FGF-dependent Notch signaling maintains the spinal cord stem zone

Jun Akai, Pam A. Halley, and Kate G. Storey[1]

Division of Cell and Developmental Biology, Faculty of Life Sciences, University of Dundee, Dundee DD1 5EH, United Kingdom

Generation of the spinal cord relies on proliferation of undifferentiated cells located in a caudal stem zone. Although fibroblast growth factor (FGF) signaling is required to maintain this cell group, we do not know how it controls cell behavior in this context. Here we characterize an overlooked expression domain of the Notch ligand, *Delta1*, in the stem zone and demonstrate that this constitutes a proliferative cell group in which Notch signaling is active. We show that FGF signaling is required for expression of the proneural gene *cash4* in the stem zone, which in turn induces *Delta1*. We further demonstrate that Notch signaling is required for cell proliferation within the stem zone; however, it does not regulate cell movement out of this region, nor is loss of Notch signaling sufficient to drive neuronal differentiation within this tissue. These data identify a novel role for the Notch pathway during vertebrate neurogenesis in which signaling between high *Delta1*-expressing cells maintains the neural precursor pool that generates the spinal cord. Our findings also suggest a mechanism for the establishment of the cell selection process, lateral inhibition: Mutual inhibition between Delta/Notch-expressing stem zone cells switches to single *Delta1*-presenting neurons as FGF activity declines in the newly formed neuroepithelium.

[*Keywords*: Notch signaling; FGF signaling; proneural genes; spinal cord; stem cells; lateral inhibition]

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The vertebrate spinal cord is generated progressively as the body axis extends caudally. This process relies on the provision of new cells from a unique region of the neural plate known as the caudal neural plate or stem zone and also on convergent extension movements within the newly generated neuroepitheluim (Mathis et al. 2001; Diez del Corral and Storey 2004). Cell labeling experiments demonstrate the presence of a resident cell population in the stem zone in both chick and mouse embryos (Brown and Storey 2000; Mathis and Nicolas 2000; Mathis et al. 2001). Furthermore, in the mouse, there is also evidence for a stem cell mode of division taking place in this region (Mathis and Nicolas 2000) and this has now also been observed in the chick (S.E. Fraser, pers. comm.), indicating that the mechanisms underlying spinal cord generation are conserved between higher vertebrates. Once cells leave the stem zone they enter the forming neural tube and here neuronal differentiation and ventral patterning commence.

Numerous studies have established that fibroblast growth factor (FGF) signaling is required for the generation of the vertebrate trunk and some progress has been made in the identification of FGF-regulated tissues within this region. FGFs are expressed in the stem zone and are also presented by neighboring tissues, the primitive streak, and the caudal paraxial mesoderm (Diez del Corral and Storey 2004). FGF signaling is required to inhibit onset of neuronal differentiation and ventral patterning genes in the stem zone (Bertrand et al. 2000; Diez del Corral et al. 2002, 2003). Furthermore, FGF signaling serves to retain cells within this region, as cells lacking such signals quickly move into the neural tube (Mathis et al. 2001). Recent work has established that a balance between opposing FGF signals in the stem zone region and retinoic acid (RA) signals provided by more rostrally located somitic mesoderm controls differentiation onset and segmentation in the extending body axis (Diez del Corral et al. 2003). While RA drives differentiation in part by inhibiting *Fgf8* expression, FGF signaling inhibits retinoid synthesis and thereby helps to preserve the precursor cell pool that forms the stem zone (Diez del Corral et al. 2003). FGF signaling thus regulates multiple activities in the stem zone region, maintaining an undifferentiated cell state that must involve continued cell cycling and also the cohesion of the stem zone cell population; however, little is known about how FGF mediates these activities.

[1]Corresponding author.
E-MAIL k.g.storey@dundee.ac.uk; FAX 44-1382-345386.
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Notch signaling is another pathway that regulates the differentiation status of cells in many contexts (Lewis 1998; Artavanis Tsakonas et al. 1999; Lai 2004). The *trans*-membrane receptor Notch is activated by binding of the Delta or Serrate ligand presented by an adjacent cell; this triggers cleavage of the intracellular domain of Notch, which then translocates to the nucleus together with the transcription factor Suppressor of Hairless SuH/RBP-Jk and there activates target genes, such as *Hairy*/*Hes* basic helix–loop–helix (bHLH) factors (Artavanis Tsakonas et al. 1999). Notch signaling is best known for its role in the cell selection process, lateral inhibition (Simpson 1997; Lewis 1998; Schweisguth 2004). Within the vertebrate neuroepithelium this mechanism operates to regulate neuron production; *Delta1*, expressed by individual cells that are differentiating into neurons, stimulates Notch signaling and *Hes* gene expression in neighboring cells, which are thereby inhibited from differentiating and continue to proliferate (Chitnis et al. 1995; Henrique et al. 1995, 1997a; Lewis 1996). The phenotypes of mice with loss-of-function mutations in Notch pathway genes (Kageyama et al. 1997; Yoon and Gaiano 2005) support these initial results in frog and chick embryos and similar findings have been obtained in studies of neuroepithelial stem cells in vitro. These data indicate a requirement for Notch signaling to maintain these cells and to enhance their self-renewal properties by inhibiting differentiation (Nakamura et al. 2000; Hitoshi et al. 2002, 2004).

There is some evidence that Notch signaling is also active in the chick stem zone. FGF signaling maintains expression of stem zone-specific genes such as *Sax1* and *cash4*, a chick homolog of *Drosophila achaete-scute* complex proneural genes that encode a bHLH transcription factor (Henrique et al. 1997b; Storey et al. 1998; Bertrand et al. 2000; Diez del Corral et al. 2002). Misexpression of *cash4* in heterologous contexts in the fly and frog embryo demonstrates that *cash4* retains the transcriptional specificity that mediates proneural activity in the fly and can function as a proneural gene in a vertebrate embryo (Henrique et al. 1997b). In *Drosophila*, *achaete-scute* genes directly promote expression of *Delta* (Hinz et al. 1994; Kunisch et al. 1994; Heitzler et al. 1996) and a similar regulatory relationship has been demonstrated between vertebrate proneural gene homologs and *Delta1* expression in differentiating neuroepitheluim (Chitnis and Kintner 1996; Fode et al. 1998; Casarosa et al. 1999; Cau et al. 2002). The endogenous function of *cash4* in the chick stem zone, however, has yet to be elucidated, but these findings raise the possibility that *cash4* promotes *Delta* expression in the stem zone.

