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Fluorescent protein expression driven by *her4* regulatory elements reveals the spatiotemporal pattern of Notch signaling in the nervous system of zebrafish embryos

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Abstract

Notch activation inhibits neuronal differentiation during development of the nervous system; however, the dynamic role of Notch signaling in individual cell lineages remains poorly understood. We have characterized 3.4 kb 5′-regulatory sequence of a Notch target gene, *her4*, and used it to drive fluorescent gene expression in transgenic lines where the spatiotemporal pattern of Notch activation can be examined *in vivo*. The 3.4 kb *her4* promoter contains five predicted Su(H) binding sites of which two proximal sites were confirmed to be required for Notch-mediated transcriptional activation. Without Notch, Su(H) effectively represses transcription regulated by the promoter. Analyses of transgenic zebrafish showed that while the expression of proneural genes and Notch activation were both critical for endogenous *her4* expression, reporter gene expression was primarily regulated by Notch activity. This study also showed that *her4* may be differently regulated in sensory cranial ganglia, where Notch activity is not essential for *her4* expression and where Su(H) may repress *her4* expression. The establishment of a reporter line with Notch-Su(H)-dependent fluorescent gene expression provides a tool to explore the complex role of Notch signaling in the development of vertebrate nervous system.

Keywords: Notch reporter; Transgenic; Su(H); CBF1; CSL; *her4*; *mind bomb*; *Neurogenin1*; *zath3*; Neurogenesis

Introduction

In both vertebrates and invertebrates, expression of basic helix–loop–helix (bHLH) proneural transcription factors defines domains where cells acquire the potential to become neurons (Ma et al., 1996; Campos-Ortega, 1995; Ghysen et al., 1993). Expression of these factors also gives cells the potential to inhibit neighboring cells from adopting the same fate by a process called lateral inhibition, mediated by the Notch signaling pathway (Campos-Ortega, 1995; Greenwald and Rubin, 1992; Artavanis-Tsakonas and Simpson, 1991; Campos-Ortega, 1988; Taghert et al., 1984). Neurogenin1 (Ngn1) and Zath3 are bHLH factors related to *Drosophila* Atonal that help define neurogenic domains in the zebrafish neural plate, where cells acquire the potential to become neurons (Park et al., 2003). These proneural factors also drive the expression of the Notch ligand Delta (Kunisch et al., 1994). Delta binds and activates Notch on the surface of neighboring cells; it facilitates proteolysis in the Notch receptor, which results in the release of a Notch intracellular fragment, NICD (Artavanis-Tsakonas et al., 1995). NICD translocates into the nucleus and functions as a transcriptional activator where it forms a transcriptional activator complex with a member of a family of CSL DNA-binding factors, named after *C*BF1/RBP-jkappa in human, Suppressor of hairless (*S*u(H)) in *Drosophila*, and *L*ag1 in *Caenorhabditis elegans* (Christensen et al., 1996; Schweisguth and Posakony, 1992; Furukawa et al., 1991; Matsunami et al., 1989). In *Drosophila*, the Notch activator complex drives expression of bHLH *Enhancer-of-split* [*E(spl)*] factors that

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inhibit the transcription of proneural factors and deter the adoption of a neural fate in the neighboring cells (Lecourtois and Schweisguth, 1995; Bailey and Posakony, 1995). In this way proneural bHLH factors facilitate neural fate in one cell, while indirectly activating *E(spl)* genes in the adjacent cells, where these inhibitory bHLH factors deter cells from adopting a neural fate. In vertebrates, homologues of the *E(spl)* related genes have been identified and since they are similar to *Drosophila hairy* they have been called *hairy E(spl)*-related *HER* or *HES* genes (Davis and Turner, 2001).

E(spl) and Hairy-related proteins contain a conserved Orange domain located C-terminal to the bHLH domain, and together with Hey and Stra13 family proteins they are part of the bHLH-Orange (bHLH-O) superfamily (Davis and Turner, 2001). The HER and HES nomenclature has led to some confusion because they can have very distinct functions in neurogenesis, where their function is either more related to Hairy or E(spl) in *Drosophila*. Zebrafish *her* genes are divided into three groups based on responsiveness to Notch signaling. Expression of Group I genes, which includes *her1* and *her4*, is up-regulated by Notch (Takke and Campos-Ortega, 1999; Takke et al., 1999) and, like *E(spl)* genes in *Drosophila* these genes are more likely to participate in lateral inhibition. In contrast, expression of *her3* and *her5* in group II is down-regulated (Hans et al., 2004), and that of *her6* and *her9* in group III is unaffected by Notch (Hans et al., 2004). Like *hairy* in *Drosophila*, Group II and III genes may function as pre-patterning genes in defining non-neurogenic domains (Fisher and Caudy, 1998).

A number of studies have shown that Notch signaling prevents neuronal progenitors from becoming early neurons (also referred to as primary neurons) and permits them to become late differentiating "secondary" neurons, adopt alternate fates or to remain as undifferentiated progenitors (Itoh et al., 2003; Appel et al., 2001). In *mind bomb* (*mib*) mutant embryos, for example, there is a neurogenic phenotype characterized by an excess of early neurons and reduction of a number of late differentiating neurons (Itoh et al., 2003; Jiang et al., 1996). However, the role of Notch signaling in determining the fate of cells in individual spinal cord lineages remains poorly defined. Our goal was to create a transgenic line in which the pattern of Notch activation in individual cells could be monitored. Such a line could eventually be used to visualize how Notch signaling determines cell fate of siblings in individual lineages and to identify mechanisms that maintain Notch activity in the growing nervous system.

