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Separating Early Sensory Neuron and Blood Vessel Patterning

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The anatomical association between sensory nerves and blood vessels is well recognised in the adult, and interactions between the two are important during development. Here we have examined the relationship between developing blood vessels and sensory neuronal cell bodies, which is less well understood. We show in the chick that the nascent dorsal root ganglia (DRG) lie dorsal to the longitudinal anastomosis, adjacent to the developing neural tube at the level of the sulcus limitans. Furthermore, the blood vessel is present prior to the neurons suggesting that it may play a role in positioning the DRG. We use the zebrafish *cloche* mutation to analyse DRG formation in the absence of blood vessels and show that the DRG are positioned normally. Thus, despite their close anatomical relationship, the patterning of the blood vessel and DRG alongside the neural tube is separable rather than interdependent. *Developmental Dynamics 239:3297–3302, 2010.* © 2010 Wiley-Liss, Inc.

Key words: DRG; sensory neuron; neural crest; blood vessel

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INTRODUCTION

Interactions between the nervous system and blood vessels have been shown to play an important role in a number of aspects of development. In the cortex, the close anatomical relationship between the neural tissue and blood vessels has been shown to regulate neurogenesis both in the adult and during embryogenesis. Neural stem cells are localised to vascular niches where the endothelial cells provide signals that maintain self-renewal and expand neurogenesis (Shen et al., 2004; Stubbs et al., 2009). These blood vessels also provide a substrate for neurite outgrowth (Stubbs et al., 2009). In the periphery of the body, nerves and blood vessels are also juxtaposed, sharing complex branching patterns. One line of evidence points to this neurovascular congruence arising as a result of signals from the peripheral sensory nerves driving alignment and arteriogenesis of the vessels (Mukouyama et al., 2002, 2005). Further evidence points to a shared mechanism of patterning with the blood vessels and nerves both expressing molecules involved in axon guidance and thus responding to the same cues (Bates et al., 2003; Larrivee et al., 2009). Although many studies have addressed peripheral nerve patterning, interactions with the sensory neuron cell bodies have not been examined. Previous studies have demonstrated a role for blood vessels in neuronal cell migration. For example, they provide a scaffold for newly migrating neurons of the rostral migratory stream to enter the adult olfactory bulb (Bovetti et al., 2007). Furthermore, in the peripheral nervous system blood vessels play a key role in positioning the sympathetic ganglia and enteric neural crest providing cues for migration and differentiation (Reissmann et al., 1996; Kasemeier-Kulesa et al., 2005; Nagy et al., 2009). Here to address a potential interaction driving positioning of sensory neurons, we analyse the relationship between

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blood vessels and developing sensory ganglia, the dorsal root ganglia (DRG).

The DRG containing the cell bodies of the primary sensory neurons are organised metamerically along the axis of the vertebrate trunk, located bilateral to the neural tube. The neurons and supporting glia that make up the DRG are derived from neural crest cells, a population of highly migratory multipotent cells that emigrate from the dorsal aspect of the neural tube and migrate into the periphery of the embryo (Le Douarin and Kalcheim, 1999). Neural crest cells give rise to a wide range of derivatives, dependent in part on the migratory route that they follow. In the trunk there are three main routes: an early ventromedial intersomitic route taken largely by neural crest cells that will go on to give rise to the neurons and glia of the sympathetic ganglia; a later developing ventromedial route through the anterior half of the somitic sclerotome, giving rise to sensory and sympathetic ganglia; and a final dorsolateral route giving rise to melanocytes (Gammill and Roffers-Agarwal, 2010). On the later ventromedial route, a sub-population of neural crest cells aggregate within the sclerotome and form the neurons and glia of the DRG, while the remainder continue to migrate ventrally and give rise to the sympathetic ganglia in close proximity to the dorsal aorta (Kasemeier-Kulesa et al., 2005).

The DRG are generated as segmental structures as neural crest cells migrate preferentially through the anterior half of the somite largely driven by a sequential combination of Sema3a and Sema3F signalling (Gammill et al., 2006; Schwarz et al., 2009). However mechanisms that cause a sub-population of neural crest cells to stop and form the DRG at a specific point on the dorso-ventral axis are less well understood. Previous studies have implicated the neural tube in some aspects of DRG development. For example, barrier insertion between the neural tube and neural crest cells showed that signals from the neural tube are necessary for DRG survival (Kalcheim and Le Douarin, 1986). Similarly, Wnt1 signalling from the dorsal neural tube is required for sensory neuron differentiation (Lee et al., 2004). However, this general requirement for the neural tube does not fully account for the positioning of the DRG at a specific dorsoventral site.