Notch1 expression is detected in the neuroepitheluim commencing at Hamburger and Hamilton stages 7–8 (HH7–8) (Henrique et al. 1995; Caprioli et al. 2002), and a number of *Hes5* family bHLH transcription factors, established targets, and effectors of Notch signaling in the neural tube, are also expressed in the stem zone (Fior and Henrique 2005). These observations therefore suggest that Notch signaling is active in the chick stem zone and may be promoted downstream of FGF. Furthermore, the neural phenotype in Notch pathway mutant mice involves precocious neuronal differentiation and loss of neural progenitors/stem cells, but strikingly is also accompanied by truncation defects in mice lacking RBP-Jk, Presenilin1 and Presenilin2, and just Presenilin 1 (de la Pompa et al. 1997; Shen et al. 1997; Wong et al. 1997; Donoviel et al. 1999; Herreman et al. 1999) consistent both with a failure of lateral inhibition in the neural tube, but also with a possible role for the Notch pathway in maintaining the neural precursor pool in the stem zone.

Here we reveal that *Delta1* is expressed in a broad and uniform domain in the chick stem zone, prior to the establishment of lateral inhibition in the differentiating neuroepithelium. We demonstrate that Notch signaling is active within this cell population and investigate the regulation and function of this pathway in the stem zone.

Results

Spinal cord stem zone cells experience Notch signaling

Examination of *Delta1* expression in serial sections of the segmenting chick embryo reveals that this gene is expressed not only in nascent neurons adjacent to somites, but is also present in a distinct uniform domain in epiblast cells next to the anterior primitive streak (Fig. 1A–E). Using cell labeling techniques we (and others) have shown previously that cells in this region progressively give rise to the entire spinal cord (Brown and Storey 2000) and studies at later stages indicate that this region behaves as a stem zone (Mathis et al. 2001). Consistent with this activity we find that these *Delta1* positive cells are mitotically active, as indicated by their incorporation of BrdU following only a brief (1 h) exposure (Fig. 1F–F″). This contrasts with individual *Delta1*-expressing cells located in the neural tube flanked by somites, which have left the cell cycle (Henrique et al. 1995) (Fig. 1G–G″).

Delta1 expression in the stem zone is accompanied by that of its receptor *Notch1* (Henrique et al. 1995; Caprioli et al. 2002), and by the expression of *Hes5-1*, a known target of Notch signaling (Fig. 1H–L; Fior and Henrique 2005), and these observations suggest that the Notch pathway is active in stem zone cells. To test this possibility, Notch signaling was blocked by misexpression of a dominant-negative Delta1 construct (DnDelta–IRES–GFP, which works cell autonomously) (Sakamoto et al. 2002, see Discussion) directly into stem zone cells (Fig. 1M). *gfp*-positive cells were then assessed for *Hes5-1* expression 16 h later (Fig. 1M). *Hes5-1* is expressed heterogeneously within its domain, but is absent in most cells expressing DnDelta, while cells expressing a control *gfp*-only vector can coexpress *Hes5-1* (Fig. 1N–P). These findings indicate that cells in the stem zone normally experience Notch signaling and raise the possibility that the Notch pathway acts within this cell group to maintain its undifferentiated state. We therefore next addressed the role of Notch signaling in this cell population, assessing how such signaling is regulated by both

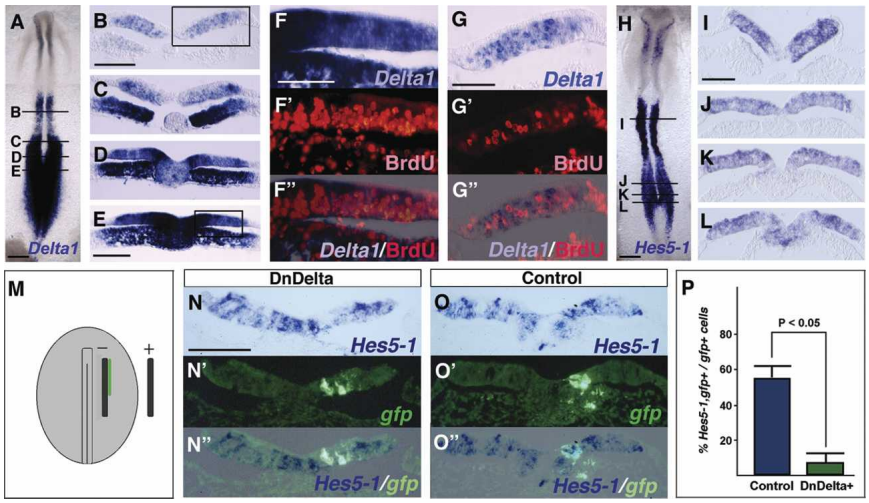

Figure 1. Stem zone cells experiencing Notch signaling are mitotically active. (*A–E*) *Delta1* at HH8 stage (*A*) and in transverse sections (TS) (*B–E*). (*B*) Closing neural tube. (*C*) Transition zone. (*D*) Hensen's node level. (*E*) Stem zone. (*F*) Higher magnification of boxed region in *E*. (*F′*) BrdU in the same section. (*F″*) Merged *Delta1*/BrdU image. (*G*) Higher magnification of boxed region in *B*. (*G′*) BrdU in the same section. (*G″*) Merged *Delta1*/BrdU image. (*H–L*) *Hes5-1* at HH8+ (*H*) and in TS (*I–L*). (*M*) HH4 embryo indicating placement of electrodes and DNA solution (green) for direct targeting of stem zone cells. (*N–N″*) Stem zone-level TS of a DnDelta–IRES–GFP-expressing embryo. (*N*) *Hes5-1*. (*N′*) *gfp*-positive cells in the same section. (*N″*) Merged image. (*O–O″*) Control IRES–GFP-only-expressing embryo. *Hes5-1* (*O*) and *gfp*-positive (*O′*) cells in the same section. (*O″*) Merged *Hes5-1/gfp* image. (*P*) Frequency of *Hes5-1* expression in *gfp*-positive cells obtained in DnDelta and control conditions. In controls, 55.3% (SD 6.6%; 16 sections from three embryos; blue bar) of *gfp*-positive cells had *Hes5-1* gene expression, while only 7.3% (SD 5.5%; 13 sections from three embryos; green bar) of *gfp*-positive cells expressed *Hes5-1* in DnDelta transfected embryos. Bars: *A*,*H*, 200 μm; *B*,*E*,*I*,*N*, 50 μm; *F*,*G*, 20 μm.

intrinsic and extrinsic factors and its effects on cell behavior in the stem zone.