In this study we have taken advantage of the fact that *her4* is thought to be part of a regulatory feedback loop that down-regulates the transcription of the proneural gene, *neurogenin1* (*ngn1*) (Takke et al., 1999). We confirm that *her4* is a target of Notch signaling: its expression is induced by ectopic Notch activation and is reduced by manipulations that reduce Notch signaling. We established zebrafish transgenic lines by using a 3.4-kb 5′-flanking region of *her4* to drive expression of green fluorescent protein (EGFP) or monomeric red fluorescent protein fused with a PEST amino acid sequence that targets the protein for degradation (dRFP). We have compared the spatial expression of EGFP under the control of the zebrafish *her4* promoter to that of endogenous *her4* both in control embryos and embryos in which Notch signaling or proneural function was altered. Our analysis shows that while key elements of *her4* expression are recapitulated in the transgenic embryos, differences have also been identified. These transgenic lines will be useful to characterize how changes in the level of Notch signaling determine the identity of cells *in vivo*.

Materials and methods

Zebrafish maintenance and mutant strain

Zebrafish were maintained as described in Westfield (1995). The embryos were staged according to Kimmel et al. (1995). An allele of *mind bomb* (*mib*^{ta52b}) was previously described (Itoh et al., 2003; Jiang et al., 1996). *Tg[hsp:GAL4]* and *Tg[UAS:NICD]* transgenic zebrafish were obtained from Campos-Ortega (Scheer et al., 2001). Reporter lines were *Tg[huC:EGFP]* (Park et al., 2000).

Isolation of 5′-flanking region of her4 genomic DNA and sequencing

Zebrafish genomic contigs from the Sanger Center (http://www.ensembl.org/Danio_rerio/) were searched for *her4* genomic sequence by using the *her4* cDNA sequence (Takke et al., 1999). A contig, z06s016987, was located containing the *her4* cDNA sequence. 3.4 kb of 5′-flanking region of *her4* genomic DNA was amplified by polymerase chain reaction (PCR) using zebrafish genomic DNA as templates with the following sets of oligonucleotide primers; her4P5, 5′-TGT TAA GCA ACA CTA AAT CCA TTG-3′; her4P7, 5′-TCA AGG GTG TGC GCT GAA TTC AAT-3′. The amplified DNA fragments were subcloned into pCR2.1 (Invitrogen) and sequenced. The accession number for the zebrafish 5′-flanking region of *her4* genomic sequence is AY691485.

Luciferase assay

To make the luciferase reporter plasmids, the various fragments of the amplified *her4* promoter were introduced into pGL3Basic (Promega). The PCR amplifications were performed using the following oligonucleotide primers: p2.4kb, 5′-TTA CCT TCA TTG AAG GTT TTT-3′; p1.7kb, 5′-TGT TAA GCA ACA GTA AAT CCA-3′; p1.3kb, 5′-GTT AAA GCT ACT GAG AGC TCA-3′; p0.6kb, 5′-CCC ATA AAA AAA CTT AAA CTG-3′; p0.2kb, 5′-AAC AGC CAA CCC TCC ACC TGC-3′; p0.1kb, 5′-CCT CCA GCA AAC TCC AGA CTC-3′.

0.5 μg of the reporter plasmid of the firefly luciferase gene, under the control of p3.4kb of *her4* promoter, was transfected into P19 cells with or without 0.1 μg of the expression vector encoding a constitutively active form of Notch5 (NICD) and *Xenopus* Su(H). 0.3 μg of the reporter plasmid of the firefly luciferase gene under the control of the various constructs of *her4* promoter were transfected into P19 cells with or without 0.1 μg of NICD. 0.025 μg of the plasmid containing the Renilla luciferase gene under the control of the herpes simplex virus thymidine kinase promoter (pHStk-RL) was also transfected as an internal control to normalize the transfection efficiency. The total DNA amount was adjusted to 0.7 μg or 0.4 μg with the control expression vector, pCS2+. The firefly luciferase activity of each promoter in the absence of the NICD vector was taken to be 100, and relative activities were measured. Cells were harvested after 1 day of transfection, and luciferase assays were carried out as described in Promega's instruction manual using a TD-20/20 luminometer (Turner designs).

Plasmid construction and generation of the transgenic zebrafish

To make the transgene, the amplified 3.4 kb *her4* promoter was used to replace the sCMV IE94 promoter of pCS2-NLS-Myc-EGFP, pCS2-EGFP, or pCS2-Myc-dRFP was made by inserting mRFP (Yeo et al., 2001; Campbell et al., 2002) fused to the PEST domain (from Tyr^{2386} to Ala^{2468}) from Notch5 cDNA (GenBank Accession Number: NM131549) between *Eco*RI and *Xba*I

sites of pCS2MT. DNA injection for making transgenic zebrafish was performed as described previously (Yeo et al., 2001). Three lines, termed *Tg[her4:EGFP]*, *Tg[her4:nlsEGFP]* and *Tg[her4:dRFP]* were obtained by screening embryos for fluorescence. *Tg[UAS:NICD];Tg[her4:EGFP]* fish was obtained by mating heterozygous *Tg[UAS-NICD]/+* and *Tg[her4-EGFP]/+* adults. Images of EGFP or dRFP fluorescence of transgenic embryos were taken using a confocal laser-scanning microscope (LSM510Meta, Carl Zeiss) and processed using PhotoShop.

