all three of these are expressed in the spinal cord (Lukowski et al., 2011; Neyt et al., 2000; Ochi and Westerfield, 2009; Wotton et al., 2008), although the specific cells types that express each of these genes has not been identified. In this paper we confirm that in contrast to amniotes, not only are all three zebrafish *lbx* genes expressed in the spinal cord but their expression domains are also distinct from each other. Our data suggest that zebrafish *lbx1a*, like mouse *Lbx1* is expressed by dI4, dI5 and dI6 spinal interneurons. In contrast, zebrafish *lbx1b* is expressed by spinal cord progenitor cells in the dP4 and maybe also the dP5 domain, whereas zebrafish *lbx2* is expressed in two distinct spinal cord domains, progenitor cells, probably in the P1 domain, and late progenitor / early post-mitotic cells in the dI4-dI6 domain.

To address where the differences in *Lbx* spinal cord expression evolved we also examined *lbx* expression in both an anamniote tetrapod *Xenopus tropicalis* and a shark, *Scyliorhinus canicula* (also known as lesser-spotted dogfish). These results show that spinal cord expression of *Lbx1* in *X. tropicalis* and *S. canicula* closely resembles spinal cord expression of *Lbx1* in mouse and chick and *lbx1a* in zebrafish, suggesting that this expression pattern is ancestral. In contrast, *Lbx2* is not expressed in *S. canicula* spinal cord, suggesting that the spinal cord expression of *lbx2* that we see in zebrafish was probably acquired somewhere in the ray-finned vertebrate lineage. As the spinal expression of zebrafish *lbx1b* also differs from Lbx1 expression in any of the other vertebrates examined by us or others, it is likely that this expression pattern evolved in teleosts, after the teleost

duplication of *lbx1* into *lbx1a* and *lbx1b* (Wotton et al., 2008; Wotton et al., 2010). Consistent with the similarities between the spinal expression of *lbx1a* in zebrafish and *Lbx1* in mouse, we also demonstrate that zebrafish *lbx1a*, like mouse *Lbx1*, is required for correct specification of a subset of dorsal interneurons. In zebrafish *lbx1a* mutants there is a reduction in the number of inhibitory dorsal spinal cord interneurons and an increase in the number of excitatory dorsal interneurons, just like in mouse *Lbx1* mutants (Cheng et al., 2005; Gross et al., 2002; Müller et al., 2002). Interestingly, we also see a reduction of inhibitory spinal neurons in *lbx1b* mutants, although in this case there is not a corresponding increase in the number of excitatory neurons and *lbx1a;lbx1b* double mutants do not have a more severe spinal cord phenotype than *lbx1a* single mutants, suggesting that *lbx1a* and *lbx1b* do not act redundantly in spinal neuron development. This suggests that *lbx1b* and *lbx1a* may be required in succession for specification of inhibitory fates, although only *lbx1a* is required for suppression of excitatory fates in dI4 and dI6 interneurons. Taken together, our findings demonstrate evolutionary conservation of *Lbx1/lbx1a* spinal cord expression and function between zebrafish and amniotes, while also identifying novel spinal cord expression patterns for zebrafish *lbx1b* and *lbx2*.

Material and methods

Ethics approval

All zebrafish experiments in this research were carried out in accordance with the recommendations of, and were approved by, either the UK Home Office or the Syracuse University IACUC committee.

Zebrafish husbandry and fish lines

Zebrafish (*Danio rerio*) were maintained on a 14 hour light/10 hour dark cycle at 28.5°C. Zebrafish embryos were obtained from natural, paired and/or grouped spawnings of wild-type (WT; AB, TL or AB/TL hybrids), *Tg(evx1:EGFP)*^{SU1} (Hilinski et al., 2016), *smoothened*^{b641} (Varga et al., 2001), *lbx1a*^{hu3569}, *lbx1a*^{sa1496}, *lbx1b*^{hu3534}, (Kettleborough et al., 2013), *mindbomb1*^{ta52b} (*mib1)* (Jiang et al., 1996), *Tg(lbx1b:EGFP)*^{ua1001} (Lukowski et al., 2011) or *Tg(1.6 lbx1a:EGFP)*^{SU33} (this paper) adults. Embryos were then reared at 28.5°C and staged by hours post fertilization (h) and/or prim staging as described in (Kimmel et al., 1995).

lbx1a^{hu3569}, *lbx1a*^{sa1496} and *lbx1b*^{hu3534} mutant alleles were received from the Welcome Trust Sanger Zebrafish Mutation Project, (https://www.sanger.ac.uk/resources/zebrafish/zmp/#t_about) (Kettleborough et al., 2013). Each of these three mutations is a single base pair change (C to T) that results in an immediate premature stop codon. In the case of *lbx1a*^{hu3569} and *lbx1a*^{sa1496}, the stop codon is located 148 bp and 34 bp after the beginning of the homeobox respectively. In the case of *lbx1b*^{hu3534}*,* the stop codon is located 145 bp before the homeobox. Therefore, in *lbx1a*^{sa1496} and *lbx1b*^{hu3534} homozygous mutants, if truncated mutant proteins are made, they will lack either all or most of the DNA-binding domain, whereas in *lbx1a*^{hu3569} homozygous mutants, any truncated Lbx1a protein will lack part of the homeobox C- terminal.

Creation of *Tg(1.6 lbx1a:EGFP)*^{SU33} transgenic line

Potential *lbx1a* enhancer regions were identified by multispecies sequence comparisons using the global alignment program Shuffle-LAGAN (Brudno et al., 2003) and visualized using VISTA (Mayor et al., 2000). Zebrafish (*Danio rerio*) *lbx1a* genomic sequence (ENSDARG00000018321) and orthologous sequences from human (ENSG00000138136) and mouse (ENSMUSG00000025216) were obtained from Ensembl (http://www.ensembl.org). *Danio rerio* sequence was used as the baseline and annotated using exon/intron information from Ensembl. The alignment was performed using a 100 bp window and a cutoff score of 70 % identity. A multi-species comparison of approximately 22Kb of *Danio rerio* genomic DNA sequence containing *lbx1a* and extending 10 Kb into flanking regions revealed high conservation in both coding and non-coding sequences among

compared species. We identified a major Conserved Non-coding Element (CNE) located 3' to *lbx1a*, 3021 bp downstream of the stop codon, that extends over 1686 bp (Fig. 1). This region was PCR-amplified from genomic DNA, using the primers Forward: cttcgtcgcaactatga and Reverse: tattagcccagtaatca. PCR conditions were: 94 °C for 3 min followed by 30 cycles of 98 °C for 30 s, 98 °C for 10 s, 62°C for 30 s, 72 °C for 30s and then a final extension step of 72 °C for 10 min. The 1.6 Kb amplicon was cloned into the pDONR™ P4-P1R vector from Invitrogen using Gateway technology (Sasaki et al., 2004; Suzuki et al., 2005). The *cfos minimal promoter:Gal4VP16:UAS:EGFP* middle entry construct (Juárez-Morales et al., 2016; Koster and Fraser, 2001) was modified by removing the *Gal4VP16:UAS* amplification cassette. The final construct was generated using the 1.6 Kb *lbx1a* 5′ pDONR, the modified *cfos minimal promoter:EGFP* middle entry vector and the pCSDest2 vector (Villefranc et al., 2007). This resulted in a vector containing *Tol2:1.6 Kb 3' zfish lbx1a:cfos minimal promoter:EGFP:Tol2*.

Plasmid DNA and *transposase* mRNA for microinjection was prepared as in (Juarez-Morales et al., 2017; Kwan et al., 2007). Approximately 10 nl of a combination of Plasmid DNA [60-80 ng/µL] and *transposase* mRNA [30 ng/µL] was injected into both blastomeres of 1-2-cell stage zebrafish embryos. Embryos were raised to adulthood and out-crossed to identify founders to generate the stable *Tg(1.6 lbx1a:EGFP)*^{SU33} line.

Genotyping

Genotyping of mutant alleles was performed on live adults and fixed embryos using DNA from fin clips and dissected heads respectively. Fin clips and DNA extraction from fins were performed as in (Schulte et al., 2011). To extract DNA from embryos, yolk was removed from the rostral embryo and the head was dissected at the hindbrain border in 70% glycerol/ 30% PBS with insect pins. Embryonic trunks were stored in 70% glycerol/30% PBS at 4ºC for later analysis. Heads were incubated in 50 µL of Proteinase K solution for 2hrs at 55°C. Proteinase K was heat inactivated at 100°C for 10 minutes and tubes were centrifuged for 20 minutes at 13000 rpm. DNA was precipitated from supernatant with 100% ice-cold ethanol at -20°C overnight. DNA was re-suspended in 20 µL of water. Alternatively, DNA was extracted using the HotSHOT method (Truett et al., 2000), adding 20 µL of 50 mM NaOH and 2 µL of 1M Tris-HCl (pH 7.5). From the resuspended or extracted DNA, 2 µL was used for each PCR.

The *lbx1a*^{hu3569} mutation creates a *XbaI* recognition site. Therefore, to genotype *lbx1a*^{hu3569} mutants, a genomic region flanking the mutation was PCR amplified using the following conditions: 94°C for 60 seconds, followed by 5 cycles of 92°C for 30 seconds, 54°C for 30 seconds, 72°C for 60 seconds;

followed by 40 cycles of 92°C for 20 seconds, 52°C for 30 seconds, and 72°C for 60 seconds, followed by a final extension at 72°C for 5 minutes. Forward primer: TTTACAGGCCTCTGCTGTTC. Reverse primer: AACACTCTTTGCTCGTTGTG. PCR products were digested with *XbaI* and analyzed on a 1% TAE agarose gel. The WT product is 510 bp. Mutant amplicons are cut into 256 bp and 254 bp fragments, which are close enough in size that they are usually detected as one band on the gel.

The *lbxlb*^{hu3534} mutation creates a *AccI* recognition site. To genotype *lbx1a*^{hu3534} mutants, a genomic region flanking the mutation was PCR amplified with the same conditions as *lbx1a*^{hu3569} above using the following primers, Forward primer: GCTATAGACAAAGGCTGGAATG. Reverse primer: GCCTACAATATACCCAGAATTG. PCR products were digested with AccI and analysed on a 1% TAE agarose gel. The WT product is 477 bp. Mutant amplicons are cut into 233 bp and 214 bp fragments. Alternatively, the *lbx1b*^{hu3534} allele was detected using KASP assays designed by Biosearch Technologies. KASP assays use allele-specific PCR primers, which differentially bind fluorescent dyes that we quantified with a Bio-Rad CFX96 real-time PCR machine to distinguish genotypes. The proprietary primers used were: *lbx1b_hu3534*.

To genotype *lbx1a*^{sa1496} mutants, a base pair change adjacent to the mutation was introduced in the forward PCR primer to create a *ScaI* recognition site only in the mutant DNA. The genomic region flanking the mutation was PCR amplified using the following conditions: 94°C for 60 seconds, followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 45 seconds, followed by a final extension at 72°C for 5 minutes. Forward primer: GAGAAAGTCGAGAACAGCTTTCACCAAGTAC. Reverse primer: CCTTCATCTCCTCTAGGTCTCTTTTGAGTT. PCR products were digested with *ScaI* and analysed on a 2.5 % TBE agarose gel. The WT product is 191 bp. Mutant amplicons are cut into 162 bp and 29 bp fragments, which are close enough in size that they are usually detected as one band on the gel. Alternatively, the *lbx1a*^{sa1496} allele was detected using KASP assays designed by Biosearch Technologies. The proprietary primers used were: *lbx1a_sa1496*.

in situ* hybridization on *Danio rerio

Embryos were fixed in 4 % paraformaldehyde (PFA) and single and double *in situ* hybridizations were performed as previously described (Batista et al., 2008; Concordet et al., 1996). Embryos older than 24 h were usually incubated in 0.003% 1- phenyl-2-thiourea (PTU) to prevent pigment formation. RNA probes were prepared using the following templates, *lbx1a* and *lbx2* (Ochi and

Westerfield, 2009), *lbx1b* (Thisse et al., 2004; Thisse and Thisse, 2004), *dbx2* (Gribble et al., 2007), *pax2a* (Pfeffer et al., 1998).