A stem zone-specific proneural gene, cash4*, induces* Delta1

Proneural genes promote *Delta* expression in flies and vertebrates, and so we assessed which proneural gene(s) are expressed at the right time and place to mediate *Delta1* expression in the stem zone. *Delta1* is initially restricted to the primitive streak but expands laterally into the adjacent stem zone epiblast as node regression commences from HH6–7 and is accompanied at this time by the spread of *Notch1* expression into this region (Fig. 1D,E; Henrique et al. 1995; Caprioli et al. 2002). As noted above, this expansion of *Delta1* nicely follows the onset of the proneural gene homolog *cash4* at HH5 in the stem zone region (Henrique et al. 1997b). We also compared *cash4* expression at these stages with that of other key proneural gene homologs, the *Neurogenin* genes. Expression of neither *Ngn1* nor *Ngn2* prefigures *Delta1* in the stem zone; *Ngn1* first appears in neural tissue opposite somite 1 at HH7 (Fig. 2A,B), while *Ngn2* commences at HH8 (Fig. 2C) and only in the lateral edges of the open neural plate. In fact, these *Ngn* expression patterns appear complementary to that of *cash4* (Fig. 2D–J; Henrique et al. 1997b), which is clearly the best candidate proneural gene for regulation of *Delta1* in the stem zone.

To test whether *cash4* can promote *Delta1* expression, the full *cash4* coding region was cloned into an expression vector containing an IRES–GFP sequence. This was then introduced into HH9–10 closing neural tube (Fig. 3A) where *cash4* is still expressed, but where *Delta1* expression is dispersed rather than uniform. We first ascertained that this vector drives both the GFP and CASH4 protein using antibodies raised against GFP and CASH4 (Fig. 3B–D″). We then assessed the effect of ectopic maintenance of CASH4 in the elongating spinal cord. In the majority of cases we found that *cash4* misexpression promotes *Delta1* expression (14 out of 17 embryos) after 8 h incubation, while a control empty vector (containing only an IRES–GFP sequence) does not elicit *Delta1* (10 embryos) (Fig. 3E–F″). We next generated constructs that allow us to test whether CASH4 works as an activator or a repressor in this context. Misexpression of *cash4* fused to the activator domain VP16 (cash4VP16) promotes *Delta1* expression (12 out of 13 embryos), while *cash4* fused to the repressor domain of the Engrailed protein (cash4EnR) does not elicit ectopic *Delta1* (11 embryos) (Fig. 3G–H″). These findings indicate that cash4 works as an activator to promote *Delta1* expression.

One interpretation of this finding is that *cash4* works in this neural tube assay by inducing expression of proneural genes, which then induce *Delta1*. Surprisingly, however, 8 h after *cash4* misexpression both *Ngn1* and *Ngn2* transcripts are suppressed (*Ngn1*, six out of eight embryos; *Ngn2*, four out of six embryos), while this is not observed with a control IRES–GFP-only vector (*Ngn1*, four out of four embryos; *Ngn2*, four out of four embryos) (Fig. 3I–L″). This absence of *Ngn* expression could indicate that *cash4* drives neuronal differentiation in this context and that it leads to only transient *Ngn* expression. We therefore next assessed whether *cash4* misexpression promotes *Ngns* after a shorter period of only 5 h, (the time at which GFP first appears following introduction of cash4–IRES–GFP) (data not shown). However, *Ngn* levels are unaffected at this time (*Ngn1*, three out of three embryos; *Ngn2*, three out of three embryos) (data not shown). These findings therefore suggest that CASH4 does not promote *Delta1* expression via induction of these proneural genes.

In addition, we found that cash4EnR expression in this

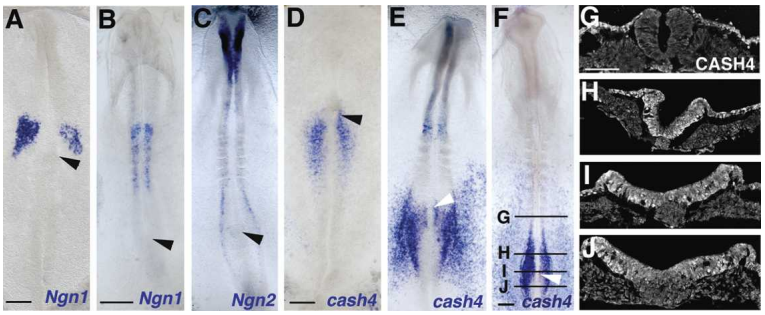

Figure 2. *Cash4* and *Ngn2* are expressed in complementary domains in the extending neural axis. *Ngn1* at HH7 (*A*) and HH8 (*B*). (*C*) *Ngn2* at HH8. *cash4* at HH5+ (*D*), HH8 (*E*), and HH9 (*F*). CASH4 at HH9 seen in TS (indicated on a different embryo in *F*), in the last formed somite (*G*), in the transition zone (*H*), anterior to the Hensen's node (*I*), and in the stem zone (*J*). Bars: *A*,*D*, 100 µm; *B*,*F*, 200 µm; *G*, 50 µm. (Arrowheads) Hensen's node.

same assay leads to *Ngn1* up-regulation (five out of five embryos) (Fig. 3M–M″). This suggests that CASH4 also works as a transcriptional activator to suppress *Ngn1* and that cash4EnR works as a dominant-negative construct that blocks CASH4 function. Utilizing this construct, we then assessed whether CASH4 is required for *Delta1* expression in the stem zone. To achieve this we introduced constructs into the epiblast lateral to the primitive streak at HH4 and cultured embryos for 10 h (Fig. 4A). Cells in this region move medially and are included in the stem zone as demonstrated by the location of IRES–GFP-only transfected cells (Fig. 4B–C′). By targeting these cells we aimed to block CASH4 function as this gene begins to be expressed. The majority of embryos expressing the control vector had normal levels of *Delta1* expression (seven out of 10 embryo) (Fig. 4C,C′) and this clearly contrasts with the loss of *Delta1* expression in stem zone epiblast cells expressing cash4EnR in most cases (five out of seven embryos) (Fig. 4D–F). Further, in cash4EnR-expressing embryos, *Delta1* loss is restricted to the stem zone epiblast, while *gfp*-positive cells located centrally within the primitive streak where *cash4* is not expressed continue to express *Delta1* (Fig. 4D–F). Cell counts were performed to quantify the difference between typical control IRES–GFP and cash4–EnR-expressing cells in the stem zone and in the primitive streak (Fig. 4F). Together these findings suggest that *cash4* is required for *Delta1* expression in the stem zone.