Given the evidence for blood vessels playing a role in positioning neural crest derivatives (sympathetic and enteric), and studies that show that endothelial cells can influence neurogenesis in the DRG (Mompeo et al., 2003), we hypothesised that blood vessels could play a role in positioning the DRG on the dorsoventral axis. Using intracardiac lectin perfusion, we show that the longitudinal anastomosis of the blood vessels along the neural tube is adjacent to the forming ganglia and arises prior to the development of the ganglia. We have investigated a possible relationship between the blood vessel and positioning of the ganglia using the zebrafish *cloche* mutant that lacks blood vessels and find that the ganglia are positioned normally. Our results suggest that although they are positioned at the same anatomical location, the patterning of sensory ganglia and blood vessels arises independently.

RESULTS AND DISCUSSION

Early Anatomical Relationship Between Blood Vessels and Dorsal Root Ganglia

The DRG are derived from a sub-population of neural crest cells that migrate on the ventromedial pathway through the anterior somite. To determine the dorso-ventral position of the early sensory neuron progenitors in relation to the general neural crest population, we used in situ hybridisation with *Isl1*, a marker of sensory neurons, and *Sox10*, a pan-neural crest marker. *Sox10* expression shows that neural crest migration in the trunk is well underway at HH17 in the chick (Fig. 1A, C). Furthermore, a population of *Isl1*-positive neural crest cells can be seen arrested adjacent to the neural tube dorsal to the presumptive sulcus limitans at the midpoint of the dorso-ventral axis (Fig. 1B, D).

To establish the position of forming blood vessels in the chick at HH17, we used intracardiac injection of a biotinylated form of the lectin, concanavalin A (ConA). ConA has been shown previously to bind well to the vasculature of early chick embryos (Jilani et al., 2003). Here visualisation of the lectin binding with fluorescein-conjugated avidin staining of the whole embryo highlights the developing blood vessels in the head and trunk (Fig. 2). In the trunk, the lectin clearly labels the dorsal aorta and the inter-somitic vessels (ISV) sprouting from it (Fig. 2B). The development of the vasculature shows a rostro-caudal gradient as expected. In the more rostral trunk, where the ISVs are more developed, a connection between neighbouring ISV can be seen at their dorsal extent (Fig. 2B). This connection corresponds to the longitudinal anastomosis of the neural tube (LA), which runs alongside the neural tube at the midpoint of the dorso-ventral axis before elaborating to become the DRG capillary plexus (Keibel and Mall, 1912). In the more caudal trunk and less mature ISV, looping can be seen where the LA is forming (Fig. 2B). In the head, the lectin clearly labels the aortic arches (I, II, III) of the pharyngeal arches (PA) and the dorsal aorta (Fig. 2A, C). The internal carotid artery (IC) can be seen extending rostrally from the dorsal aorta, as well as the capillary plexus (CP) surrounding the developing brain (Fig. 2C). Furthermore, the anterior cardinal vein (ACV) can be seen running dorsal to the dorsal aorta in the pharyngeal region (Fig. 2C).

To determine the relationship between the developing DRG and the vasculature, we combined the lectin binding with antibody staining for the neuronal markers, neurofilament medium chain (NFM), and Elavl3/4 (also known as HuC/D). NFM labelling is distributed throughout the cell body and axon, and can be seen in the central nervous system and extending out into the periphery of the embryo (magenta in Fig. 3). In the periphery, NFM staining is detectable adjacent to the ISVs (green), which corresponds to axons running alongside the ISV (white arrowhead, Fig. 3C). Furthermore, it appears that the development of the ISVs precedes that of the surrounding neurons as the ISVs can be seen more caudally than the NFM labelling of axons (orange arrowhead, Fig. 3C). However, as