To determine neurotransmitter phenotypes, we used the following markers: *slc32a1* (formerly called *viaat*), which encodes a vesicular inhibitory amino acid transporter, labels all inhibitory cells (Kimura et al., 2006), a mixture of two probes (*glyt2a* and *glyt2b*) for *slc6a5* (previously called *glyt2*) which encodes a glycine transporter necessary for glycine reuptake and transport across the plasma membrane labels glycinergic cells (Higashijima et al., 2004b; Higashijima et al., 2004c) and a mixture of two probes to *gad1b* (previously called *gad67*, probes used to be called *gad67a* and *gad67b*) and one probe to *gad2* (previously called *gad65*) was used to label GABAergic cells (Higashijima et al., 2004b; Higashijima et al., 2004c). *gad1b* and *gad2* encode for glutamic acid decarboxylases, necessary for the synthesis of GABA from glutamate. Glutamatergic (excitatory) cells were labelled with a mixture of *slc17a6b* (formerly called *vglut2.1*) and *slc17a6a* (formerly called *vglut2.2*; (Higashijima et al., 2004b; Higashijima et al., 2004c). These genes encode proteins responsible for transporting glutamate to the synapse. In all of these cases, a mix of equal concentrations of the relevant probes was used.

in situ* hybridization on *Scyliorhinus canicula

Scyliorhinus canicula (*S. canicula*, lesser spotted dogfish) egg cases were obtained from the Marine Biological Association of the United Kingdom in Plymouth. Fertilized eggs were stored at 17°C in a 10 L aerated sea water container until appropriate stages were reached. Staging was performed according to Ballard's staging series (Ballard et al., 1993). The anterior and posterior tendrils from each egg case were cut and the position of the embryo was determined by shining a bright light behind the egg case. Using a scalpel, a large window was cut in the side where the embryo was located. The yolk stalk was pulled out using a pair of tweezers and cut with a pair of dissection scissors. The embryo was spooned out and placed into PBS to wash, then into PBS with tricaine methanesulfonate (Sigma Aldrich, A5040) until the embryo was still, followed by fixation in 4% PFA and 3 washes for 5 minutes each in PBS.

Cryosections were prepared by incubating fixed embryos in 30% sucrose in PBS solution at 4°C overnight. Embryos were then trimmed and set into OCT on dry ice, followed by sectioning after 30 minutes on dry ice or stored at -20°C for later sectioning. Sections were cut on a Leica Jung Frigocut 2800E cryostat set at approximately 20-40µm thickness and collected on SuperFrost® Plus (Menzel-Gläser) slides and stored at -20°C until used. The zebrafish *in situ* protocol (Batista et al., 2008; Concordet et al., 1996). was used with the following modifications: cryosection slides were

rehydrated in PBS or PBT, immediately followed by placing 200µl of RNA probe in hybridization buffer onto the section and covering with a coverslip, slides were incubated at 70°C in a sealed box overnight. Slides were then placed in Coplin jars and washed as in the zebrafish protocol but with the first formamide washes omitted. For staining, 500µl of the NBT/BCIP solution diluted in NTMT were placed on the sections, covered with a coverslip and the slides were placed in the dark until staining developed. After staining, slides were washed in NTMT and PBS and fixed with 4% PFA.

S. canicula lbx1 and *lbx2* were identified from ESTs sequenced by Sanger sequencing. *S. canicula lbx1* probe was made from EST E217018. This plasmid was cut with *SalI* and transcribed with SP6 RNA polymerase. *S. canicula lbx2* probe was made from EST E158l19. This plasmid was cut with *KpnI* and transcribed with SP6 RNA polymerase. These sequences have been deposited in Genbank.

in situ* hybridization on *Xenopus tropicalis

Xenopus tropicalis embryos were obtained from Jim Smith's Lab at the Gurdon Institute, University of Cambridge. Embryos were incubated at 25°C until the appropriate stage, when the vitelline membrane was removed by forceps, embryos were fixed in 4% PFA and kept at 4°C overnight. After fixation, embryos were washed in PBT and then dehydrated in 100% methanol and stored at -20°C until used. Embryos in methanol were transferred to 100% ethanol and rehydrated through an ethanol/PBT series (90, 70, 50, 30, 10%), 5 minutes each. Rehydrated embryos older than stage 25 were incubated in 5µg/ml proteinase K at room temperature for 15 minutes, followed by re-fixation in 4% PFA. Fixed embryos were placed into hybridization buffer (50% formamide, 5×SSC, 1mg/ml yeast tRNA (Roche # 10109223001), 100µg/ml heparin (Sigma-Aldrich # H9399), 2% Blocking reagent (Roche # 11096176001), 0.1% Tween 20, 0.1% CHAPS (Sigma-Aldrich)) until embryos sank and then incubated in fresh hybridization buffer for 5 hours at 70°C. This was followed by an overnight incubation at 70°C with DIG labelled *lbx1* RNA probe (Martin and Harland, 2006) in hybridization buffer plus 0.1% SDS to allow better probe penetration.

Embryos were washed in hybridization buffer preheated to 70°C for 10 minutes, followed by three 20-minute washes in 2×SSC, 0.3% CHAPS at 60°C. This was followed by two 30-minute washes in 0.2×SSC, 0.3% CHAPS and two 10 minute 0.3% CHAPS in PBT washes at 60°C. After a 10-minute wash in PBT at room temperature, embryos were incubated with 0.5% blocking reagent in PBT before an overnight incubation in 1:2000 anti-dig AP antibody (Roche Diagnostics # 11093274910) in 0.5% blocking reagent (Roche # 11096176001), in PBT solution at 4°C.

After antibody incubation, embryos were washed five times, for one hour each wash, in PBT. Then, they were transferred into a 24 well cell culture plate and washed twice in NTMT (0.1M Tris pH 9.5, 50mM MgCl2, 0.1M NaCl, 0.1% Tween 20) for five minutes. Color reaction was performed by adding 20µl/ml NBT/BCIP (Roche # 11681451001) per ml of NTMT buffer and the embryos were placed in the dark. To stop the staining, embryos were washed several times in PBT and fixed in 4% PFA. When required, pigment was removed by washing the embryos four times in 70% ethanol in PBS for one hour and then leaving them in bleach (3% H_2O_2, 5% formamide, 0.5×SSC) for an initial 5 minutes, followed by incubating for 2 hours on a light box with fresh bleach and then washing several times with PBS. Prior to staining visualization, embryos were dehydrated in several washes of methanol and transferred into glass watch glasses where they were cleared in Murray's solution (2:1 benzyl benzoate : benzyl alcohol) (Klymkowsky and Hanken, 1991).

in situ* hybridization plus imunohistochemistry on *Danio rerio

Primary antibodies used were chicken polyclonal anti-GFP (Abcam ab13970, 1:500), rabbit anti-GFP (Molecular Probes A6465, 1:500) and rabbit anti-activated Caspase-3 (Fisher Scientific/BD, BDB559565, 1:500). Antibodies used for fluorescent *in situ* hybridization were mouse anti-Dig (Jackson ImmunoResearch 200-002-156, 1:5000), detected with Invitrogen Tyramide #5 (T20915) or rabbit anti-Flu. Secondary antibodies used were Alexa Fluor 568 goat anti-mouse (Molecular Probes 11031, 1:500), Alexa Fluor 488 goat anti-rabbit (Molecular Probes A11034, 1:500) and Alexa Fluor 488 goat anti-chicken IgG (H+L) (Invitrogen A11039, 1:500).

Embryos for immunohistochemistry only were treated with acetone for 20 min (30h embryos) to permeabilize them, washed for 5 min in distilled water, then washed 2 x 10 min in PBS. Embryos were treated with Image-iT Signal Enhancer (Invitrogen, I36933) for 30 min, then incubated in block solution (2 % goat serum, 1 % BSA, 10 % DMSO and 0.5 % Triton) for 1h at RT followed by incubation in primary antibody in fresh block solution at 4 °C overnight. Embryos were washed with PBT (PBS + 0.1 % Triton) for 2h at RT and incubated with secondary antibody in block solution at 4 °C overnight. Embryos were then washed with PBT for at 2h at RT and stored in 2 % DABCO (Acros Organics, AC11247-1000).

In several cases, we were unable to perform double fluorescent stainings due to very weak labeling with our RNA probes. In these cases, we combined single fluorescent labeling with NBT/BCIP chromogenic staining. However, whereas stronger *in situ* signal from weak RNA probes can be obtained using NBT/BCIP, visualization of co-expressing cells becomes more difficult with this method. To preserve the integrity of both the NBT/BCIP chromogenic *in situ* signal and the weaker

IHC signal, embryos were stored and mounted in Vectashield Mounting Medium (Vector Laboratories, H-1000-10).

Image acquisition and processing

Whole-mount tadpoles were placed in a 1% agarose plate and covered in PBS for imaging using an Olympus SZX16 stereomicroscope and a Q-Imaging Micropublisher 5.0 RTV camera. Whole mount zebrafish embryos and *S. canicula* cross-sections were mounted in either 70% glycerol or Vectashield (NBT/BCIP staining) or 2% DABCO (fluorescent staining) on a microscope slide. DIC pictures were taken using an AxioCam MRc5 camera mounted on a Zeiss Axio Imager M1 compound microscope. Fluorescent images were taken on a Zeiss LSM 710 confocal microscope. In some cases, dual NBT/BCIP ISH + anti-GFP immunohistochemistry images were captured in parallel using a Zeiss LSM 710 confocal microscope equipped with a T-PMT. Images were processed using Adobe Photoshop software (Adobe, Inc) and Image J software (Abràmoff et al., 2004). Combined fluorescent and brightfield images were merged in Photoshop by placing the fluorescent image on top of the brightfield image and then adjusting the opacity and/or fill of the fluorescent image.

Cell counting and statistical analyses

In all cases, cells counts are for both sides of a five-somite length of the spinal cord adjacent to somites 6-10. Most values are an average of at least 5 embryos. To determine whether differences in values are statistically significant, data were first analyzed for normality using the Shapiro-Wilk test. All data sets analyzed had normal distributions. For the pairwise comparison of *slc17a6* expression in 30h WT and *lbx1a*^{hu3569} mutant embryos, the F-test for equal variances was performed, and since the variances were equal, a type 2 (for equal variances) student's *t*-test was performed, and the p value is indicated by $^{+}$ in Table 1. To control for type I errors in all other data sets comparing WT, *lbx1a* single mutant, *lbx1b* single mutant and *lbx1a;lbx1b* double mutant embryos, a one-way analysis of variance (ANOVA) test was performed. Since all data sets for ANOVA analysis had normal distributions, they were first assessed for homogeneity of variances using Bartlett's test. All of the data sets had homogeneous (homoscedastic, Bartlett's test p value >0.05) variances and so standard ANOVA analysis was performed. ANOVA results are reported as F(dfn,dfd) = f-ratio, p value = *x*, where F is the F-statistic, dfn = degree of freedom for the numerator of the F-ratio, dfd = degree of freedom for the denominator of the R-ratio, and *x* = the p value. For statistically significant ANOVA, to determine which specific genotypic group or groups differed, post hoc testing was performed. Since all ANOVA data sets had homogeneous (homoscedastic) variances, Tukey's honestly significant difference post hoc test for multiple comparisons was performed. P values generated by Tukey's honestly significant difference test are indicated by ‡ in Table 1. Data are depicted as individual value plots and the *n*-

values for each experimental group are also shown. For each plot, the wider red horizontal bar depicts the mean and the narrower red horizontal bars depict the standard error of the mean (SEM). SEM values are listed in Table 1. Shapiro-Wilk testing was performed in R version 3.5.1 (R_Development_Core_Team, 2005). The F-test, and student's t-test were performed in Microsoft Excel version 16.41). Bartlett's testing, standard ANOVA, and Tukey's honestly significant difference testing were performed in Prism version 9.0.0 (GraphPad Software, San Diego, California USA, www.graphpad.com).