RNA and morpholinos injection

For mRNA injection, the fragments encoding *N5ICD* (from Met^{1659} to Ala^{2468}) were subcloned into pCS2. Injection was performed at the two-cell stage as described (Yeo et al., 2001). Morpholinos (MOs) (Gene Tools, LLC) were stored at a stock concentration of 10 mg/ml at −20°C. The sequences of the MOs used were 5′-GAA ACG GTT CAT AAC TCC GCC TCG G-3′ for *notch1a* (*N1a-MO*), 5′-A TCG TAT AGT GGA CTA GGA GAA AGA-3′ for *notch1b* (*N1b-MO*) and 5′-ATA TCC AAA GGC TGT AAT TCC CCA T-3′ for *notch5* (*N5-MO*). Mixed MOs of 2.5 ng of *notch1a*, *notch1b* and *notch5* were injected into one- or two-cell stage embryos. *ngn1*-, *zath3*- and *Su(H)1*-MO were injected into the embryos of transgenic fish (Park et al., 2003; Sieger et al., 2003).

Whole-mount in situ hybridization and immunohistochemistry

Whole-mount *in situ* hybridization and immunohistochemistry was performed as described previously (Yeo et al., 2001, 2004). Antisense riboprobes were synthesized from *her4* cDNA (Takke et al., 1999), *egfp* (ClonTech) and *mRFP* (Campbell et al., 2002). We used the following primary antibodies: mouse anti-Islet1/2 (1:100, Developmental Studies Hybridoma Bank (DSHB)), mouse znp1 (1:250, DSHB), mouse zn8 (1:250, DSHB). For fluorescent detection, we used Alexa Fluor 568 goat anti-mouse conjugate (1:2000, Molecular Probes).

Results

her4 expression is dependent on Notch activation

To investigate if *her4* expression is dependent on Notch signaling in zebrafish, we performed whole-mount *in situ* hybridization with *her4* riboprobe on embryos in which Notch function had either been knocked-down with morpholinos or in which expression of the active Notch intracellular fragment had been induced. *notch1a* and *notch5* are expressed in the developing neural plate during early neurogenesis. Previous studies had shown that while knockdown of *notch1a* or *5* alone has little effect on neurogenesis, combined *notch1a* and *5* knockdown produces a prominent neurogenic phenotype consistent with a failure of lateral inhibition mediated by Notch signaling (Cheol-Hee Kim and Di Jiang, unpublished, data not shown). Consistent with *her4* being a target of Notch activation, embryos in which *notch1a* and *notch5* function has been simultaneously knocked-down show a dramatic reduction of *her4* expression at the 3-somite stage (Figs. 1A and B). In contrast, overexpression of the N1a intracellular domain (N1ICD) by heat-shock at 70% epiboly stage in the *Tg[hsp-GAL4];Tg[UAS:NICD]* embryo causes broad ectopic *her4* expression in the neural plate (Figs. 1C and D), although the induced expression is less prominent on the ventral side of the embryo at bud stage (10 hours post fertilization (hpf)). These observations are consistent with *her4* being a target gene of Notch signaling pathway in the neural plate.

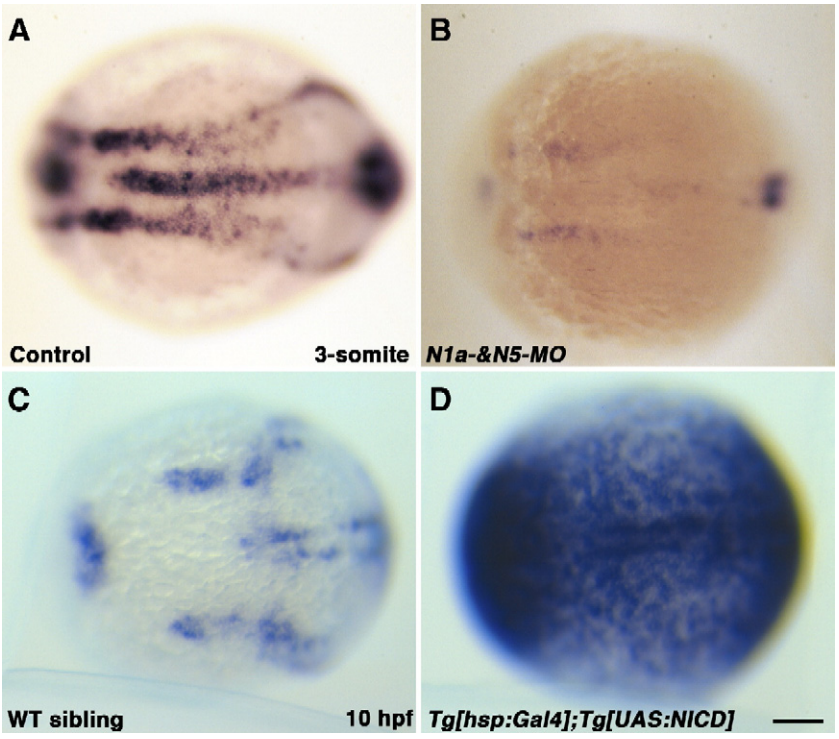

Fig. 1. *her4* is a target of the Notch signaling pathway. (A, B) *her4* expression is reduced in *notch1a* and *notch5* morphants (MO). Compared to control embryos at the 3-somite stage (A), *N1a*- and *N5-MO*-injected embryos (B) have a reduction in *her4* expression in the neural plate. (C, D) *her4* is ectopically expressed in embryos with induced NICD expression. Compared to wild-type sibling (C), *Tg[hsp-GAL4;UAS-NICD]* embryos (D), which had been heat-shocked at 70% epiboly, displayed ectopic expression of *her4* in the neural plate at 10 hpf. The control, MO-injected, and heat-shocked embryos were morphologically normal at the stage shown. Dorsal view. Anterior is left. Scale Bar, 100 μm.