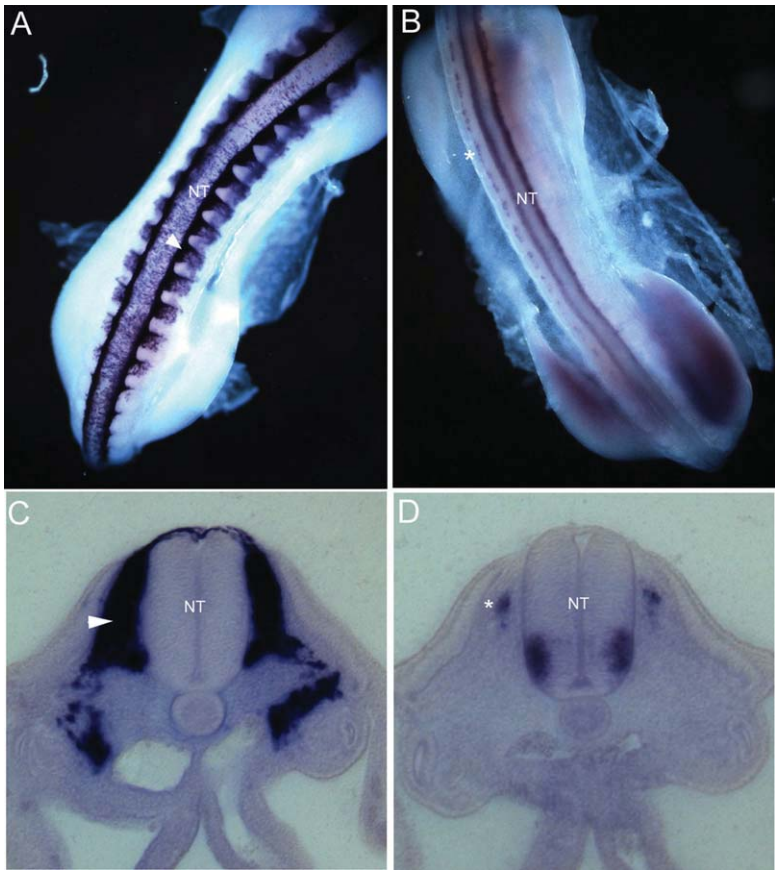

Fig. 1. Sensory neuron precursors halt dorsal to the midpoint of the neural tube dorsoventral axis. In situ hybridisation with Sox10 (**A,C**) and Isl1 (**B,D**) in HH17+ chick embryos. Sox10-positive neural crest cells migrate from the dorsal neural tube in the periphery (A) dorsal view ×2.5, (C) transverse section ×20. Isl1 shows a number of neuronal cells lying alongside the neural tube in the nascent DRG. The Isl1-positive cells are restricted to a position dorsal to the midpoint of the neural tube dorsoventral axis. (B) dorsal view ×2.5, (D) transverse section ×20. NT, neural tube; arrowhead, migrating neural crest; *, nascent DRG.

NFM labels efferent motor axons as well as developing sensory neurons, we turned to Elavl3/4 to determine the position of the DRG. Elavl3/4 labels post-mitotic neurons and is restricted to the cell body (Marusich et al., 1994). In cross-section at HH17, Elavl3/4-positive cells can be seen at the site of DRG formation (asterisks, Fig. 3E). Comparison with the lectin staining shows that the Elavl 3/4 cells are found dorsal to the LA within the sclerotome of the somite (Fig. 3F, G). By HH18, the vasculature has become more robust and it can be seen that the relationship between blood vessels and DRG is maintained (Fig. 3H). The rostro-caudal development of the vasculature and ganglia can be used to determine which structure is in position first. Nascent DRG are not seen beyond somite 30 of HH18 embryos (n = 3) while the vasculature is evident in more caudal somites (Fig. 3I, J). As the blood vessels are in position first, this suggests that they may play a role in positioning the DRG.

Blood Vessels Are Not Required for Correct Positioning of the DRG in Zebrafish

To address the potential role for the blood vessels in positioning the DRG, we wanted to perturb blood vessel development. The close anatomical