Results

zebrafish *lbx* genes have different spinal cord expression patterns

In contrast to mammals which have two *Lbx* genes, *Lbx1* and *Lbx2,* teleosts have two *lbx1* genes, *lbx1a* and *lbx1b*, and one *lbx2* gene (Wotton et al., 2008; Wotton et al., 2010). In addition, while only *Lbx1* is expressed in mammalian spinal cord, all three of the zebrafish *lbx* genes are expressed in the spinal cord (Fig. 2; Lukowski et al., 2011; Neyt et al., 2000; Ochi and Westerfield, 2009). To examine and compare the spinal cord expression of the three zebrafish *lbx* genes we performed *in situ* hybridizations at different stages of embryonic development. Our data show that *lbx1a*, *lbx1b* and *lbx2* are all expressed in the zebrafish spinal cord by mid-somitogenesis stages. At 18h (18-somites) *lbx1a* is expressed in a subset of post-mitotic cells in the dorsal spinal cord. This expression is stronger in the rostral spinal cord, and decreases more caudally (Fig. 2a). As development proceeds, additional cells in the same dorsal/ventral spinal cord domain start to express *lbx1a* and the expression extends more caudally (Fig. 2b & d). By 30 h, *lbx1a* is expressed along the whole rostral-caudal axis of the spinal cord (Fig. 2d). Analysis of spinal cord cross-sections shows that *lbx1a*-expressing cells are located at the lateral edge of the dorsal spinal cord, consistent with expression by post-mitotic neurons (Fig. 2c; at these stages of development progenitor cell are located medially next to the ventricule and post-mitotic neurons are located laterally at the edge of the spinal cord). Further confirming that this expression is in post-mitotic neurons, *lbx1a* is expressed in an expanded number of spinal cells in *mindbomb1*^{ta52b} mutants at 24h (Fig. 2e & f). *mindbomb1* is a ubiquitin-ligase essential for efficient Notch signaling. When Notch signaling is disrupted or lost, spinal cord progenitor cells precociously differentiate as early forming neurons, resulting in a loss of progenitor gene expression and expanded expression of most post-mitotically-expressed genes (Batista et al., 2008; Itoh et al., 2003; Jiang et al., 1996; Park and Appel, 2003; Schier et al., 1996).

lbx1b is also expressed in the dorsal spinal cord at 18h, but unlike *lbx1a,* it is expressed by a continuous row of cells along the rostral-caudal axis, more reminiscent of progenitor domain expression and the expression extends more caudally than *lbx1a* expression (Fig. 2g). This expression pattern persists at 24 and 30h (Fig. 2h & j). Analysis of spinal cord cross-sections suggests that *lbx1b* is expressed in both medial progenitor and lateral post-mitotic dorsal spinal cord cells. This suggests that *lbx1b* expression may persist, at least for a short time, in post-mitotic neurons (Fig. 2i). However, in *mindbomb1*^{ta52b} mutants at 24h most of the spinal cord expression of *lbx1b* is lost, consistent with it being expressed in progenitor cells and suggesting that if it is expressed in post-mitotic cells, it is very quickly turned off in these cells (Fig. 2k & l).

In contrast to *lbx1a* and *lbx1b*, *lbx2* is expressed in two different dorsal-ventral spinal cord domains. At 18h, the dorsal row of *lbx2* expression consists of more spaced/sporadic cells than the more continuous ventral row (Fig. 2m) and this expression pattern persists at 24h and 30h (Fig 2n & p). The ventral row of expression is about 3 cell diameters dorsal to the notochord and the more dorsal row of expression is approximately another 2-3 cells dorsal. The most dorsal row of *lbx2*-expressing cells is more clearly clearly visible in the rostral spinal cord but sometimes it extends more caudally. *lbx2* is also expressed in rostral somites at 18h and this expression extends caudally and increases substantially by 24h and 30h, making it harder to clearly see the spinal cord staining (Fig. 2m-p). Analysis of spinal cord cross-sections at 24h shows that the ventral *lbx2*-expressing spinal cells are predominantly medial, although there are also occasional laterally located cells, and the dorsal *lbx2*-expressing spinal cells are located either at the lateral edges of the spinal cord or slightly medial to that – between the medial ventricle and the lateral edge of the spinal cord (Fig. 20). This suggests that the dorsal *lbx2* expression domain consists of cells that are becoming post-mitotic and the ventral expression domain is predominantly progenitor cells. However, in *mindbomb1*^{ta52b} mutants at 24 h, most of the spinal cord expression of *lbx2* is lost (Fig. 2 q & r), although there is an expansion in the number of cells expressing *lbx2* in the very caudal region of the spinal cord and a small number of cells continue to express *lbx2* in more rostral regions of dorsal spinal cord (Fig. 2r). This is consistent with the ventral *lbx2*-expressing cells being predominantly progenitor cells and it suggests that even in the more dorsal domain of expression, *lbx2* is only expressed transiently in post-mitotic cells: its expression is turned off soon after these cells become post-mitotic.

Unfortunately, all of the zebrafish *lbx in situ* probes are relatively weak and, as a result, we were unable to successfully perform double *in situ* hybridizations with combinations of these genes. Therefore, to compare the different *lbx* spinal expression domains, we identified a putative enhancer region downstream of *lbx1a* and used this to construct a *Tg(1.6 lbx1a:EGFP)*^{SU33} transgenic line (see materials and methods & Fig. 1). This line at least partly recapitulates endogenous *lbx1a* expression (Fig. 3a & b). The EGFP expression is in the same dorsal-ventral spinal cord region as endogenous *lbx1a* and at least most of the EGFP-positive cells co-express *lbx1a* mRNA. We also confirmed that a previously published *Tg(lbx1b:EGFP)*^{ua1001} line (Lukowski et al.) is expressed in a similar dorsal spinal cord domain to endogenous *lbx1b* expression (Fig. 3d; Lukowski and colleagues reported that this line recapitulates endogenous *lbx1b* expression but did not show the data).

When we compare the expression of *lbx1a* and *lbx1b* to *Tg(lbx1b:EGFP)*^{ua1001} it is clear that the *lbx1a* spinal expression is, in the main, more ventral than that of *lbx1b* although the two genes overlap in the most dorsal region of the *lbx1a* expression domain (Fig. 3c-e). Comparing expression of *lbx1a*

and *lbx2* to *Tg(1.6 lbx1a:EGFP)*^{SU33} also confirms that the ventral row of *lbx2* expression is more ventral than *lbx1a* expression although the dorsal *lbx2*-expressing cells are within the *lbx1a* expression domain (Fig. 3g). While we did not observe any co-labeled *lbx2* and *Tg(1.6 lbx1a:EGFP)*^{SU33} cells, we cannot rule out the possibility that some of the dorsal *lbx1a*-expressing cells may co-express *lbx2*.

Zebrafish *lbx1a*-expressing cells develop into commissural bifurcating interneurons (CoB)

Previous work in mouse has shown that *Lbx1* is expressed by three early-forming dorsal interneuron populations, dI4, dI5 and dI6 cells, and subsequently by later forming dIL_A and dIL_B neurons (Gross et al., 2002; Müller et al., 2002). However, the axon trajectories and morphologies of these cells have not been described in detail, although the work of Gross and colleagues suggests that many of the later-born cells are ipsilateral (Gross et al., 2002). When we examined *Tg(1.6 lbx1a:EGFP)*^{SU33} embryos we found that by 35 h, at least most of the EGFP-positive cells have extended their axons ventrally and crossed the midline to the other side of the spinal cord (Fig. 3f, h & i). We analyzed 96 neurons and were able to determine the axon trajectories of 66 (69%) once they crossed the midline. All of these (66/66) turned slightly dorsally and then bifurcated. This suggests that many *lbx1a*-expressing spinal neurons have a commissural bifurcating, or CoB morphology (Fig. 3 f, h & i(Bernhardt et al., 1990).

Zebrafish *lbx1a* is expressed by dI4, dI5 and dI6 spinal interneurons

In mouse, *Lbx1* is expressed in dI4, dI5 and dI6 spinal interneurons, which are located immediately dorsal to V0 interneurons and develop both from, and dorsal to, the *dbx2*-expressing progenitor domain (Lewis, 2006 and references therein). As zebrafish *lbx1a* seemed to be expressed in a similar spinal cord domain to mouse *Lbx1* we used *in situ* hybridization and immuno-histochemistry double-labeling experiments to investigate whether it was also expressed by dI4, dI5 and dI6 interneurons. We found that some *Tg(1.6 lbx1a:EGFP)*^{SU33} expressing cells are at the same dorso-ventral spinal cord level as *dbx2*-expressing cells and some are more dorsal, although, as expected for post-mitotic interneurons the EGFP-expressing cells are more lateral than the *dbx2*-expressing cells (Fig. 4ª & b). In addition, when we compared the expression of *lbx1a* to *Tg(evx1:EGFP)*^{SU1}, which labels V0v interneurons (Juárez-Morales et al., 2016), we found that most of the *lbx1a*-expressing cells are dorsal to V0v interneurons and we did not observe any co-expression of *lbx1a* and EGFP (Fig. 4c & d). We also compared expression of *Tg(1.6 lbx1a:EGFP)*^{SU33} to *pax2a,* which is expressed by V1, $V0_D$, dI4 and dI6 spinal interneurons (Batista and Lewis, 2008). We found that most of the *lbx1a*-expressing cells are located in the same dorso-ventral spinal region as *pax2a*-expressing cells, and more importantly, a subset of the EGFP-positive cells co-express *pax2a* (Fig. 4e). Finally, as dI4 and dI6

interneurons are inhibitory and dI5 neurons are excitatory (Cheng et al., 2005; Gross et al., 2002; Müller et al., 2002) we also examined the neurotransmitter phenotypes of *Tg(1.6 lbx1a:EGFP)*^{SU33} expressing cells. We found that many of the *Tg(1.6 lbx1a:EGFP)*^{SU33} expressing cells co-express the inhibitory marker *slc32a1* (used to be called *viaat;* Fig. 4f), and a smaller number co-express the excitatory marker *slc17a6* (used to be called *vglut2;* Fig. 4g; see materials and methods for a more detailed discussion of the neurotransmitter markers used). Taken together, these data suggest that, like mouse *Lbx1*, zebrafish *lbx1a* is expressed in dI4, dI5 and dI6 spinal interneurons. Consistent with this conclusion, in a previous report we showed co-expression of *lmx1bb* and *lbx1a*, suggesting that *lbx1a* is expressed in at least some dI5 cells (Hilinski et al., 2016).

Consistent with our comparisons of *lbx1a*, *lbx1b* and *lbx2* expression discussed above, *Tg(lbx1b:EGFP)*^{ua1001} is expressed immediately dorsal to *dbx2* (Fig. 4h), and the ventral row of *lbx2*-expressing cells is located ventral to V0v interneurons and the dorsal row of *lbx2*-expressing cells is dorsal to these cells. (Fig. 4i). We did not observe any co-expression of *Tg(evx1:EGFP)*^{SU1} and *lbx2.* These data suggest that *lbx1b* is probably expressed in the dP4 progenitor domain and the ventral *lbx2*-expressing cells are probably in the P1 progenitor domain.

Lbx1a and Lbx1b are required to specify correct neurotransmitter fates of a subset of dorsal spinal interneurons

In mouse *Lbx1* mutants there is a reduction in the number of spinal GABAergic neurons and a corresponding increase in the number of spinal glutamatergic neurons (Gross et al., 2002; Müller et al., 2002). To test whether this function of Lbx1 is conserved in zebrafish, we analyzed the neurotransmitter phenotypes of *lbx1a* mutants.