Cloning of the 3.4 kb 5′-flanking region of her4

Since *her4* expression is dependent on Notch activation in the neural plate, we cloned its 5′ flanking genomic fragment with the goal of identifying regulatory sequence capable of driving Notch responsive *her4* expression in the embryo. The zebrafish genomic database at the Sanger Center was searched and contig, z06s016987 was identified to contain *her4* cDNA sequence. Polymerase chain reaction (PCR) fragments obtained using primers directed against 3.409 kb 5′-flanking region of *her4* genomic DNA were subcloned, sequenced and analyzed by the Promoter 2.0 prediction server (http://www.cbs.dtu.dk/services/Promoter/) and by MATINSPECTOR (http://www.genomatix.de/products/MatInspector/index.html). Predicted sequences were also compared to Su(H) DNA-binding consensus sequence, YGTGRGAA, where Y is C or T, and R is A or G (Nellesen et al., 1999).

3.4 kb 5′-flanking region of *her4* genomic DNA contains 22 bp of 5′-untranslated region (UTR) sequence of *her4* cDNA. The putative transcription start site A is referred to as +1 and all subsequent nucleotide positions were numbered relative to this location (Fig. 2 and Supplementary Fig. S1). A putative cellular and viral TATA box element is located in the 30 bp upstream region (−36/−20) suggesting that the core promoter for *her4* is located around this region. The 5′-flanking region of *her4* genomic sequence includes five predicted vertebrate CBF1/Suppressor of hairless [Su(H)] binding sites (CBSs), CBS#1 from −2332 bp to −2346 bp, CBS#2 from −1443 bp to −1457 bp, CBS#3 from −1248 bp to −1261 bp, CBS#4 from −225 bp to −239 bp, and CBS#5 from −89 bp to −75 bp (Figs. 2A, B), consistent with this 5′ sequence mediating, at least in part, transcription in response to Notch activation (Tun et al., 1994). In addition, there were putative bHLH transcription factor-binding sites including two predicted Neurogenin1