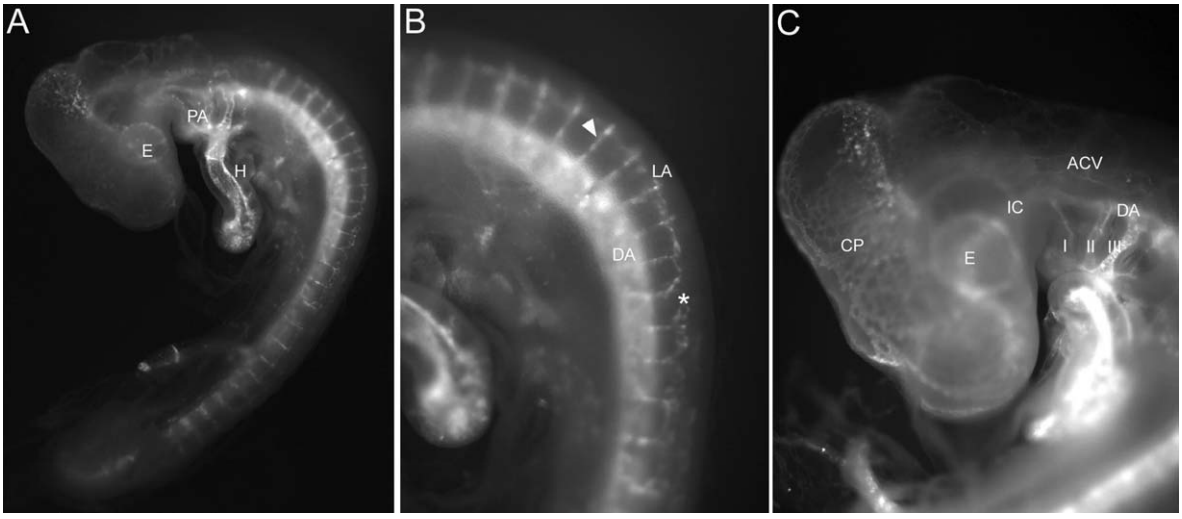

Fig. 2. Intra-cardiac injection of lectin conA labels developing blood vessels in the chick embryo. **A**: Lateral view of whole embryo shows developing vasculature. **B**: Higher magnification of trunk region shows the rostrocaudal development of the ISV (arrowhead) with more rostral ISV connected by the LA, which is still forming more caudally (*). **C**: Higher magnification of the head shows the aortic arches (I, II, III), IC, ACV, and the capillary plexus associated with the developing brain. ACV, anterior cardinal vein; DA, dorsal aorta; E, eye; H, heart; IC, internal carotid; LA, longitudinal anastomosis; PA, pharyngeal arches.

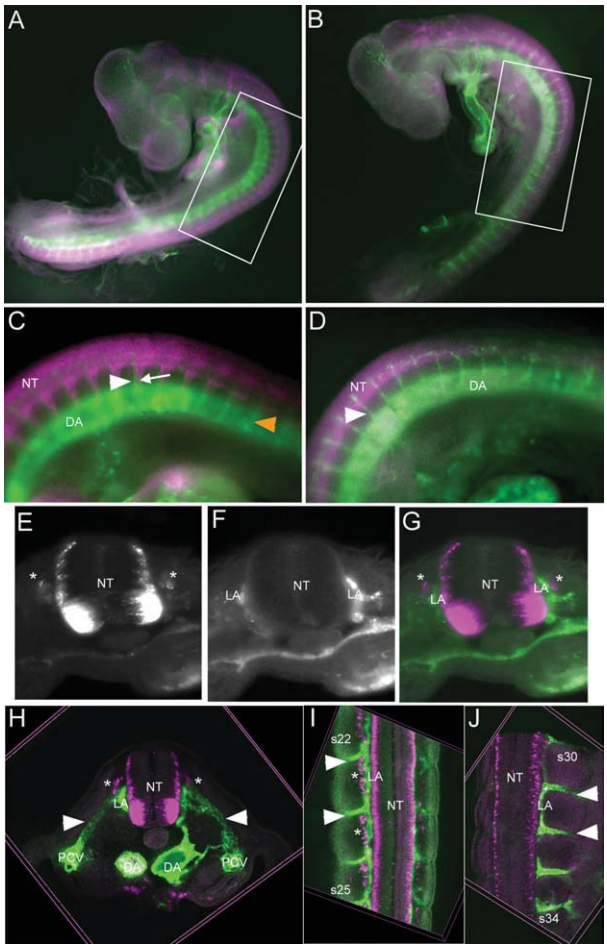

Fig. 3.

