AN INVESTIGATION INTO THE DEVELOPMENT AND PATTERNING OF DORSAL LONGITUDINAL ASCENDING INTERNEURONS IN *DANIO RERIO*

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Dedicated to

Terry and Casey

You left before your time was up

Contents

Abstract

The dorsal longitudinal ascending (DoLA) interneurons are an uncommon, seemingly irregularly distributed interneuron type of the developing embryonic zebrafish spinal cord. For reasons not yet understood DoLA interneurons express *tbx16*, a T-box transcription factor originally recognised for its important role in mesodermal development. This is the only cell type expressing *tbx16* in the developing spinal cord, making DoLA neurons one of the few neuronal types that can be identified by expression of a unique molecular marker.

Throughout the natural world regularity in pattern formation is frequent; mechanisms that direct the production of regular patterns have been studied and many are well understood. The creation of irregular "patterns", especially in embryo development has been subjected to far less analysis. This is largely because studies in developmental biology frequently involve methods that disrupt regular patterning while the disruption of an irregular pattern is likely to result in similarly irregular pattern. The DoLA interneurons with their unique genetic marker offer a rare opportunity to investigate the mechanisms behind irregular patterns in development. This is of particular importance in the development of the spinal cord, as most of the known vertebrate spinal interneurons appear to have irregular distributions.

The main focus of the research presented in this thesis has been to try to understand how the distribution pattern of DoLA interneurons is generated. This knowledge may then be extended to other spinal neurons and possibly to other irregular developmental patterns.

In the work described in this thesis the distribution of DoLA interneurons has been extensively examined statistically. It was found that there is an underlying cryptic organisation to their peculiar distribution. This led to the surprising discovery that these neurons migrate rostrally a significant distance along the spinal cord. These neurons were also found in larval zebrafish at much older times than has previously been described, suggesting that they may play a role in post-embryonic stages.

Notch signalling appears to have an influence on DoLA interneuron distribution since perturbing Presenilin (Psen) function affects the number of these cells. Interestingly, DoLA cell number is not affected when Psen1 function is inhibited but increases when Psen2 function is inhibited. Furthermore the wild type level of DoLA interneuron number can be partially rescued by inhibiting Psen1 function in combination with inhibition of Psen2 function.

The creation of transgenic zebrafish lines where GFP is transcribed from *tbx16* promoter sequence is described. These animals were produced to attempt to discover more about the patterning of DoLA interneurons and the function of *tbx16* during development. Serendipitously, one of these transgenic lines expresses GFP in the commissural primary ascending (CoPA) interneurons. This led to the discovery that the CoPA interneurons are marked by *mafba*/*valentino*, revealing a new unique spinal neuron molecular marker.

Declaration

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The identity and distribution of neural cells expressing the mesodermal determinant *spadetail* (Authors: Tamme, R., Wells, S., Conran, J.G., Lardelli, M.)

Independent and cooperative action of Psen2 with Psen1 in zebrafish embryos (Elsevier B.V., Amsterdam)

Cryptic organisation within an apparently irregular rostrocaudal distribution of interneurons in the embryonic zebrafish spinal cord (Elsevier B.V., Amsterdam)

Transgenic zebrafish recapitulating *tbx16* gene early developmental expression (Submitted manuscript, under review)

Signed

.....Date.....

Introduction

1. Zebrafish as a model system

The zebrafish is an attractive model system for studying questions about development (reviewed in [1]). The early stages of development produce an optically clear embryo providing easy viewing of internal tissues. This occurs within a clear protective chorion that may be removed by microdisection with no detriment to the developing animal. The zebrafish, as with many other fish produce eggs that will develop external to the mother, obviating the need to euthanise the mother that may well be a valuable genetic resource. An obvious advantage of this system is that females can consistently produce hundreds of eggs each week. Furthermore zebrafish adults are small (~ 3cm) and are tolerant of housing at quite high densities [2, 3], allowing for large-scale genetic and chemical screening [4-6]. Generation time is relatively short (3-4 months) allowing rapid screening procedures. They are cheap to feed and maintain. The zebrafish community has released version 9 of the genome assembly and has recently handed the data to the Genome Reference Consortium for long-term maintenance.

Embryonic development is rapid, with most tissues and organs including the spinal cord generated within the first 24 hours [7]. There are now many mutant lines available and most molecular biology techniques have been adapted to the zebrafish [8]. Many new technologies have also been developed in the fish; for example morpholino oligonucleotides have proven to be a relatively cheap, reliable and accessible alternative to the creation of mutant lines [9]. They can be used to reduce translation or disrupt splicing of most protein coding genes during embryogenesis and have recently been shown to be amenable to photoactivation [10, 11].

2. The *spadetail* mutation

The *spadetail* (*spt*) mutation was first identified in a screen for recessive mutations affecting neuronal development in zebrafish [12]. The mutation causes severe developmental deficiencies in the trunk region of affected embryos, which generally die within the first week of life. Somites in the trunk area fail to form, resulting in a disordered scattering of mesenchymal cells in this region and the establishment of a large ball of undifferentiated cells which enter the tail bud and remain throughout tail extension [13, 14]. Additionally, *spt* mutants have restricted hematopoesis and vasculogenesis [15] and some individuals fail to develop pronephric kidneys and pectoral fins [14]. Other structures located in the trunk region, such as the notochord and the spinal cord, form normally but become kinked once developed [14]. Interestingly, by 24 hours post fertilisation (hpf), myotomes become evident in the trunk where somites did not form, although they are severely disrupted [14]. It therefore appears that cells in *spt* mutant embryos can be recruited to alternate, incorrect tissues.

Early observations of developing tissue within wild type embryos by injection of a lineage tracer dye have revealed that trunk somitic mesoderm normally arises from convergence of cells in the lateral part of the germ ring during gastrulation [16, 17]. However in *spt* mutant embryos, cells from the lateral germ ring can become located in the notochord; a tissue normally derived from the dorsal side of the early embryo, not the lateral side [14]. Thus it seems that expression of the wild type gene affected by the *spt* mutation is required for correct convergence movements of the lateral marginal zone in the germ ring during gastrulation.

A set of experiments by Ho and Kane (1990) further demonstrated that the *spt* mutation acts cell-autonomously. In this study, wild-type cells transplanted into a mutant background in the lateral marginal zone of the blastoderm were able to migrate to a wild-type position in the trunk. However, *spt* mutant cells transplanted in a wild-type background were unable to migrate normally and were instead found in the tail [13].

3. The *tbx16* gene is mutated in *spadetail* embryos

The full-length cDNA for the gene mutated in *spt* embryos was cloned simultaneously by two separate groups in 1998 [18, 19]. After identifying a T-box sequence within the gene, Ruvinsky *et al*. suggested the name *tbx16* in accordance with T-box gene nomenclature. They also published the first examination of *tbx16* expression patterns through early development [19], noting the earliest expression of *tbx16* throughout the blastoderm at 2.75 hpf. At sphere stage (4 hpf) *tbx16* is downregulated at the animal pole, becoming restricted to the marginal zone of the blastoderm. Gastrulation begins with the involution of cells at the dorsal shield region, followed by involution around the entire blastoderm margin. After involution of cells that become head mesoderm, *tbx16* is no longer expressed in dorsal marginal cells that are fated to become notochord. During involution, *tbx16* is expressed in the epiblast in cells close to the margin, as well as extensively in the hypoblast at a distance of up to several cell diameters from the point of involution. After the completion of epiboly, *tbx16* expression is initiated in the polster - tissue that becomes the hatching gland - as well as in the paraxial and lateral mesoderm of the tail bud [19].

Strong expression of *tbx16* is also detected in adaxial cells flanking the developing notochord. Expression of *tbx16* is downregulated in the presomitic mesoderm before cells form somites, eventually becoming restricted to the most posterior tail region by 24 hpf. At this stage *tbx16* transcripts are also detected within a small population of cells lying dorsolaterally in the developing spinal cord. These cells do not appear to be segmentally arranged, and thus were originally postulated to be Rohon-Beard neurons [19]. We have since shown that *tbx16* expressing cells in the spinal cord are the dorsal longitudinal (DoLA) interneurons (Paper I [20]).

Interestingly, the *Xenopus laevis* orthologue of *tbx16*, *VegT*, is expressed in a similar pattern to *tbx16* in the developing spinal cord. Based on the spatial expression of *VegT* in the spinal cord, it was suggested by some groups that these cells are Rohon-Beard neurons [21, 22]. However, research published simultaneously suggested that *VegT*-positive cells are located in the dorsolateral area of interneuron

formation, excluding the possibility that they are Rohon-Beard neurons [23]. Further examination will determine the identity of *VegT* expressing neurons in *Xenopus*. The possibility exists that *Xenopus* neurons expressing *VegT* are the dorsolateral interneurons, the *Xenopus* equivalent of the DoLA interneuron [24].

Expression of *tbx16* in *spt* mutant embryos is perturbed [18]. Throughout early gastrulation, *tbx16* transcripts are found in the correct locations but with lower expression levels. During gastrulation expression of *tbx16* is present in the ventral germ ring, but is absent in a wide arc of the dorsolateral germ ring. Similarly, during somitogenesis, *tbx16* transcripts are detected in the epiblast at the tail bud, but are absent from the paraxial mesoderm and adaxial cells. This expression pattern is consistent with the phenotypes observed in *spt* mutant embryos.

4. The T-box gene family

The T-box genes encode a family of around 20 transcription factors required for embryo development [25]. The gene family is named after the mutated T locus which was identified in mouse in 1927 as a mutation which is homozygous lethal, but results only in a short tail phenotype in heterozygotes [26]. The T locus was subsequently found to contain a gene, *T* [27] that encodes the protein Brachyury [28]. Brachyury can bind DNA at the partially palindromic sequence T[G/C]ACACCTAGGTGTGAAATT through a region at its amino-terminus called the T-box [28]. Since a number of T-box proteins can bind the Brachyury consensus DNA sequence, DNA binding does not appear to be an intrinsic determinant for specificity [29-31]. An alternative is that T-box protein specificity is determined by binding with cofactors.

There are five subfamilies of genes within the T-box family: Brachyury, Tbrain1, Tbx1, Tbx2 and Tbx6 [32]. Genes in the same class have arisen from duplication events and often show some overlapping expression which may indicate an element of functional redundancy in some cases [33]. Duplication events allow for divergence and diversification of gene function and therefore diversification of organisms [34, 35]. The developing zebrafish tail shows overlapping expression of

tbx16, *tbx6* and *no tail* (*ntl*) [18, 19, 36, 37]. This intimate relationship has been the focus of gene hierarchy studies, which suggest that these T-box genes can interact in combinatorial, additive or competitive relationships to influence their downstream targets [38]. However, this idea has been challenged by recent work examining downstream targets of *ntl* and *tbx16*, which shows that most interactions are additive [39].

The identification of T-box transcription factor target genes began somewhat slowly and has only recently gained momentum, with the employment of large scale screening techniques that produce significant amounts of new data (reviewed in [40]). Mueller and colleagues recently used microarray and gene-set enrichment analyses to find genes downstream of *tbx16* and discovered a large number of candidates, many of which they confirmed by expression analysis [41]. This technique, however, gives no indication as to whether the identified genes are direct binding targets. Another study extended this idea by using computer prediction to identify Ntl and Tbx16 consensus binding sites in genomic regions surrounding microarray candidates and successfully found direct targets [39].

5. T-box genes are important during development

T-box family members are expressed in many tissues and are required for the normal development of numerous organisms such as worms, flies, fish, mice, dogs, frogs and humans [25, 42, 43]. For most T-box family members, there are obvious orthologues with conserved expression and function across a range of species [32]. Other T-box genes appear to be more restricted in their appearance in organisms; for example, the human genome does not appear to have orthologues of the zebrafish *tbx6, tbx16* or *tbx24* genes [44, 45].

T-box gene family members have been shown to be important in early embryo development and a number of them are involved in the major signalling pathways. This is reflected by the numerous human developmental problems that can occur due to mutations in various T-box genes. For example, DiGeorge syndrome (TBX1 [46-

48]), Ulnar-mammary syndrome (TBX3 [49, 50]), Holt-Oram syndrome (TBX5 [51-53]) and X-linked cleft palate and ankyloglossia (TBX22 [54]) are all caused by mutations in T-box genes. As with many developmentally important genes, the T-box gene family has also been implicated in cancer development and progression, including Tbx3 [55], Tbx2 [56], Tbx5 [57], T-bet [58] and Brachyury [59].

In zebrafish, two T-box family members, *tbx16* and *ntl*, are required for the development of all posterior mesoderm [60]. These genes have been shown to rely on signalling by the FGF family members Fgf8 and Fgf24 and *vice versa* in an auto-regulatory feedback loop that is necessary for the development of posterior mesoderm [61]. This feedback mechanism was further verified when signalling by One-eyed pinhead was demonstrated to be affected by FGF signalling and also by mutations in the T-box genes *tbx16* or *ntl* [62].

Zebrafish *spt* mutants also show restricted haematopoiesis [15]. Red blood cells develop in trunk intermediate mesoderm, a tissue which is present in *spt* mutants, however the early expression of haematopoietic genes such as *stem cell leukemia (scl)* [63, 64], *LIM-only 2 (lmo2)* [15], *haematopoietically expressed homeobox (hhex)* [64] and *gata2* [65] in this tissue is absent in *spt* mutants, suggesting that early blood progenitor development is specifically disrupted [66]. Recent work has suggested that this is due to disrupted Wnt signalling in the intermediate mesoderm [41]. Expression of *tbx16* is required cell-autonomously for the expression of these genes, suggesting that they exist downstream of *tbx16* in trunk intermediate mesoderm [66]. Furthermore, the authors show that *tbx16* expression is also required non-cell-autonomously, as there is a requirement for an interaction between embryonic paraxial mesoderm – which is missing in *spt* mutants - and the intermediate mesoderm for the normal development of red blood cells.

Zebrafish *spt* mutants show disruptions to the development of the organiser, an organ critical for generating embryonic polarity and the CNS [67]. The organiser is a mesodermal structure found in the gastrula that is the source of morphogens that stimulate embryonic axial polarity [68, 69]. Recent work has shown that the organiser begins as two, side-by-side cellular fields that subsequently coalesce to form the mature organiser tissue [67]. In *spt* mutant embryos this coalescing action is

disrupted, resulting in a malformed midline. Furthermore, reducing Wnt11 in these embryos accentuates the malformation, indicating that Wnt11 and Tbx16 act together to shape the organiser [67].

Defects in left-right patterning have also been observed in some T-box family mutants. It is known that *tbx16* and *ntl* are required in dorsal forerunner cells (DFC's) that become Kupffer's vesicle [70], in order to bring about correct left-right patterning [71]. In DFC's the gene *pkd2* has been shown to be regulated by *tbx16* to ensure correct expression of left-side-specific genes such as *southpaw*, *lefty1* and *lefty2* [71]. Mis-expression of these genes results in randomisation of heart and gut looping during development [71]. More recent work has suggested that *tbx16* and *ntl* are required at multiple distinct steps to drive the progression of the development of Kupffer's vesicle from the DFC's [72]. Interestingly, asymmetric left-right patterning has also been shown to require sources of Nodal signalling that are dependent on *tbx16* and *ntl* expression and are found close to, but outside of, Kupffer's vesicle suggesting a broader role for these genes in patterning in the embryo [73].

6. Expression of *tbx16* in the central nervous system

At around 16 hpf, strong expression of *tbx16* begins in a tract of dorsolateral cells in the neural tube that do not appear to be segmentally arranged (Paper I). Double-labelling experiments staining for *tbx16* and the early neuronal marker *huC* [74] show that *tbx16* positive cells of the spinal cord are also *huC* positive, therefore verifying that these cells are neurons (Paper I).

The seemingly irregular rostrocaudal distribution, along with the particular dorsolateral position of these cells, has led to the proposal that they are Rohon-Beard neurons in both zebrafish [19] and *Xenopus* [21, 22]. Rohon-Beard neurons are primary mechanosensory neurons [75] with large somata, which extend two thin, ipsilateral longitudinal axons: one ascending and one descending [76]. They are the dorsal-most primary neurons found in both zebrafish and *Xenopus* [24]. Double-labelling of zebrafish embryos confirms that *tbx16*-expressing spinal cord cells co-

express neural markers *islet-1*, *islet-2*, or *islet-3* further verifying that these cells are neurons (Paper I). The pattern of staining reveals that the *tbx16*-expressing cells actually lie slightly ventral to the dorsal-most neurons (Paper I), suggesting that these cells are probably DoLA neurons rather than Rohon-Beard neurons. DoLAs are interneurons with a single ascending longitudinal axon (Paper I). Only longitudinal ascending projections are observed in *tbx16* stained neurons, supporting the suggestion that these cells are DoLAs and not, as previously thought, Rohon-Beard neurons. The discovery of a unique marker of DoLA interneurons was the beginning of the investigation of the distribution of these cells.

7. How to build a spinal cord

The control of patterning the developing vertebrate spinal cord has been examined in a number of model organisms and the pathways and molecules involved show extensive conservation from zebrafish to chick to mouse [77-79].

7a. Gross morphogenetic movements as the tail extends

In vertebrates, the spinal cord originates from the neural plate during gastrulation [80]. Neural plate invagination occurs progressively as the body axis lengthens caudally [81]. Invagination of this tissue results in meeting and fusion of the dorsal regions to form the neural tube, following which initial patterning and differentiation of the neural tube is controlled by a collection of interacting and opposing morphogens [82, 83].

Rapid cell proliferation drives the caudal extension of the body axis. The caudal-most region is maintained in a proliferative and non-differentiating state [84, 85]. Cellular differentiation of this naïve, stem cell-like tissue takes place somewhat later and proceeds caudally following the extending body axis. To allow this to occur, two distinct zones are sustained in the developing neural tube by the presence of opposing morphogens: Fibroblast Growth Factor (FGF) and retinoic acid (RA) (Fig.

1A, [86]). The FGF morphogen is found in the caudal zone and maintains cells in the proliferative non-differentiating state, while the RA zone is rostral [87]. Neural differentiation can only begin when RA inhibits FGF signalling, a signal that is produced by the flanking somites [88, 89]. The capacity of tissue to respond to the opposing FGF and RA signals appears to be regulated by the anteroposterior *hox* genetic code [90].

Fig. 1 - Schema of vertebrate spinal cord structure (based on chick studies) (A) Diagrammatic representation of a dorsal view of an early neural tube. Neuronal differentiation only occurs when Fgf signalling is inhibited by the action of Retinoic acid signalling coming from the somitic mesoderm (for more details see Diez del Corral and Storey 2004). Dorsoventral patterning commences as progenitors leave the stem zone. (B) Schema of a section of the spinal cord. Proliferative neuroprogenitors are located medially adjacent to the central canal (CC) in a region known as the ventricular zone (VZ). Postmitotic differentiated neurons are located laterally in a region known as the mantle zone (MZ). Distinct neuronal subtypes are generated from different domains of progenitors arrayed along the DV axis. Individual progenitor domains are identified by the expression of different combinations of transcription factors (shown in C). The spatial pattern of transcription factor expression in progenitors depends on the action of counteracting gradients of Bmp, Wnt and Shh. N, notochord; RP, roof plate; FP, floor plate. (Reprinted with permission from Ulloa and Briscoe 2007 [91]).