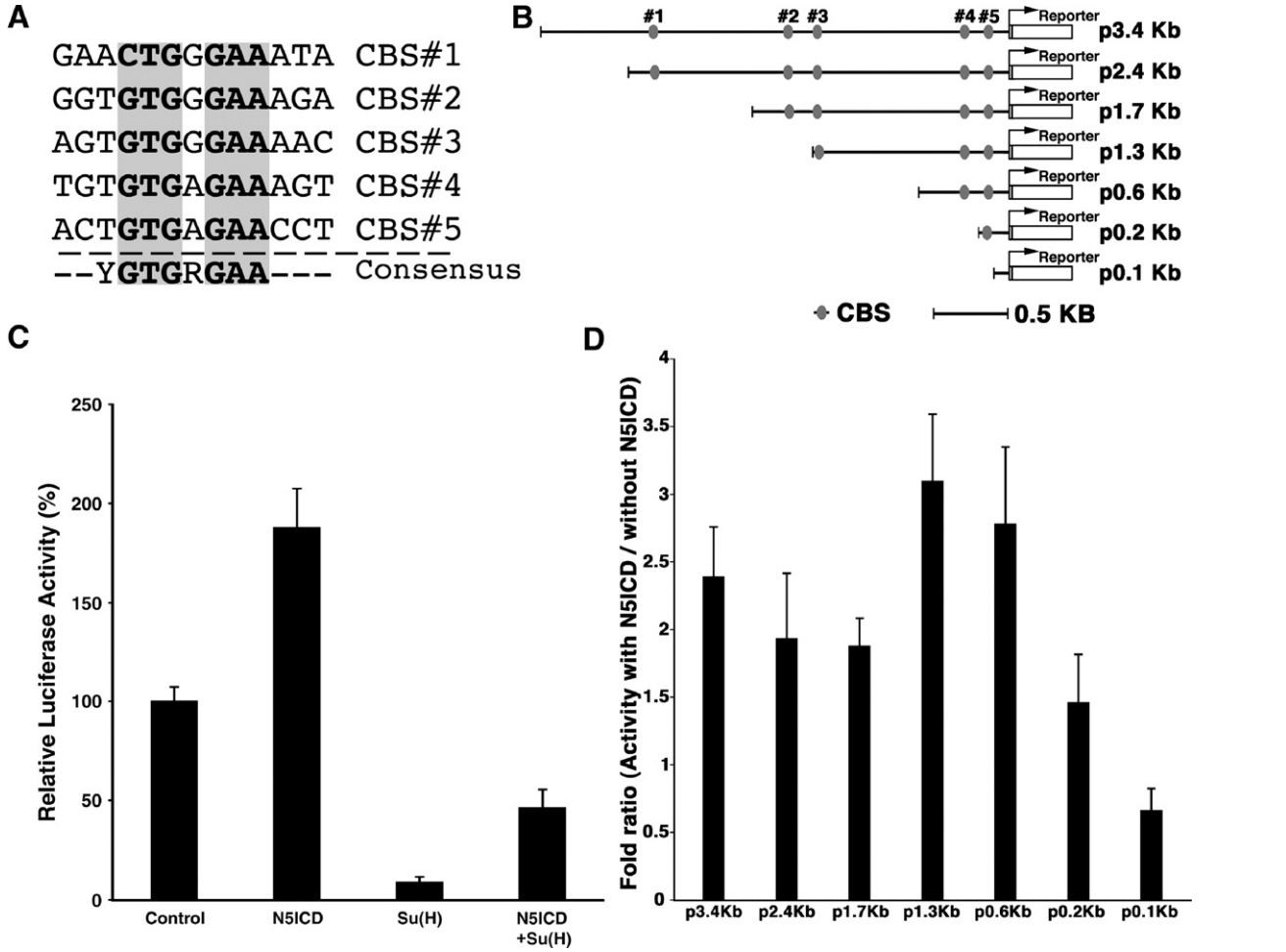

Fig. 2. Responsiveness of *her4* promoter to Notch activation *in vitro*. (A) Putative CBF1/Su(H) binding sites (CBS) in the zebrafish 3.4 kb *her4* promoter. CBS#1, CBS#2, CBS#3, CBS#4, CBS#5 are putative Su(H) binding sites identified by MatInspector and by comparison to the Su(H) binding consensus sequence YGTGRGAA defined in Nellesen et al. (1999), where Y is C or T, and R is A or G. CBS#1 was predicted by MatInspector but does not conform to the consensus sequence. (B) Diagram of 5′-flanking region of *her4*. Ovals indicate putative CBSs. (C–D) Responsiveness of *her4* promoter to Notch5ICD-Su(H) mediated transcription. Luciferase activity under the control of the 3.4 kb promoter is increased in the presence of NICD, while its activity is decreased in the presence of Su(H) (C). Luciferase activity mediated by truncated promoters reveals contribution of predicted CBSs to Notch responsiveness (D). Each value with a standard deviation is the average of at least two independent experiments performed in triplicate.

binding sites (−1190/−1202, −490/−478), consistent with activation also being regulated by proneural genes.

The 3.4 kb fragment mediates a response to Notch signaling in a luciferase assay

For an evaluation of the functional significance of CBSs in the 3.4 kb putative promoter, the genomic fragment was cloned into a reporter plasmid to examine its ability to drive transcription of luciferase. Luciferase activity, assayed in P19 cells co-transfected with the 3.4 kb promoter–reporter plasmid and N5ICD, showed an approximately 1.9±0.2-fold (mean± SD, $p<0.05$) increase in relative luciferase activity compared to that of control (Fig. 2C, see Materials and methods for details), suggesting that the 3.4 kb *her4* promoter has elements responsive to Notch signaling. Co-transfection of the reporter plasmid with Su(H) led to effective suppression of the response (∼0.1±0.0-fold, $p<0.005$), consistent with this DNA binding factor mediating basal repression of *her4* transcription (Fig. 2C). Co-transfection of Su(H) with N5ICD reduced but did not completely eliminate the Su(H)-mediated repression (∼0.5± 0.0-fold, $p<0.01$). Together these observations suggest that CBSs in the 3.4 kb promoter are capable of mediating a response to NICD-Su(H)-mediated transcription. Furthermore, in the absence of N5ICD these binding domains mediate effective repression of luciferase transcription by Su(H).

For a preliminary evaluation of the contribution of different parts of the 3.4 kb genomic fragment to transcriptional regulation, 5′ deletions of the promoter were engineered by amplifying and cloning progressively smaller fragments into the luciferase reporter plasmid (Fig. 2D). Luciferase activity mediated by truncated promoters in which 5′ sequence had been progressively eliminated revealed variability in responsiveness to NICD (N5ICD) activation (Fig. 2D). Loss of CBF1/Suppressor of hairless [Su(H)] binding site CBS#1 caused no obvious decrease in responsiveness to N5ICD in the 1.7 kb promoter compared to that in the 3.4 kb promoter (p3.4kb, ∼2.4±0.4, $p<0.01$; p1.7kb, ∼1.9±0.2, $p<0.01$). Additional loss of CBS#2 in the 1.3 kb promoter led to slight increase in luciferase activity (p1.3kb, ∼3.0±0.5, $p<0.01$). Loss of CBS#3 in the 0.6 kb promoter also caused no obvious decrease in luciferase activity (p0.6kb, ∼2.8±0.6, $p<0.05$). However, subsequent loss of predicted CBF1/Suppressor of hairless [Su(H)] binding sites CBS#4 and CBS#5 produced progressive loss of N5ICD-mediated luciferase activity (p0.2kb, ∼1.5±0.4, $p<0.05$; p0.1kb, ∼0.7±0.2, $p<0.1$). Together, these observations suggest that these two CBSs are sufficient to mediate the Su(H)-mediated Notch activation in the 3.4 kb *her4* promoter. It is harder to evaluate the contribution of other predicted sites, they may not actually contribute *in vivo* or their contribution may be regulated by additional flanking regulatory sequence.