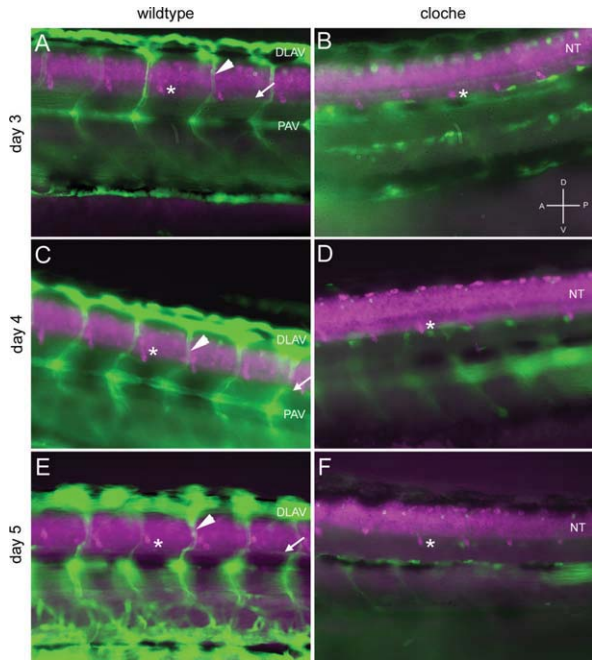

Fig. 4.

relationship between the developing blood vessels and ganglia means that using embryo manipulation to physically remove the vasculature in the chick embryo would potentially damage the adjacent structures making it difficult to determine the direct effects. Furthermore, as many of the molecules that have been implicated in blood vessel patterning are also involved in nervous system development, it is difficult to use these to reroute blood vessels with no direct

Fig. 3. Anatomical relationship between blood vessels and DRG in the chick embryo. **A–D**: Lateral view of whole embryos stained for lectin and NFM (A,C) and Elavl3/4 (B,D). C,D represent higher magnifications of boxed regions in A,B. A,C: At HH18, NFM staining shows axons (arrow) extending into the periphery alongside the ISV (arrowhead). B,D: at HH17+ Elavl3/4-positive neurons can be seen in the neural tube, and in the cranial sensory ganglia, but in the trunk are not distinguishable from the neural tube in this lateral view. **E–G**: Elavl3/4/lectin transverse section at HH17 ×20. (E) Elavl3/4 alone, (F) lectin alone, (G) overlay of Elavl3/4 and lectin shows that Elavl3/4-positive neurons are in the periphery at the midpoint of the dorsoventral axis of the neural tube, and they show a complementary relationship with the blood vessels. **H**: Elavl3/4/lectin at HH18. 3D visualisation of a transverse section confocal stack (×10) shows that the vasculature is more robust at this stage than earlier stages, and the neurons are aggregating at the midpoint of the dorso-ventral axis of the neural tube just dorsal to the LA. **I, J**: Dorsal view of Elavl3/4/lectin at HH18: 3D visualisation of confocal stacks, ×10. **I**: Analysis at somites 22–25 shows neurons in the periphery adjacent to the LA. **J**: Analysis at somites 30–34 in the same embryo shows that there are no neurons in the periphery although the vasculature is present. DA, dorsal aorta; LA, longitudinal anastomosis; NT, neural tube; PVC, posterior cardinal vein; s, somite; *, nascent DRG; arrowhead, intersomitic vessel.

Fig. 4. Loss of blood vessels in the *cloche* mutant zebrafish does not affect the positioning of the DRG. **A,C,E**: Lateral view of fli1:gfp transgenic embryos stained for Elavl3/4 at 3 (A), 4 (C), and 5 (E) dpf. **B,D,F**: Lateral view of tg(fli1:gfp) *cloche* mutant embryos stained for Elavl3/4 at 3 (B), 4 (D), and 5 (F) dpf. The fli1:gfp labelling indicates the vasculature and in the normal embryos the intersomitic vessels (arrowhead), dorsal longitudinal anastomotic vessel (DLAV), parachordal vessel (PAV), and vertebral artery (arrow) can be seen, whereas these are all absent in the *cloche* embryos. Elavl3/4-positive neurons can be seen in the neural tube (NT) and in the developing DRG (*). The dorsoventral position of the DRG is the same in both normal and mutant embryos.