7b. Patterning the dorsoventral axis

Proliferative cells of the spinal cord are located flanking the central canal of the neural tube, in a region called the ventricular zone. When cells stop proliferating in the developing spinal cord and become post-mitotic, they move away from the ventricular zone towards more lateral positions in a region known as the mantle zone of the neural tube (Fig. 1B). A number of well defined progenitor domains in the ventricular zone generate the array of neuronal cells positioned along the dorsoventral axis of the spinal cord [92, 93]. Each of the 11 progenitor domains has a unique "address", defined by expression of a small number of transcription factors (Fig. 1C). The transcription factor addresses depend on opposing gradients of the morphogens Bone Morphogenetic Protein (BMP), Wingless/Integration-1 (Wnt), and Sonic Hedgehog (SHH) [77, 78, 93]. SHH is produced by the floor plate - at the ventral side of the neural tube - and by the notochord - found adjoining the neural tube on the ventral side [94, 95]. The roof plate of the neural tube produces BMPs and Wnts [91, 95]. The unique transcription factor addresses in each of the progenitor domains produce neurons appropriate to that dorsoventral level [96]. It appears, at least in zebrafish, that the Wnt signal has a dual purpose in the developing spinal cord: Wnt is needed for patterning the dorsoventral axis, but at early stages it is also needed for cell proliferation [97].

7c. Patterning the rostrocaudal axis

While genetic partitioning of the dorsoventral axis of the vertebrate spinal cord is well known and has been extensively studied, there appears to be very little genetic partitioning of the rostrocaudal axis [98-100]. The rostral section of the rostrocaudal axis of the vertebrate CNS displays both genetic and morphological divisions: early in development the rostrocaudal axis of the CNS is progressively divided into sections, which will become the forebrain and midbrain, the hindbrain and spinal cord, and the midbrain-hindbrain boundary (reviewed in [80]). The action of various transcription factors, including the *hox* genes, on these regions subsequently produces varying neuronal cell types [80, 101, 102]. In contrast, the

spinal cord, although somewhat genetically segmented, displays no obvious morphological segmentation [103, 104]. Additionally, it appears that neuronal types do not vary along the rostrocaudal axis of the spinal cord [24, 105] with the exception of the poorly characterised IC neurons [106] and some motoneurons subtypes [107]. In the case of the motoneurons, the overlying lateral somitic mesoderm imparts patterning instructions [108] and guides outgrowth of axonal projections from these cells [109, 110].

Another prominent signalling mechanism in the spinal cord is Notch signaling (A whole issue of Current Topics in Developmental Biology has been devoted to Notch signalling [111]). The *presenilin* genes, *PSEN1* and *PSEN2*, encode components of γ-secretase complexes that have been shown to cleave transmembrane proteins such as Notch and the amyloid precursor protein [112]. Cleavage of Notch in this manner releases the Notch intracellular domain (NICD), which facilitates signalling by instigating gene expression changes through its interaction with other DNA binding proteins [113]. Notch signalling has been shown to be important in lateral inhibition and the establishment of correct numbers and types of neurons in the spinal cord [114, 115]. Lateral inhibition occurs between neighbouring cells that influence each other to become specialised cells with different fates. For example, Rohon-Beard neuron numbers are controlled through inhibition of Notch signalling resulting in an upregulation of *neurogenin1* and subsequently *neurogenic differentiation* (*neuroD)* and ultimately leading to an increase in RB cell numbers [116-119].

While motoneurons appear regularly, with patterning information imparted from overlying somitic mesoderm, the bulk of known neurons appear to exist in an irregular rostrocaudal distribution [76]. Rohon-Beard neurons, for example, are not affected by signalling from the mesoderm, but are affected by lateral inhibition during Notch signaling [116, 118]. The Rohon-Beard neurons develop in sufficient numbers to appear to be touching each other along the length of the rostrocaudal axis at a rate of much greater than 1 per hemisegment [76]. The signalling processes that result in the distribution of motoneurons or Rohon-Beard neurons are relatively easy to understand. A third type of distribution in the spinal cord is displayed by most of the other neuronal cell types. They appear irregularly distributed and not close enough to

have arisen through lateral inhibition from a progenitor field [105]. Since most spinal cord neurons seem to have this type of distribution, it is clearly important to discover how this type of distribution arises. I have attempted to dissect the mechanisms behind the seemingly irregularly patterned DoLA interneuron and in the process, have discovered that the distribution is, actually, non-random and can be partially explained by the fact that these neurons begin to migrate rostrally along the spinal cord shortly after their birth (Paper III).

8. Zebrafish spinal cord interneurons

The zebrafish spinal cord is an attractive tissue to study due to the optical clarity of the developing animal, but also due to the relatively low number of neurons present (Fig. 2, [120]). The earliest classifications of neural cells in the developing zebrafish spinal cord described cells based on their morphology, spatiotemporal position and direction of projections originating from the cells [76, 105, 121]. Work since then has further classified the various cells by their neurotransmitter properties [122, 123].

The development of the zebrafish spinal cord and neural circuitry occurs rapidly. By 18 hpf the embryo shows spontaneous coiling, at 27 hpf a robust touch response can induce swimming events, and by 52 hpf the embryo has hatched and exhibits swimming behaviour [124, 125].

Studying the various neural types has been hindered by the fact that there are very few genetic markers unique to individual neural types. Examples where unique markers have been identified include Circumferential Ascending (CiA) interneurons, which express *Engrailed-1b (Eng1b)* [126] while Circumferential Descending (CiD) interneurons express *alx/Chx10/vsx2*, although it appears that perhaps not all CiD neurons express this gene [127]. Ventral Longitudinal Descending (VeLD) interneurons have been shown to express *scl* and *gata3* although these genes are simultaneously expressed by the ventrally positioned Kolmer-Agdur neurons [128]. Paper I shows that DoLA interneurons uniquely express *tbx16* and Paper IV shows

Commissural Primary Ascending (CoPA) interneurons express *mafba/valentino* [129, 130]. The discovery of further unique genetic markers will assist the advancement of the study of zebrafish spinal cord neurons and the circuits they form.

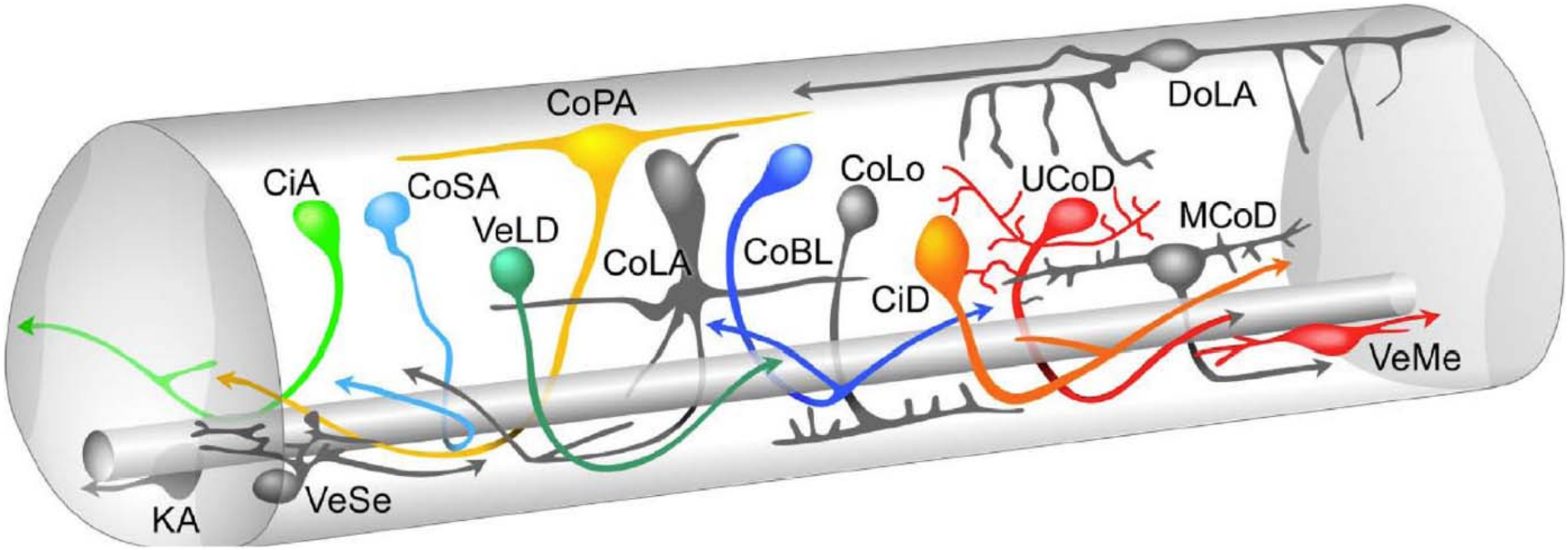

Fig. 2 - Identified interneurons in the embryonic zebrafish spinal cord. Rostral is left and dorsal is up. Reprinted by permission from Macmillan Publishers Ltd: *Nat. Rev. Neurosci.* (2009 July; 10(7): 507-518. Doi:10.1038/nrn2608), copyright (2009)

9. DoLA neurons

The *tbx16* expressing interneurons in the neural tube have been identified as the DoLA neurons (Paper I). DoLA neurons are a class of interneuron extending a single ascending longitudinal axon, sometimes over a distance of five or more segments [131]. By 26 hpf, processes can be seen arising from the soma or the proximal part of the ascending axon [121]. The majority of these project longitudinally, but some have been noted to project ventrally. By 4 dpf the cell appears to have developed extensive branching including numerous ventrally projecting processes that have been postulated to be dendrites [122].

The DoLA cell body is found in the dorsal longitudinal fasciculus (DLF) immediately ventral to the Rohon-Beard cell body [76]. Relatively few DoLA neurons are seen throughout development and until recently it was believed that they apoptose at around 4 dpf [105]. Their distribution along the anterior-posterior axis appears to be irregular, although there is a cryptic organisation to their distribution (Paper III). Expression of *tbx16* in DoLA neurons begins at around 16 hpf, (Paper I) and disappears by 5 dpf (results not published). This corresponds with findings by Bernhardt *et al*. (1992) and Roberts (2000) using alternate methods of marking these cells [24, 131]. The function of DoLA neurons is unknown.

DoLA interneurons have been shown to be GABAergic since they express the genes *glutamic acid decarboxylase 65* and *67* (*GAD65* and *GAD67*) and the neurotransmitter gamma-amino butyric acid (GABA) is present [122, 131]. Additionally, these cells are negative for expression of the gene *glycine transporter 2* (*GLYT2*), indicating that they are not glycinergic [132]. However, it has not been definitively determined whether DoLA interneurons are glutamatergic [122]. Furthermore DoLAs do not show expression of the neurotransmitters dopamine, noradrenaline or serotonin [123].

10. Spatial analysis of geometric distributions

One of the major aims of this project was to examine the distribution of cells along the spinal cord and determine whether this distribution is random, clumped or regular. Several mathematical and statistical procedures must be employed. Clark-Evans nearest-neighbour analysis can be applied to determine which particular distribution a set of data points has taken [133]. However, to obtain an accurate result, this method requires at least 30 data points. The number of DoLA interneurons in each embryo is 21.4 ± 3.4 at 24 hpf (Paper I). For smaller quantities of data points, the Poisson law of small numbers may be used to determine if a data set is distributed randomly [134, 135]. If a data set does not fit a Poisson distribution, this is evidence that the distribution is uniform or clumped. Fourier analysis can identify periodic signals and thus indicate uniform distributions. Consequently a negative response to a Fourier analysis indicates that the data set is non-uniform [136]. In Paper III the distribution of DoLA cells in each embryo was examined for the existence of a Poisson distribution and then the data set underwent Fourier analysis.

Other mathematical tools may also be used to reveal hidden repeating patterns within linear distributions. Cross-correlation is a technique that is able to detect patterns between two related data sets [137]. While autocorrelation is the correlation of a data set with itself [137] and is usually used exclusively for measurements in time, spatial autocorrelation measures the correlation of data points in a set within geometric space. Spatial autocorrelation is more complex than autocorrelation because it is multidirectional [137]. The statistical analysis of the degree of dependence among data points can be undertaken by various means. Common methods are Moran's *I* [138, 139] and Geary's *C* [140]. Moran's *I* is a measure of global spatial autocorrelation while Geary's *C* is more adept at gauging local spatial autocorrelation [141]. In searching for patterns, it is possible to subject a data set to partial autocorrelation, which lowers the influence of intervening data points for that particular interval space [142]. In Paper III the distribution of cells within each embryo underwent analysis for partial autocorrelation followed by spatial autocorrelation and also auto-correlation. Moran's *I* was used to test statistical validity.

11. Generating transgenic animals for investigation of DoLA interneurons

Another aspect of this project was to develop transgenic zebrafish lines expressing GFP under the control of the *tbx16* promoter region. Genetic modification of organisms by the intentional introduction of exogenous DNA has been performed for many years. The first transgenic organisms were developed to confer advantageous attributes to agriculturally important crops or to generate medically important drugs (reviewed in [143, 144]). The first organisms to undergo transgenesis were prokaryotes due to their obvious simplicity. In the early 1980's the first animal transgenics became available – mice [145, 146], *Drosophila* [147] and goldfish [148]. These events paved the way for an explosion in the creation of transgenic organisms that would be valuable for molecular and genetic studies.

The addition of "reporter genes" such as β-galactosidase, chloramphenicol acetyltransferase or firefly luciferase to introduced genes allowed the tracing of gene expression and the dissection of cell lineage progression and tissue development [149-151]. These reporter genes are enzymes that require the addition of substrates or cofactors that limits their function. The ability to trace gene expression became infinitely more practical with the production of a physiologically stable green fluorescent protein (GFP) that allowed researchers to observe tissue development in real time [152, 153]. This was rapidly followed by the production of enhancements to the physical properties of the protein and colour variations to allow wider biological application [154, 155] which peaked with, perhaps, the pinnacle of scientific capitalism, the Glofish [156]. A further recent development has been the cloning of the gene that produces Kaede. Kaede can be irreversibly photoconverted from fluorescing green to fluorescing red using ultra violet light [157]. This allows the observation of the behaviour of cells as the cell recycles red fluorescing Kaede and slowly reverts to fluorescing green.

The first stable transgenic zebrafish lines were created as proof of principle in 1988 [158]. This was quickly followed by the construction of transgenic zebrafish to study tissue development and gene expression [159, 160]. The reporter genes

previously common to transgenic animals were superseded by the demonstration of GFP as a practical reporter gene in zebrafish [161]. For the first ten years, transgenic zebrafish lines were produced by the inelegant method of injection of large quantities of naked linear or circular DNA. This process, while successful, was laborious due to low germline transmission rates (2-5%) and often resulted in the undesirable production of concatemers at the insertion site or occasional chromosomal rearrangements [162, 163]. Therefore, the zebrafish community turned to known transposable elements that have been used in other organisms to successfully create transgenics. To date we have seen attempts with the Tc3 transposon from *Caenorhabditis elegans* [164], mariner from *Drosophila mauritiana* [165], the synthetic transposon Sleeping beauty [166] and Tol2 from *Oryzias latipes* [167]. These transposable elements have been shown to increase the rate of transgene insertion into the genome. Paper IV describes the method used to create transgenic zebrafish lines: the injection of a linearised DNA construct resulting in successful but low germline transmission rates.

Summary of papers and continuity

Paper I presents multiple lines of evidence that the cells labelled by *tbx16* expression in the developing zebrafish spinal cord are the DoLA interneurons. Prior to this paper, *tbx16*-expressing cells in the spinal cord were proposed to be the Rohon-Beard sensory neurons. While Rohon-Beard neurons are found dorsal of the dorsal longitudinal fasiculus (DLF), *tbx16*-expressing cells are shown to be in intimate contact with the DLF. Co-staining for *tbx16* transcripts and multiple different neuronal markers such as *huC*, *isl-1*, *-2* and *-3* indicate that the cells co-expressing these genetic markers consistently lie immediately ventral to the Rohon-Beard neurons. While a number of neurons are found at this dorsoventral position in the spinal cord, there is only one neuron type that exhibits the longitudinal ascending projection required to explain the regular appearance of *tbx16* transcripts in putative projections rostral to the main cell body. The paper concludes that *tbx16*-expressing cells in the spinal cord are the DoLA interneurons.

Statistical analysis reveals a tendency of DoLA interneurons to appear more frequently at rostral levels than caudal levels in the spinal cord. Additionally, it was found that the most rostral position of these cells progresses towards the head as embryonic development proceeds. This led to several of hypotheses: that the DoLA interneurons continue to be born at rostral positions as the CNS matures, or that these cells undergo programmed cell death at caudal positions, or that that they may migrate rostrally in the spinal cord subsequent to their differentiation. Furthermore, extended staining for *tbx16* transcripts revealed previously unreported expression in the dorsocaudal region of newly formed somites that consistently neighbours DoLA interneurons. This led to the hypothesis that there may be an "inefficient" signal produced by the regularly placed somites which induces irregular *tbx16* expression in the spinal cord.

Notch signalling is a common mechanism to generate the correct number and distribution of various neurons in the CNS. Presenilin proteins are involved in catalytic processing of a number of transmembrane proteins such as Notch to instigate their activation. Paper II investigates the independent and cooperative action of

Presenilin1 (Psen1) and Presenilin2 (Psen2) in zebrafish embryos. This paper shows that DoLA cell number is unaffected when Psen1 function is inhibited but increases when Psen2 function is inhibited. Furthermore, the wild type number of DoLA neurons can be partially rescued by additionally reducing Psen1 function. Additionally this suggests a role for Notch signalling in the development of DoLA interneurons.

Paper III further examines the patterning of the DoLA interneurons. Statistical analysis reveals that the apparent irregular pattern is actually likely to be a clumped distribution and is therefore not irregular. Additionally the left and right sides of the embryo display a significant cross-correlation: that is, if a DoLA neuron is found at a particular rostrocaudal level in the spinal cord with respect to the overlying somites, then there is a significant chance that there will be a DoLA neuron on the opposite side of the spinal cord at the same rostrocaudal level.

Using a novel labelling technique it was discovered that the DoLA interneurons migrate a substantial distance rostrally within the spinal cord after differentiating. Furthermore these are the only cells in the spinal cord that migrate during this period of embryonic development. Further examination shows that most of the DoLA interneurons will migrate a distance of up to 5 somite lengths. This correlates with data presented in Paper II showing the difference in the most rostral position of DoLA cells at various embryonic developmental times. Taken together, these findings suggest a mechanism explaining the pattern of the DoLA interneurons. We propose that there is simultaneous birth of pairs of cells in the tailbud, which then move to either side of the spinal cord and then vary in their rostralwards migration distance.

Additionally the uncaging labelling technique described in Paper III was able to detect DoLA interneurons in 8-day-old larva. This is a significant increase on the reported life span of these cells since prior analyses have reported that these cells disappear from the spinal cord before 5 dpf. This suggests that the DoLA cells may play a role in post-embryonic stages.

Paper IV describes the creation and analysis of transgenic zebrafish expressing GFP under the control of the *tbx16* promoter region. These transgenic embryos display faithful recapitulation of early endogenous *tbx16* expression in the presomitic mesoderm, the polster and subsequently the hatching gland. Later expression of GFP diverges somewhat from endogenous expression of *tbx16* indicating that the genomic region required to fully duplicate expression is larger than the 5 kilobases of DNA that was used. Serendipitously, one of the transgenic lines expresses GFP in CoPA interneurons. This led to the discovery that CoPA interneurons express *mafba/valentino*, revealing a new unique spinal neuron molecular marker.