At 24h, we observed a slight reduction in the number of cells expressing *slc32a1* (previously called *viatt*) although this decrease was not statistically significant (Fig. 5b & o; Table 1). However, the reduction in the number of cells expressing *slc32a1* became more pronounced and statistically significant at 30h (Fig. 5d & p; Table 1). In contrast, there was a statistically significant increase in the number of spinal cells expressing *slc17a6* (previously called *vglut2*) at 30h (Fig. 5f & q, Table 1). The increase in the number of excitatory spinal cells was similar and statistically significant in both *lbx1a*^{sa1496} and *lbx1a*^{hu3569} single mutants (Fig. 5 f, h, q & r, Table 1).

As the spinal expression patterns of *lbx1a* and *lbx1b* suggest that at least some *lbx1a*-expressing interneurons may develop from *lbx1b*-expressing progenitor cells, we also tested whether there was any redundancy between *lbx1a* and *lbx1b* by examining *lbx1a;lbx1b* double mutants. There was no

statistically significant difference between the number of spinal cord cells expressing either *slc32a1* or *slc17a6* in *lbx1a* single mutants compared to *lbx1a;lbx1b* double mutants (Fig. 5p & q). However, while there was no increase in the number of spinal cord cells expressing *slc17a6* in *lbx1b* single mutants compared to WT sibling embryos (Fig. 5q), there was a decrease in the number of spinal cord cells expressing *slc32a1* that was equivalent to the decrease *in lbx1a* single mutants and *lbx1a;lbx1b* double mutants (Fig. 5p). This suggests that both *lbx1b* and *lbx1a* are required, presumably in succession, for the inhibitory fates of at least some dI4 and dI6 interneurons but only *lbx1a* is required to repress excitatory fates in these cells.

To determine whether the reduction in the number of inhibitory cells in *lbx1a* and *lbx1b* single and double mutants represents a reduction in the number of GABAergic or glycinergic neurons, we examined expression of markers of these neurotransmitter phenotypes in both single and double *lbx1a;lbx1b* mutants compared to WT embryos. There was no significant difference in the number of cells expressing GABAergic markers at 30h (Fig. 5j & s, Table 1). However, in contrast, there is a statistically significant decrease in the number of spinal neurons expressing glycinergic markers in *lbx1a;lbx1b* double mutants (Fig. 5l & t, Table 1), although this reduction is slightly less than the reduction in the number of cells expressing *slc32a1*.

To test whether the reduction in inhibitory neurons might be caused by cell death, we performed activated caspase-3 immunohistochemistry. However, we did not observe any difference in the number of activated caspase-3 cells when comparing WT and double mutant embryos ($p = 0.68$; n= 3; Fig. 6). This is also consistent with the fact that there is an increase in the number of glutamatergic cells in the spinal cord of these mutants, which suggests that cells are changing aspects of their fate rather than dying.

Evolution of *lbx* spinal cord expression

As discussed above, in zebrafish there are three *lbx* genes and all three of them are expressed in the spinal cord, with different expression patterns, whereas in mouse, only *Lbx1* is expressed in the spinal cord. In addition, while the spinal cord expression and function of zebrafish *lbx1a* seems to be conserved with that of Lbx1 in mouse, zebrafish lbx1b and lbx2 have different spinal cord expression patterns. To investigate where these differences in gene expression evolved in the vertebrate lineage, we examined *lbx* gene expression in *Scyliorhinus canicula* (lesser-spotted dogfish) and *Xenopus tropicalis*.

Our analyses of a *Scyliorhinus canicula* (dogfish) EST library identified two *lbx* genes*: lbx1* and *lbx2* (see materials and methods). We were unable to analyze spinal cord expression of these two genes using *in situ* hybridizations on whole-mount specimens, due to lack of penetration into the spinal cord. Therefore, we performed *in situ* hybridization on embryo cross-sections at stages 25, 28, 31 and 32. Similar to mouse (Cheng et al., 2005; Gross et al., 2002; Kruger et al., 2002; Müller et al., 2002), we observed *lbx1* expression laterally in the dorsal spinal cord, just above the mid-point of the dorsal-ventral axis (Fig. 7a). Interestingly, and again similar to mouse (Chen et al., 1999; Moisan et al.), we did not observe any *lbx2* spinal cord expression at any of these stages (Fig. 7b, and data not shown). However, *lbx2* is clearly expressed in both the hindbrain (black arrowheads in Fig. 2c) and the gut (black arrowheads in Fig. 2d), indicating that our *in situ* hybridization experiment clearly worked.

Xenopus tropicalis only has one *lbx* gene, *lbx1* (Wotton et al., 2008). Using whole-mount *in situ* hybridization, we analyzed expression of this gene from stage 22 to stage 37 (Fig 7e & f and data not shown). *lbx1* is expressed in the rostral spinal cord at stage 22 and expression extends more caudally as development proceeds. By stage 35, *lbx1* is clearly expressed along the whole rostral-caudal extent of the spinal cord (Fig. 7e). Spinal cord cross-sections show that the *lbx1*-expressing cells are clearly lateral, consistent with them being post-mitotic (Fig. 7f). Similar to dogfish and mouse, *lbx1* expression in *X. tropicalis* is also located just above the mid-point of the dorsal-ventral axis (Fig. 7f).

Discussion

Lbx genes have crucial functions in mesoderm and nervous system development in a wide range of animals as demonstrated by studies in the fruitfly *Drosophila melanogaster*, the marine annelid *Platynereis dumerilii*, teleosts, mouse, chick and humans (Blader et al., 2004; Jagla et al., 1998; Lukowski et al., 2011). As previously discussed, amniotes have two *Lbx* genes (*Lbx1* and *Lbx2*), although only *Lbx1* is expressed in the spinal cord (Gross et al., 2002; Jagla et al., 1995; Müller et al., 2002; Schubert et al., 2001; Chen et al., 2001; Chen et al., 1999; Kanamoto et al., 2006). In contrast, zebrafish have three *lbx* genes (*lbx1a*, *lbx1b* and *lbx2*), as both teleost duplicates of *lbx1* have been retained (Wotton et al., 2008). All three of these zebrafish genes are expressed in the spinal cord (Lukowski et al., 2011; Neyt et al., 2000; Ochi and Westerfield, 2009; this report) but before this paper their spinal expression had not been analyzed in detail. Our data show that all three of these genes have distinct spinal cord expression patterns.

In a previous study we showed that *lbx1a* is co-expressed with *lmx1bb* in the zebrafish spinal cord, suggesting that some of the *lbx1a*-expressing spinal cells are dI5 interneurons (Hilinski et al., 2016). In this study, our double-labeling experiments between zebrafish *Tg(1.6 lbx1a:EGFP)*^{SU33} and *dbx2* and *lbx1a* and *Tg(evx1:EGFP)*^{SU1} suggest that zebrafish *lbx1a*-expressing cells are located in the dI6-dI4 region of the spinal cord as the *Tg(1.6 lbx1a:EGFP)*^{SU33}-expressing cells are either within the *dbx2* expression domain or slightly dorsal to it and most of the *lbx1a*-expressing cells are dorsal to *Tg(evx1:EGFP)*^{SU1}-expressing cells (Fig. 4; zebrafish *dbx2* is expressed by dP6, P0 and P1 progenitor domains (Gribble et al., 2007; Lewis, 2006) and *Tg(evx1:EGFP)*^{SU1} is expressed by V0v cells (Juárez-Morales et al., 2016); the small size of the zebrafish spinal cord means that different domains overlap slightly and are not as clearly separated as in larger amnite spinal cords). Finally, our data also demonstrate that several of the *lbx1a*-expressing spinal cells co-express *pax2a* (which is expressed by V1, $V0_D$, dI4 and dI6 spinal interneurons (Batista and Lewis, 2008)) and several of the *lbx1a*-expressing spinal cells are inhibitory, while a smaller number are excitatory (Fig. 4). Taken together, these analyses suggest that zebrafish *lbx1a* is expressed in dI4, dI5, dI6 spinal interneurons, like Lbx1 in amniotes.

In contrast to *lbx1a*, *lbx1b* is expressed by progenitor cells, probably in the dP4 or both the dP4 and dP5 domains as *lbx1b* expression is dorsal to *dbx2* (Fig. 4h) and also dorsal to and overlapping with *lbx1a* (Fig. 3c & e). Consistent with *lbx1b* being expressed in progenitor cells, spinal cord expression of this gene is lost in *mindbomb1*^{ta52b} mutants (Fig. 2k and l), in which progenitor spinal cord cells precociously differentiate into post-mitotic neurons, unlike *lbx1a* expression, which is expanded (Fig, 2e & f). This result also suggests that *lbx1b* expression is turned off as cells become post-mitotic, as

(in contrast to *lbx2*, see discussion below) there is not even any expanded expression in the caudal spinal cord, where the "youngest" post-mitotic neurons are located at this stage (Fig, 2l; the spinal cord develops in a rostral – caudal gradient).

lbx2 is expressed in two distinct spinal cord domains. The ventral domain appears to correspond to progenitor cells located below *Tg(evx1:EGFP)*^{SU1}-expressing V0v interneurons (Fig. 4i), suggesting that it is probably the P1 domain and the dorsal *lbx2*-expressing cells are located in the same dorsal/ventral domain as *lbx1a* expression (Fig. 3g), suggesting that *lbx2* may be expressed briefly in some dI4, dI5 or dI6 neurons or the progenitor cells that give rise to them. Analysis of *lbx2* expression in spinal cord cross-sections suggests that some of the more dorsal *lbx2*-expressing cells are post-mitotic, whereas others are located between the progenitor and post-mitotic domains (Fig. 2o and data not shown). However, expression of this gene in *mindbomb1*^{ta52b} mutants, suggests that *lbx2* is predominantly expressed in progenitor cells, as its expression is lost throughout most of the spinal cord of *mindbomb1*^{ta52b} mutants (Fig. 2q). However, there is some expanded expression of *lbx2* in the caudal spinal cord (Fig. 2r) where more recently differentiated spinal cells are located suggesting that *lbx2* expression persists into some post-mitotic cells, although it is also turned off relatively quickly.

To understand how *lbx* spinal expression has evolved in the vertebrate lineage and, in particular, to investigate whether spinal expression of *lbx2* and/or the spinal progenitor domain expression pattern of *lbx1b* have been gained in the ray-finned lineage or lost in the lobe-finned lineage, we examined expression of *lbx1* and *lbx2* in the lesser spotted dogfish *Scyliorhinus canicula* and *lbx1* in the African clawed frog *Xenopus tropicalis* (*X. tropicalis* does not have a *lbx2* gene). *S. canicula* is ideally placed to distinguish between ancestral and derived characteristics, as it is a member of the *chondrichthyes* (cartilaginous fishes), which as the sister group to the *osteichthyes* (bony fish) provides an outgroup to the major *osteichthyan* taxa (Coolen et al., 2008). Our results show that *lbx1* expression in *S. canicula* and *X. tropicalis* is most similar to zebrafish *lbx1a* expression (cf Fig. 2 & Fig. 7) and to mouse Lbx1 (Gross et al., 2002; Jagla et al., 1995; Müller et al., 2002; Schubert et al., 2001). Expression of *lbx1* in all these species is in cells at the lateral edge of the same dorsal-ventral region of the spinal cord. All together, these data suggest that *Lbx1/ lbx1a* spinal cord expression is conserved in all vertebrates. However, in contrast, as Lbx1 is not expressed by spinal cord progenitor cells in amniotes, *S. canicula* or *X. Tropicalis, lbx1b* spinal cord expression presumably evolved in the teleost lineage after the teleost duplication of *lbx1* into *lbx1a* and *lbx1b* (Wotton et al., 2008; Wotton et al., 2010). Our data also suggest that *lbx2* spinal expression has been acquired somewhere in the ray-finned lineage, as this gene is not expressed in the spinal cord of either amniotes or *S. canicula*. It would be interesting in future studies to examine expression of *lbx2* in other teleosts and

other extant vertebrates in the ray-finned lineage such as paddlefish, to determine more precisely when this expression domain evolved. One intriguing possibility is that the spinal cord expression of *lbx2* in zebrafish reflects a rostral extension of the hindbrain expression that is seen in *S. canicular*, although interestingly *Lbx2* is not expressed in the amniote hindbrain (Chen et al., 2001; Chen et al., 1999; Kanamoto et al., 2006).