Expression of egfp and drfp in transgenics is similar but not identical to endogenous her4 expression

To evaluate the pattern of transcriptional expression driven by the 3.4 kb promoter *in vivo*, transgenic lines, *Tg[her4:EGFP]* and *Tg[her4:dRFP]*, were established in which the promoter drives expression of a fluorescent protein. The spatiotemporal pattern of *egfp* and *drfp* transcript expression in the *Tg[her4:EGFP]* and *Tg[her4:dRFP]* embryos was compared to that of endogenous *her4*. At the 5-somite stage, *her4* is expressed in lateral, intermediate and medial neurogenic domains in the caudal neural plate where cells have the potential to become neurons (Fig. 3A). At this stage, *egfp* and *drfp* transcripts in the transgenic zebrafish were also expressed in the intermediate and medial domains but not in the most lateral domain in the caudal neural plate where Rohon Beard sensory neurons differentiate (Figs. 3B and C). Expression of *egfp* and *drfp* was also not present in the trigeminal sensory ganglia where endogenous *her4* transcripts are seen at this stage. Endogenous *her4* is also expressed in characteristic domains of the forebrain, midbrain, and hindbrain. *egfp* and *drfp* transcripts are expressed in a similar pattern with the important distinction that ectopic broad expression was observed in the caudal diencephalon where no endogenous *her4* is expressed (Figs. 3B and C). By 25 hpf, expression of endogenous *her4,* and EGFP and dRFP in the transgenics is most intense and conspicuous in the central nervous system (CNS) (Figs. 3D–F). At this stage, *her4* is expressed in an extensive set of cells in the embryonic brain and spinal cord (Fig. 3D). Expression in the brain includes the ventrocaudal cluster of the telencephalon, the ventrorostral cluster of the diencephalon, the epiphysis, the tegmentum and the rhombencephalon. The spatial expression of EGFP and dRFP under the control of the zebrafish 3.4 kb *her4* promoter was similar to endogenous *her4* at this stage. However, an individual cell-by-cell comparison was not possible because of the complexity of the expression pattern.

Accumulation of EGFP was expected to fill the cytoplasm and reveal the morphology of cells with relatively high Notch activation. To identify the cell body easily, *Tg[her4-nlsEGFP]* was established in which the 3.4 kb promoter drives expression of nuclear-localized EGFP. However, the stability of EGFP was expected to prevent effective reporting of dynamic changes in Notch activity. To overcome this disadvantage, *Tg[her4:dRFP]* was established in which the 3.4 kb promoter drives expression of a destabilized red fluorescent protein (dRFP). A PEST sequence of Notch5 was introduced into the C-terminal of monomeric RFP (Campbell et al., 2002) to create a destabilized form of this rapidly maturing RFP. It is important to note that EGFP, nlsEGFP and dRFP were expressed in the identical pattern under the control of the 3.4 kb promoter in the three transgenic lines although, consistent with its engineered destabilization and consequent inability to accumulate to high levels in the cells, dRFP did not appear to fill cells and label cell processes as effectively as EGFP (data not shown). Western blot analysis of protein lysates of transgenic embryos showed that Myc-tagged dRFP was detected at 10 hpf when EGFP was still not detected (Fig. 3G). It is unclear whether EGFP is not expressed at this stage, since the sensitivity of anti-EGFP antibody differs from that of anti-Myc antibody. Although the sensitivity of anti-Myc antibody differs from that of anti-GFP antibody, EGFP was more strongly detected at 12.5 hpf than Myc-tagged dRFP in the double transgenic of *Tg*