effect on neurons. We, therefore, used the zebrafish mutant *cloche* in which blood and endothelial cell development is severely compromised leading to a lack of blood vessels along the length of the body (Fig. 4) (Stainier et al., 1995). We visualised the developing vasculature using a fli1:GFP transgenic line (Fig. 4, green) combined with whole-mount immunostaining for Elavl3/4 (Fig. 4, magenta) to identify the DRG. In zebrafish, the DRG (Fig. 4, asterisks) form more ventrally than in the chick, but can be seen to form in close proximity to the longitudinal vertebral vessel (Fig. 4A, C, E, arrow) (Kamei and Weinstein, 2005). Comparison of wildtype and *cloche* mutant embryos at 3, 4, 5dpf shows that the DRG are positioned as normal in the absence of blood vessels (Fig. 4). Occasionally, ectopic DRG neurons were seen in the mutant embryos (data not shown); however, these are also seen in wildtype embryos as has been described previously (An et al., 2002). Previous in vitro studies using rat tissue has suggested that endothelial cells may play a role in sensory neuron proliferation (Mompeo et al., 2003). In our study, it was not possible to determine whether the blood vessels play any later role in proliferation or survival of the DRG neurons as 5dpf is the limit of viability for the *cloche* mutant embryos.

Our results demonstrate that there is an anatomical relationship between the developing DRG and vasculature, but that this does not reflect a functional interaction at least at early stages of ganglion formation. Neurovascular congruency is well recognised in adult anatomy and a number of theories have suggested that peripheral nerves and blood vessels pattern each other (Carmeliet and Tessier-Lavigne, 2005). However, our results suggest that the blood vessel and DRG are independently positioned adjacent to the neural tube and reinforce studies that demonstrate that the patterning arises due to common guidance mechanisms used by both systems (Bates et al., 2003). This raises the question of the source of the signal for positioning both the vasculature and DRG. Studies have shown that the neural tube acts as a signalling centre to recruit blood vessels (Hogan et al., 2004). We suggest that the signal for more specific positioning may also be provided by the neural tube.

EXPERIMENTAL PROCEDURES

Lectin Labelling of Blood Vessels

Fertile hens' eggs (Winter Egg Farm, UK) were incubated at 38°C to Hamburger Hamilton stage 17 (HH17) (Hamburger and Hamilton, 1992). Eggs were opened to expose the embryos and the vitelline arteries cut. Subsequently, 0.5 μl biotinylated Concanavalin A (Vector, UK 1 μg/μl) with fast green (2.5% w/v in Ringers) was introduced into the vasculature by intracardial injection (Jilani et al., 2003). The solution was allowed to circulate for 15 min before embryo fixation in MEMFA. Post-fix embryos were washed in PBSTx (PBS: 1% Triton-X100) and blocked (PBSTx: 10% serum). Embryos were incubated in block solution containing fluorescein-conjugated avidin (1:1,000), anti-HuC/D antibody (16A11, also known as Elavl3/4) (1:500), sodium azide (0.02%) for 5 days at 4°C. Embryos were washed in block solution and incubated with secondary antibody (anti-mouse IgG Alexa-568) overnight at 4°C. Embryos were washed in block solution followed by PBS alone. Whole-mount images of embryos were captured using a Leica stereomicroscope. For transverse sections, embryos were mounted in 20% gelatine: 20% sucrose in PBS and the blocks fixed overnight in MEMFA before vibratome sectioning at 50 μm. To determine where in the embryonic axis neurons or blood vessels were first seen, embryos were cut into 4-somite chunks using Oban bioscissors and imaged by confocal analysis (Zeiss 510). 3D visualisation was carried out using Volocity (Perkin Elmer, Waltham, MA).

Whole-Mount Antibody Labelling

Zebrafish (*Danio rerio*) embryos were obtained from a wild-type strain and raised at 28.5°C (Westerfield, 1993). Hemizygous transgenic embryos expressing GFP under the control of the Fli1 gene Tg(fli1:EGFP)y1 were used (Lawson and Weinstein, 2002). Normal and mutant zebrafish embryos were fixed at 3, 4, 5 days post-fertilisation in 4%PFA overnight at 4°C. Embryos were washed in PBSTx (PBS: 1% Triton-X100), then treated with 0.25% trypsin for 8 min to permeablise. Following further washing, embryos were blocked (PBSTx: 10% serum) for 1 hr. Embryos were incubated in block containing anti-GFP (1:250) and anti-HuC/D (1:500) antibodies (Molecular Probes, Eugene OR) for 5 days at 4°C. Following washing, the embryos were incubated with secondary antibodies, anti-rabbit Alexa 488, and anti-mouse Alexa 568 overnight at 4°C. Embryos were washed in block solution followed by PBS alone. Whole-mount antibody labelling and in situ hybridisation of chick embryos was carried out as described previously (Graham et al., 2007).

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