The fact that both *lbx1a* and *lbx1b* have been maintained in zebrafish and other teleosts suggests that either the functions of Lbx1 have been subdivided between these two duplicates or that one or both of these genes have acquired novel function(s). The observation that *lbx1b* is expressed in different cells to *lbx1a* in zebrafish spinal cord might suggest the latter. However, our mutant studies suggest that both of these *lbx1* genes are required for the spinal cord to have the correct number of inhibitory spinal interneurons, although interestingly only *lbx1a* is required for the spinal cord to have the correct number of excitatory spinal interneurons (Fig. 5). This suggests that *lbx1b* and *lbx1a* are both required, presumably in succession, for correct specification of dI4 and dI6 interneurons, although only *lbx1a* is required for suppression of excitatory fates in these cells. It also suggests that the specification of inhibitory fates and the inhibition of excitatory fates are regulated by independent mechanisms, with different requirements for Lbx1b function. One possible explanation that could explain this, is if the acquisition of an excitatory fate occurs after the loss of the inhibitory fate, and the influence of Lbx1b on the cells does not extend long enough to affect the former. While some of the analyses in mouse have focused on Lbx1's role in specifying neurotransmitter phenotypes (e.g. Cheng et al., 2005), others suggest that in the absence of Lbx1, dI4-dI6 cells transfate into dI1-dI3 interneurons (Gross et al., 2002; Müller et al., 2002). This would also cause a reduction in inhibitory neurons and an increase in excitatory neurons as dI4 and dI6 interneurons are inhibitory whereas dI1, dI2, dI3 and dI5 interneurons are excitatory. In this case, the change in cell fate might be a multistep process, with both *lbx1a* and *lbx1b* being required for the early steps, and only *lbx1a* for the latter steps. As some *lbx2*-expressing spinal cord cells are also located in the dI4-dI6 domain, it is possible that this gene also functions in these processes. Investigation of this possibility was outside of the scope of this study as it would require creating a *lbx2* mutant and combining it with our existing *lbx1a* and *lbx1b* mutants.

The similarity between some aspects of the phenotypes of *lbx1a* and *lbx1b* single mutants and *lbx1a;lbx1b* double mutants suggest that *lbx1a* and *lbx1b* are expressed by at least some of the same spinal cord cells, and hence, that the post-mitotic *lbx1a*-expressing cells may derive from the *lbx1b*-expressing progenitor domain. Consistent with this, as discussed above, the *lbx1b* expression domain overlaps with the most dorsal *lbx1a*-expressing cells. If *lbx1a*-expressing cells do indeed derive from

the *lbx1b*-expressing progenitor domain, this would suggest that these two genes are expressed by the same spinal cord cells, with *lbx1b* being expressed before *lbx1a*. This would further suggest that some of the cell-type specific regulatory elements that control *lbx1* spinal expression may have been retained by *lbx1b* and there has just been a change in the regulation of the temporal specificity of its expression. It would also imply that the more ventral location of many of the *lbx1a*-expressing cells may be due to ventral migration. Interestingly, this would be consistent with mouse, where some of the Lbx1-expressing spinal cells migrate ventrally (Gross et al., 2002).

In conclusion, our data suggest that zebrafish *lbx1a* is expressed by dI4, dI5 and dI6 spinal interneurons and that this expression pattern is conserved throughout the vertebrate lineage. In contrast, *lbx1b* and *lbx2* have novel spinal cord expression patterns that have probably evolved in the ray-finned vertebrate lineage (*lbx2*) or in teleosts (*lbx1b*). Our mutant analyses demonstrate that Lbx1a and Lbx1b are redundantly required for the correct number of inhibitory neurons to be present at 30h, although, only *lbx1a* is required for the correct number of spinal excitatory neurons to be present at this stage. Taken together, the data in this paper have increased our knowledge both of spinal cord evolution and of the genetic mechanisms that establish correct neurotransmitter phenotypes within the spinal cord.

Acknowledgements

We thank Ginny Grieb, Angelica Kowalchuk and Sophie Lutter for help with *in situ* hybridizations. Sophie Lutter also performed some early experiments that led to this project. We thank ZFIN for providing information on nomenclature and other essential zebrafish resources and the Sanger Zebrafish Mutation Project and Stemple lab for providing us with *lbx* mutant alleles. We also thank Andrew Waskiewicz for providing us with the *Tg(lbx1b:EGFP)*^{ua1001} transgenic line and Patrick Wincker, Corinne Da Silva and Hélène Mayeur for help in identifying and sequencing the *S. canicula lbx* sequences. We are grateful to Adrian McNabb, Kirsty Scott, Tomasz Dyl, Henry Putz, Jessica Bouchard, Paul Campbell, Annika Swanson, Ginny Grieb and several SU undergraduate fish husbandry workers for help with maintaining zebrafish lines. We also thank the Marine Biological Association of the United Kingdom, Plymouth and Clare Baker's lab (Department of Physiology, Development and Neuroscience, University of Cambridge) for providing us with *Scyliorhinus canicula* egg cases and Jim Smith's Lab (The Gurdon Institute, University of Cambridge) for providing us with *Xenopus tropicalis* embryos. This research was funded by NIH NINDS R01:NS077947 and NSF IOS 1755354.

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Figure 1. Construction of *Tg(1.6 lbx1a:GFP)^{SU33}* transgenic line.

Schematic showing Shuffle-LAGAN analysis of *lbx1a* genomic region with zebrafish sequence as baseline compared to orthologous regions in mouse and human genomes. Conserved coding sequences are indicated in blue, arrow indicates 5'→3' orientation. Conserved non-coding elements (CNEs) in 3' region are indicated in pink. The region amplified to create the *Tg(1.6 lbx1a:EGFP)^{SU33}* transgenic line is indicated with a red dotted box.

Figure 1

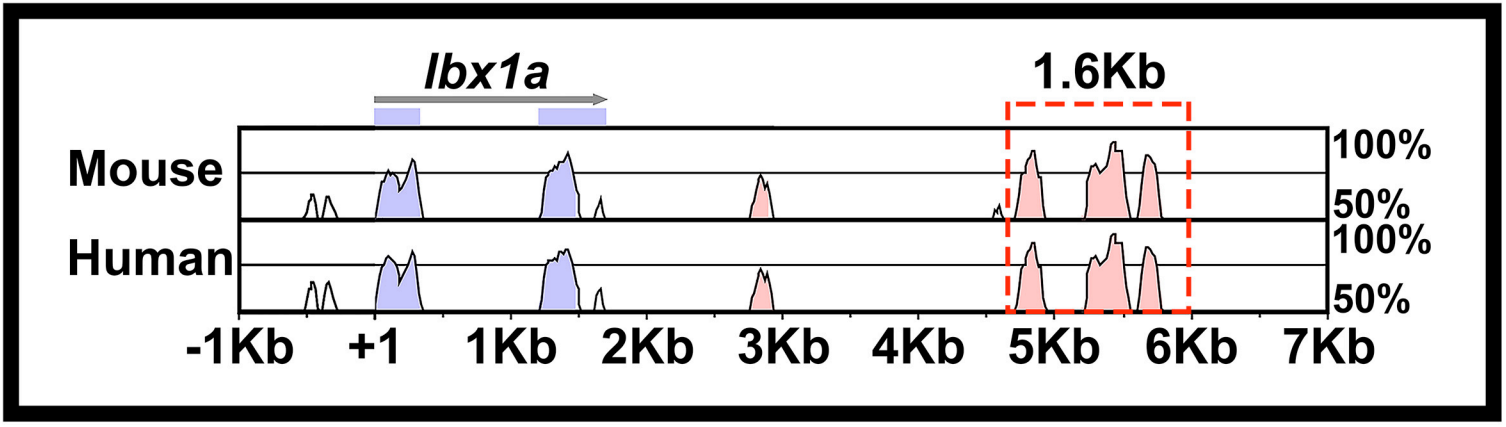

lbx1a
1.6Kb
Mouse
Human
100%
50%
100%
50%
-1Kb
+1
1Kb
2Kb
3Kb
4Kb
5Kb
6Kb
7Kb

Figure 2. Expression of *lbx* genes in zebrafish spinal cord.

(a, b, d, e, g, h, j, k, m, n, p & q) Lateral views of spinal cord expression of *lbx* genes at 18h (18-somites; **a, g, m)**, 24h **(b, e, h, k, n** & **q)** and 30h **(d, j, p)** in WT embryos **(a-c, g-i** & **m-o)** and *mindbomb1*^{ta52b} mutants **(e, k** & **q)** and lateral views of whole *mindbomb1*^{ta52b} mutants at 24h (**f, l & r**). Rostral is left and dorsal up in all cases. **(c, i & o)** Spinal cord cross-sections of 24h WT embryos. **(a)** *lbx1a* is expressed in the hindbrain and rostral spinal cord at 18h, and caudally in a few scattered dorsal spinal cord cells. At 24h **(b)** and 30h **(d)**, expression extends more caudally. **(c)** Cross-section of WT spinal cord confirms that *lbx1a*-expressing cells are located laterally in a post-mitotic dorsal spinal cord domain. **(e)** *lbx1a* spinal expression is expanded in *mindbomb1*^{ta52b} mutant embryos at 24h, again suggesting that the cells expressing this gene are post-mitotic. **(f)** At 24h, this expanded expression is most pronounced in the rostral spinal cord. **(g)** *lbx1b* is expressed in an almost continuous row of cells in the hindbrain and dorsal spinal cord at 18h. This expression persists at 24h and 30h **(h** & **j)**. **(i)** Cross-section of WT spinal cord shows *lbx1b* expression both medially and laterally in the dorsal spinal cord, suggesting it is expressed by both post-mitotic and progenitor cells. **(k)** *lbx1b* expression is lost throughout most of the spinal cord in *mindbomb1*^{ta52b} mutants, suggesting that this gene is expressed by progenitor cells that differentiate precociously in these mutants. **(l)** A small number of cells still express *lbx1b* in the very rostral spinal cord (black arrow head). It is unclear why this region differs from the rest of the spinal cord. **(m)** At 18h, *lbx2* is expressed in a continuous row of cells in the ventral spinal cord and discontinuously in a more dorsal row of cells (indicated with black arrowheads, which point to some of the expressing cells in this dorsal row). **(n)** This expression remains at 24h. **(o)** Cross-sections of WT spinal cord at this stage confirm that the ventral *lbx2*-expressing spinal cord cells are mainly located medially (suggesting they are likely progenitor cells), whereas dorsal *lbx2*-expressing cells (black arrow head) are more lateral (suggesting they are either becoming, or are already, post-mitotic cells). Some are (like in O) located slightly medial to the lateral edge of the spinal cord and some are located at the lateral edge. Somite staining can also be observed outside of the spinal cord (indicated with black asterisks). **(p)** At 30h, expression of *lbx2* in the dorsal spinal cord becomes more difficult to see due to strong somite staining (seen here as out of focus repeated blocks). **(q)** *lbx2* expression is lost in the ventral spinal cord domain in *mindbomb1*^{ta52b} mutants, although a small number of *lbx2*-expressing cells remain more dorsally. This suggests that if *lbx2* is expressed by any postmitotic cells, then it is only for a short period of time. (**r**) There is also expanded expression of *lbx2* in the hindbrain and caudal dorsal spinal cord. The caudal expression is likely to be post-mitotic cells that have not yet turned *lbx2* expression off. Scale bar: 50 µm (**a, b, d, e, g, h, j, k, m, n, p & q**), 200 µm (**f, l & r**), 30 µm (**c, i, o**).