FGF signaling is required for expression of Delta1 *and the proneural gene* cash4

To establish the regulatory relationship between FGF and Notch signaling in the stem zone, we misexpressed a DnFGFR1-eYFP construct in epiblast cells lateral to the primitive streak at HH4 and assessed *Delta1* expression 10 h later. Loss of *Delta1* was observed in all cases (nine out of nine embryos) (Fig. 4G–H″) with transcripts absent in the majority of DnFGFR1-expressing cells (Fig. 4I), while most cells transfected with the IRES–GFP-only vector continue to express *Delta1* (IRES–GFP controls as above) (Fig. 4I). This finding places FGF signaling upstream of *Delta1* expression in the stem zone. Interestingly, we also found a small number of DnFGFR1 cells located within the primitive streak and noticed that these cells also lack *Delta1* (Supplementary Fig. 1).

It has been shown previously that FGF signaling can ectopically maintain expression of the proneural gene *cash4*, (Henrique et al. 1997b; Storey et al. 1998; Bertrand et al. 2000; Diez del Corral et al. 2002) and as *cash4* can induce *Delta1*, we next assessed whether FGF signaling is required for *cash4* expression in the stem zone. Misexpression of DnFGFR in this cell population for 10 h leads to loss of *cash4* (four out of four embryos) (Fig. 4J–L″) while control, *gfp*-only transfected cells coexpress *cash4* and *gfp* in all cases (four embryos) (Fig. 4J,M–N″). Together these data define the regulatory relationship between FGF and Notch signaling and indicate that FGF acts upstream of *cash4*, which is in turn required for *Delta1* expression in the stem zone.

Notch signaling does not regulate cell movement out of the stem zone, but maintains proliferation within this cell group

Previous work has shown that cells made deaf to FGF signaling are more rapidly displaced out of the stem zone than GFP-only-expressing cells, and so FGF helps to regulate cohesion of the stem zone cell population (Mathis et al. 2001). To see whether Notch signaling in the stem zone is contributing to this regulatory mechanism, we compared cell movement out of the stem zone following loss of FGF or Notch signaling. DnFGFR1 or DnDelta constructs were misexpressed in the stem zone at HH4–6 and embryos were assessed 24 h later (see Fig. 1M). As shown previously, cells expressing DnFGFR1 leave the stem zone region and are located in the neural tube (seven out of seven embryos), but those expressing DnDelta (seven out of seven embryos) or a control IRES–GFP-only construct (five out of five embryos) are distributed more evenly along the rostro–caudal axis and many cells are still located in the stem zone (Fig. 5). This demonstrates that Notch signaling does not account for the ability of FGF to regulate movement of cells out of the stem zone and into the forming neural tube.

Cell proliferation is required to maintain the stem zone cell population and so we next assessed whether Notch signaling is necessary for this process by examining incorporation of BrdU in cells lacking Notch signaling. DnDelta–IRES–GFP was expressed in the HH4–6 stem zone (see Fig. 1M) and embryos examined 16 h later following exposure to BrdU for the final hour. Most stem zone cells expressing DnDelta do not incorporate BrdU (Fig. 6A–F,M), while many IRES–GFP-only transfected

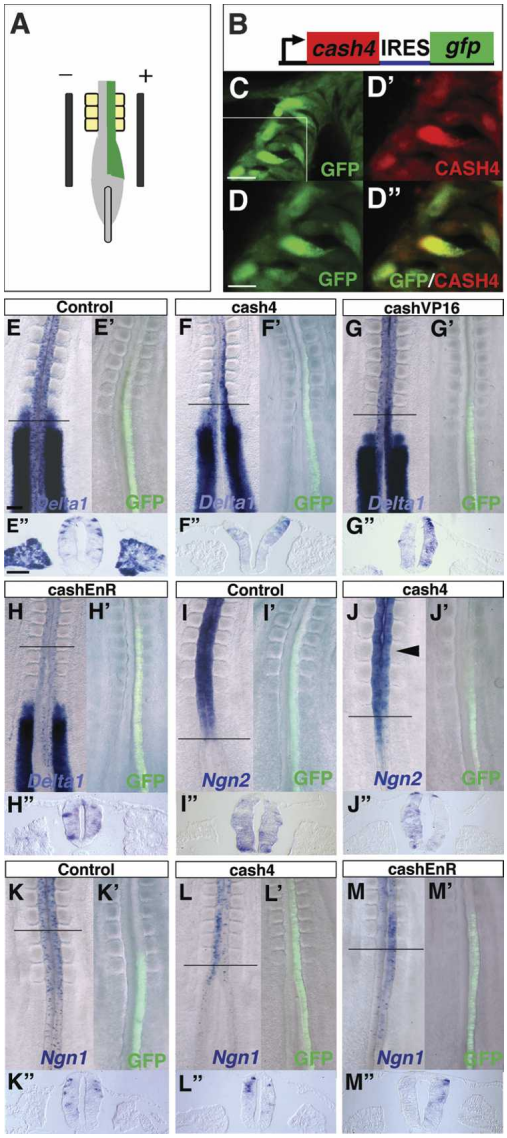

Figure 3. CASH4 works as an activator to induce *Delta1*. (*A*) Electroporation at HH10. (*B*) *cash4* expression vector (cash4–IRES–GFP). (*C*) GFP-positive cells following misexpression of cash4–IRES–GFP in the neural tube. (*D–D″*) Colocalization of GFP and CASH4 proteins. (*D*) Higher magnification of the boxed region in *C*. (*D′*) CASH4 detected with a CASH4 antibody in the same section. (*D″*) Merged image shows CASH4 in the nuclei of the GFP-positive cells. (*E–M″*) Misexpression experiments. In all cases, the first panel shows in situ of gene of interest, the second panel shows GFP localization prior to fixation, and the third panel shows TS through the region of misexpression indicated in the first panel. (*E–E″*) *Delta1* following control vector misexpression. (*F–F″*) *Delta1* following *cash4* misexpression. (*G–G″*) *Delta1* following cashVP16–IRES–GFP misexpression. (*H–H″*) *Delta1* following cashEnR–IRES–GFP misexpression. We saw no change in *Delta1* expression (apparent asymmetry is due to compression of tissue in whole-mount prep). (*I–I″*) *Ngn2* following control vector misexpression. (*J–J″*) *Ngn2* following cash4–IRES–GFP misexpression. (*K–K″*) *Ngn1* following control vector misexpression. (*L–L″*) *Ngn1* following misexpression of cash4–IRES–GFP. (*M–M″*) *Ngn1* following cashEnR–IRES–GFP misexpression. Bars: *C*, 20 μm; *D*, 10 μm; *E*, 100 μm; *E″*, 50 μm.

cells are labeled with this marker of S-phase progression (Fig. 6G–M). This indicates that Notch signaling is required for cell proliferation in the stem zone.