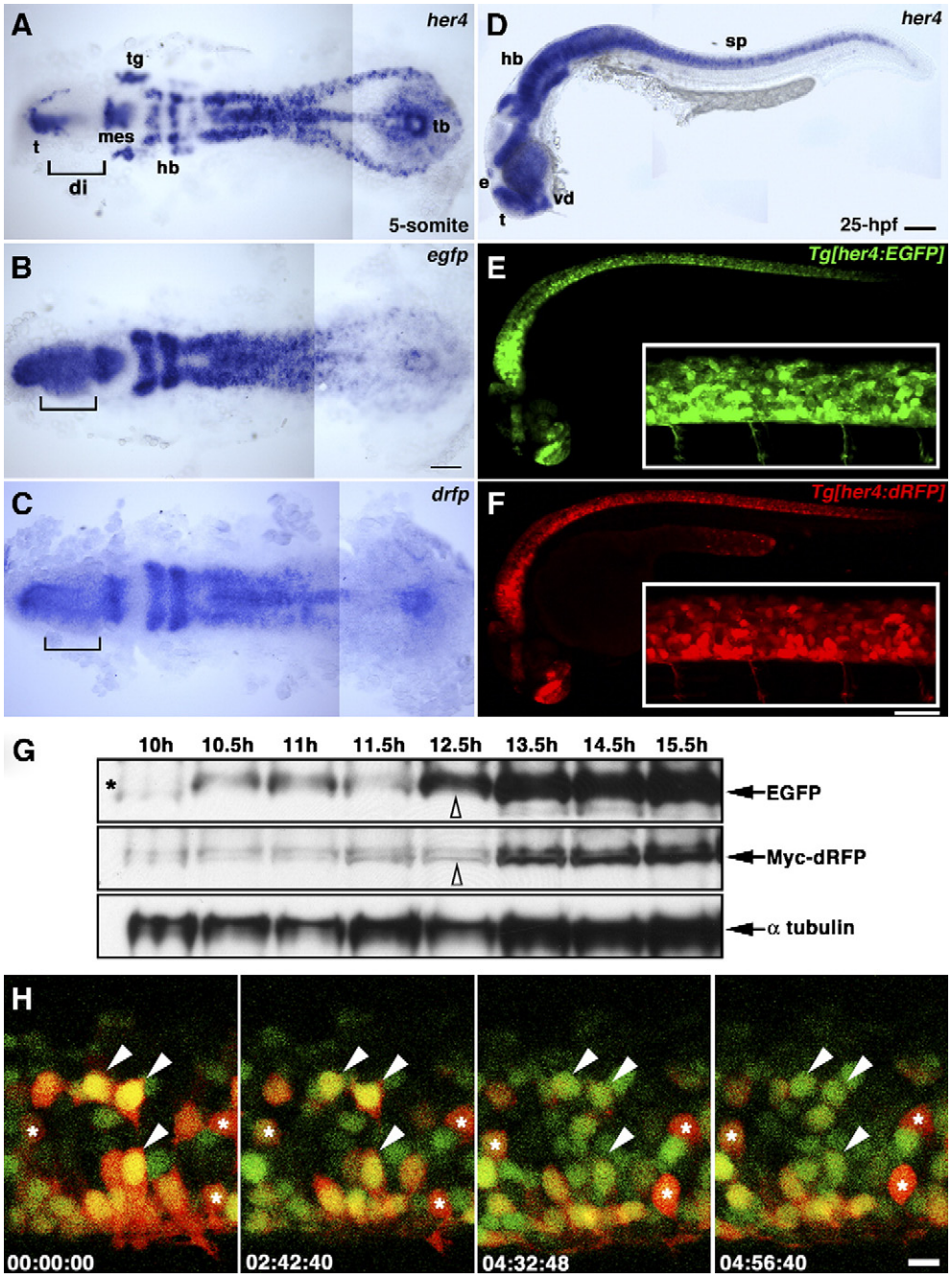

Fig. 3. Expression of fluorescent reporters in the heterozygous transgenic zebrafish. Dorsal views (A–C) and lateral views (D–E). Anterior to the left. Expression of *her4* (A), detected by whole-mount *in situ* hybridization at the 5-somite stage, is similar to *egfp* (B) and *drfp* (C), with some exceptions. *egfp* and *drfp* expression is not observed in trigeminal ganglia (tg) at this stage and ectopic *egfp/drfp* expression is induced in the diencephalon (di, bracket). The pattern of CNS *her4* expression in a wild-type sibling at 25-hpf (C) is similar to EGFP in *Tg[her4:EGFP]* (D) or dRFP in *Tg[her4:dRFP]* (E) embryos. High magnification views are inset in panels E and F. (G, H) Protein stability of reporters *in vivo*. Western blot analysis using anti-EGFP and anti-Myc antibody showed that EGFP and Myc-tagged dRFP were detected at various stages in the *Tg[her4:EGFP]:[her4:dRFP]* embryos (G). Total protein was prepared from 30 progenies at 10, 10.5, 11, 11.5, 12.5, 13.5, 14.5 and 15.5 hpf (h). Myc-tagged dRFP was detected at 10 hpf whereas EGFP was undetected (asterisk). At 12.5 hpf, higher level of EGFP could be observed in the *Tg[her4:EGFP]:[her4:dRFP]* embryos than that of Myc-tagged dRFP. The values of total protein were expressed with respect to the α-tubulin control. Time-lapse images showed that Myc-tagged dRFP was more rapidly degraded than EGFP *in vivo* in the *Tg[her4:nls-EGFP]:[her4:dRFP]* embryos (arrowheads in H). The *Tg[her4:EGFP]:[her4:dRFP]* embryo was imaged in the spinal cord region, from a lateral view, beginning at approximately 30 hpf and extending through 36 hpf. White asterisks indicate the cells in which Myc-tagged dRFP and EGFP were persistently expressed. t, telencephalon; mes, mesencephalon; hb, hindbrain; tb, tailbud; e, epiphysis; vd, ventral diencephalon; sp, spinal cord. Scale bar: 100 μm (A–F), 20 μm (H).

[her4:EGFP] and *Tg[her4:dRFP]* (Fig. 3G). Time-lapse images of the double transgenic of *Tg[her4:nlsEGFP]* and *Tg[her4:dRFP]*, beginning at approximately 30 hpf and extending through 36 hpf, showed that dRFP started to degrade after 2.5 h and largely diminished after 4.5 h while EGFP was persistently expressed in the same cells of the spinal cord (Fig.

3H). These data demonstrated that dRFP is less stable than EGFP *in vivo*.

Together, the comparison of endogenous *her4* expression with that of *egfp* and *drfp* transcripts and protein in *Tg[her4:EGFP]* and *Tg[her4:dRFP]* transgenic zebrafish embryos at the 5-somite stage showed that the 3.4 kb promoter was capable of driving expression of fluorescent proteins in a spatiotemporal pattern similar to that of endogenous *her4*, however, there were clear differences. First, *egfp* transcripts driven by the 3.4 kb promoter were not seen as early as *her4* in lateral domains where RB and trigeminal sensory neurons differentiate, suggesting the lack of regulatory elements capable of driving early or high enough expression in the these domains. Second, ectopic expression of fluorescent protein transcripts was observed in the diencephalon suggesting that critical regulatory elements responsible for repressing expression in this part of the brain are not included in the 3.4 kb promoter. Finally, there is much less expression of *egfp* in the most caudal part of the neural plate and in the tailbud where the corresponding *her4* expression is observed.