Figure 2

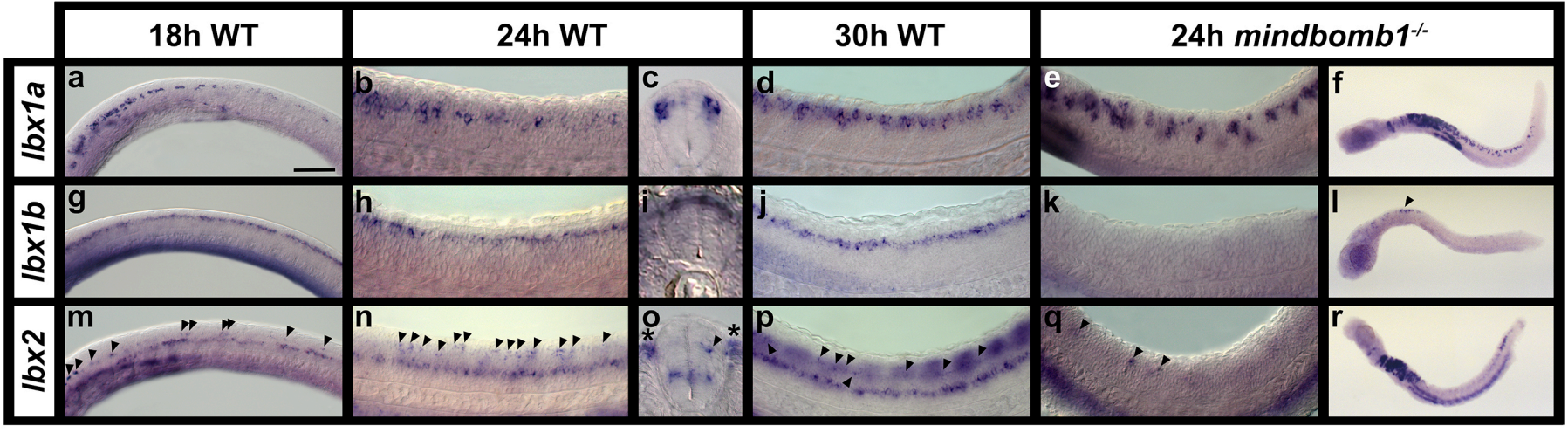

18h WT
24h WT
30h WT
24h mindbomb1-/-
lbx1a
lbx1b
lbx2
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r

Figure 3. Comparisons of zebrafish *lbx1a*, *lbx1b* and *lbx2* spinal cord expression.

Immunohistochemistry for EGFP (green) in *Tg(1.6 lbx1a:EGFP)*^{SU33} (**a, b & f-i**) and *Tg(lbx1b:EGFP)*^{ua1001} (**c-e**) embryos, coupled with *in situ* hybridization (blue) for *lbx1a* (**a-c & e**), *lbx1b* (**d**) or *lbx2* (**g**). Lateral views of spinal cord with dorsal up and anterior left at 27h (**c-d**), 30h (**a, b, g**) and 35h (**f**), cross-section of spinal cord at 27h (**e**) and dorsal views of two different focal planes of the spinal cord at 35h (**h & i**). White dotted box in (**a**) indicates the region shown in magnified view in (**b**). White asterisks in (**a & b**) indicate co-labeled cells. Occasional single-labeled EGFP cells may be the result of weak *lbx1a* expression not being detected in the double staining experiment or they may be cells that used to express *lbx1a* and the EGFP expression has persisted. Single-labeled *lbx1a* cells may be cells that have turned on *lbx1a* expression but have not yet made EGFP protein. Dotted line (**e**) indicates edge of the spinal cord. Co-expression of *Tg(lbx1b:EGFP)*^{ua1001} and *lbx1a* can be seen in the dorsal region of the lbx1a-expression domain. In contrast we did not observe any co-labeled *lbx2* and *Tg(1.6 lbx1a:EGFP)*^{SU33} cells (**g**). (**f**) Dotted white line (drawn slightly to the right of the axon so EGFP expression is still visible) indicates commissural bifurcating axon trajectory. White arrow head indicates where the axon starts to cross the midline. (**h & i**) white arrow head indicates the same neuron whose axon goes ventral in the spinal cord (**h**), and crosses the midline and bifurcates on the other side of the spinal cord (**i**). Scale bar: 50 µm (**a, c, d & g**), 35 µm (**e**), 25 µm (**f, h & i**).

Figure 3

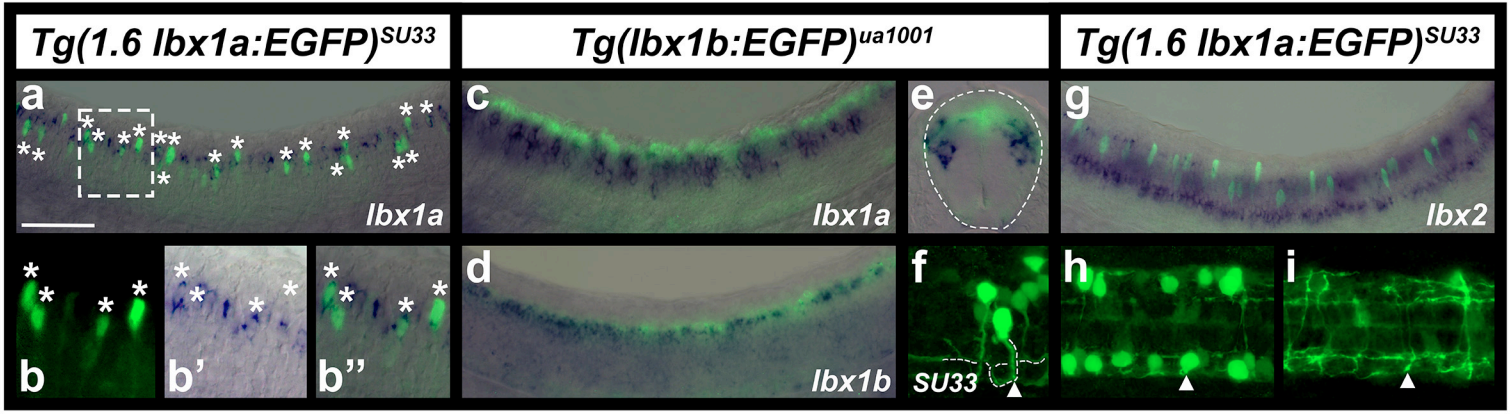

Tg(1.6 lbx1a:EGFP)SU33
Tg(lbx1b:EGFP)ua1001
Tg(1.6 lbx1a:EGFP)SU33
a
lbx1a
c
lbx1a
e
g
lbx2
b
b'
b''
d
lbx1b
f
SU33
h
i

Figure 4. Zebrafish *lbx1a* is expressed by dI4, dI5 and dI6 spinal interneurons.

(a-f) Immunohistochemistry for EGFP (green) in *Tg(1.6 lbx1a:EGFP)*^{SU33} (**a, b & e-g**), *Tg(evx1:EGFP)*^{SU1} (**c, d & i**), and *Tg(lbx1b:EGFP)*^{ua1001} (**h**) embryos, coupled with *in situ* hybridization (blue) for *dbx2* **(a, b** & **h)**, *lbx1a* **(c** & **d)**, *slc32a1* **(f)**, *slc17a6* **(g)**, *lbx2* **(i)**, and *in situ* hybridization (red) for for *pax2a* **(e)**. *dbx2* **(a, b** & **h)** is expressed in dP6, P0 and P1 progenitor domains, whereas *pax2a* **(e)** is expressed by V1, $V0_D$, dI4 and dI6 spinal interneurons and *evx1* **(c, d & i** is expressed by V0v spinal inteneurons. Lateral views with dorsal up and anterior left of spinal cord at 30h **(a & e-h)** and 24h **(c & i)** and cross-sections with dorsal up at 30h (**b**) or 24h (**d**). (**e-g)** panels on the right are magnified views of white dotted box region in left hand panel. White asterisks indicate co-labeled cells. **(b** & **d)** White dotted lines indicate the edge of the spinal cord. Scale bar: 50 µm (**a, c & e-i**), 35 µm (**b & d**), 35 µm (**e', e'', e''', f', f'', f''', g', g'', g'''**).

Figure 4

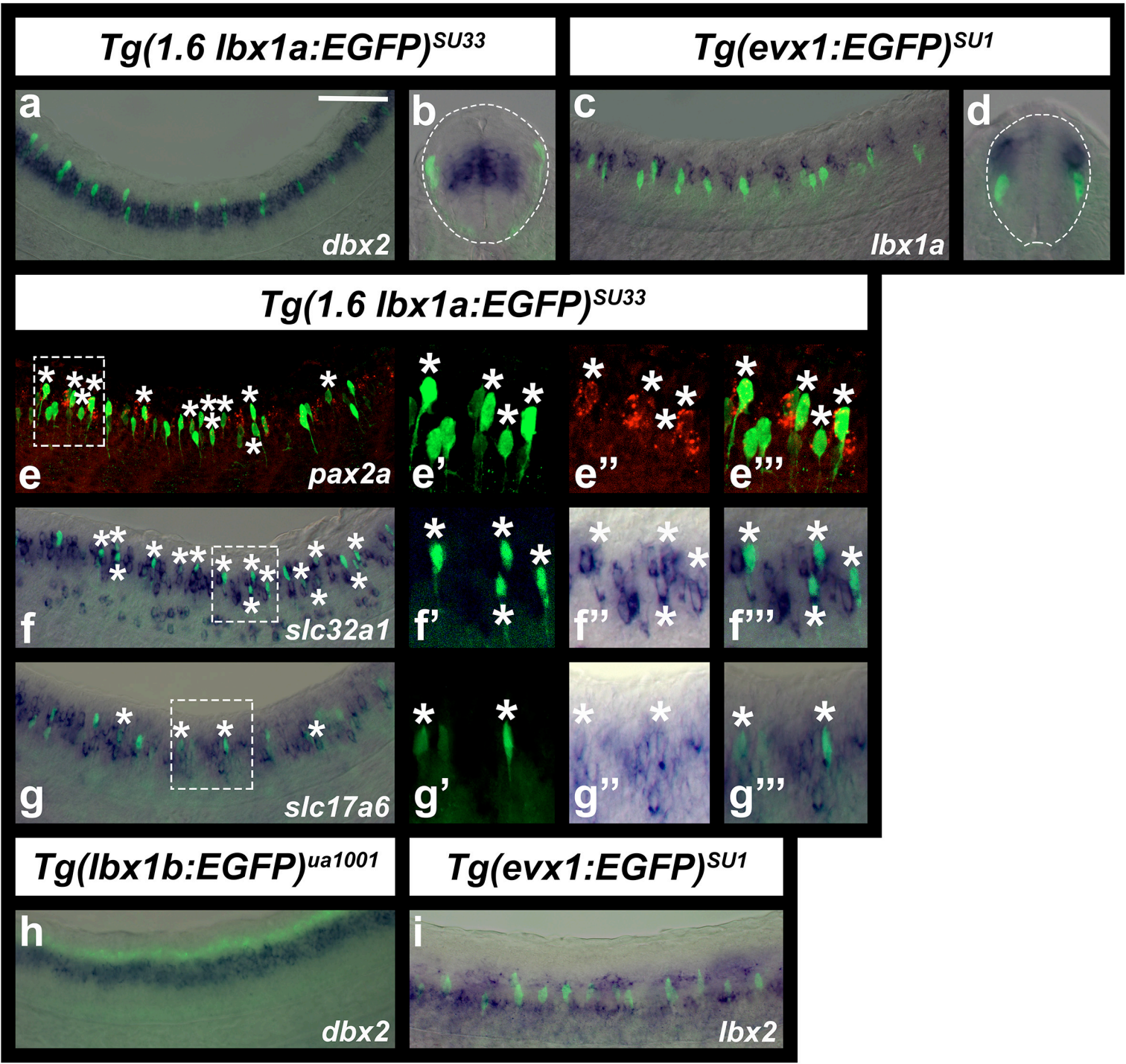
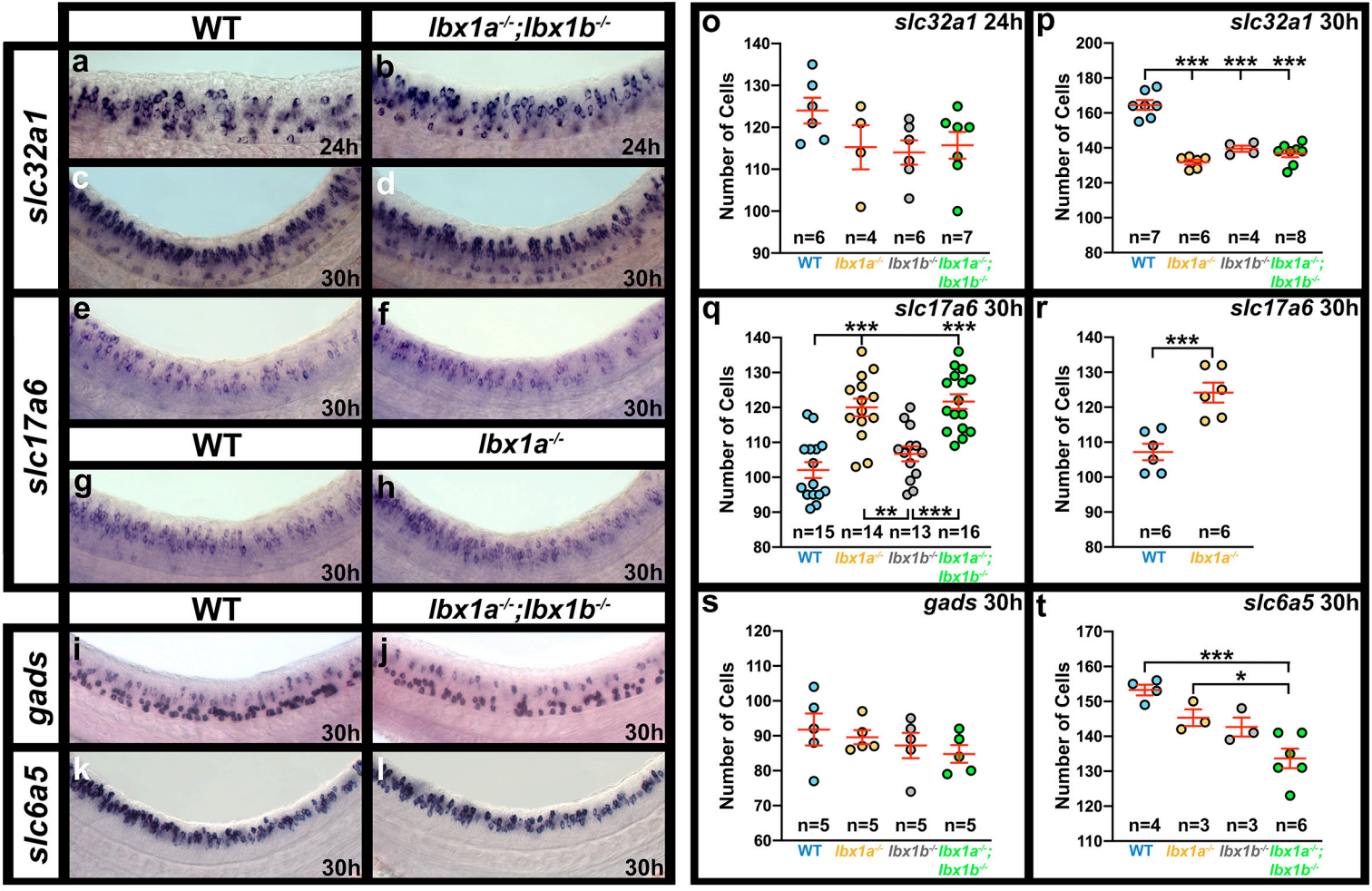