Paper I

The identity and distribution of neural cells expressing the mesodermal determinant *spadetail*

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NOTE:
Statements of authorship appear on pages 27-28 in the print copy of the thesis held in the University of Adelaide Library.

BMC Developmental Biology

Research article

The identity and distribution of neural cells expressing the mesodermal determinant *spadetail*

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Abstract

Background: The *spadetail* (*spt*) gene of zebrafish is expressed in presomitic mesoderm and in neural cells previously suggested to be Rohon-Beard neurons. The mechanism(s) generating the apparently irregular rostrocaudal distribution of *spt*-expressing cells in the developing CNS is unknown.

Results: *spt*-expressing neural cells co-express *huC*, a marker of neurons. These cells also co-express the genes *islet-1*, *-2* and *-3* but not *valentino*. The *islet-1* gene expression, irregular distribution and dorsolateral position of *spt*-expressing cells in the developing CNS are characteristic of dorsal longitudinal ascending (DoLA) interneurons. Shortly after their birth, these neurons extend processes rostrally into which *spt* mRNA is transported. At 24 hours post fertilisation(hpf), *spt*-expressing neurons occur most frequently at rostral levels caudal of the 5th-formed somite pair. There is no apparent bias in the number of *spt*-expressing cells on the left or right sides of embryos. Extended staining for *spt*-transcription reveals expression in the dorsocaudal cells of somites at the same dorsoventral level as the *spt*-expressing neurons. There is frequent juxtaposition of *spt*-expression in newly formed somites and in neurons. This suggests that both types of *spt*-expressing cell respond to a common positional cue or that neurons expressing *spt* are patterned irregularly by flanking somitic mesoderm.

Conclusions: *spt*-expressing cells in the developing CNS appear to be DoLA interneurons. The irregular distribution of these cells along the rostrocaudal axis of the spinal cord may be due to "inefficient" patterning of neural *spt* expression by a signal(s) from flanking, regularly distributed somites also expressing *spt*.

Background

The spinal cord of vertebrates shows no apparent morphological metamerism. However, the pattern of motor and sensory axonal projection from the spinal cord shows a metameric distribution that is patterned by the flanking somites [1,2].

In developing zebrafish, both metameric and non-metameric patterns of neuron distribution can be observed.

When primary motoneurons first arise in the developing ventral spinal cord, three such cells are present per hemisegment [3,4]. Mutation of the gene *spadetail* (*spt*) causes changes in somite formation that affect this pattern of motoneuron formation. This shows that motoneuron patterning is controlled by signals from the somites [5–7]. In contrast, the Rohon-Beard sensory neurons in the dorsal central nervous system (CNS) show no segmental distribution and are not affected by mutations affecting somite formation [5]. However, mutations such as *bmp2b*/*swirl*, *bmp7*/*snailhouse* affecting signalling by members of the bone morphogenic protein (BMP) family, [8]) and changes in Notch signalling [9][10][11] can affect the number/differentiation of these cells.

Rohon-Beard neurons, when they arise, are sufficiently numerous to be found adjacent to every somite (i.e. in each "hemisegment"). However, a third type of neural cell distribution exists with less than one cell per hemisegment. For example, dorsal longitudinal ascending (DoLA) interneurons are found at a frequency of 0.06 per hemisegment for the 5^{th}- to 8^{th}-formed flanking somite pairs in embryos at 18 hpf [12]. The mechanisms that control these irregular distributions are unknown.

The *spt* mutation was originally described by Kimmel et al. in 1989 [13] as a γ ray-induced mutation affecting trunk development including somite formation. Closer analysis of the effect of this mutation on development has shown that *spt* controls convergence movements and the differentiation fate of mesodermal precursors of the trunk [13–17].

The locus for *spt* mutations was identified by Griffin et al. in 1998 [18]. They showed that the *spt* gene encodes a T-box protein similar to those encoded by the *Xenopus* gene *Xombi* (also known as *Antipodean*, *BraT* or *VegT*) and the chick gene *Tbx6L*. *spt* is transcribed in caudal paraxial mesoderm before its differentiation to somitic mesoderm. *spt* is also expressed in irregularly distributed neural cells that have been suggested, on the basis of their position and distribution, to be Rohon-Beard neurons [19].

In the work described in this paper, we show that the neural cells expressing *spt* have the characteristics of DoLA interneurons. We then examine the distribution of *spt*-expressing neurons on the rostrocaudal axis and on the left and right sides of embryos. Intriguingly, we have discovered low-level expression of *spt* in the dorsocaudal extremities of newly formed somites that corresponds in dorsoventral level and, frequently, rostrocaudal position, to newly formed neurons expressing *spt*. This distribution of *spt* expression suggests the possible existence of an "inefficient" mechanism producing an irregular pattern of neuron distribution based on a regularly patterned flanking structure (somitic mesoderm).

Results

Neural spt-expressing cells have the characteristics of DoLA neurons

Cells expressing *spt* in the developing central nervous system have previously been suggested to be Rohon-Beard neurons [18,19]. To confirm their neuronal nature, we double-stained embryos for *spt* expression and the neuronal marker gene *huC*[20]. We observed coexpression of *spt* with *huC* confirming that these cells are neurons (Figure 1D).

To test the idea that *spt*-expressing neurons are Rohon-Beard neurons we double-stained embryos for expression of *spt* and the *islet* (*isl*)-*1*, *-2* or *-3* genes [6,7,21] or *valentino* (*val*, [22]) that have been stated to be expressed in these cells. Interestingly, the *spt*-expressing neurons also express all three known *isl* genes but not *val* (Figures 1E,1G,1H,1I). In embryos at 22 hours post fertilisation (hpf, at 28.5°C), *spt*-expressing cells express *isl-1* from the moment of their first detection at the caudal end of the developing CNS. *isl-2* and *isl-3* coexpression with *spt* is more easily visible at more rostral levels. In every case, the cells co-expressing the *spt* and *isl* genes are located just ventral to dorsally located cells expressing *isl* genes alone, i.e. Rohon-Beard neurons. The *isl-1* expression, rostrocaudal distribution and dorsolateral position of these cells are characteristic of DoLA interneurons [6,21]. We cannot state with certainty that all DoLA neurons express *spt*, only that all DoLA neurons expressing *isl-1* also appear to express this gene. Contrary to an earlier report [7], we observed expression of *isl-2* and *isl-3* in these interneurons. This might be explained by difficulty in distinguishing DoLA neurons from Rohon-Beard neurons at the rostral levels where *isl-2* and *-3* expression is more easily observed.

spt mRNA is transported into neurite-like structures

Soon after their differentiation in the central nervous system, a rostrally-projecting process of the *spt*-expressing neurons can be observed to contain *spt* mRNA (Figure 1B). This process may, in fact, become the future ascending axon of the DoLA neurons. The transport of *spt* mRNA into this process presumably is an active rather than passive process since other mRNAs, such as those of *huC* and the *islet* genes, are not similarly localised (data not shown).

We attempted to observe the pattern of axonal projection from *spt*-expressing neurons at later times after their differentiation. We stained embryos at 22 hpf to reveal both *spt*-transcription and the presence of acetylated tubulin (that labels axons). Confocal imaging of *spt*-expressing neurons

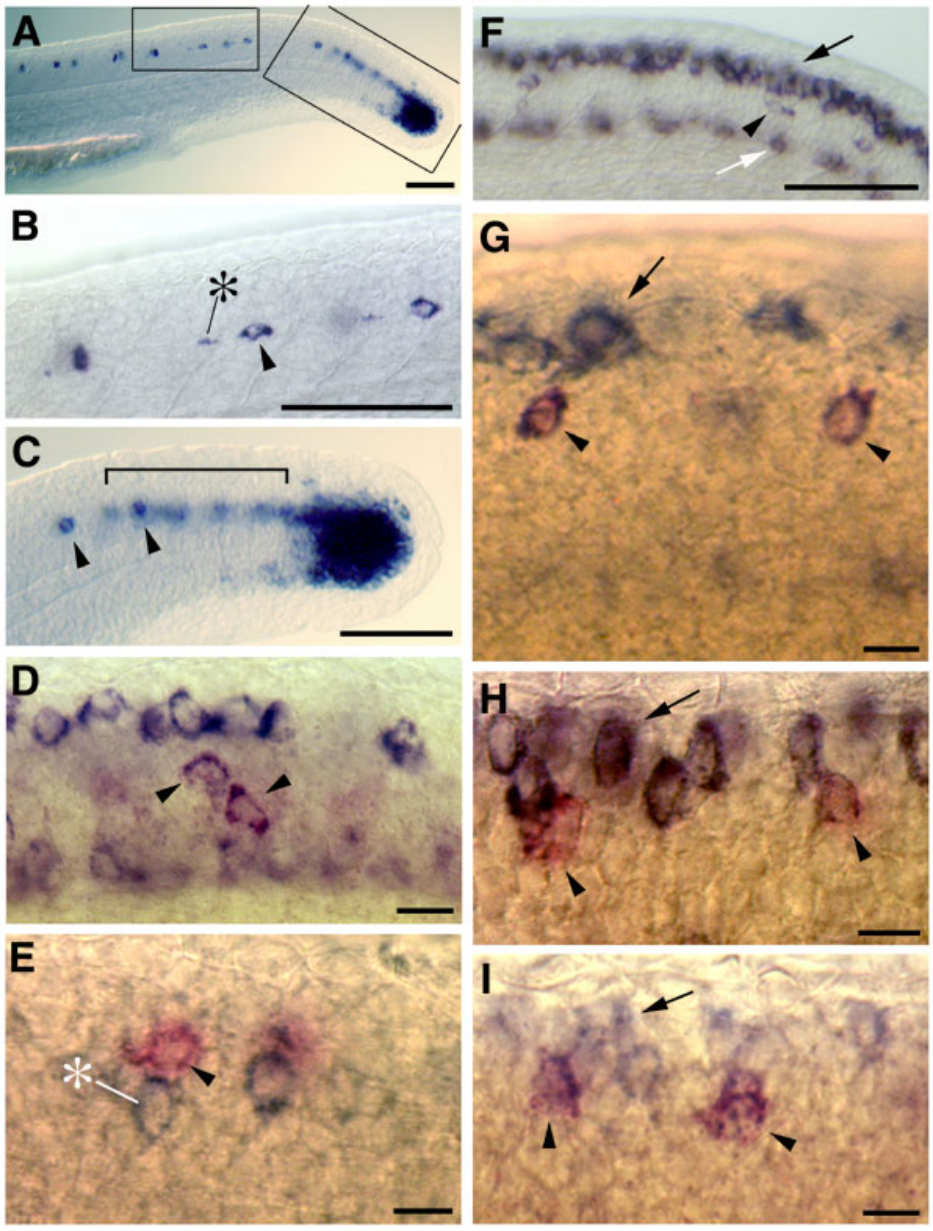

Figure 1
Whole mount *in situ* transcript hybridisation analysis of the expression of *spt* and other genes in the tail and trunk of zebrafish embryos at approximately 22 hpf. In all images, dorsal is up and rostral is to the left. An apparently irregular rostrocaudal distribution of *spt*-expressing cells is seen in the developing CNS rostral to the domain of expression in the presomitic mesoderm of the extending tail (A). Boxed areas in A indicate parts of the image magnified in B and C. Shortly after their birth, these cells extend a process rostrally (indicated by a black asterisk in B) into which *spt* transcript is transported. *spt* is expressed in newly formed somites in a restricted region, the "somitic trail" (bracketed in C), at the same dorsoventral level as *spt*-expressing cells in the developing CNS (black arrowheads in any panel). The *spt*-expressing cells in the developing CNS (red stain) co-express *huC*, a marker of neurons (blue stain in D). A probe that identifies cells transcribing *val* (blue stain) shows that the *spt*-expressing neurons (red stain) are not identical with these (E). Transcription of the *isl-1* gene (see F) is seen dorsally in Rohon-Beard neurons (black arrows in any panel), and ventrally in motoneurons (white arrow). Intermediate between these two levels are DoLA neurons that also express *isl-1* (black arrowhead). Double staining with *isl-1* (blue) and *spt* (red) shows that these intermediate-level neurons express *spt* (G). Costaining of *spt* (red) with *isl-2* (blue in H) and *isl-3* (blue in I) shows that the DoLA neurons also apparently express these genes, although the onset of expression occurs more rostrally than for *isl-1*. Scale bars equal 100 μm in A, B, C and F and 20 μm in D, E, G, H, I.

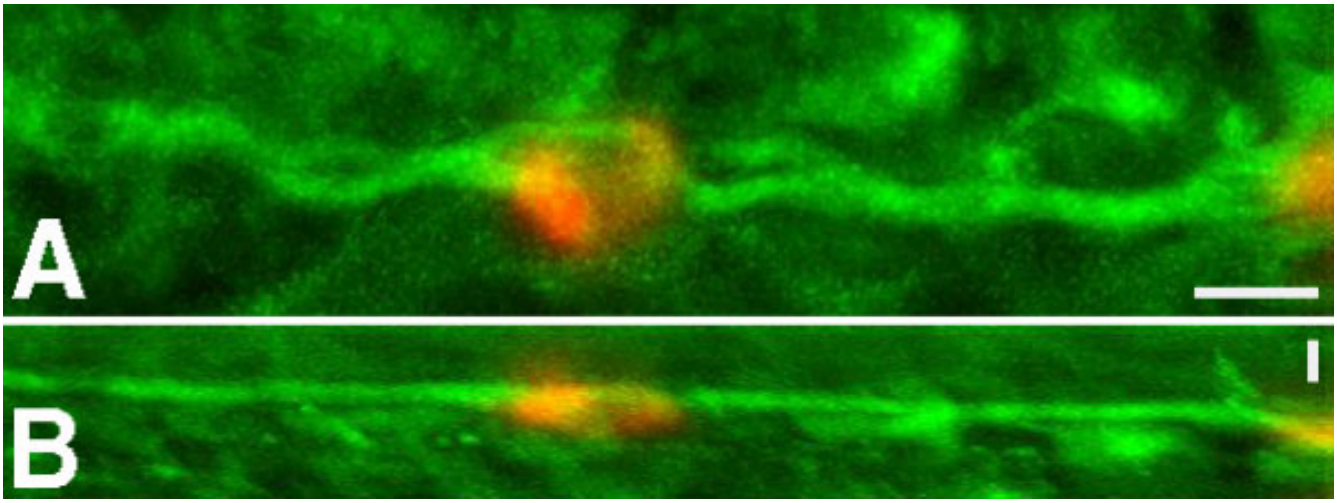

Figure 2
Close association of neurons expressing *spt* with the dorsal longitudinal fasciculus. Images shown are projections of serial 0.5 μm optical sections through a 22 hpf embryo stained to reveal *spt* transcripts (red) and acetylated tubulin (green) that marks axons. The cell shown lies in that part of the developing spinal cord midway along the yolk extension. Rostral is to the left in both images. A shows a lateral projection with dorsal to the top. B shows a dorsal projection with medial to the bottom and lateral to the top. The size bar in A indicates 10 μm. B has an identical rostrocaudal dimension but the mediolateral dimension is compressed. The size bar in B indicates 10 μm in the mediolateral dimension.

in the region of the spinal cord dorsal to the yolk extension showed that these cells lie alongside the dorsal longitudinal fasciculus (DLF, Figure 2). Their proximity to the DLF obscured the pattern of axonal projection from these cells. We did not observe the presence of *spt* transcript in axons near these cells.

Dorsoventral and rostrocaudal correspondence of caudal spt expression in the somitic mesoderm and developing CNS

Extended staining for *spt* expression allowed us to observe *spt* mRNA in recently-formed somites at 24 hpf just rostral to the previously observed, high-level expression of *spt* in the presomitic mesoderm. This expression is not present throughout the somites but, rather, only at the same dorsoventral level as *spt*-expressing cells in the developing spinal cord. From a lateral perspective, this gives the impression of a "trail" of *spt*-expressing cells in the somitic mesoderm left behind by the extending tail tip (Figures 1C, 3A,3B,3D).

The somitic expression of *spt* is strongest in the dorsocaudal cells of these structures (Figure 3). Observation of this region from a dorsolateral perspective shows that cells expressing *spt* in the developing spinal cord most commonly form so that they are in direct juxtaposition with these cells across the basal lamina (Figures 3C,3E; at least 76% of observed cases, n = 25). However, they do not form adjacent to every somite. This distribution suggests that: 1) the *spt*-expressing neurons are either generated in response to signals from the dorsocaudal cells of each somite or, 2) that neural and somitic cells express *spt* in response to a common patterning signal(s). In either case, an "inefficient" stimulation of neural cells to transcribe *spt* would result in the observed distribution of *spt*-expressing neurons.

Occasionally, neural cells transcribing lower levels of *spt* can be observed adjacent to the most posterior somites (see asterisk in Figure 3E). We have not observed such cells at more rostral levels so these might represent cells in the process of activating *spt* transcription. Alternatively, neural cells transcribing *spt* at lower levels might be lost or might repress *spt* transcription later in spinal cord development.

The earliest formation of spt-expressing neurons

The somitic expression of *spt* at 24 hpf is only seen in the most recently formed somites. We wished to observe whether newly born *spt*-expressing neurons are always flanked by *spt* expression in somitic mesoderm, and to determine the earliest time at which *spt*-expressing neurons could be observed.

To gain an indication of the time at which *spt*-expressing neurons might first arise, we observed the somitic juxtaposition of the most rostral *spt*-expressing neuron in 11 embryos at approximately 24 hpf. The majority of the embryos (n = 10) possessed at least 6 somite pairs rostral to the most rostral *spt*-expressing neural cell (Figures 4A,4B).

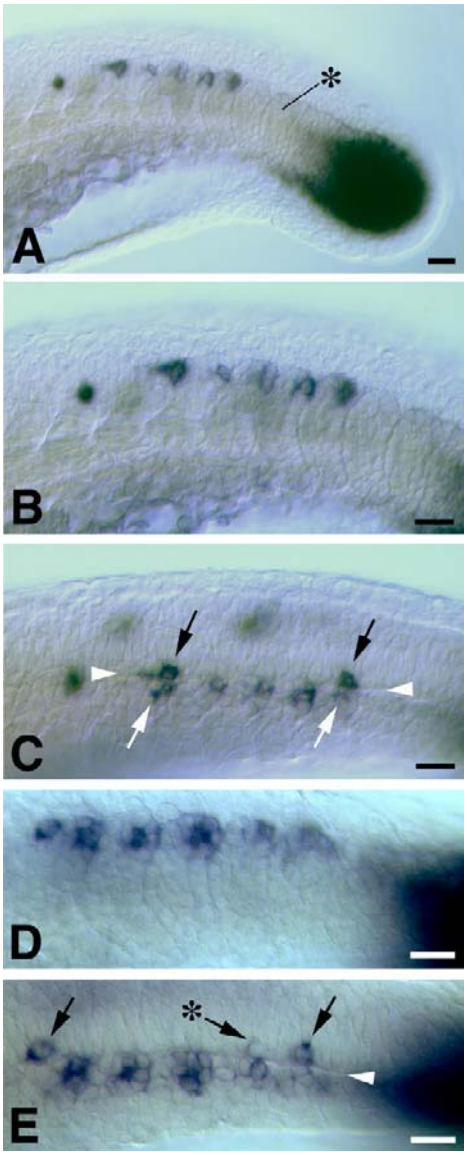

Figure 3
The juxtaposition of *spt* expression in newly formed somites and the developing CNS at approximately 22 hpf. In all images dorsal is uppermost and rostral is to the left. A, B and C are views from one embryo. A and B show the appearance from a lateral view of the tail in the region of the "somitic trail" of *spt* expression. A black asterisk indicates the most recently formed somite. *spt* expression is concentrated to the dorsocaudal extremity of somites. In an optical (DIC) section through the same embryo viewed from a dorsolateral perspective (C), the basal lamina separating the developing CNS and the somitic mesoderm can be seen clearly (arrowheads). Cells expressing *spt* in the developing CNS (black arrows) are juxtaposed to somitic cells expressing *spt* (white arrows). The "somitic trail" region of a second embryo is shown in D (lateral view) and E (dorsolateral view). The black asterisk in E indicates a neural cell expressing a lower level of *spt*. Scale bars equal 20 μm.