Blocking Notch signaling is insufficient for neuronal differentiation

As loss of Notch signaling blocks cell proliferation, it may also lead to precocious differentiation of cells in the stem zone. To assess this possibility the DnDelta construct was expressed in the stem zone at HH4–6 (see Fig. 1M) and expression of *NeuroM*, a marker of newly born neurons (Roztocil et al. 1997; Diez del Corral et al. 2002) was examined 16 h later. *NeuroM* expression is normally confined to single cells within the neural tube flanked by somites and is never expressed in the stem zone (Diez del Corral et al. 2002). *NeuroM* transcripts were not detected in stem zone cells expressing DnDelta–IRES–GFP (eight out of eight embryos) (Fig. 6N–O″) (the same result was also obtained by blocking Notch signaling by overexpression of DnSuH construct, two out of two embryos; data not shown). This finding is identical to that observed following expression of IRES–GFP-only control vector (nine embryos) (Fig. 6P–Q″). We also assessed whether loss of Notch signaling in the stem zone leads to expression of an earlier marker of neural differentiation, *Ngn1*. As with *NeuroM*, no *Ngn1*-positive cells were detected in the stem zone following misexpression of DnDelta1 or the control GFP-only construct in this region (five out of five embryos DnDelta1; five embryos IRES–GFP-only) (Supplementary Fig. 2). These findings indicate that loss of Notch signaling and cell cycle exit are not sufficient to promote neuronal differentiation in the stem zone.

Discussion

We have identified a new domain of uniform *Delta1* expression localized in the spinal cord stem zone. These cells are mitotically active and transduce Notch signaling as indicated by the loss of the Notch effector gene *Hes5-1* when Delta/Notch signaling is blocked. *Delta1* expression in the stem zone is promoted by the proneural gene *cash4* and we further demonstrate that FGF signaling is required for *cash4* and *Delta1* expression. This defines the regulatory cascade by which FGF promotes Notch signaling; FGF induces *cash4*, which promotes *Delta1*, which in turn mediates Notch signaling and expression of *Hes5-1*. Significantly, we show that Notch activity is in part responsible for FGF functions in the stem zone; it does not regulate movement of cells out of this region, but maintains proliferation within this cell population. Strikingly, as cells leave the stem zone we see that *Delta1* expression resolves to single cells within the newly generated neuroepithelium. This is where lateral inhibition first begins to operate and this observation suggests that declining levels of FGF instigate the establishment of this mechanism in the neuroepithelium. Finally, we reveal that loss of Notch signaling is insufficient for neuronal differentiation in the stem

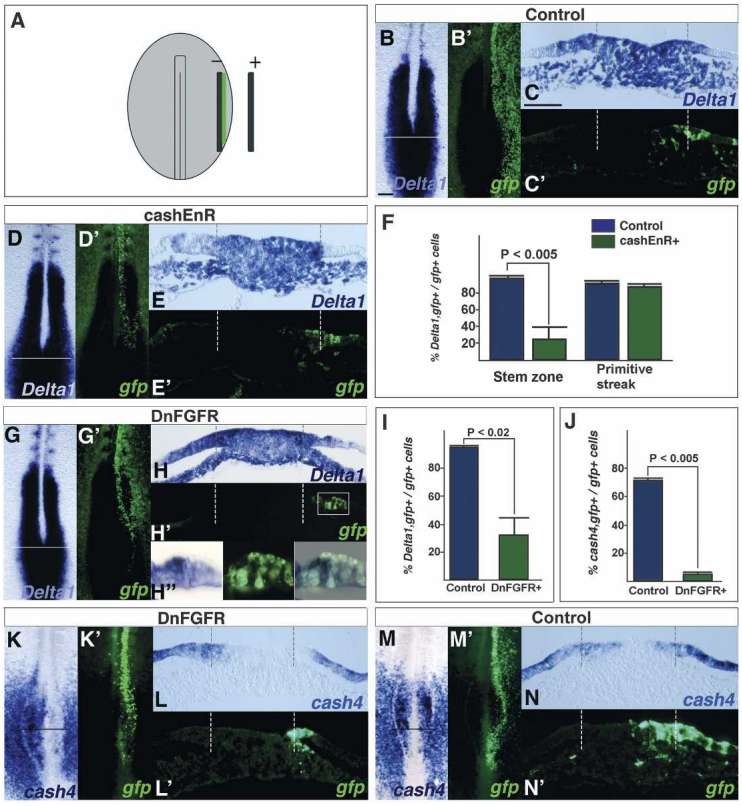

Figure 4. FGF-dependent *cash4* expression is required for *Delta1* expression in the stem zone. (*A*) HH4 embryo indicating placement of electrodes and DNA solution (green) for targeting prospective stem zone cells in the lateral epiblast. (*B*,*B*′) Control IRES–GFP-only transfected embryo. *Delta1* (*B*) and *gfp* (*B*′) expression. (*C*–*C*″) TS of *Delta1*. (*C*′) *gfp*. *Delta1* (*D*) and *gfp* (*D*′) expression following cashEnR misexpression. (*E*–*E*′) TS showing the loss of *Delta1* expression in the stem zone. (*F*) The proportion of *gfp*-positive cells expressing *Delta1* in the stem zone and primitive streak. In cashEnR misexpressing embryos, 25.6% of *gfp*-positive cells express *Delta1* (SD 14.8%; 10 slides from three embryos; green bar). This is significantly lower than the proportion observed in the stem zone of control embryos (99% ± 1.4%, 13 sections from three representative embryos; blue bar). In contrast, in the primitive streak the proportion of *gfp*-positive cells expressing *Delta1* in cashEnR embryos (90.4% ± 1.72%) is not significantly different from that in controls (93.3% ± 1.3%). (*G*,*G*′) DnFGFR1-*gfp* misexpressing embryo. (*H*–*H*″) TS and high-power views (boxed regions) showing loss of *Delta1* expression in DnFGFR1-*gfp* positive cells in the stem zone. (*I*) The proportion of *gfp*-positive *Delta1*-expressing cells following DnFGFR1 misexpression (32.9% ± 12.1%; nine sections from three embryos; green bar) is significantly lower than in control embryos (13 sections from three embryos; blue bar). (*J*) *cash4* expression in the stem zone following DnFGFR1 misexpression. In control embryos, 72.1% (SD 2.1%; 9 sections from two embryos; blue bar) of *gfp*-positive cells in the stem zone express *cash4*, whereas only 5.7% (SD 1.1%; eight sections from two embryos; green bar) of cells in the stem zone have *cash4* expression in the DnFGFR1 expression embryo. DnFGFR-misexpressing embryo (*K*,*K*′) and TS of *cash4* (*L*–*L*′) in *gfp*-expressing cells. (*M*,*M*′) Control embryo. (*N*–*N*′) TS of *cash4* and *gfp* expression. Dashed lines indicate the border between the primitive streak and the stem zone epiblast. Bars: *B*, 100 μm; *C*, 50 μm.

zone. We propose that this reflects the requirement for retinoid signals provided by rostrally located somitic mesoderm, which we have shown previously attenuate FGF signaling and drive neuron production (for review, see Diez del Corral and Storey 2004).