Tg(1.6 lbx1a:EGFP)SU33
Tg(evx1:EGFP)SU1
a
dbx2
b
c
lbx1a
d
Tg(1.6 lbx1a:EGFP)SU33
e
pax2a
e'
e''
e'''
f
slc32a1
f'
f''
f'''
g
slc17a6
g'
g''
g'''
Tg(lbx1b:EGFP)ua1001
Tg(evx1:EGFP)SU1
h
dbx2
i
lbx2

Figure 5. A subset of spinal interneurons have changed neurotransmitter phenotypes in the absence of Lbx1a and Lbx1b function.

Expression of markers of different neurotransmitter phenotypes *slc32a1* (also called *viaat,* marker of all inhibitory interneurons)*, slc17a6* (also called *vglut,* marker of all excitatory interneurons)*, gads* (marker of GABAergic inhibitory interneurons) and *slc6a5* (also called *glyt2,* marker of glycinergic inhibitory interneurons) in $lbx1a^{-/-}$; $lbx1b^{-/-}$ single and double mutant embryos. Lateral views of zebrafish spinal cord at 24h **(a & b)** and 30h **(c-l),** showing *in situ* hybridization expression for genes indicated on the left. Anterior left, dorsal up. Mutant alleles are **(b)** $lbx1a^{hu3569}$;$lbx1b^{hu3534}$, **(d, f, j & l)** $lbx1a^{sa1496}$;$lbx1b^{hu3534}$ and **(h)** $lbx1a^{hu3569}$. **(o-t)** Number of cells (y-axis) expressing indicated genes (indicated at top right) in spinal cord region adjacent to somites 6-10 at 24h **(o)** and 30h **(p-t)**. All data were first analyzed for normality using the Shapiro-Wilk test. All data sets are normally distributed. For the pairwise comparison shown in (**r**), the F test for equal variances was performed. This data set has equal variances and so a type 2 (for equal variances) student's t-test was performed. To accurately compare the 4 different data sets shown in each of panels **o, p**, **q, s, and t**, a one-way analysis of variance (ANOVA) test was performed. All data sets for ANOVA analysis have both normal distributions and homogeneous (homoscedastic, Bartlett's test p value >0.05) variances and so standard ANOVA analysis was performed. The ANOVA results are as follows, only the ANOVA for panels **p, q and t** are significant (**o**: ANOVA ($F(3,197) = 1.812$, $p = 0.1793$), **p**: ANOVA ($F(3,21) = 45.60$, $p = <0.0001$), **q**: ANOVA ($F(3,54) = 18.79$, $p = <0.0001$), **s**: ANOVA ($F(3,16) = 0.8174$, $p = 0.5030$) and **t**: ANOVA ($F(3,12) = 11.05$, $p = 0.0009$) , and so to determine which specific experimental group or groups differed, Tukey's honestly significant difference post hoc test for multiple comparisons was performed. Data are depicted as individual value plots and the *n*-values (number of embryos counted) are also indicated for each genotype. In each plot, the wider, middle red horizontal bar depicts the mean number of cells and the narrower red horizontal bars depict the standard error of the mean (S.E.M.). Statistically significant comparisons are indicated with brackets and asterisks. $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***. Mean, SEM and P values for comparisons are provided in Table 1. Scale bar: 50 µm **(a-n)**.

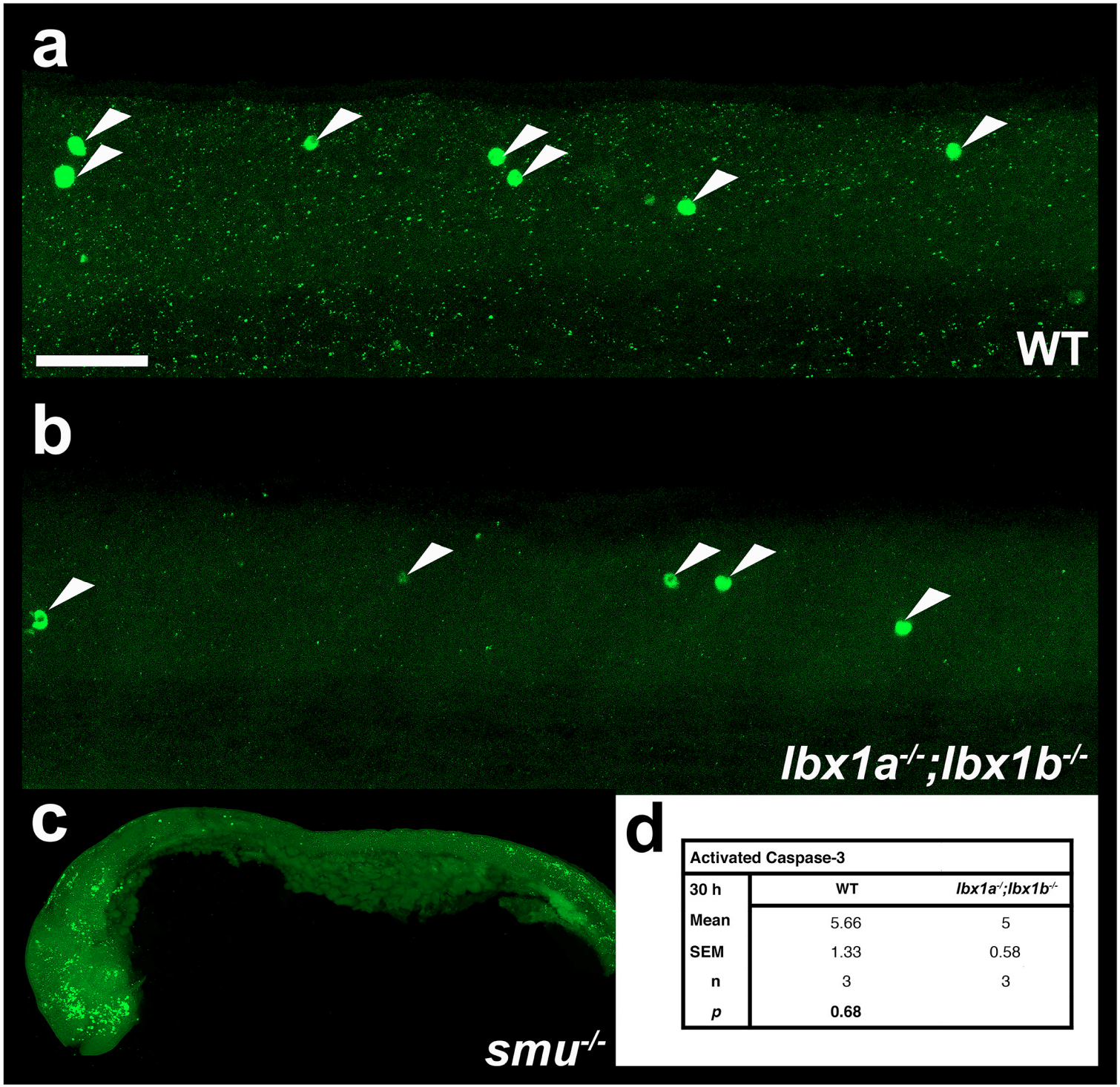
Figure 5


WT
lbx1a-/-;lbx1b-/-
slc32a1
a
b
24h
c
d
30h
slc17a6
e
f
30h
WT
lbx1a-/-
g
h
30h
WT
lbx1a-/-;lbx1b-/-
gads
i
j
30h
slc6a5
k
l
30h
o
slc32a1 24h
Number of Cells
n=6 n=4 n=6 n=7
p
slc32a1 30h
***
n=7 n=6 n=4 n=8
q
slc17a6 30h
**
n=15 n=14 n=13 n=16
r
slc17a6 30h
n=6 n=6
s
gads 30h
n=5 n=5 n=5 n=5
t
slc6a5 30h
*
n=4 n=3 n=3 n=6
WT
lbx1a-/-
lbx1b-/-
lbx1a-/-; lbx1b-/-

<u>Figure 6. There is no increase in apoptosis in *lbx1a;lbx1b* double mutant spinal cords.</u>

(a-c) Lateral views of activated caspase-3 immunohistochemistry in zebrafish spinal cord (**a & b**) or whole embryo (**c**) at 30h in **(a)** WT embryo, **(b)** *lbx1a*^{sa1496};*lbx1b*^{hu3534} double mutant and **(c)** *smoothened*^{b641} mutant. The latter was used as a positive control as apoptosis is increased in the head and tail regions. In all cases anterior is left, dorsal top, **(a** & **b)** White arrowheads indicate Caspase-3 positive cells. **(d)** Numbers of Caspase-3 positive cells in spinal cord region adjacent to somites 6-10 in *lbx1a*^{sa1496};*lbx1b*^{hu3534} double mutants and WT siblings. Values shown are the mean from 3 different embryos, the S.E.M. and the P value from a student's t-test. Scale bar = 50 µm **(a & b)** and 200 µm **(c)**.