Only one embryo showed a lower number (at least 5 rostral somite pairs). Thus, *spt*-expression in the spinal cord is flanked by the region of somitic mesoderm that shows slower somite formation (occurring after the initial rapid formation of the first six somite pairs, [23]). Since the 5th somite pair forms at approximately 12 hpf (at 28.5°C), we examined embryos between 12 hpf and 16 hpf for *spt* staining in the CNS. The CNS primordium is relatively flattened at this time and the basal laminae separating CNS, mesoderm and individual somites are difficult to observe in fixed embryos. Nevertheless, the earliest time at which we could observe *spt* expression confidently in the developing CNS was 15.5 hpf (13 somite pairs). At 16 hpf (14 somite pairs), five of six embryos examined for which *spt*-expressing neurons could be seen had at least 9 somite pairs rostral to the most rostral *spt*-expressing neuron (see Figure 5). This observation implies that the *spt*-expressing neurons at more rostral positions (i.e. adjacent to the 6th to 9th somite pairs) differentiate at later times or that *spt*-expressing cells migrate rostralwards after their birth (see later). At 16 hpf, the *spt*-expressing neural cells are also flanked by low level *spt* expression in somites (white arrowheads in Figure 5). Thus, low level somitic expression of *spt* occurs during most of somitogenesis. Low level *spt* expression is observable at 14.5 hpf in laterocaudal cells. However, we could not determine whether these cells were neural or mesodermal (data not shown).

Analysis of left-right bias in spt-expressing neuron number
The irregular distribution of *spt*-expressing neurons may conceal a left or right bias in the number of these neurons. To investigate this we examined the numbers of neurons on the left and right sides of 48 embryos at 24 hpf. The mean number of cells on the left sides of embryos was found to be 10.5 with a standard deviation of 2.1. The mean number of cells on the right sides of embryos was found to be 10.7 with a standard deviation of 1.9. The differences in the mean number of *spt*-expressing neurons on the left and right sides of the embryos is considerably smaller than the standard deviations of left and right. This argues against any left-right bias.

The analysis above might not reveal a left or right bias when the variability in the number of *spt*-expressing neurons in each embryo is high. Thus, we also examined the difference in the numbers of *spt*-expressing neurons between the left and right sides of individual embryos. For each of the 48 embryos, the number of *spt*-expressing cells on the left of the embryo was subtracted from the number on the right. The mean difference was +0.2 with a standard deviation of 2.0. Since the standard deviation is far larger that the mean difference, this also argues against any left or right bias in *spt*-expressing neuron number. Finally, we tested whether there is simply a tendency for an absolute difference in the numbers of *spt*-expressing cells

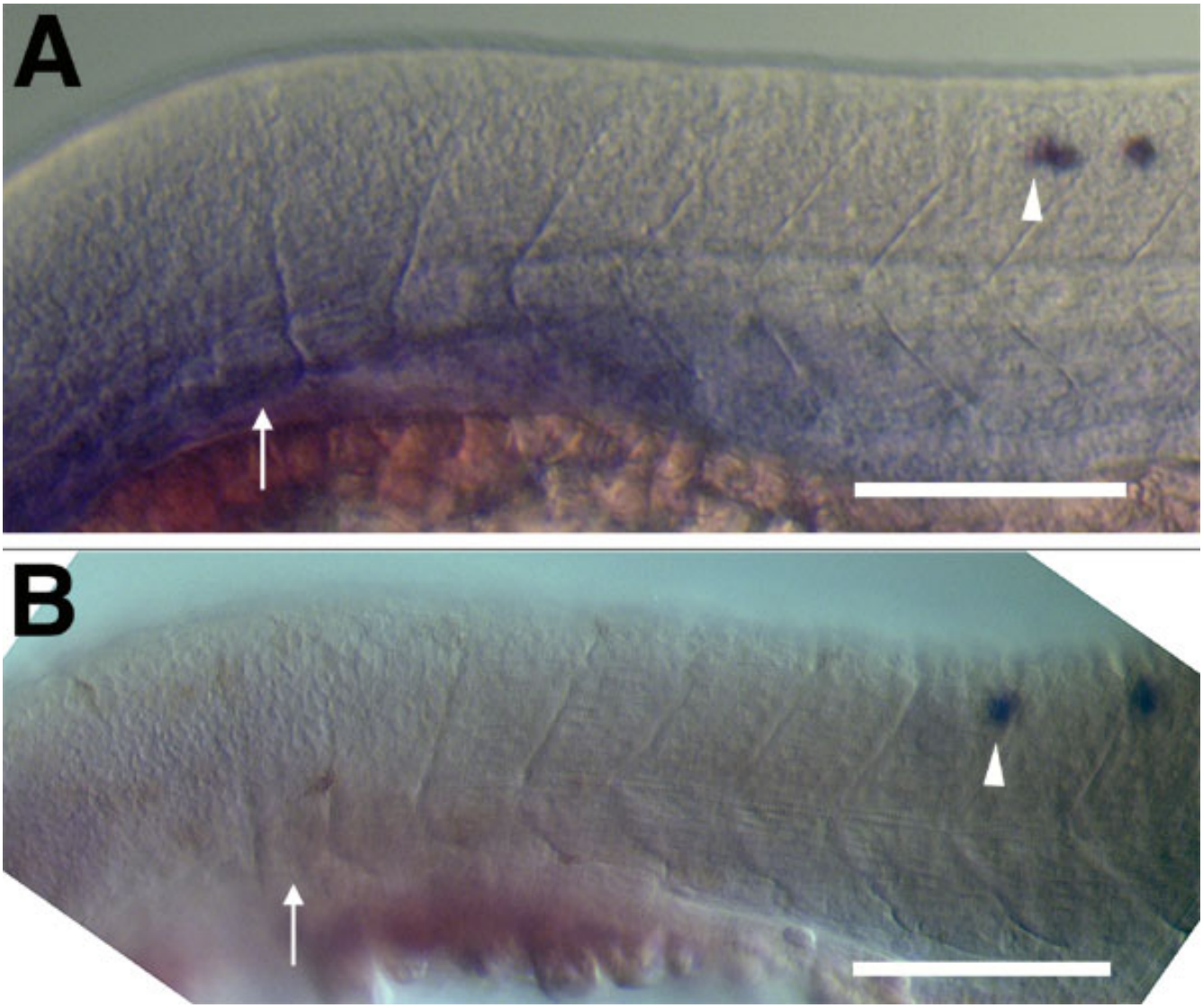

Figure 4
Lateral views of two embryos (A and B) at approximately 24 hpf stained to reveal *spt* transcription. Dorsal is up and rostral is to the left. DIC microscopy was used to reveal somite boundaries. Consequently, *spt*-expressing cells in the developing CNS are not seen clearly because they lie in a different focal plane. However, the most rostral cell in each embryo is indicated by a white arrrowhead. The most rostral visible discernible somite is indicated by a white arrow. In both cases there are 6 somites rostral to the most rostral *spt*-expressing neuron. Scale bars equal 100 μm.

to exist between the two sides of the embryo, regardless of any left-right bias. The mean absolute bilateral difference for the 48 embryos was 1.5 cells. The standard deviation for this value was 1.4. Thus, there is no significant difference in the numbers of *spt*-expressing cells between the two sides of embryos.

Preferred positions of spt-expressing neurons on the rostrocaudal axis

While the distribution of spt-expressing neurons along the rostrocaudal axis of the spinal cord appears to be irregular, preferred positions may, nevertheless, exist. To analyse this, 20 embryos were fixed at 24 hpf and stained to reveal expression of spt. The left and right sides of the trunk and tail of the embryos were then photographed under differential interference contrast (DIC) optics to show simultaneously the spt-expressing neurons and the boundaries between the flanking somitic tissue. We then counted the neurons occurring adjacent to each particular somite on the left and right sides of the embryo. Since we have shown that there is no left-right bias in the number of spt-expressing neurons, we combined the data from the two

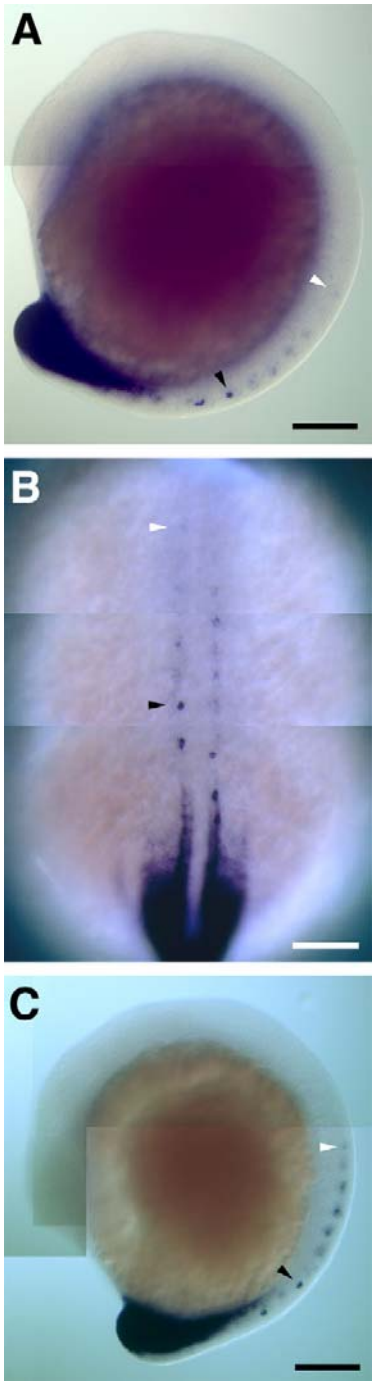

Figure 5
Early *spt* expression in the developing CNS and somites. A and C show lateral views of two embryos at 16 hpf. Rostral is up and dorsal is to the right. B shows a dorsal view of the embryo in A. Rostral is up. White arrowheads indicate the most rostral somitic domain of *spt* transcription visible. Black arrowheads indicate the most rostral neural cell expressing *spt*. For B, the light source was concentrated behind the yolk to give greater visibility of staining. All images are composites of smaller images. Scale bars equal 100 μm.

sides. The number of somite pairs present in embryos at 24 hpf can vary [23], as can the visibility in fixed embryos of the most anterior somite boundaries and the most recently formed somite boundaries. Therefore, to make the results from each embryo comparable, we identified the somite pair directly dorsal to the most caudal extent of the yolk extension as somite level 0. We then numbered the other somite pairs according to this reference point (Figure 6). Somite pairs rostral to somite level 0 were given a "+" designation while caudal somite pairs were given a "-" designation. The mean number of cells present at each somite level was then calculated (Table 1).

A tendency to higher numbers of cells at rostral somite levels is evident. The highest mean number observed was at somite level +11 (1.9 cells per embryo for left and right sides combined). At 24 hpf, somite level +11 commonly corresponds to the 7th somite pair formed. Lower numbers of *spt*-expressing neurons are observed at somite levels caudal to somite level 0 (commonly the 18th somite pair formed). However, there is great variability between embryos in the number of cells at any somite level (as indicated by the large standard deviation values in Table 1). The increase in cell number at rostral levels is not explained by the increase in the rostrocaudal dimension of somites as they mature since the segmental pattern of neuron distribution in the spinal cord expands correspondingly [2]. The higher number of *spt*-expressing neurons found rostral to somite level 0 could be due to: 1) continuing birth of these neurons at rostral positions as the CNS develops, 2) programmed cell death of neurons at caudal positions, or 3) rostralwards migration of neurons after their birth. Two observations support the last possibility. First, the mean number of *spt*-expressing neurons along the entire rostrocaudal axis per embryo was determined for 76 embryos at 24 hpf (21.4 neurons, standard deviation 3.4) and 45 embryos at 30 hpf (22.7 neurons, standard deviation 2.9). Somitogenesis ends at approximately 24 hpf but differentiation along the rostrocaudal axis continues in a rostral to caudal manner. Thus, any later, rostral generation of *spt*-expressing neurons or programmed cell death of caudal neurons as spinal cord development continues after 24 hpf might be expected to alter the average number of neurons by a greater number than that observed. Second, ipsilateral juxtaposition of *spt*-expressing neurons (which we defined as instances in which the cell bodies of the neurons appear to contact each other) occurred for 5.5% of cells in the region of somite levels -11 to +4, but for 11.3% of these cells in the region of somite levels +5 to +12. These data, together with the observation of greater neuron numbers at rostral levels, suggest that these neurons accumulate at rostral levels due to rostralwards migration after their birth.

Table 1: Numbers of *spt*-expressing neurons per somite level (pair of hemisegments) at 24 hpf

Somite level	Somite number	Number of embryos	Mean cell number	Standard deviation
-14	32	3	0	0
-13	31	7	0	0
-12	30	12	0	0
-11	29	15	0.07	0.26
-10	28	19	0.16	0.50
-9	27	19	0.47	0.61
-8	26	19	0.47	0.61
-7	25	19	0.63	0.76
-6	24	20	0.55	0.69
-5	23	20	0.50	0.61
-4	22	20	0.70	0.92
-3	21	20	0.55	0.69
-2	20	20	0.55	0.60
-1	19	20	0.95	0.83
0	18	20	0.95	1.05
+1	17	20	0.70	0.66
+2	16	20	0.70	0.73
+3	15	20	0.95	1.00
+4	14	20	0.75	0.79
+5	13	20	1.00	0.92
+6	12	20	0.75	0.72
+7	11	20	1.40	0.68
+8	10	20	1.45	1.19
+9	9	19	1.11	0.88
+10	8	17	1.47	1.01
+11	7	13	1.92	1.38
+12	6	7	1.14	0.69
+13	5	4	0.75	0.50
+14	4	2	0	0

Somite level 0 represents the somite pair immediately dorsal to the most posterior extremity of the yolk extension. Negative values are more caudal to somite level 0 and positive values are more rostral. The common identity of each somite pair (i.e. disregarding variability between embryos) in terms of its order of formation is given as the somite number

No *spt*-expressing cells were observed rostral of somite level +13, commonly corresponding to the 5th somite pair formed. This could be an artefact of the low number of embryos for which these somite levels could be distinguished during observation. However, this result is consistent with our earlier failure to observe *spt*-expressing neurons more rostral than the 5th most rostral somite pair (see Figure 4 and above).

At first glance, the numbers of *spt*-expressing neurons we observe at each somite level (i.e. per two hemisegments) at 24 hpf does not appear to be comparable to the previous observations of Bernhardt et al. in 1990 [12] of 0.06 DoLA interneurons per hemisegment (0.12 per somite level) flanked by the 5th- to 8th-formed somite pairs in embryos at 18 hpf. However, the fact that we rarely observe *spt*-expressing neurons anterior to the 6th-formed somite pair at 24 hpf combined with the possibility that these neurons migrate rostrally after birth (see above) suggests that fewer DoLA neurons may be found in the region flanked by the 5th- to 8th-formed somite pairs at 18 hpf compared to 24 hpf. Also, these authors identified DoLA neurons by their pattern of arborisation whereas we have identified these cells by *spt* expression. At 18 hpf many *spt*-expressing cells may not yet have developed characteristic DoLA arborisation patterns. In contrast, in a study of GABAergic DoLA neurons in embryos at 27 hpf by Bernhardt et al. in 1992 [24], a mean of 3.89 cells (standard deviation 1.17) were observed in the region of hemisegments 6 to 10. At 24 hpf, we observed a mean of 3.64 cells (standard deviation 1.08) in the same region. The close correspondence of these figures supports that *spt*-expressing neural cells are DoLA neurons.

Discussion

The identity of spt-expressing neural cells

The *spt*-expressing cells in the developing spinal cord show coexpression of a number of neural markers such as

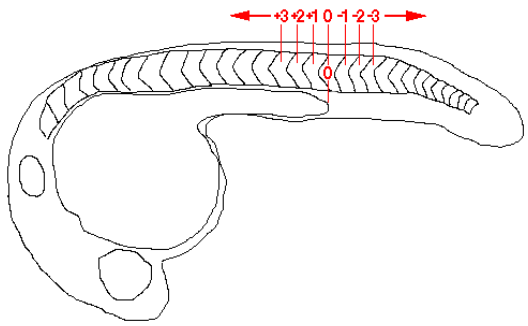

Figure 6
Diagram of somite level designations relative to the caudal tip of the yolk extension in a 24 hpf embryo.

huC and the *islet* genes. This, together with the position of these cells just ventral to the Rohon-Beard neurons and their rostrocaudal distribution establishes that these cells are likely to be the DoLA neurons originally described by Bernhardt et al. [12]. Indeed, we are able to observe a rostrally projecting process of these cells similar to the ascending axon of DoLA neurons due to the active transport of *spt* mRNA along this process.

That DoLA neurons express *spt* conflicts with observations of the expression in *Xenopus* embryos of the *spt* orthologous gene, *Xombi*. *Xombi* is transcribed in a very similar pattern to *spt* in the developing spinal cord. In 1996, Stennard et al. [25] and Zhang and King [26] suggested that this gene (they named it *Antipodean* and *VegT* respectively) might be expressed in Rohon-Beard neurons based on the dorsal/dorsolateral position of expressing cells in the spinal cord. However, in a simultaneous publication, Lustig et al. [27] suggested that *Xombi* expression was in the dorsolateral area of interneuron formation. We expect that closer examination will show that *Xombi* is expressed in *Xenopus* DoLA-equivalent cells, probably dorsolateral interneurons (see review by Roberts [28]).

The observation of *spt* mRNA in an anterior growth process/axon suggests a number of possibilities. First, the mRNA may not be translated but may perform some other (or no) role in the process. Second, Spt protein may be required in this process for a function other than gene regulation. Third, *spt* mRNA may be required in the process for production of protein that is used to signal back to the nucleus. There is some precedence for the expression of transcription factors in neurites since these are known to be found in dendrites where it is thought that they may be involved in activities such as long term potentiation [29]. Finally, and most intriguing, is the possibility that *spt* mRNA might be involved in signalling to cells with which the process makes contact. It has been demonstrated that the transcription factor Engrailed and the homeodomains of other proteins can be transported between cells [30–33]. Testing of these possibilities will require observation of the distribution of Spt protein.

spt-expressing DoLA neurons possibly migrate rostrally

Higher numbers of *spt*-expressing neurons are observed rostrally compared to caudally in the spinal cord. It may be that *spt*-expressing neurons continue to be born as the developing CNS matures in a rostral to caudal progression or that caudal neurons undergo programmed cell death. However, the marginal change in the number of these neurons between 24 and 30 hpf argues against this. Also, ipsilateral juxtaposition of these neurons is more common at rostral compared to caudal sites. The increased juxtaposition rostrally could be caused by rostral migration of *spt*-expressing neurons when an anterior limit exists for the migration. *spt*-expressing neurons are rarely seen anterior of the 6th-formed somite pair suggesting that this position on the rostrocaudal axis may represent such a limit.

spt is expressed in somitic mesoderm

Extended staining for *spt* mRNA revealed that this gene is transcribed at low levels in the dorsocaudal cells of recently formed somites. It has previously been assumed that *spt* expression marks only presomitic mesoderm. The function of *spt* expression in these somitic cells is unknown. Discovery of other genes expressed in a similar pattern in newly formed somites may reveal more of the function or fate of these cells.