Notch and FGF signaling in the regulation of stem zone activity

Within the vertebrate central nervous system Notch signaling is usually considered in the context of lateral inhibition, during which a single *Delta1*-expressing cell stimulates Notch signaling in its neighbors and thereby inhibits their differentiation. Here we describe a new situation in the stem zone, where uniform expression of *Delta1* is observed. BrdU incorporation by *Delta1*-positive stem zone cells indicates that they are mitotically active and this contrasts with single *Delta1*-expressing cells in the neural tube, which have left the cell cycle. Further, we show that Notch signaling is required for the proliferation of stem zone cells. Together these findings suggest that uniform and high *Delta1* expression results in uniform, mutually inhibitory, Notch signaling between stem zone cells, rather than localized stimulation of this pathway delivered by scattered *Delta1*-expressing cells, characteristic of lateral inhibition.

This Notch signaling context in the stem zone contrasts with that observed in other cell groups that express high levels of *Delta1*, such as epidermal stem cells (Lowell et al. 2000). These cells do not transduce Delta/Notch signaling, but stimulate this pathway in neighboring cells at the group edge (Lowell et al. 2000). This may occur because cells expressing high levels of Notch ligand can make homodimers (Klueg and Muskavitch 1999), which can work in a dominant-negative fashion and lead to cell autonomous suppression of Notch signaling (Micchelli et al. 1997; Sakamoto et al. 2002). Interestingly, the DnDelta construct that we used appears to work by enhancing this cell autonomous inhibition of Notch signaling (Sakamoto et al. 2002). Our finding that this construct suppresses the Notch target gene *Hes5-1* in stem zone cells thus suggests that these cells do not normally experience such a cell autonomous inhibition mechanism and clearly demonstrates that Notch signaling is normally active within stem zone cells.

FGF signaling is required to maintain the cohesiveness of the stem zone, as attenuation of this pathway drives cells out of this domain and into the neural tube (Mathis et al. 2001) and Notch signaling also regulates cohesion of cell groups in a variety of contexts (Lowell et al. 2000; Pourquie 2000). However, we found that cells expressing DnDelta are still able to stay in the stem zone, indicating

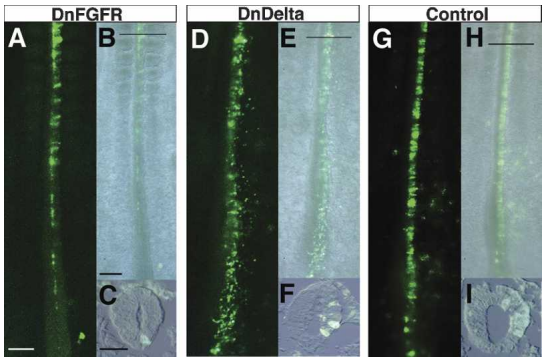

Figure 5. Notch signaling is not required to retain cells in the stem zone. Distribution of GFP-positive cells 24 h after misexpression of constructs in the stem zone at HH4 (imaged prior to fixation). (*A*) DnFGFR1–IRES–GFP-expressing cells. (*B*) Merged bright-field/GFP image. (*C*) TS of GFP cells in the neural tube. (*D*) DnDelta–IRES–GFP-expressing cells. (*E*) Merged bright-field/GFP image. (*F*) TS of GFP cells in the neural tube. (*G*) Control IRES–GFP-expressing cells. (*H*) Merged bright-field/GFP image. (*I*) TS. Bars: *A*,*B*, 100 µm; *C*, 50 µm.

that Notch signaling does not mediate this FGF activity in this context. FGF has also been shown to act upstream of Notch signaling to maintain proliferation and the undifferentiated state of a number of stem cell populations, including neural and dental stem cells (Harada et al. 1999; Hitoshi et al. 2002; Yoon et al. 2004). We show here that FGF signaling is required for the broad expression of *Delta1* in the stem zone, which we propose then leads to a state of mutual inhibition. This regulatory relationship differs from the action of FGF within a neuroepithelium in which lateral inhibition is operating, as here FGF up regulates *Notch1* and suppresses *Delta1* expression, which most likely represents differentiating neurons (Faux et al. 2001). The group of *Delta1*-expressing stem zone cells thus appears to represent a novel mode of Notch signaling operating during vertebrate neurogenesis, which is delivered between a group of FGF-dependent Delta/Notch-expressing cells and which acts to maintain the neural precursor cell pool that gives rise to the spinal cord.

Notch signaling mediates body axis extension across species

Cell populations equivalent to the chick stem zone in other vertebrates embryos express Notch pathway genes, and there is evidence that axis extension also relies on Notch signaling in these animals. Expression of *Delta1* in a cell group adjacent to the organizer/anterior primitive streak is apparent in mouse and frog embryos (Bettenhausen et al. 1995; Ma et al. 1996; Beck and Slack 1998; Przemeck et al. 2003). Further, in the mouse, although a caudally located proneural gene has yet to be described, *Hes5* is detected in the stem zone region and strikingly, is absent in mice lacking *Notch1*, *RBP-Jk*, or *Presenilin1* and *Presenilin2* (Figs. 3P, 4B,C; de la Pompa et al. 1997; Donoviel et al. 1999). As noted above, reduction of cell numbers within the neural tube of RBP-Jk and Presenilin1 single-knockout or Presenilin1 and Presenilin2 double-knockout mice is also accompanied by a truncation phenotype (de la Pompa et al. 1997; Shen et al. 1997; Wong et al. 1997; Donoviel et al. 1999; Herreman et al. 1999) supporting a role for the Notch pathway in the maintenance of the neural precursor cell pool in the stem zone. Furthermore, Notch signaling is required for tail bud outgrowth and specifically for regeneration of the spinal cord in the frog (Beck and Slack 2002; Beck et al. 2003). Although established later in development, the frog tailbud appears analogous to the stem zone of higher vertebrates (as well as homologous to the later forming tailbud). The Notch pathway thus plays a conserved role in the maintenance of the cell state necessary for extension of the neural axis.