Figure 6



Activated Caspase-3		
30 h	WT	*lbx1a*$^{-/-}$;*lbx1b*$^{-/-}$
Mean	5.66	5
SEM	1.33	0.58
n	3	3
p	**0.68**	

Figure 7. *lbx* expression in *Scyliorhinus canicula* (dogfish) and *Xenopus tropicalis* (frog) spinal cords.

(a-c) Expression of *Scyliorhinus canicula (S. canicula) lbx1* and *lbx2* in spinal cord and gut (**d**) of cryo-sectioned embryos at stage 25, dorsal top. **(a)** *S. canicula lbx1* is expressed laterally just above the mid-point of the dorsal-ventral axis. **(b)** *lbx2* is not expressed in the spinal cord although it is expressed in the hindbrain (black arrowheads in **c**) and gut (black arrowheads in **d**). **(e)** whole mount and **(f)** cross-section of *in situ* hybridisation in *Xenopus. tropicalis* (*X. tropicalis*) at stage 35 and 32 respectively. **(e)** *lbx1* is expressed in a line of cells along the whole rostral-caudal axis of the spinal cord. **(f)** As in *S. canicula*, *Xenopus lbx1* is expressed laterally just above the mid-point of the dorsal-ventral axis of the spinal cord.

Figure 7

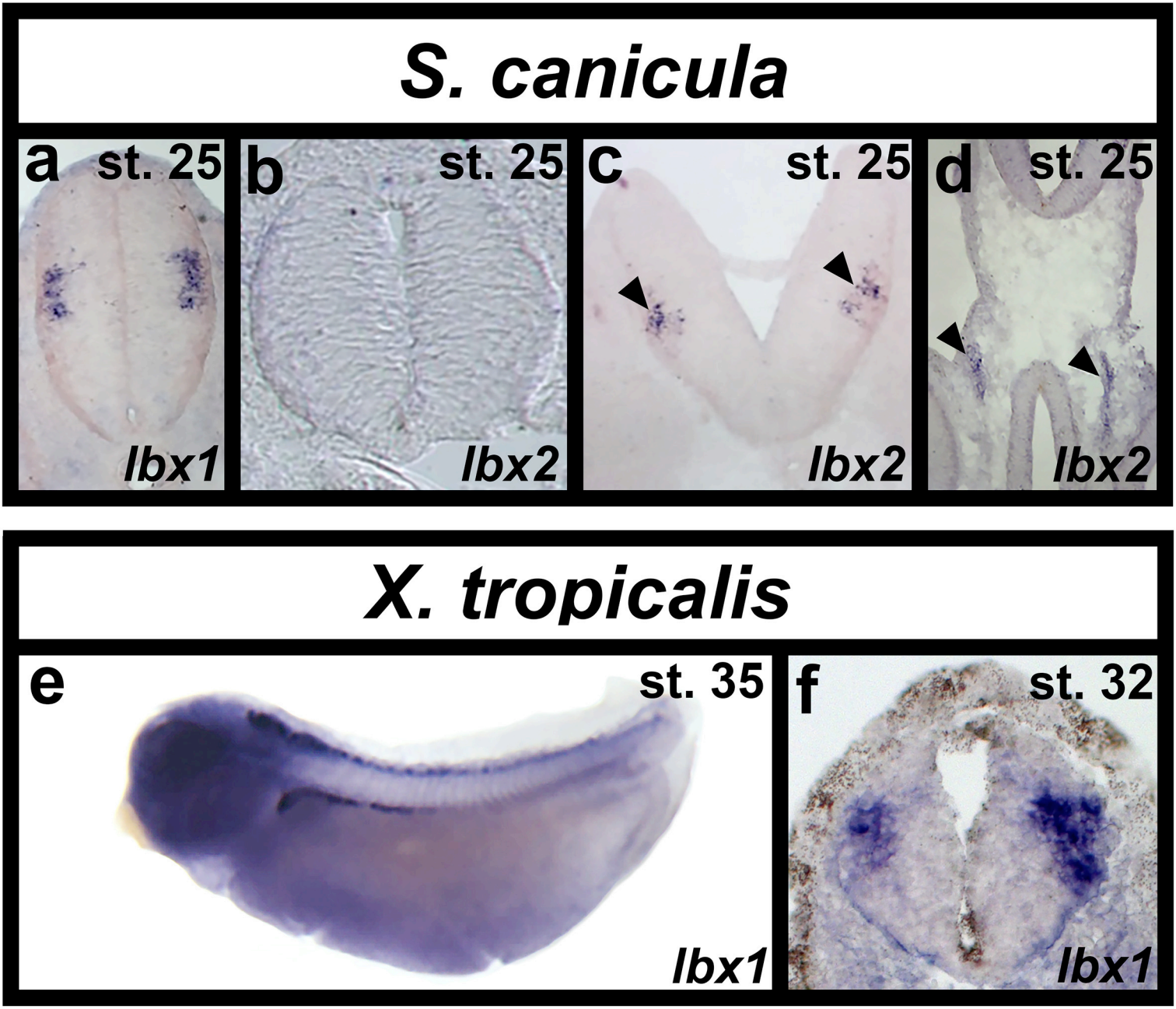

S. canicula
a st. 25
lbx1
b st. 25
lbx2
c st. 25
lbx2
d st. 25
lbx2
X. tropicalis
e st. 35
lbx1
f st. 32
lbx1

Table 1. Number of cells expressing particular genes in WT and *lbx* mutant embryos.

Numbers of cells expressing particular genes (row 1) in spinal cord region adjacent to somites 6-10 and P values of comparisons between embryos with different genotypes (rows 6, 9, 12, 15, 18 and 21). Row 2 indicates the panels in Figure 5 that show the data for that comparison and row 3 indicates the developmental stage assayed. Rows 4, 7, 10, 13, 16 and 19 indicate the genotypes being compared. Values (rows 5, 8, 11, 14, 17 and 20) indicate the mean from at least 3 different embryos +/- standard error of the mean (S.E.M.) for each genotype in that particular comparison. For all genes assayed in both *lbx1a/b* single and double mutants, a standard ANOVA was performed with Tukey's honestly significant post-hoc testing. P values generated by this method of testing are indicated by (‡). For the 30h *slc17a6* experiment performed on *lbx1a*^{hu3569} mutant embryos, a type 2 student's t-test was performed, with p values indicated by ($^{+}$). Statistically significant (P<0.05) values are indicated in bold. For a discussion of why particular tests were used, see materials and methods. P(a) (row 6) compares WT *with* lbx1a single mutant embryos, P(b) (row 9) compares WT with *lbx1b* single mutant embryos, P(c) (row 12) compares WT with *lbx1a;lbx1b* double mutant embryos, P(d) (row 15) compares *lbx1a* single mutant embryos with *lbx1a;lbx1b* double mutant embryos, P(e) (row 18) compares *lbx1b* single mutant embryos with *lbx1a;lbx1b* double mutant embryos and P(f) (row 21) compares *lbx1a* single mutant embryos with *lbx1b* single mutant embryos. Mean cell count values are provided to one decimal place, S.E.M. values to 2 decimal places and P values to three decimal places. The *lbx1a*^{sa1496} allele was used in all experiments except the 24h *slc32a1* double mutant experiment and the 30h *slc17a6* single mutant experiment, in which the *lbx1a*^{hu3659} allele was used instead. In all cases, the *lbx1b* allele used was l*bx1b*^{hu3534}.

	slc32a1	***slc32a1***	***slc17a6***	***slc17a6***	***gads***	***slc6a5***	***lbx1a***
Figure 5 Panels	a, b, o	c, d, p	e, f, q	g, h, r	i, j, s	k, l, t	m, n, u
Stage	24h	30h	30h	30h	30h	30h	30h
WT vs *lbx1a*$^{-/-}$							
Mean Number of Cells + S.E.M.	124.0 ± 3.06 vs 115.3 ± 5.27	164.6 ± 2.80 vs 131.8 ± 1.40	102.1 ± 2.28 vs 120.0 ± 2.54	107.2 ± 2.34 vs 124.2 ± 2.85	91.8 ± 4.59 vs 89.6 ± 2.04	153.3 ± 1.55 vs 145.3 ± 2.40	74.8 ± 1.44 vs 56.1 ± 1.45
***p-value* (a)**	0.379‡	**<0.001**‡	**<0.001**‡	**0.001**+	0.966‡	0.266‡	**<0.001**‡
WT vs *lbx1b*$^{-/-}$							
Mean Number of Cells + S.E.M.	124.0 ± 3.06 vs 114.0 ± 2.89	164.6 ± 2.80 vs 139.5 ± 1.76	102.1 ± 2.28 vs 106.7 ± 2.14	Not applicable	91.8 ± 4.59 vs 87.2 ± 3.62	153.3 ± 1.55 vs 142.7 ± 2.73	74.8 ± 1.44 vs 60.0 ± 2.07
***p-value* (b)**	0.188‡	**<0.001**‡	0.499	Not applicable	0.766‡	0.096‡	**<0.001**‡
WT vs *lbx1a*$^{-/-}$;*lbx1b*$^{-/-}$							
Mean Number of Cells + S.E.M.	124.0 ± 3.06 vs 115.7 ± 3.19	164.6 ± 2.80 vs 136.8 ± 2.08	102.1 ± 2.28 vs 121.7 ± 2.11	Not applicable	91.8 ± 4.59 vs 84.8 ± 2.52	153.3 ± 1.55 vs 133.7 ± 2.81	74.8 ± 1.44 vs 55.6 ± 2.50
***p-value* (c)**	0.301‡	**<0.001**‡	**<0.001**‡	Not applicable	0.471‡	**<0.001**‡	**<0.001**‡
***lbx1a*$^{-/-}$ vs *lbx1a*$^{-/-}$;*lbx1b*$^{-/-}$**							
Mean Number of Cells + S.E.M.	115.3 ± 5.27 vs 115.7 ± 3.19	131.8 ± 1.40 vs 136.8 ± 2.08	120.0 ± 2.54 vs 121.7 ± 2.11	Not applicable	89.6 ± 2.04 vs 84.8 ± 2.52	145.3 ± 2.40 vs 133.7 ± 2.81	56.1 ± 1.45 vs 55.6 ± 2.50
***p-value* (d)**	0.999‡	0.392‡	0.951‡	Not applicable	0.743‡	**0.042**‡	0.998‡
***lbx1b*$^{-/-}$ vs *lbx1a*$^{-/-}$;*lbx1b*$^{-/-}$**							
Mean Number of Cells + S.E.M.	114.0 ± 2.89 vs 115.7 ± 3.19	139.5 ± 1.76 vs 136.8 ± 2.08	106.7 ± 2.14 vs 121.7 ± 2.11	Not applicable	87.2 ± 3.62 vs 84.8 ± 2.52	142.7 ± 2.73 vs 133.7 ± 2.81	60.0 ± 2.07 vs 55.6 ± 2.50
***p-value* (e)**	0.982‡	0.855‡	**<0.001**‡	Not applicable	0.956‡	0.136‡	0.429‡
***lbx1a*$^{-/-}$ vs *lbx1b*$^{-/-}$**							
Mean Number of Cells + S.E.M.	115.3 ± 5.27 vs 114.0 ± 2.89	131.8 ± 1.40 vs 139.5 ± 1.76	120.0 ± 2.54 vs 106.7 ± 2.14	Not applicable	89.6 ± 2.04 vs 87.2 ± 3.62	145.3 ± 2.40 vs 142.7 ± 2.73	56.1 ± 1.45 vs 60.0 ± 2.07
***p-value* (f)**	0.995‡	0.183‡	**0.001**	Not applicable	0.956‡	0.927‡	0.384‡

Table 1. Number of cells expressing particular genes in WT and *lbx* mutant embryos.