The irregular pattern of spt-expressing neurons may be based on an underlying regularity

The question of how irregular patterns of cell distribution or gene expression are controlled is not commonly addressed in studies of developmental biology. Nevertheless, these patterns are common in the central nervous systems of most animals and occur in many other tissues. In the spinal cord of the developing embryo, Rohon-Beard neurons occur at a frequency of more than one per hemisegment [12]. Their positions are not highly ordered and do not depend upon signals from mesoderm [5]. Instead, short-range intercellular interactions controlled by Notch signalling appear to play a role in their differentiation from a field of progenitor cells [10,11].

The ascending commissural neurons that are located just ventral to Rohon-Beard neurons are also found at a frequency of more than one per hemisegment. However, subclasses of these neurons exist with lower frequency. For example, anti-CON1 antibody labels a subclass of ascending commissural neurons in the embryo that probably become commissural primary ascending (CoPA)

interneurons in the larva. These are present in an irregular pattern on the rostrocaudal axis at a frequency of 0.87 per hemisegment flanking the 6th- to 11th-formed somite pairs at 28 hpf [12]. Ascending commissural neurons are located at a similar dorsoventral level to the DoLA neurons. We have shown that *val* expression in the spinal cord occurs just ventral to *spt*-expressing neurons. Thus, it is possible that *val* labels a subclass of ascending commissural neurons.

The neuromasts of the posterior lateral line – while part of the peripheral nervous system – are, nevertheless, an example of a neural cell type distributed at a frequency of less than one per hemisegment. These neurons are deposited by the migrating lateral line primordia along the myoseptum at the boundary between somites at four or five positions along each side of the embryo. While their rostrocaudal distribution is not completely irregular, there is considerable variation in the actual position of any one neuromast. The position at which a neuromast is deposited appears to depend more strongly on the distance from the previously deposited neuromast rather than the precise position on the rostrocaudal axis [34]. Interestingly, the recessive, homozygous viable mutation *hypersensitive* (*hps*) results in neuromast deposition at nearly every somite boundary [35]. The fact that this (presumably) loss-of-function mutation can increase the regularity of a pattern indicates that the distribution of neuromasts probably results from the combined effect of at least two patterning mechanisms – one controlling inter-neuromast distance and one controlling neuromast localisation to intersomitic boundaries. This raises the question as to whether mutations might exist that increase the frequency of generation of *spt*-expressing neurons, for example, by increasing the strength of a patterning signal from the mesoderm to the developing CNS.

The dorsoventral and rostrocaudal correspondence of *spt* expression in newly formed somites and the CNS suggests a functional connection between the *spt* expression in these two tissues. The somitic and neural cells may be responding to a common patterning signal. Alternatively, the somitic *spt* expression may mark the source of a signal from the somite to neural tissue. A precedent for the latter alternative exists in the influence of flanking mesoderm on primary motoneuron formation [5–7]. However, the formation of most primary motoneurons occurs with complete regularity (one neuron per hemisegment). An interesting exception to this is the Variable Primary (VaP) motoneuron that occurs at a frequency of less than 0.5 per hemisegment. VaPs arise adjacent to Caudal Primary (CaP) motoneurons midway between hemisegment boundaries [36]. VaPs normally extend an axon to the horizontal myoseptum in the myotomes after which the VaP dies. In contrast, the CaP axon continues from the myoseptum into ventral muscle. These two neurons actually represent an equivalence pair since ablation of a CaP causes the neighbouring VaP to develop a CaP-like arborisation pattern [37]. Thus, rather than VaP formation occurring with less than complete regularity, we can regard this situation as CaP formation at greater than one cell per hemisegment followed by regulation to one cell per hemisegment.

We suggest that the *spt*-expressing DoLA interneurons might be "inefficiently" patterned by flanking somitic mesoderm. Thus, the initial distribution of these neurons would represent an incomplete pattern based on a regular template. Migration and tissue growth might then scramble this pattern. We are currently testing this hypothesis by examining the role of *spt* expression and mesodermal signals in DoLA neuron differentiation and distribution.

Conclusions

spt-expressing cells in the developing central nervous system appear to be DoLA interneurons. The irregular distribution of these cells along the rostrocaudal axis of the spinal cord may be due to "inefficient" patterning of neural *spt* expression by flanking, regularly distributed somites also expressing *spt*. Rostral migration of *spt*-expressing neurons might then scramble any residual regularity in their distribution. The idea that irregular patterns of neuron distribution may arise in partial correspondence to regular templates is a parsimonious explanation for the evolution of such patterns.

Materials and Methods

Double whole mount in situ transcript hybridisation

(Cloning of probe sources)

A cDNA clone, (26 M), corresponding to transcription from *spt* was isolated in a whole mount *in situ* transcript hybridisation screen of zebrafish embryos [38]. cDNAs corresponding to parts of transcripts from the genes *huC*, *isl-2* and *valentino* were amplified by RT-PCR from embryos at 24 hpf using the oligonucleotide primers described in Table 2. All cDNA fragments were cloned into the pGEMT vector (Promega Corporation, Madison, WI, USA). The inserts of these clones were amplified by PCR using M13 primers and then transcribed with T3 or SP6 RNA polymerase to produce digoxigenin- or fluorescein-labelled antisense RNA probes (see [38]). The clones for production of probes against *isl-1* and *isl-3* transcripts were obtained from Hitoshi Okamoto [6,7].

Double whole mount *in situ* transcript hybridisation was performed essentially as described in [39] but the first staining reaction was with BCIP/NBT, inactivation of the first alkaline phosphatase staining reaction was by heating

Table 2: Oligonucleotides used for cDNA fragment isolation for probe synthesis

Gene transcripts detected	oligo name	PCR oligonucleotide sequence
huC	#277	5' CAG ATG ACA GCA AAA CTA ACC 3'
	#278	5' AGA GCA ATA GTG ACT AGG CC 3'
isl2	#351	5' GAC GGC AAG ACT TAT TGC 3'
	#352	5' CAT CTT CGG AGA TCA TGC 3'
val	#322	5' GGT CCC CCT GTC GCC TC 3'
	#323	5' CCA CGA GCG ACA ACC CG 3'

to 65°C for 45 min in PBS and the second staining reaction used the Alkaline Phosphatase Substrate Kit I (Vector Laboratories Inc., Burlingame, CA, USA).

Combined whole mount in situ transcript hybridisation and immunohistochemistry

Staining for the presence of *spt* transcript and acetylated tubulin was performed essentially as described above for double whole mount *in situ* transcript hybridisation except that *spt* staining using the Alkaline Phosphatase Substrate Kit I ("Vector Red", Vector Laboratories Inc.) was performed first followed by washing for 10 min in 100 mM Tris HCl pH 8.5 then 10 min in PBS + 0.1% Tween 20 (Sigma, St. Louis, MO, USA) (PBT) before fixation in 4% formaldehyde in PBT. Embryos were then washed 4 × 5 min in PBT, then 3 h in PBT + 0.3% IPEGAL (Sigma) (PBTI) + 2% BSA (Fraction V, Sigma), then 1 h in PBTI + 2% BSA at 4°C before incubation overnight at 4°C in a 1:2500 dilution of anti-Acetylated Tubulin antibody (Sigma Cat. No. T6793) in PBTI + 0.2% BSA. Embryos were then washed 6 × 1 h in PBTI then 2 × 30 min in PBTI + 2% BSA before incubation overnight at 4°C in a 1:200 dilution of anti-mouse IgG labelled with Alexa Fluor 488 (Molecular Probes Inc., Eugene, OR, USA) in PBTI + 0.2% BSA. Finally, embryos were washed 7 × 1 h in PBTI before equilibration with 80% glycerol in PBT before imaging. Note that all wash series were preceded by three rinses in the wash solution and were at room temperature unless otherwise indicated.

Observation and statistical analysis of cell distribution

Embryos were dechorionated at 15–18 hpf, 22 hpf, 24 hpf or 30 hpf and fixed in 4% formaldehyde in PBS at 4°C before *in situ* transcript hybridisation with a probe for *spt*. To ensure observation of all cells expressing *spt* including any expressing *spt* at low levels, the staining reaction was allowed to proceed overnight at 4°C before the embryos were fixed in 4% formaldehyde in PBS and then equilibrated with 80% glycerol.

Light field observation of the embryos was conducted under a Zeiss Axiophot™ microscope (Carl Zeiss Jena GmbH, Jena, Germany) at 200× magnification using DIC optics. For examination of cell positions, the trunk-tail region of an embryo was removed from the rest of the body and then laid flat on a slide. Photographs were taken such that the intersomitic boundaries and the *spt*-expressing neural cells were simultaneously visible. Confocal imaging of embryos was conducted on a Bio-Rad MRC-1000 UV Confocal Laser Scanning Microscope System (Bio-Rad Laboratories Inc., Hercules, CA, USA) using a Nikon Diaphot 300 inverted microscope (Nikon Instech Co., Ltd., Kawasaki, Kanagawa, Japan). Fluorescence was observed using a krypton/argon laser with excitation at 488/10 nm and emission at 522/35 nm excitation for Alexa 488 and with excitation at 568/10 nm and emission at 605/32 nm for Vector Red. Images were processed with Adobe Photoshop version 5.0 (Adobe Systems Inc. San Jose, California, USA) and Confocal Assistant version 4.02 (Todd Clark Brelje).

List of abbreviations used

BCIP, 5-Bromo-4-chloro-3-indolyl-phosphate.p-toluidine-salt

BSA, bovine serum albumin

CaP, Caudal Primary

CNS, central nervous system

CoPA, Commissural Primary Ascending

DIC, differential interference contrast

DLF, dorsal longitudinal fasciculus

DoLA, dorsal longitudinal ascending

isl, *islet*

hpf, hours post fertilisation

NBT, Nitroblue tetrazolium chloride

PBS, phosphate buffered saline

PBT, PBS + 0.1% Tween 20

PBTI, PBT + 0.3% IPEGAL

RT-PCR, reverse transcription polymerase chain reaction

spt, *spadetail*

val, *valentino*

VaP, Variable Primary

Authors' contributions

RT carried out the majority of the *in situ* transcript hybridisation, cell counting, and statistical analyses and some photography.

SW performed the *in situ* transcript hybridisation analyses on 12 – 16 hpf embryos and staining for acetylated tubulin.

JGC contributed to the statistical analysis

ML directed the research, performed observation of staining patterns, some cell counting and statistical analysis, some photography and drafted the manuscript.

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Paper II

Independent and cooperative action of Psen2 with Psen1 in zebrafish embryos

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Paper III

Cryptic organisation within an apparently irregular rostrocaudal distribution of interneurons in the embryonic zebrafish spinal cord

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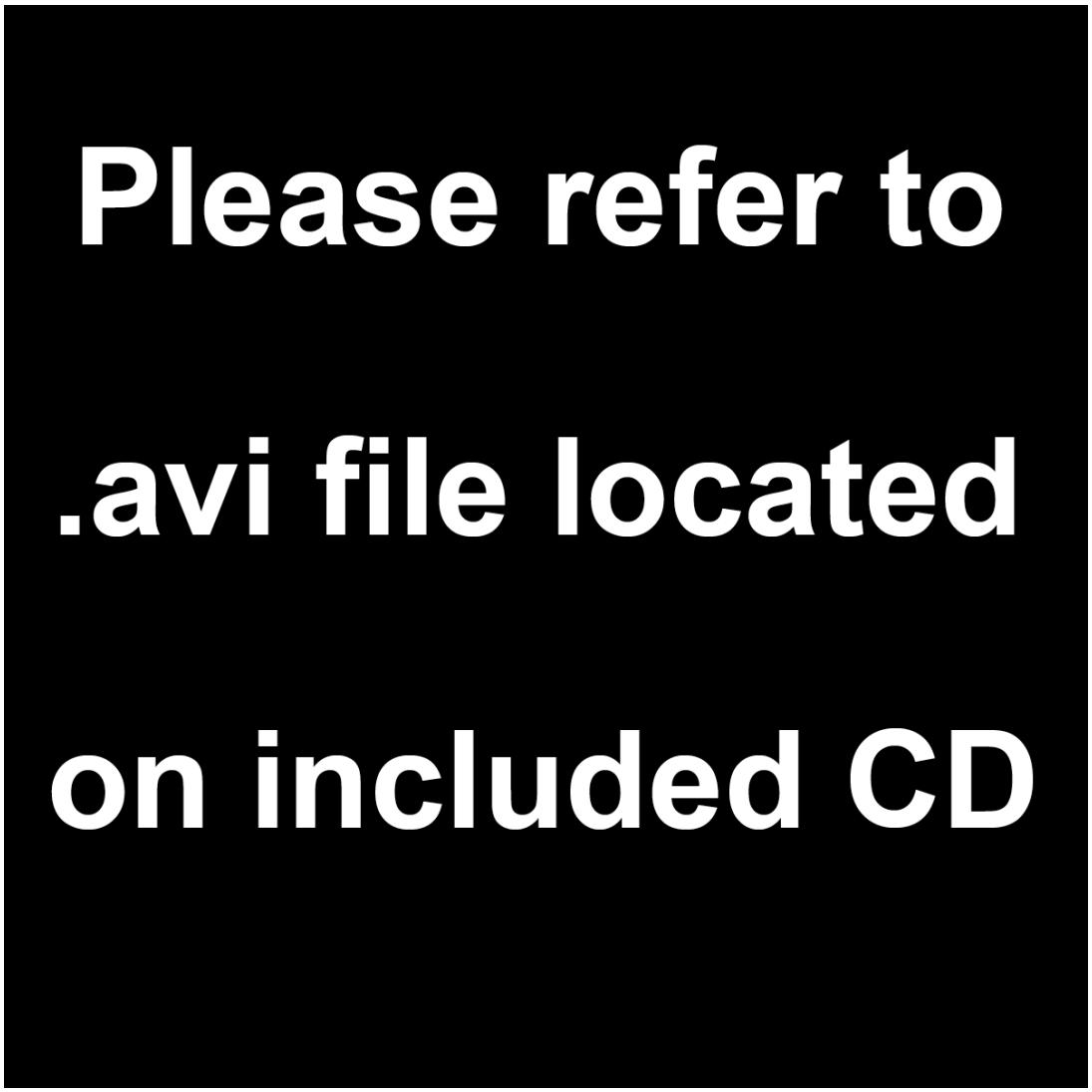

Supplementary File S2 - DoLA interneurons migrate rostrally after birth. Time-lapse imaging of DoLA interneurons migrating along the dorsal longitudinal fasciculus of the spinal cord between 16-23 hpf. The tailbud of a *HuC-Kaede* transgenic embryo was photoconverted at 16 hpf and development was captured every 5 minutes for 370 minutes. Photoconverted cells appear red before slowly returning to green as photoconverted Kaede is cleared in the cells. Over this time 3 cells can be seen to migrate rostrally in the spinal cord (I, II, III). Rostral is to the right and dorsal is down. Scale bar 100 μm.

Paper IV

Transgenic zebrafish recapitulating *tbx16* gene early developmental expression

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Submitted to: Transgenic Research

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Dear Editor of Transgenic Research,

We wish to submit a manuscript for consideration for publication.

A long-standing project in our laboratory has been the dissection of the mechanisms behind the distribution of a particular subset of spinal neurons, the Dorsal Longitudinal Ascending (DoLA) interneurons in zebrafish. We discovered that the DoLA interneurons are the only cells in the spinal cord that uniquely express the mesodermal determinant *tbx16/spadetail* (Tamme *et al*. *BMC Developmental Biology* 2002, **2**:9). These cells are found in restricted numbers, with less than one cell per flanking somite and are distributed in a seemingly irregular distribution in the spinal cord. However, we have previously shown that this distribution includes a form of cryptic organisation axis (Wells *et al*. *Exp Cell Res.* 2010, 316:3292).

In the work described in this manuscript we attempted to extend our examination of the organisation and behaviour of DoLA interneurons by creating transgenic zebrafish expressing GFP under the control of the *tbx16* promoter. These animals show accurate replication of endogenous *tbx16* expression during early stages of development with strong expression in the polster, hatching gland and presomitic mesoderm. We found that later expression of GFP in the transgenic lines diverged somewhat from endogenous *tbx16* expression and that the transgene is not expressed in DoLA neurons, as we had desired. However, serendipitously, one of our transgenic lines shows GFP expression in commissural primary ascending (CoPA) interneurons, which will facilitate future studies of the differentiation and function of that cell type. Indeed, we show that CoPA interneurons are marked by the transcription factor gene *mafba/valentino*. Our transgenic lines may be of interest to researchers interested in the development of tissues showing early expression of *tbx16*.

We hope that this manuscript will be of interest to the audience of Transgenic Research
Sincerely,
Simon Wells

Title Page

Title of Article:

Transgenic zebrafish recapitulating *tbx16* gene early developmental expression

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Abstract: We describe the creation of a transgenic zebrafish expressing GFP driven by a 7.5kb promoter region of the *tbx16* gene. This promoter segment is sufficient to recapitulate early embryonic expression of endogenous *tbx16* in the presomitic mesoderm, the polster and, subsequently, in the hatching gland. Expression of GFP in the transgenic lines later in development diverges to some extent from endogenous *tbx16* expression with the serendipitous result that one line expresses GFP specifically in commissural primary ascending (CoPA) interneurons of the developing spinal cord. Using this line we demonstrate that the gene *mafba (valentino)* is expressed in CoPA interneurons.

Keywords: spadetail, *tbx16*, commissural primary ascending, CoPA, *mafba*, *valentino*

Introduction

The production of transgenic organisms for research purposes has been a powerful method for accelerating the study of cell lineages in embryo development. This fact in combination with the genetic amenability and accessibility of zebrafish (*Danio rerio*) has stimulated production of an increased number of germline transgenic fish stocks available for such investigations. In particular, using zebrafish promoters linked to green fluorescent protein (GFP) has enabled recapitulation of the expression of various genes and the tracing of cells in embryos as they develop (Gong, Ju et al. 2002; Mione, Baldessari et al. 2008; Chen and Chiou 2010).

The zebrafish *spadetail* (*spt*) mutation was first discovered in a screen for recessive mutations affecting neuronal development (Streisinger, Walker et al. 1981) The gene affected by the *spt* mutation (*tbx16*) was subsequently found to be required for normal morphogenetic cell movement during mesoderm development (Kimmel, Kane et al. 1989). The disruption to early development caused by the *spt* mutation results in a lack of trunk mesoderm with cells normally directed to this region accumulating to create a mass of cells at the distal end of the extending tail, the "spade" structure characteristic of these mutants (Ho and Kane 1990; Molven, Wright et al. 1990).

tbx16 encodes a member of the T-box family of transcription factors (Griffin, Amacher et al. 1998; Ruvinsky, Silver et al. 1998). *tbx16* expression is found in presomitic paraxial mesoderm, the polster and hatching gland cells (Ruvinsky, Silver et al. 1998) and a subset of spinal cord cells (first reported by Ruvinsky et. al. (1998) but mistakenly identified as Rohon-Beard neurons). We later demonstrated that these cells are dorsal longitudinal ascending (DoLA) interneurons (Tamme, Wells et al. 2002).