Interestingly, while most stem zone cells form neural tissue, some cells located caudally within this region contribute to paraxial mesoderm (Catala et al. 1996; Brown and Storey 2000) and these cells coexpress panneural and early mesodermal genes as well as *Delta1* (Kispert and Herrmann 1994; Kispert et al. 1995; Charrier et al. 1999; Delfino-Machin et al. 2005). Paraxial mesoderm is generated largely by cells located in the primitive streak, but the presence of some mesodermal precursors in the stem zone (for discussion, see Delfino-Machin et al. 2005), where Notch signaling is required for proliferation, suggests that the Notch pathway may also regulate generation of paraxial mesoderm. Consistent with this possibility we also observed loss of *Delta1* transcripts in primitive streak cells expressing DnFGFR1.

The stem zone as a persisting domain of mutual inhibition

The spinal cord is generated progressively in a rostral to caudal sequence such that the temporal events of neurogenesis are spatially separated in the extending axis. *Delta1* expression in a uniform domain is found in the caudal end of the stem zone, which contains cells that reside in the stem zone as it regresses, and which give rise to the caudal-most parts of the spinal cord (Brown and Storey 2000; Mathis et al. 2001). This uniform *Delta1* expression resolves as cells leave this region, into individual cells within the neuroepithelium rostral to the primitive streak/node, which have been shown to be post-mitotic neuronal precursors (see Fig. 1A; Henrique et al. 1995). This temporal sequence is similar to that described within a proneural cluster in the fly epithelium (Simpson 1997). Here, all cells initially express both *Notch* and *Delta* and experience mutual inhibition. As differences in levels of *Delta* appear between cells, these differences are then reinforced by lateral inhibition and individual *Delta*-expressing cells are singled out. The caudal stem zone could therefore be considered a persisting proneural domain in which mutual inhibition operates. Cells near the edge of the broad *Delta1* domain experience less FGF. As a result they become displaced

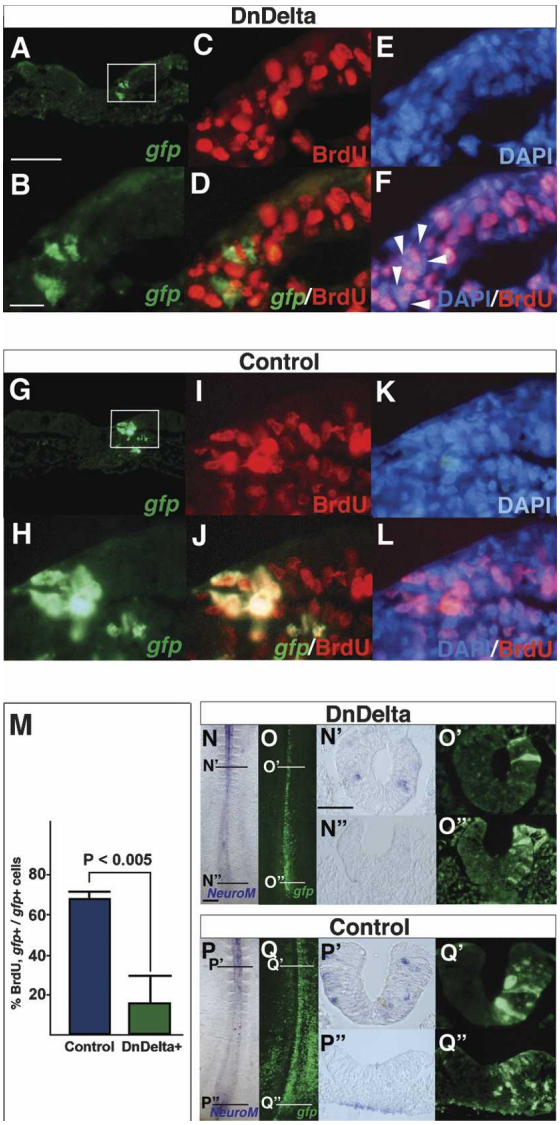

Figure 6. Notch signaling is required for proliferation of stem zone cells. (*A*) TS at stem zone level of DnDelta–IRES–GFP-expressing cells showing *gfp*. (*B*) Higher magnification of boxed region in *A*. (*C*) BrdU-incorporating cells (red). (*D*) gfp/BrdU merged image. (*E*) DAPI. (*F*) DAPI/BrdU merged image; note nuclei of *gfp*-positive cells (indicated by arrowheads) are BrdU-negative. (*G–L*) TS at stem zone level in control IRES–GFP embryo. (*G*) *gfp*. (*H*) Higher magnification of boxed field in *G*. (*I*) BrdU-incorporating cells (red). (*J*) Merged *gfp*/BrdU image. (*K*) DAPI. (*L*) DAPI/BrdU merged image. (*M*) In the stem zone, proportion of *gfp*-expressing/BrdU-positive cells is 16.8% (SD 14.7%; 13 sections from three embryos; green bar) in DnDelta-expressing embryos. This is significantly lower than in control embryos (68.3% ± 3.0%; seven sections from three embryos; blue bar). (*N–Q*″) *NeuroM* expression following misexpression of DnDelta–IRES–GFP at HH4. (*N*) *NeuroM* in neural tube. TS of the neural tube (*N*′) and stem zone (*N*″). (*O*) DnDelta–IRES–GFP cells in the same embryo as *N*, and in TS of the neural tube (*O*′) and stem zone (*O*″). *NeuroM* in control IRES–GFP-expressing embryo (*P*) and in TS of the neural tube (*P*′) and stem zone (*P*″). (*Q*) *gfp*-only-expressing cells in the same embryo as *P*. TS of the neural tube (*Q*″) and stem zone (*Q*″). Bars: *A*, 50 µm; *B*, 10 µm; *N*, 200 µm; *N*′, 50 µm.

into the forming neuroepithelium where extensive cell mixing takes place (Mathis et al. 2001) and where they begin to lose *cash4* and *Delta1* expression. Slight differences in the levels of Notch signaling between cells in this transition zone may then lead to the establishment of lateral inhibition and the birth of neurons (Fig. 7).