DoLAs have a seemingly irregular distribution along the rostrocaudal axis of the spinal cord, a pattern that is particularly difficult to dissect. We have recently shown that there is an underlying cryptic organisation to the rostrocausal and contralateral distribution of these cells (Wells, Conran et al. 2010). Furthermore, we have shown that this distribution

is, to some extent, created by the rostralwards migration of these cells shortly after their birth in the developing spinal cord (Wells, Conran et al. 2010). Subsequently, we endeavoured to use these neurons to examine the molecules and mechanisms that establish irregular distributions of cells along the rostrocaudal axis in the spinal cord. The creation of a GFP transgenic zebrafish line under the control of the *tbx16* promoter might allow us to examine aspects of DoLA neuron development and migration and examine paraxial mesoderm development in real time.

In this paper we describe an attempt to track the developmental expression of *tbx16*-expressing cells using a transgene possessing 7.5kb of DNA sequence surrounding the site of transcription initiation of the *tbx16* gene. Four transgenic lines of fish all displayed expression of GFP in the hatching gland progenitors, presomitic mesoderm, newly formed somites, and the hatching gland similar to endogenous *tbx16*. However no expression was observed in DoLA interneurons. Surprisingly one transgenic line of fish expressed GFP in commissural primary ascending (CoPA) interneurons and these cells are shown to be marked by transcripts of the gene *v-maf musculoaponeurotic fibrosarcoma oncogene family, protein B-avian (mafba)/valentino*.

Materials and Methods

Embryos and staging

Zebrafish were maintained as described (Westerfield 2000). Embryos were collected and allowed to develop at 28.5˚C to the required stage. Morphological features of embryos were consistent with the zebrafish staging guide (Kimmel, Ballard et al. 1995).

Generation of *tbx16*: GFP transgenic lines

We obtained *tbx16* genomic DNA by screening a zebrafish BAC construct library (Genome Systems, Inc., St. Louis, MI, USA) using a *tbx16* cDNA probe. The BAC clone was analysed by Southern blot, and an *EcoRI-BamHI* fragment containing 5kb of 5′

promoter DNA, the first exon and part of the first intron was cloned in frame upstream of GFP in the vector pEGFP-N1 (Clontech, Mountain View, CA, USA). The region for injection was excised from vector sequence, gel purified and was microinjected into the cytoplasm of embryos at the 1-cell stage. GFP expression was analysed before 24hpf by observation under a fluorescence dissection microscope and embryos positive for GFP expression were raised to sexual maturity. Germline transgenic founders were identified by screening their F1 progeny for GFP fluorescence. Four founders (192A, 512B, 812A, 812C) were isolated, mated with wild-type fish, and their GFP positive offspring were raised to adulthood.

Detailed investigation of developing embryos by fluorescence microscopy was undertaken using a Zeiss Axioplan 2 deconvolution microscope with an AxioCam MRm camera (Carl Zeiss Jena GmbH, Jena, Germany).

Whole mount *in-situ* transcript hybridisation

The clones for production of probes against *tbx16* and *mafba* transcripts have been described previously (Tamme, Wells et al. 2002). The GFP sequence was excised from pIRES2-EGFP (Clontech) and subcloned into pBluescript (Stratagene Products Division, La Jolla, CA, USA). The insert containing regions from these plasmids were amplified by PCR with M13 primers and then transcribed with T7 or T3 RNA polymerases to produce digoxigenin- (Sigma-Aldrich Corp., St. Louis, MO, USA) or fluorescein- (Sigma) labelled antisense riboprobes. Whole mount *in situ* transcript hybridisation was carried out essentially as described (Jowett 1997) but the two-colour staining reactions were undertaken initially with BCIP/NBT (F. Hoffmann-La Roche Ltd, Basel, Switzerland), and subsequently, the second staining reaction used the Vector Red Alkaline Phosphatase Substrate Kit I (SK-5100, Vector Laboratories Inc., Burlingame, CA, USA). Inactivation of the first alkaline phosphatase reaction was achieved by heating to 65^{o}C for 1 hour in phosphate buffered saline. Older embryos were treated in proteinase K as described (Jowett 1997). Light field observations were conducted under a Zeiss Axiophot

microscope (Carl Zeiss) with a 20x objective using differential interference contrast (DIC) optics.

Results

Generation of *tbx16*:GFP transgenic zebrafish – including construct

We have previously shown that *tbx16*-expressing neurons are migratory and this migration contributes to their distribution in the developing spinal cord (Wells, Conran et al. 2010). To examine further these cells including their origins and interaction with other cells of the spinal cord we attempted to label them with GFP by creating transgenic fish expressing GFP under the control of the *tbx16* promoter. A 7.5kb genomic DNA fragment including 5kb of 5' promoter (Fig 1a) and 2.5kb of transcribed *tbx16* gene sequence (Fig 1b) – the "*tbx16* promoter" (Fig 1c) was cloned upstream of EGFP in the pEGFP-N1 vector (Fig. 1d, see Materials and Methods). This promoter region included the site of insertion in the enhancer trap line CLGY6 that was shown to recapitulate *tbx16* expression at 24hpf (Ellingsen, Laplante et al. 2005). Independent lines of transgenic zebrafish were generated by injecting linearised construct into single-cell embryos. Germline transmission was found in 2.6% of examined surviving adults (not all mosaic adults were screened). Four founders (192A, 512B, 812A, 812C) were isolated, mated with wild-type fish, and their GFP positive offspring were raised to adulthood. Founder germline mosaicism was determined to be between 4 and 40%. To confirm germline integration of the transgene, heterozygous GFP expressing F1 embryos were raised to sexual maturity and outbred to wild-type fish. Transgenic F2 progeny were generated at approximately 50% (49-52%, $n > 870$ for each line) indicating a single insertion site in each founder, and a typical Mendelian inheritance pattern. Each line has consistently retained its pattern of GFP expression for at least 3 generations.

Consistent expression of *tbx16*:GFP in embryonic zebrafish

We examined transgenic lines for temporal and spatial expression of GFP. All examined transgenic lines displayed extensive GFP fluorescence consistent with the expression of *tbx16* transcript in wild type embryos. Transgene expression was first detected at 12hpf in the hatching gland progenitors, presomitic mesoderm and the tail bud (Fig 2a). GFP expression at this stage was also noted throughout the somitic mesoderm indicating that the protein persists longer in the developing mesoderm than endogenous *tbx16* transcripts. Expression at 24hpf is seen in the hatching gland and in the posterior somites while expression has faded from the earliest formed somites (Fig 2b). By 48hpf GFP expression in somitic mesoderm has diminished in all lines except 192A, allowing cells of the spinal cord to be viewed (Fig 2c). It is unknown when spinal cord neurons commence expression of GFP due to the masking effect of overlying somitic mesoderm obscuring more medial tissues. At 48hpf all lines strongly express GFP in the hatching gland. Peculiarly, all lines show some level of expression in the notochord (Fig 2d). Line 192A displays persistent low-level somitic mesodermal expression at 96hpf (not shown).

Tissue specific GFP fluorescence patterns

The earliest GFP expression appears to be consistent among the lines, with line-specific expression beginning later in development. At 24hpf line 192A displays GFP expression in cells in the midbrain (not shown). At 48hpf (and 96hpf) this expression pattern has coalesced into two distinct regions that appear to consist of distinct cells in the midbrain and the epiphysis (Fig 3a). At 24hpf line 512B expresses GFP in the dorsal aorta (Fig 3b) and later at 48hpf in ventrally projecting neurons in the ventral spinal cord (Fig 3c). These neural cells persist until at least 96hpf (not shown). 24hpf embryos of the 812A line have a large portion of the midbrain expressing GFP along with the epiphysis and show a distinct banding pattern in the hindbrain (Fig 3d) that appears to be related to the rhombomeres (Fig 3e). This pattern disappears by 48hpf. At 96hpf the line 812A expresses GFP in the floor plate of the spinal cord (Fig 3f). In line 812C at 48hpf we

observe expression of GFP in the epiphysis and regions of the midbrain. This line also shows expression in dorsal neurons in the spinal cord in an apparently irregular rostrocaudal distribution that appears similar to that of DoLA interneurons (Fig 3g). These embryos display expression in distinct neural cells in the hindbrain (Fig 3h).

Neurons expressing GFP in the spinal cord of the 812C line are comissural primary ascending interneurons (CoPAs)

We have previously shown that the *tbx16*-expressing cells of the spinal cord are the dorsal longitudinal ascending (DoLA) interneurons. GFP positive spinal cord cells observed in the 812C line (Fig. 3g) exhibit characteristics consistent with DoLA cells; both cell types show an apparent irregular rostrocaudal distribution of approximately 20-25 cells per embryo with similar dorsoventral positioning and they occur at similar developmental times. Upon closer examination, the GFP expressing cells have properties differing from the published morphology of DoLA cells (Kuwada, Bernhardt et al. 1990; Roberts 2000). Although the GFP expressing neurons are distributed in an apparently irregular rostrocaudal pattern and can be found along the dorsal longitudinal fasciculus (Fig. 4a) they have ascending and descending longitudinal projections, and a single ventral projecting axon that crosses the midline and ascends in the DLF contralateral to the cell body (Fig. 4a, b). The morphology and distribution of these cells is consistent with them being commissural primary ascending (CoPA) interneurons.
To confirm that the 812C transgenic line is not expressing GFP in the DoLA neurons, we undertook *in situ* transcript hybridisation using probes against *GFP* and *tbx16* simultaneously. This showed that the neurons expressing *GFP* do not simultaneously express *tbx16*, although the two cell types are found at the same dorsoventral level of the developing spinal cord (Fig 4c). We examined the expression of another transcript, *mafba* known to be expressed in a similar distribution in the developing spinal cord but not in *spt*-expressing DoLA neurons {Tamme, 2002 #8} and discovered that the *mafba*-expressing cells show coexpression of *GFP* (Fig 4d). Thus expression of *mafba* appears to mark CoPA interneurons.

Discussion

In this paper we have described an attempt to produce transgenic zebrafish to track the developmental expression of *tbx16* by using GFP fused to *tbx16* promoter sequence. We used a 7.5kb segment of genomic DNA that encompassed 5kb of the 5′ promoter and includes the translation start site, the first exon, and part of the second exon of the *tbx16* gene. Extensive outbreeding has shown that these lines are all single insertion site transgenic lines that show typical Mendelian inheritance.

The four lines of fish examined display expression of GFP in the hatching gland progenitors (the polster), presomitic mesoderm, newly formed somites, and the hatching gland consistent with endogenous *tbx16* expression and perdurance of GFP from expression in progenitor tissues. However, none of the lines express GFP in the DoLA neurons which was the original purpose for which they were created. Later in development expression of GFP in the four lines diverges somewhat from endogenous *tbx16* expression and from each other. Persistent expression of enhanced GFP in lateral tissues can mask expression in deeper medial tissues, suggesting that destabilised GFP might have been a better choice for creating these particular transgenic zebrafish.

The common differences in expression between the transgenic lines and endogenous *tbx16* expression (e.g. absence of expression in DoLAs in the transgenic fish) are most likely due to the absence of some *tbx16* promoter regulatory sequences from the construct. Our construct includes 5kb of 5′ promoter sequence, an amount that has been shown to be sufficient to replicate the expression patterns of many endogenous genes in transgenic fish (Park, Kim et al. 2000; Ju, Chong et al. 2003; Yeo, Kim et al. 2007). However, the work of Kikuta *et. al.* (Kikuta, Laplante et al. 2007) has shown that genes can be classified into two broad types depending on whether their transcription is regulated by elements close to the transcription initiation site (“bystander genes”) or dispersed over relatively large genetic distances defining “genomic regulatory blocks” (GRBs).

Typically, the genes regulated by elements dispersed over GRBs are those regulating embryo development such as *tbx16.*

The particular differences in transgene expression between the individual lines are more likely due to the influence upon their transcription of different regulatory elements flanking the transgene insertion sites in the genome. Interestingly, one transgenic line, 812C, uniquely expresses GFP in a subset of spinal interneurons. These cells are distributed in a seemingly irregular pattern and can be found along the dorsal longitudinal fasciculus (Fig. 4a). They have ascending and descending longitudinal projections, and a single ventral projecting axon that crosses the midline and ascends in the DLF contralateral to the cell body (Fig. 4a, b). The morphology and distribution of these cells is consistent with them being commissural primary ascending (CoPA) interneurons. Furthermore these cells have been shown to be marked by transcripts of the *mafba* gene. The 812C transgenic line may be useful for studies of the birth and development of CoPAs and their projections.

The zebrafish spinal cord consists of a limited number of discrete neuronal cell types. Although it is possible to distinguish each of these types by their spatiotemporal position and morphology few unique molecular markers of these cells have been discovered. Circumferential Ascending (CiA) interneurons express *Eng1b* transcripts (Higashijima, Masino et al. 2004). *alx* is expressed in Circumferential Descending (CiD) interneurons although it appears, perhaps, that not all CiD neurons express this gene (Kimura, Okamura et al. 2006). We previously showed that DoLA cells express *tbx16* (Tamme, Wells et al. 2002). We believe that the gene *mafba* – the expression pattern of which superficially resembles that of *tbx16* that marks DoLAs in the spinal cord - may be a unique marker of another zebrafish spinal neuron, the CoPA interneuron. Since DoLAs and CoPAs share very similar dorsoventral positions and rostrocaudal distributions in the spinal cord, it is interesting to speculate that regulatory elements within our transgene are cooperating with elements flanking the transgene insertion site to alter activation of transcription away from DoLAs so that it occurs instead in CoPAs. Identification of the GRB in which our 812C insertion has occurred may help to explain this phenomenon.

However, the idea that some elements required for neuronal expression occur within the 7.5kb of DNA from the *tbx16* locus within our transgene is supported by the fact that all of our transgenic lines show expression at some stage in neurons.

Figures

Fig. 1 Genomic region of the *tbx16* gene and transgenic construct. (a) *Eco*RI and *Bam*HI restriction sites in the genomic region of the *tbx16* gene surrounding the transcription initiation site. The interval marked "CLGY6" indicates the published enhancer trap insertion site resulting in GFP expression that recapitulates *tbx16* gene expression (Ellingsen, Laplante et al. 2005). (b) Boxes indicate the first three exons and filled boxes are protein-coding regions. The start of translation is shown (ATG). (c) The *Eco*RI/*Bam*HI fragment of the *tbx16* promoter used in the transgenic construct includes the translation start site, the first exon, and part of the second exon of the *tbx16* gene. (d) The promoter fragment was cloned in-frame upstream of enhanced GFP (EGFP) and a SV40 polyadenylation signal (A) in the multiple cloning site of the pEGFP-N1 vector

Fig. 2 GFP expression in stable transgenic lines consistent with *tbx16* expression in wild type embryos. (a) At 12hpf strong expression is seen in the polster (arrowhead), and the presomitic mesoderm (p). Expression persists in mesoderm that has formed somites (s). (b) At 24hpf GFP expression is seen in the hatching gland (arrowhead) and in posterior somites, while it has faded from the earliest formed somites. (c) At 48hpf expression remains in the hatching gland that has formed from the polster (arrowhead) and persists in the posterior somites. (d) The same embryo as in (c). GFP expression is observed throughout the notochord (n) highlighting cell extremities. Lateral views of embryos, anterior is left and dorsal is up. Scale bars in a-c = 250μm, in d = 100μm

Fig. 3 Expression unique to individual stable transgenic lines. (a) Line 192A at 48hpf. GFP expression is observed in regions of the midbrain (arrowhead) and in the epiphysis (e). Line 512B at 24hpf (b) and 48hpf (c). Expression is seen in the dorsal aorta and in ventral neurons (n) of the spinal cord. Line 812A at 24hpf (d-e) and at 48hpf (f).

Expression can be seen in the epiphysis, the midbrain and in a banding pattern in rhombomeres (boxed). White box indicates the area enlarged in (e). Later expression is observed in the floor plate of the spinal cord (f). (g-h) Line 812C at 48hpf. Expression is seen in the epiphysis and the midbrain. GFP is also noted in a specific subpopulation of spinal cord neurons and neurons posterior of the otic vesicle in the hindbrain (boxed). White box indicates the area enlarged in (h). Lateral views of embryos, anterior is left and dorsal is up. Scale bars in a, d, g = 250μm, in b, c, e, f, h = 50μm

Fig. 4 Identification of spinal cord neurons in line 812C at 48hpf. (a) Lateral view of three GFP-positive neural cells situated in a non-uniform distribution along the dorsal longitudinal fasiculus (DLF) indicated by a tight grouping of neuronal projections. Axons projecting from neurons located in the contralateral DLF can be seen to rise from the ventral midline before joining the in-focus DLF (arrowheads). (b) Lateral view of a single GFP-positive neuron illustrating the projections originating from the cell. Two projections extend within the DLF – one ascending and one descending – and a third ventrally extending axon turns anteriorly and fades from view as it approaches the ventral midline before ascending in the contralateral DLF (not shown). (c) Two-colour *in situ* transcript hybridisation indicating that GFP-positive cells (stained red) are not the *tbx16*-positive dorsal longitudinal ascending interneurons (stained blue). (d) Two-colour *in situ* transcript hybridisation showing that neurons expressing GFP (stained red) also express *mafba* (stained blue). All embryos are positioned anterior left and dorsal up. Scale bars indicate 100 μm

Acknowledgements

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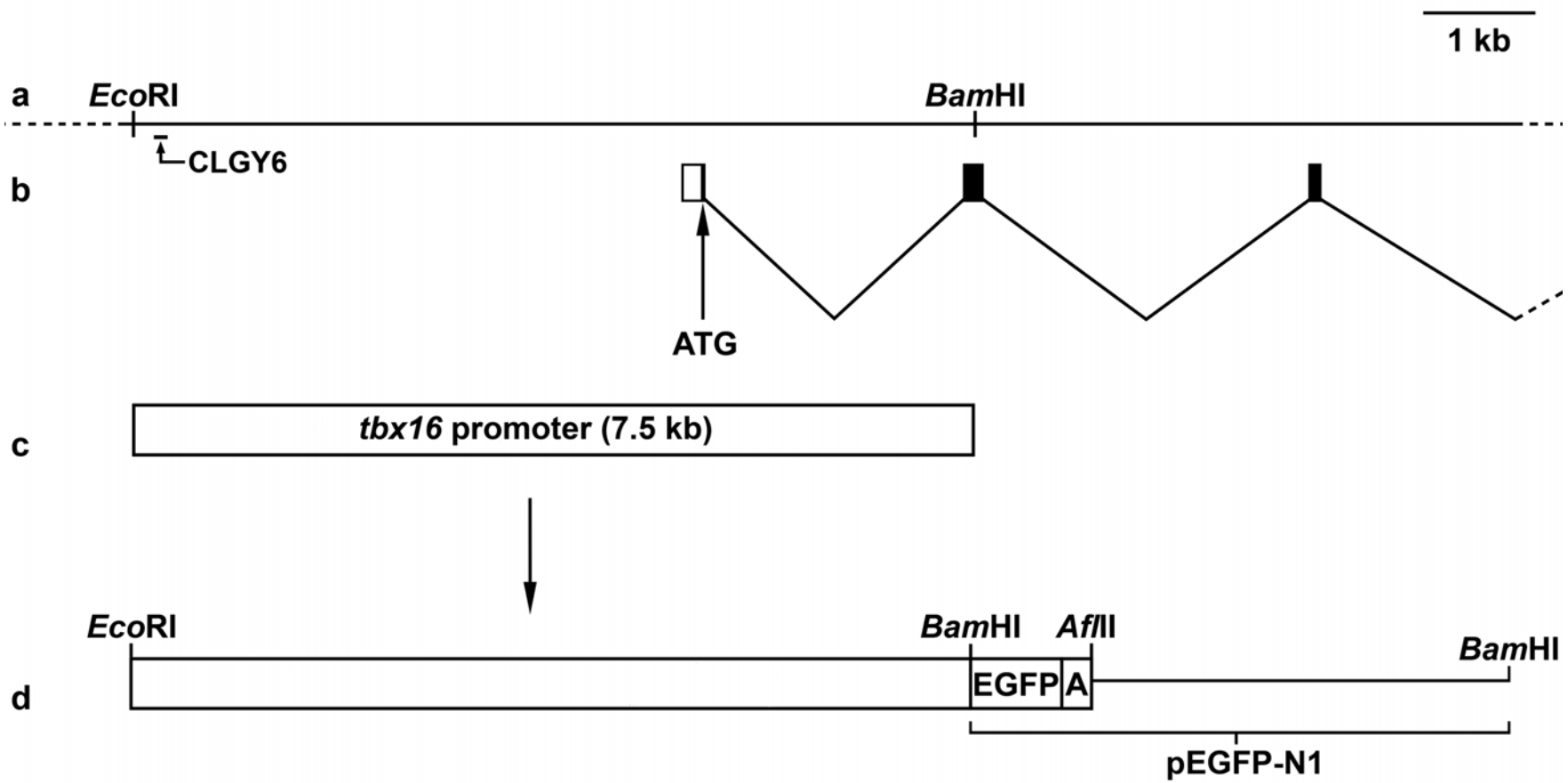

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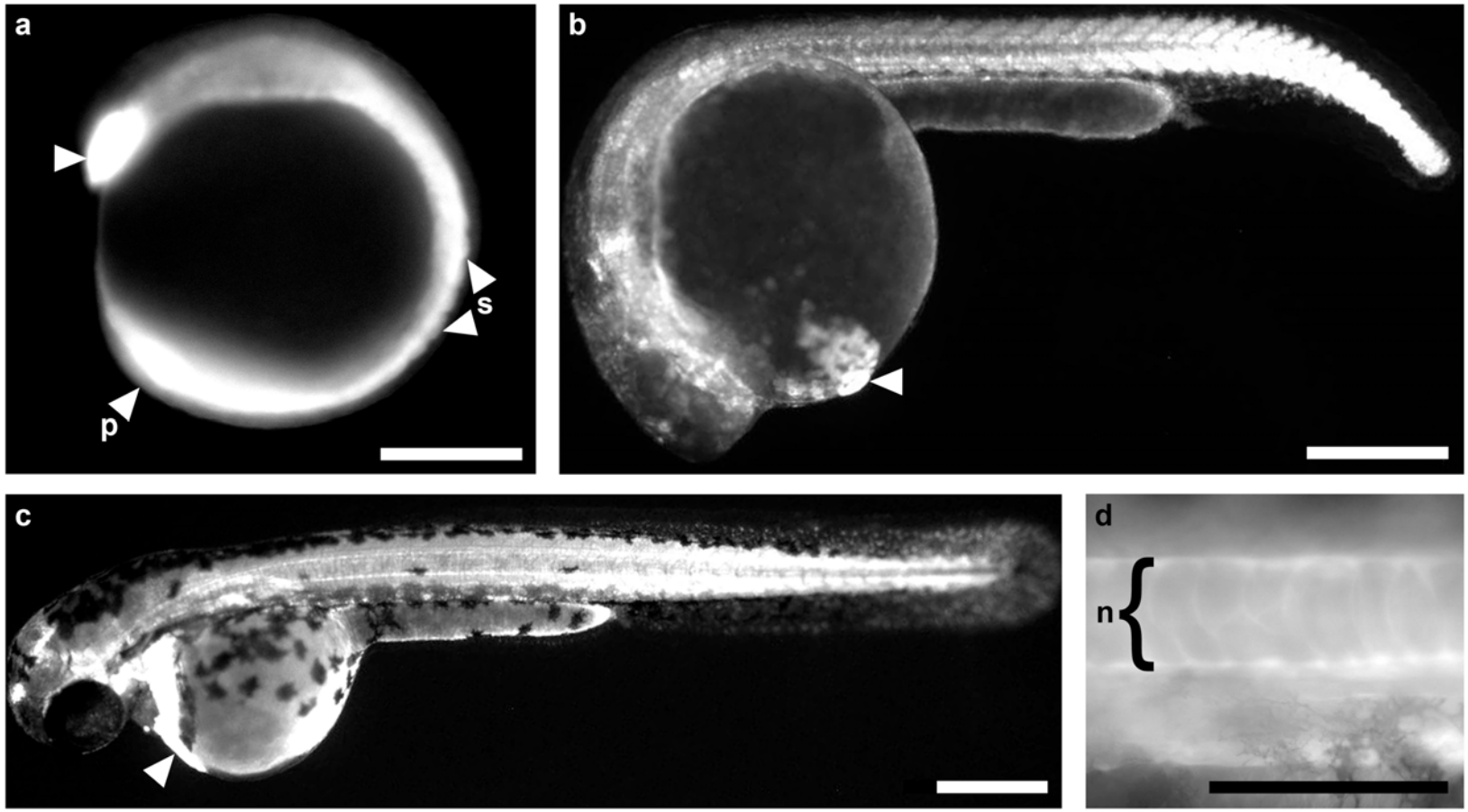

Fig. 2 GFP expression in stable transgenic lines consistent with *tbx16* expression in wild type embryos. (a) At 12hpf strong expression is seen in the polster (arrowhead), and the presomitic mesoderm (p). Expression persists in mesoderm that has formed somites (s). (b) At 24hpf GFP expression is seen in the hatching gland (arrowhead) and in posterior somites, while it has faded from the earliest formed somites. (c) At 48hpf expression remains in the hatching gland that has formed from the polster (arrowhead) and persists in the posterior somites. (d) The same embryo as in (c). GFP expression is observed throughout the notochord (n) highlighting cell extremities. Lateral views of embryos, anterior is left and dorsal is up. Scale bars in a-c = 250μm, in d = 100μm

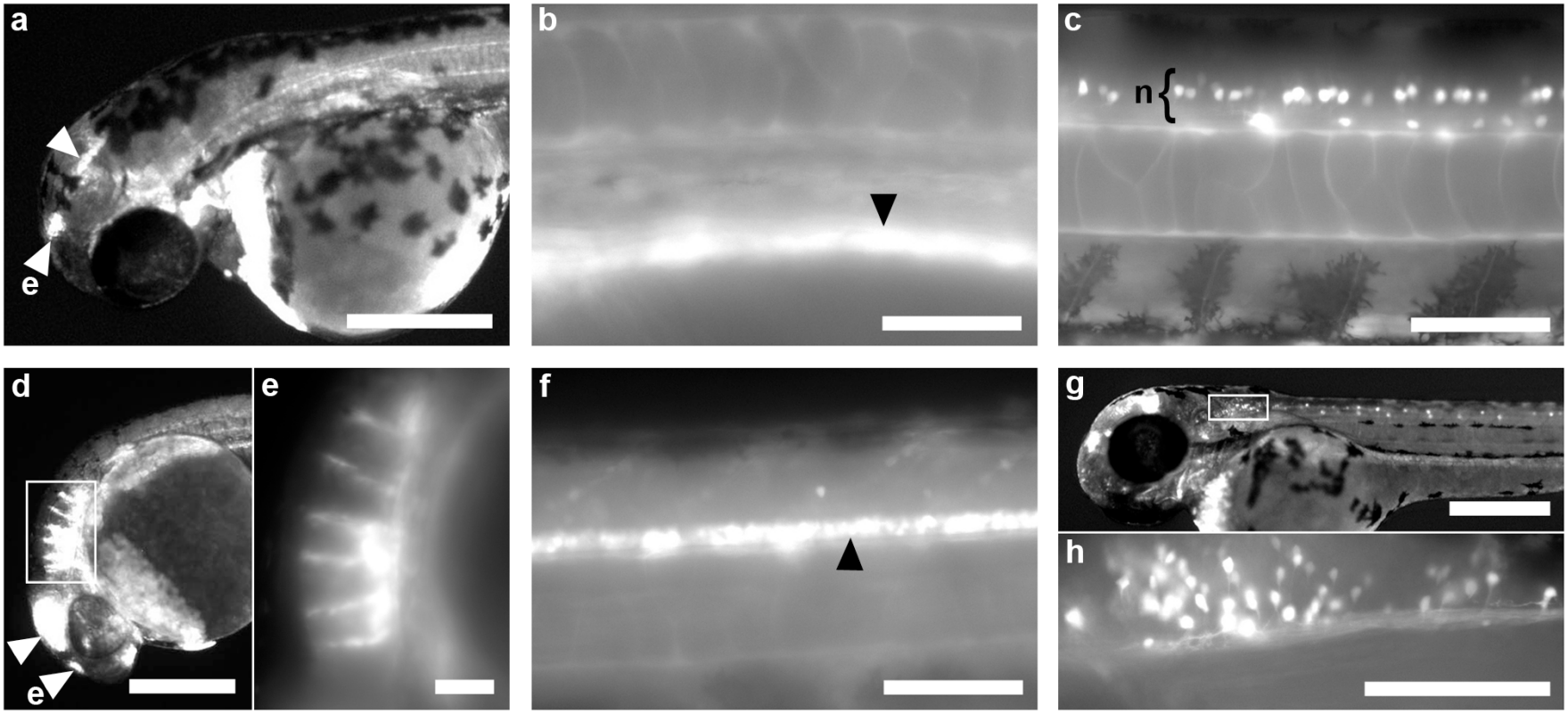

Fig. 3 Expression unique to individual stable transgenic lines. (a) Line 192A at 48hpf. GFP expression is observed in regions of the midbrain (arrowhead) and in the epiphysis (e). Line 512B at 24hpf (b) and 48hpf (c). Expression is seen in the dorsal aorta and in ventral neurons (n) of the spinal cord. Line 812A at 24hpf (d-e) and at 48hpf (f). Expression can be seen in the epiphysis, the midbrain and in a banding pattern in rhombomeres (boxed). White box indicates the area enlarged in (e). Later expression is observed in the floor plate of the spinal cord (f). (g-h) Line 812C at 48hpf. Expression is seen in the epiphysis and the midbrain. GFP is also noted in a specific subpopulation of spinal cord neurons and neurons posterior of the otic vesicle in the hindbrain (boxed). White box indicates the area enlarged in (h). Lateral views of embryos, anterior is left and dorsal is up. Scale bars in a, d, g = 250μm, in b, c, e, f, h = 50μm

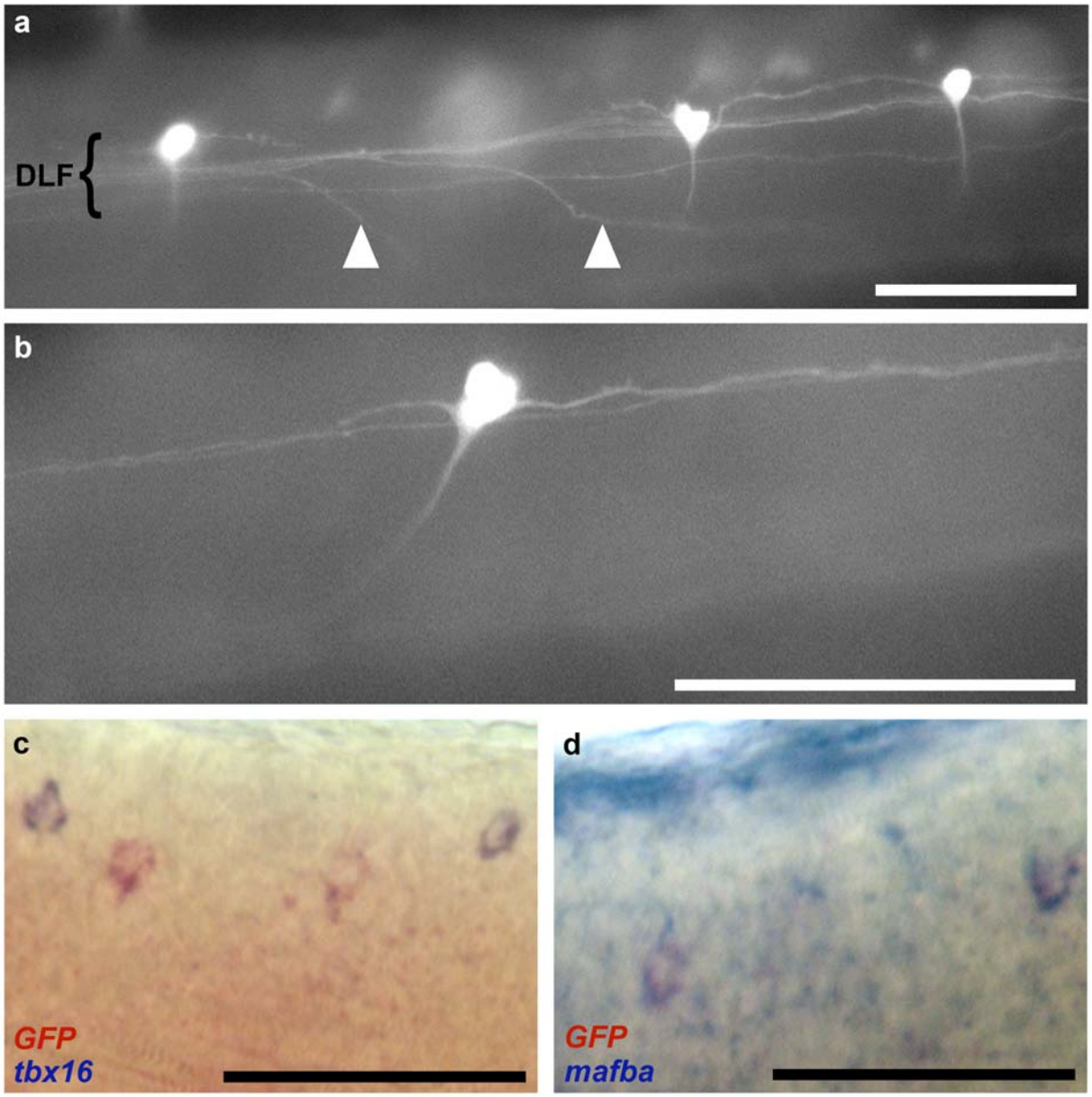

Fig. 4 Identification of spinal cord neurons in line 812C at 48hpf. (a) Lateral view of three GFP-positive neural cells situated in a non-uniform distribution along the dorsal longitudinal fasiculus (DLF) indicated by a tight grouping of neuronal projections. Axons projecting from neurons located in the contralateral DLF can be seen to rise from the ventral midline before joining the in-focus DLF (arrowheads). (b) Lateral view of a single GFP-positive neuron illustrating the projections originating from the cell. Two projections extend within the DLF – one ascending and one descending – and a third ventrally extending axon turns anteriorly and fades from view as it approaches the ventral midline before ascending in the contralateral DLF (not shown). (c) Two-colour *in situ* transcript hybridisation indicating that GFP-positive cells (stained red) are not the *tbx16*-positive dorsal longitudinal ascending interneurons (stained blue). (d) Two-colour *in situ* transcript hybridisation showing that neurons expressing GFP (stained red) also express *mafba* (stained blue). All embryos are positioned anterior left and dorsal up. Scale bars indicate 100 μm

Discussion

Paper III proposes a mechanism to explain the observed distribution of DoLA interneurons. The hypothesis states that DoLA interneurons or their precursors differentiate in the tail bud as pairs of cells before parting and moving laterally to opposite sides of the spinal cord. Here they contact the DLF and then migrate rostrally approximately 5 somite lengths resulting in statistically significant contralateral correlation. Here I would like to further justify the explanation offered in Paper III.

The DoLA interneurons migrate rostrally in the developing spinal cord. There are a number of mechanisms that could result in the imperfect but strong contralateral correlation that is observed for the final pattern of these cells.

Mechanism 1: DoLA cells may be able to communicate across the spinal cord to coordinate the position at which they stop migrating

This would allow the interpretation of a common stop-migration signal. The DoLA interneuron extends one major projection rostrally from early in its life and later will develop numerous other projections that mostly appear to be longitudinally projecting and ascending, although some project ventrally [122, 131]. While the targets of these late projections have not been defined they have only been seen developing at around 26 hpf [121], a time point after the earliest (most anterior) DoLA cells have ceased migration (Paper I). Thus migrating DoLA cells do not appear to be in direct contact through their projections at any stage.

The possibility exists that there are commissural neural connections crossing the spinal cord that can transmit information between DoLA cells. There are no documented embryonic (or larval) neurons that project across the spinal cord to contact the contralateral DLF at the same rostrocaudal level [76, 105, 121]. All neurons that project to the contralateral DLF extend neurites that ascend or descend to rostrocaudal levels that differ to the rostrocaudal position of the cell body [76, 105]. Thus there are no DoLA cells that are able to communicate directly between identical rostrocaudal levels on opposite sides of the spinal cord.

There are two neuron types that have their cell body located on the DLF, extend an axon to the contralateral DLF and exist early enough in embryonic development that they may be able to be involved with communication across the spinal cord: the CoPA and the Commissural Secondary Ascending (CoSA) interneurons [121]. The projections for both of these neurons extend several somitic lengths anteriorly before intersecting the contralateral DLF so communication directly across the spinal cord to cause the observed contralateral correlation is impossible. CoPAs begin extending growth cones before CoSAs [121] so are the more likely candidate for setting up early commissural connections. The growth cone of the earliest CoPA interneurons begins extending ventrally at around 16-17 hpf and reaches the midline by 18-19 hpf [121]. After this the projection turns and extends longitudinally and dorsally in the contralateral cord before meeting the DLF. Kuwada and Bernhardt observed that the growth cones of the earliest CoPA cells reach the level of the commissural cell bodies by about 20-21 hpf. We have observed DoLA interneurons in their most rostral position by 24 hpf (Paper I). It is possible that the DoLA neurons stop migrating prior to 24 hpf but this has not been confirmed. Thus it appears that communication across the spinal cord is available by the time we know the DoLA interneurons have ceased migrating. The timing of the stop to migration of the rostralmost DoLAs could be confirmed by observation of these neurons in *HuC:Kaede* transgenic fish described in Paper III. Alternatively, observation of DoLA cells by staining for *tbx16* transcripts in embryos of various ages between 16-24 hpf would be able to show when these cells arrive at the rostral-most position observed. This would indicate the relationship between the timing of the first commissural projections linking both DLFs and when the DoLA cells stop migrating.