It is not clear whether the first single *Delta1*-expressing cells retain expression or whether this is a new phase of *Delta1* transcription is induced by the neurogenin genes, which now begin to be expressed in this tissue (Fig. 2A–C). As *cash4* is induced by FGF and *Ngns* rely on Retinoid signaling supplied by adjacent differentiating paraxial mesoderm (Diez del Corral et al. 2003), this change in proneural gene expression reflects the switch from FGF to Retinoid signaling as differentiation progresses in the extending body axis. Importantly, these two pathways are mutually inhibitory in this context and FGF, by repressing onset of RA synthesis in the paraxial mesoderm and opposing RA activity in the neuroepithelium, maintains the stem zone as a Retinoid-free cell population (Diez del Corral et al. 2002, 2003). This may explain why blocking Notch signaling does not generate neurons in the stem zone, but does promote neuronal differentiation in the neural tube (le Roux et al. 2003; for review, see Yoon and Gaiano 2005). Indeed,

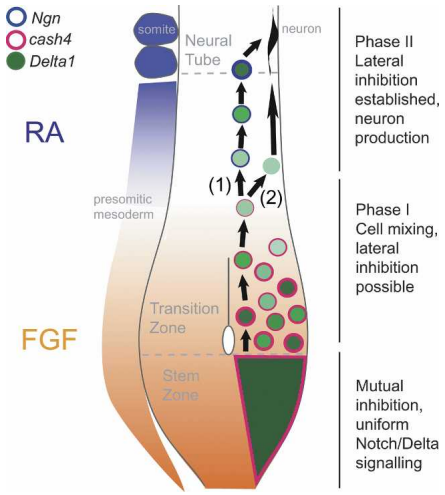

Figure 7. Model of neurogenesis progression in the extending axis. Stem zone cells all express *Delta1* and experience mutual inhibition. Cells at the rostral edge of the stem zone experience less FGF and move out of this region into the forming neural tube (transition zone). Transition zone cells mix with recently arrived stem zone cells (Mathis et al. 2001), and as a result *Delta1* expression begins to change from a uniform to a dispersed pattern. This is Phase I, and lateral inhibition is possible as differences between Notch signaling exist between neighboring cells. In Phase II, cells experience RA, and as a result FGF signaling declines (Diez del Corral et al. 2003) and hence *cash4* and *Delta1* levels are reduced. RA-dependent genes such as *Ngns* are now also expressed but are restricted to a few cells because lateral inhibition is already operating. Here *Ngn* expression promotes new *Delta1* transcription leading regulated neuron production (1); some cells may also retain *Delta1* expression from the stem zone and could differentiate into neurons (2).

while *asc* class proneural genes are expressed in proliferating cells, atonal proneural gene homologs, such as the *Ngns*, drive cell cycle exit and differentiation of neuronal progenitors (for review, see Bertrand et al. 2002). Our findings thus help to dissect the role of Notch signaling in the nervous system, indicating that neuronal differentiation is not an automatic consequence of reduced Notch activity and that additional retinoid-dependent events are required (Fig. 7).

Iterative use of the Notch signaling pathway during the generation and patterning of a tissue has been described in other contexts, including the fly eye (Blair 1999; Baonza and Freeman 2001, 2005) and chick inner ear (Daudet and Lewis 2005). Our findings demonstrate that serial requirements for Notch signaling also underlie the generation of the spinal cord and identify a new context in which to study the transition from mutual to lateral inhibition.

Materials and methods

In situ hybridization

Standard methods for whole-mount in situ hybridization were used to detect expression of endogenous genes (Wilkinson and Nieto 1993) except for *Ngn2*, for which the hybridization step was performed at 70°C instead of 65°C. *Gfp* transcripts were visualized with TSA plus fluorescence system (PerkinElmer Life Sciences) following manufacturer's instructions. Plasmids were kind gifts from Domingos Henrique (Faculdade de Medicina da Universidade de Lisboa, Lisboa, Portugal) (*Delta1*, *Hes5-1*), David Anderson (California Institute of Technology, Pasadena, CA) (*Ngn1*, *Ngn2*), and Mark Ballivet (University of Geneva, Geneva, Switzerland) (*NeuroM*).

Immunocytochemistry

Cash4 protein was detected with a cash4 antibody (1:50) raised in sheep (see below), using a biotin-conjugated anti-sheep secondary antibody (Jackson laboratories; 1:250) and Cy3-conjugated streptavidin (Jackson laboratories; 1:250) in sectioned tissue and imaged with a confocal microscope, TCS SPII (Leica). Incorporation of BrdU was detected using standard techniques (Gunhaga et al. 2000), following exposure of embryos in ovo or prepared in New culture to 1 µM BrdU for a required period. BrdU was visualized with an anti-BrdU antibody (Roche; 1:20) and anti-mouse Cy3 conjugated antibody (Jackson laboratories; 1:1000).

CASH4 antiserum production

A CASH4-CM construct was made containing 61–504 base pairs (bp) of cash4 sequence (Henrique et al. 1997b) corresponding to the bHLH and C-terminal region of the protein. This was cloned into pQE30 (Qiagen) and expressed in M15 *Escherichia coli*, which gave a 17-kDa fusion protein with a 6xHis tag. This was purified on a NiNTA agarose column and eluted with 8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris-Cl (pH 5.9). One milliliter of the denatured protein at 1.25 mg/mL was sent for immunization into sheep at the Scottish antibody production unit. Second bleed antiserum was caprylic acid fractionated, resuspended in PBS, and dialyzed. Further purification was carried out by washing over CM protein bound to nitrocellulose and elution with 200 mM glycine and 1 mM EGTA (pH 2.5). The antibody was then concentrated on a 10MWCO Microcon column (Millipore) and absorbed against HH stage 3 acetone powders prior to characterization on Western blots and by immunocytochemistry.

Plasmid construction and in vivo electroporation

The full ORF of the *cash4* gene was cloned into the pBluescript vector. Part of the gene (~309 nucleotides [nt]), including the DNA-binding site and HLH coding sequence, was subcloned and fused with the EnR repressor domain or VP16 activator domain coding sequence. Each form of the cash4 gene was then transferred to the pEFBOS–IRES–GFP expression vector. The DnDelta construct (a kind gift from K. Katsube, Tokyo Medical and Dental University, Tokyo, Japan; Sakamoto et al. 2002) was subcloned into the pIRES2–EGFP expression vector (BD Biosciences). The DnSuH construct was kindly provided by D. Henrique. The DnFGFR1–eYFP vector was a kind gift from C. Weijer (University of Dundee, Dundee, UK) (Yang et al. 2002). As a control we used pEFBOS–IRES–GFP empty vector. Standard in ovo electroporation techniques were used on HH10 stage embryos (Fig. 4). Electroporation of HH4 stage embryos was preformed on embryos in New culture; embryos were placed dorsal up for electroporation and then replaced dorsal side down for subsequent culture (details available on request). For both electroporation strategies we used an INTRACEPT TSS10 dual pulse isolated stimulator (INTRACEL) and CUY610P1.5 parallel electrodes (NEPA GENE).

Quantitative analysis

Cell counts were carried out by superimposing bright-field (BCIP/NBT in situ hybridization signal) and *gfp*/FITC images, and this was aided by nuclear labeling for BrdU or DAPI staining. The number of cells, sections, and embryos examined are indicated in the figure legends. The average percentage of cells expressing specific genes was obtained for each embryo and standard deviations were then calculated. *P* values for statistical significance of differences between experimental and control samples were determined using Student's *t*-test.

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