CoPA axonal projections continue ascending longitudinally to reach the hindbrain by around 27 hpf [121]. This observation raises the possibility that CoPAs found earlier than this may be electrically silent and may not facilitate cross spinal cord communication. However, 85% of CoPA neurons are electrically active between 20-24 hpf [168]. The electrical signals observed in this study appear to be the rhythmic electrical signals common to early neurons attempting proper connectivity rather than *bona fide* neural signalling. Chemically mediated transmission of signals between neurons can be abolished by exposing embryos to botulinum neurotoxin [168]. This would prevent neurotransmitter signalling between neurons and therefore allow assessment of the need for neural signalling on the time DoLA cells stop migrating. Botulinum toxin would be injected at the 1-cell stage and then embryos could be analysed for the existence of cross-correlation of DoLA interneurons. It should be noted that

in the study by Saint-Amant and Drapeau neurons exposed to botulinum toxin appear able to depolarise as per normal but glycinergic neurotransmitter release was undetectable. This indicates that although depolarisation was able to occur it was not linked to the release of neurotransmitters at the synaptic cleft and therefore neural signalling was effectively blocked.

Mechanism 2: Existing tissue may produce a signal to inform migrating cells when to stop

A communicating signal that coordinates DoLA cells migration termination does not necessarily need to be electrical or even associated with neurons. One option is that these signals are programmed by tissue developing on either side of the spinal cord. The overlying somitic mesoderm could be the source of these signals but a regular repeating unit such as the somites would be expected to produce a regular repeating pattern in neurons it informs to stop such as that seen in motoneurons [108]. The pattern of DoLA interneuron distribution observed is not regular and there are not DoLA cells juxtaposed to each somite (Paper I, Paper III). The signal could originate from within the spinal cord, perhaps from another neuron. Cells in the spinal cord that are present at the correct time and dorsoventral level are the CoPA and the CoSA interneurons [121]. At up to 5 per hemisegment, the CoSA neurons are too numerous to be the source of a signal that produces such a scattered pattern [76]. The CoPA neurons appear in the embryonic spinal cord at a maximum of 1/segment [24]. DoLA neurons are often juxtaposed to CoPA neurons (see Fig. 1E, Paper I) but not always (see Fig. 4C, Paper IV) suggesting that these neurons also are not the source of a signal. Any of these proposed signals that are fixed along the rostrocaudal axis generate a particular problem: it is not at all clear how some DoLAs would have the ability to bypass a signal while the next DoLA cell gets trapped.

These possibilities imply that there is patterning along the rostrocaudal axis of the developing embryo that has the ability to guide the positioning of DoLA interneurons. There is little evidence of rostrocaudal patterning in the development of the spinal cord. The vertebrate spinal cord is somewhat genetically segmented by the *hox* genes but displays very little morphological segmentation [103, 104]. The consistent *hox* gene pattern between embryos would be expected to generate a pattern of DoLA interneurons that is somewhat

consistent between embryos. This is not observed. It appears that it is unlikely that signals fixed within the trunk and tail are responsible for stopping the migration of DoLA interneurons.

Mechanism 3: Cross-correlated DoLA neurons are born simultaneously

The DoLA interneurons could be produced as a pair in the rapidly proliferating tail tip before moving to opposite sides of the spinal cord and joining the DLF on that particular side. The cells would then migrate rostrally by an imperfect analogous distance or for an invariant distance but then some cells could be lost thus disrupting perfect cross-correlation. The final model is a combination of these two possibilities. The idea that there is paired production is supported by the fact that at 24 hpf there are statistically similar numbers of DoLA neurons on the left (10.5 +/- 2.1) and the right (10.7 +/- 1.9) sides of the spinal cord (Paper I). These numbers also indicate that the left and right sides are not absolutely equivalent in each embryo, which may be a sign of cell death following paired production or that the possibility exists that both cells of a pair occasionally move to the same side of the spinal cord. Evidence that the cells begin in an axial position in the tail before moving laterally to the DLF can be seen in the supplementary movie S2 (Paper III).

Invariant migration distance followed by cell death is unlikely. Large numbers of DoLA cells would need to be generated to produce perfect correlation of paired cells on either side of the spinal cord along the rostrocaudal axis before subsequent cell death of up to 50% of the DoLA cells. Since the number of DoLA cells does not appear to dramatically change between 24 hpf (21.4 ± 3.4) and 30 hpf (22.7 ± 2.9) it appears that little cell death occurs (Paper I). This suggests that the imperfect cross correlation is not caused by cell death but rather the other possibility - imperfect analogous migration distance. There may be a small amount of cell death as shown by the differences between the cell numbers on the left and right sides of the spinal cord (Paper I).

4. A model for the mechanism underlying DoLA cell contralateral correlation

The DoLA cells or their precursors are generated medially in the rapidly proliferating tailbud (Fig. 3a). Cell division produces pairs of cells that are temporally linked (Fig. 3b). As tail outgrowth occurs the DoLA cells are deposited bilaterally into the developing spinal cord (Fig. 3c). Pairs of cells are progressively generated by the tail bud from around the time it is formed until full extension of the tail with the occasional DoLA succumbing to cell death. At some stage the separated pair of DoLA cells begin rostralwards migration. The decision to begin migration appears to be uncoordinated between the pairs of cells. The cells move laterally from a medial position to join the DLF and then rostrally along the DLF (Fig. 3d). All evidence indicates that DoLA cells have a tendency to migrate a distance slightly more than 5 somitic lengths although there is considerable variation. A DoLA cell and its common birth partner migrate rostrally a similar distance to end up in similar rostrocaudal positions on opposite sides of the spinal cord (Fig. 3e) producing a significant but imperfect contralateral correlation.

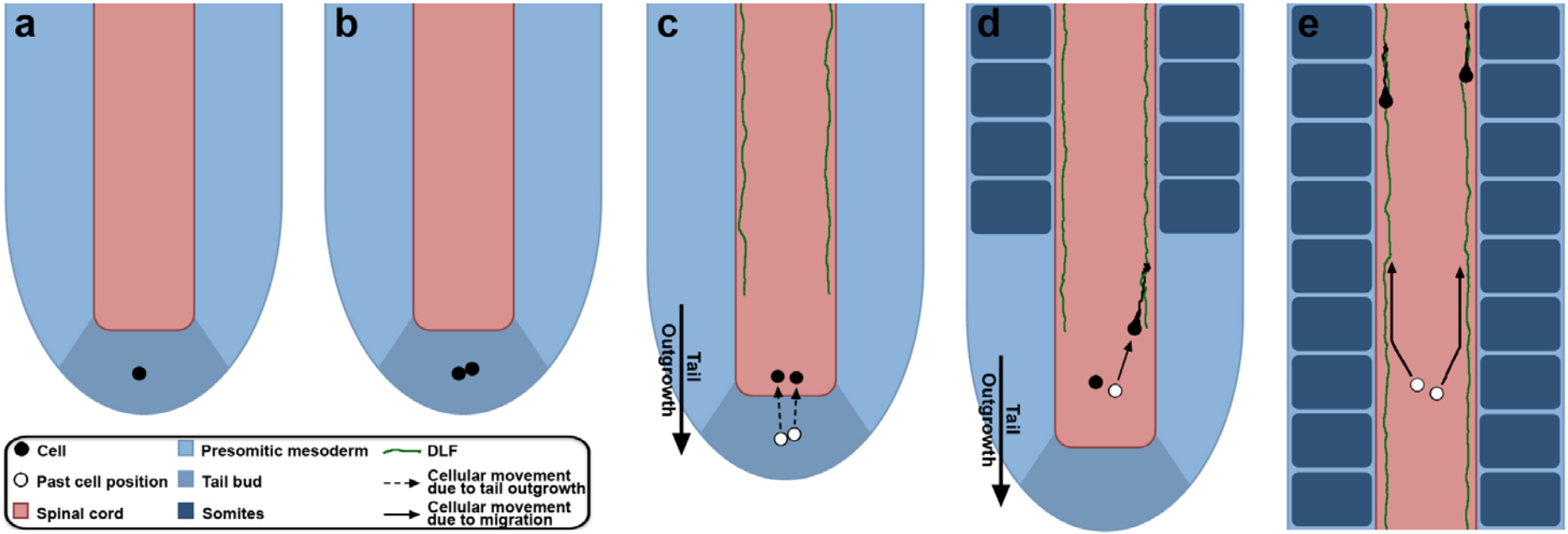

Fig. 3 - A model for the mechanism underlying DoLA cell contralateral correlation. (a) DoLA cells or their precursors are generated medially in the rapidly proliferating tail bud. (b) Cell division produces pairs of cells that are temporally linked. (c) As tail outgrowth occurs DoLA cells are deposited bilaterally (dashed arrow) into the developing spinal cord. Pairs of cells are progressively generated by the tail bud from around the time it is formed until full extension of the tail with the occasional DoLA cell succumbing to cell death. (d) At some stage the DoLA cells begin rostralwards migration; the decision to begin migration is uncoordinated between the pairs of cells. The cells move laterally from a medial position to join the DLF and the rostrally along the DLF. All evidence indicates that DoLA cells have a tendency to migrate a distance slightly more than 5 somitic lengths although there is considerable variation. (e) A DoLA cell and its common birth partner migrate rostrally an imperfect analogous distance to end up in similar rostrocaudal positions on opposite sides of the spinal cord producing a significant but imperfect contralateral correlation.

5. Future directions

The next task to confirm this hypothesis will be to determine the origin of DoLA neurons. The model proposes that DoLA interneurons or their precursors are generated medially in the rapidly dividing tail bud. Transgenic embryos are unlikely to be able to be used for this purpose. *HuC:Kaede* transgenic embryos do not have sufficient amounts of the Kaede protein within DoLA interneurons to be visualised until the cells have been deposited in the spinal cord and are rostral of the proliferative tail tip (see supplementary movie S2, Paper III). This is likely to be the case for other transgenic embryos expressing the genes that are known to mark DoLA interneurons: *tbx16, islet-1, -2,* and *-3* (Paper I). Any transgenic reporter gene needs to express for long enough to build up a recordable level of protein and it appears that this region develops too rapidly for the possible visualisation to occur. A possible solution to this may be to carry out cell labelling such as the uncaging protocol detailed in Paper III but to further restrict the area activated to solely the proliferative area in the tail tip. Once a time-lapse series has been produced the origin of the migrating DoLA cells may be determined. Uncaged embryos may also be used to observe the timing of the decision to begin migration and determine if this decision is a coordinated event between pairs of cells. Uncaging may also detect early occurrences of cell death that result in differing numbers of DoLA interneurons on the left and right sides of the embryo or occurrences of both cells of a pair remaining on one side of the spinal cord.

Previous experience with uncaging has shown that observation of embryos gradually uncages inactivate dye (Paper III, Fig. 5). Thus extended observation of the DoLA cells over their migratory life time will most likely require the use of the existing *HuC:Kaede* transgenic lines or a newly constructed *tbx16:GFP* transgenic. A transgenic that expresses *GFP* in the DoLA cells should be relatively easy to create given current molecular methods (described below). Once a transgenic line expressing GFP in the DoLA neurons is established, this will allow research into genetic and biochemical factors involved in neural migration. Additionally this transgenic line may offer some insight into the suspected ipsilateral clumping observed in developing embryos.

The zebrafish community is beginning to understand the development of the embryonic zebrafish spinal cord by using transgenic embryos that express GFP in the various neurons. The axons generated by CoPA interneurons need to be able to navigate ventrally, cross the midline and ascend in the contralateral spinal cord. Additionally these neurons extend an ascending and a descending projection in the ipsilateral spinal cord. The 812C transgenic line described in this thesis offers a unique opportunity to study the axonogenesis and pathfinding decisions undertaken by the projections produced by the CoPA interneurons.

6. The creation of a new *tbx16:GFP* transgenic line

The promoter region used for creating *tbx16:GFP* transgenic lines described in Paper IV appears to be insufficient to faithfully drive *GFP* expression to recapitulate endogenous *tbx16* expression at all developmental time points. This deficiency is probably due to the limited extent of *tbx16* promoter sequence used to create the transgenic construct. While minimal promoters driving the expression of reporter genes have been shown to faithfully reproduce the endogenous expression of genes of interest [169-171] it is often necessary to include much larger segments of promoter to encompass distant gene regulatory regions. The promoter region used to generate the *tbx16:GFP* transgenics was sourced from a commercially available BAC clone and the appropriate segment was isolated and cloned through conventional means. Currently, methods exist to create transgenic constructs with much larger promoter regions. One example is the use of bacterial artificial chromosome (BAC) recombination systems [172] one of which incorporates the ToL2 transposon system [173]

The construct used to produce the *tbx16:GFP* transgenics described in Paper IV contained enhanced GFP (EGFP). EGFP has been optimised for increased fluorescence [174, 175]. High expression of EGFP was observed in presomitic mesoderm in stable transgenic lines, recapitulating endogenous *tbx16* expression. However, EGFP was seen to persist in somites generated from presomitic mesoderm a

pattern not the same as endogenous *tbx16* expression. The expression of EGFP was to such an extent that the developing spinal cord was often obscured by the overlying somites. Thus a new transgenic line should be constructed with the destabilised version of GFP [176]. This protein shows a faster turn over due to the inclusion of a degradation domain.

The transgenic lines described in Paper IV displayed variation in the expression patterns of GFP. This is supposedly due to variation in the genomic insertion site leading to exposure to different enhancer regions. Recently, a vector has been developed for the generation of enhancer trap transgenic lines that includes insulator regions that protect the included promoter from insertion position effects [177]. This vector also contains a positive control for transgenesis. It may be possible to use these insulator regions to further protect an inserted transgene from the effects of enhancers surrounding the random insertion sites.

Original attempts at introducing exogenous DNA into a genome to create a transgenic organism consisted of simply injecting linear DNA. The subsequent screening of possible transgenics was laborious due to the low rate of germline transmission. The germline transmission rates for injected embryos have been greatly improved since the introduction of the use of transposon systems. Of the transposon systems established for use in zebrafish there are two that are now regularly used: the Sleeping beauty transposon and the Tol2 transposon. Recently a “hyperactive” Sleeping beauty transposon was created through a large-scale mutation screen [178] and shown to be 8 times more efficient at inducing transient transgenic expression [179]. The Tol2 transposon has also undergone significant modifications that appear to increase the efficiency at which transgene insertion occurs by 50-70% [180, 181]. This represents at least a tenfold increase in transgenesis efficiency over injection of non-transposon associated DNA. Recently, the Gateway cloning method has also been added to the Tol2 system to enhance the ease of construct production [182, 183].

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Thesis Alterations

Page 3: Publications - alter to show full references as on pages 26, 41, 67, 85

Page 5: microdisection (change to "microdissection")

Page 5: most tissues and organs are generated (change to "evident")

Page 6: transplanted in a wild type background (change to "into")

Page 8: This expression pattern is consistent with the phenotypes observed in spt mutant embryos. Replace this statement with: "Regions of *spt* mutant embryos that appear to lose *tbx16* expression compared to wild-type embryos are those tissues that are absent in *spt* mutant embryos."

Page 9: zebrafish *tbx6*, *tbx16* or *tbx24* genes (change to "*tbx6* or *tbx16* genes")

Page 9: At the end of the third paragraph add: "Confounding this relationship further is that the teleost lineage underwent a whole genome duplication event some 350 million years ago (Meyer and Van de Peer 2005). This has resulted in the existence of paralogues such as zebrafish *tbx15* and *tbx18* having similar levels of identity to human *TBX18* (Synorth: http://synorth.genereg.net/)."

Page 15: although somewhat genetically segmented_, (change to "although genetically segmented by *hox* gene expression,")

Page 15: motoneurons subtypes (change to "motoneuron subtypes")

Page 15: The signalling processes that result in the distribution of motoneurons or Rohon-Beard neurons are relatively easy to understand (at the end of this sentence add: "(as detailed in the previous two paragraphs)")

Page 18 (Figure 2): after the title of Fig. 2, add: CiA - circumferential ascending (v1), CiD - circumferential descending (v2a), CoBL - commissural bifurcating lateral (dl2/dl3/dl6?), CoLA - commissural longitudinal ascending, CoLo - commissural local (dl2/dl3?), CoPA - commissural primary ascending (dl1), CoSA - commissural secondary ascending (v0), DoLA - dorsal longitudinal ascending (dl3/dl4?), KA - Kolmer-Agduhr, MCoD - multipolar commissural descending, UCoD - unipolar commissural descending (v3), VeLD - ventral longitudinal descending (v2b), VeMe - ventral medial (v3), VeSe - ventral serotonergic. Terms in parentheses indicate putative zebrafish neuronal homologues generated from vertebrate neuroprogenitors (shown in Fig.1) based on position, morphology and gene expression data (reviewed in Goulding, 2009).

Page 19: apoptose (change to "undergo apoptosis")

Page 23: several of hypotheses (delete "of")

Page 24: 8-day-old larva (change to "larvae")

Page 107: Add a new paragraph that appears first: Paper I detailed key preliminary work that suggested the possibility that the DoLA neurons migrate after their birth in the spinal cord. The use of the uncaging technique and the examination of DoLA activity in HuC:Kaede fish confirmed that these cells are indeed migratory (Paper III). Work presented in Paper II indicates that control of DoLA cell number is partially under the control of Notch-signalling. Furthermore these results show that Psen2 rather than Psen1 has a greater influence in the control of Notch-signalling involved in the differentiation of DoLA interneurons.

Page 115, paragraph 2: add extra information after sentence 2: Different genomic insertion sites may be identified by sequencing regions flanking the insertion site. This could be achieved by using inverse PCR (Ochman *et al* 1988) on purified genomic DNA from each of the individual lines. While these experiments would highlight the reasons behind the observed differences in GFP expression between transgenic lines, the construction of a new transgenic line with larger promoter regions would obviate the need to undertake this procedure.

Page 115 (at the end): add Conclusions and significance:

Most spinal cord neurons have an infrequent irregular distribution along the rostrocaudal axis so the analysis of how these patterns arise is essential to our understanding of the development of the spinal cord.

The work presented in this thesis demonstrates that the seemingly irregular distribution of zebrafish DoLA interneurons has an underlying cryptic organisation. The cryptic organisation can be partially explained by the fact that these cells migrate rostrally after differentiating. Furthermore, DoLA interneurons are the only cells that we have detected that migrate rostrally during early embryonic development of the spinal cord. The uncaging technique described in this thesis work was developed specifically to visualise that DoLA interneurons migrate. This technique will be useful for the analysis of molecules and mechanisms controlling neuron migration in the spinal cord.

The DoLA interneuron distribution is in part influenced by Notch-signalling. Additionally, it seems that Psen2 rather than Psen1 has a greater influence in the control of the Notch-signalling involved in the differentiation of DoLA neurons. The assay for DoLA cell number has become important in determining the function of Psen2.

DoLA interneurons persist in the spinal cord for longer than previously known. Previously, they have not been observed in the larval spinal cord. Our observations suggest that DoLAs have an ongoing function in the larval spinal cord.

Transgenic zebrafish expressing GFP under the control of *tbx16* promoter region will allow researchers to study the early function of *tbx16*. Additionally, serendipitously, one of these lines expresses GFP in CoPA interneurons which will allow closer examination of the development of these cells and their projections. This led to the discovery that CoPA interneurons express the gene *mafba/valentino*. This information adds to the small list of unique spinal interneuron markers in zebrafish.

These results highlight the importance of investigating the molecules and mechanisms that influence "irregular" patterns of cell distribution. This is particularly important in the CNS where most cells have an irregular distribution, but is also critical in other tissues that display irregular patterning.

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