Response of her4 and egfp to altered Notch signaling at the 5-somite stage

The effect of altering Notch, Su(H) or proneural function on *egfp* expression in the *Tg[her4:EGFP]* embryos was examined to better characterize the differences in expression driven by the endogenous *her4* regulatory elements and by the 3.4 kb transcriptional regulatory module located upstream of *her4*. Effects of Notch activation were assayed by injecting embryos with mRNA encoding the intracellular fragment of Notch or by heat shock applied to *Tg[hsp:GAL4];Tg[UAS:NICD];Tg[her4:EGFP]* embryos (Fig. 4).

When synthetic mRNA encoding the intracellular domain of zebrafish Notch5, *N5ICD,* was injected into one blastomere of *Tg[her4:EGFP]* transgenic embryos at the 2-cell stage, it caused an increase in the expression of EGFP (Fig. 4A). Heterozygous *Tg[UAS-NICD];Tg[her4:EGFP]* fish were crossed with homozygous *Tg[hsp:GAL4]* fish and fluorescent progeny carrying *Tg[her4:EGFP]* were selected. Heat shock of fluorescent progeny was expected to induce NICD expression in *Tg[hsp-GAL4];Tg[UAS:NICD];Tg[her4:EGFP]* embryos but not in *Tg[hsp-GAL4];Tg[her4:EGFP]* siblings lacking *Tg[UAS:NICD]* that served as controls (Figs. 4B and C). Consistent with expectations, a dramatic increase of EGFP expression was seen in half the fluorescent embryos at 36 hpf following heat-shock at 24 hpf (Fig. 4C). Next, we performed Western blots using an anti-Myc antibody. Heterozygous *Tg[UAS-NICD];Tg[her4:dRFP]* fish were crossed with homozygous *Tg[hsp:GAL4]* fish. Total Protein was prepared from 30 progeny 0.25, 0.5, 1 and 2 h after a 40 min heat-shock at 80% epiboly (Fig. 4D). Myc-tagged NICD protein was detectable from 1 h after heat-shock induction and Myc-tagged dRFP protein was detectable 2 h after heat-shock (Fig. 4D).

Knock-down of *notch1a* and *notch5* effectively reduced both endogenous *her4* expression and *egfp* expression driven by the 3.4 kb promoter (Figs. 4F and F′). Expression of *egfp* in the caudal diencephalon, however, was only slightly reduced by knock-down of Notch function, suggesting that this domain of *egfp* expression, which does not correspond to that of endogenous *her4*, is not as dependent on Notch activation (Fig. 4F′).

Response of her4 and egfp to knock-down of Su(H)1 at the 5-somite stage

Since Su(H) binding sites are required both for mediating transcriptional activation by co-activators like NICD and for basal repression by co-repressors, we asked how the pattern of *her4* and *egfp* expression is altered by reducing Su(H) function. Embryos injected with morpholinos against Su(H)1 had a robust neurogenic phenotype consistent with failure of Notch signaling (data not shown). At the 5-somite stage, *Su(H)1-MO* reduced *her4* expression in the CNS, though the reduction was not as much as seen following knock-down of *notch1a* and *notch5*; *her4* expression was completely lost in the forebrain and its expression was maintained in a small subset of cells within domains of the hindbrain and caudal neural plate (Fig. 4G and G′). Expression in the tail bud was also completely lost. In contrast, the Su(H)1 morphant embryos had a slightly broader domain of *her4* expression in the sensory trigeminal ganglia and in cells near the rostral edge of the neural plate (Fig. 4G). This suggests that, while Su(H)1 is required to drive high levels of *her4* expression in the CNS, it may have a more essential role in repressing *her4* expression in some parts of peripheral nervous system (PNS) like the trigeminal ganglia.

The *Su(H)1* knock-down also produced a reduction of *egfp* expression in the *Tg[her4:EGFP]* embryos. As with *her4*, knock-down of *Su(H)1,* did not reduce expression of *egfp* as effectively as knock-down of *notch1a* and *notch5.* No obvious increase in *egfp* expression, corresponding to changes in endogenous *her4* expression, were seen in the trigeminal sensory ganglia or at the rostral edge of the neural plate in the transgenic fish following knock-down of *Su(H)1* (Fig. 4G′).

Response of her4 and egfp to knock-down of ngn1 and zath3 at the 5-somite stage

Morpholinos to *ngn1* and a related atonal homolog, *zath3*, were injected in the embryos to investigate the role of these proneural genes in regulating both endogenous *her4* expression and expression of *egfp* in the transgenic embryos. Consistent with the critical role of proneural genes in regulating *E(spl)* expression in *Drosophila*, the simultaneous knock-down of *ngn1* and *zath3* resulted in a dramatic reduction of *her4* expression (Fig. 4H). Knock-down of these proneural genes did not, however, significantly reduce expression in the non-neural tailbud region.

In contrast to effects on endogenous *her4*, knock-down of *ngn1* and *zath3* had variable effects on *egfp* reporter expression in the transgenic embryos. Expression of *egfp* was significantly reduced in medial expression domains of hindbrain and caudal neural plate, while its expression in other domains was less affected. The *egfp* expression in the medial domain was also

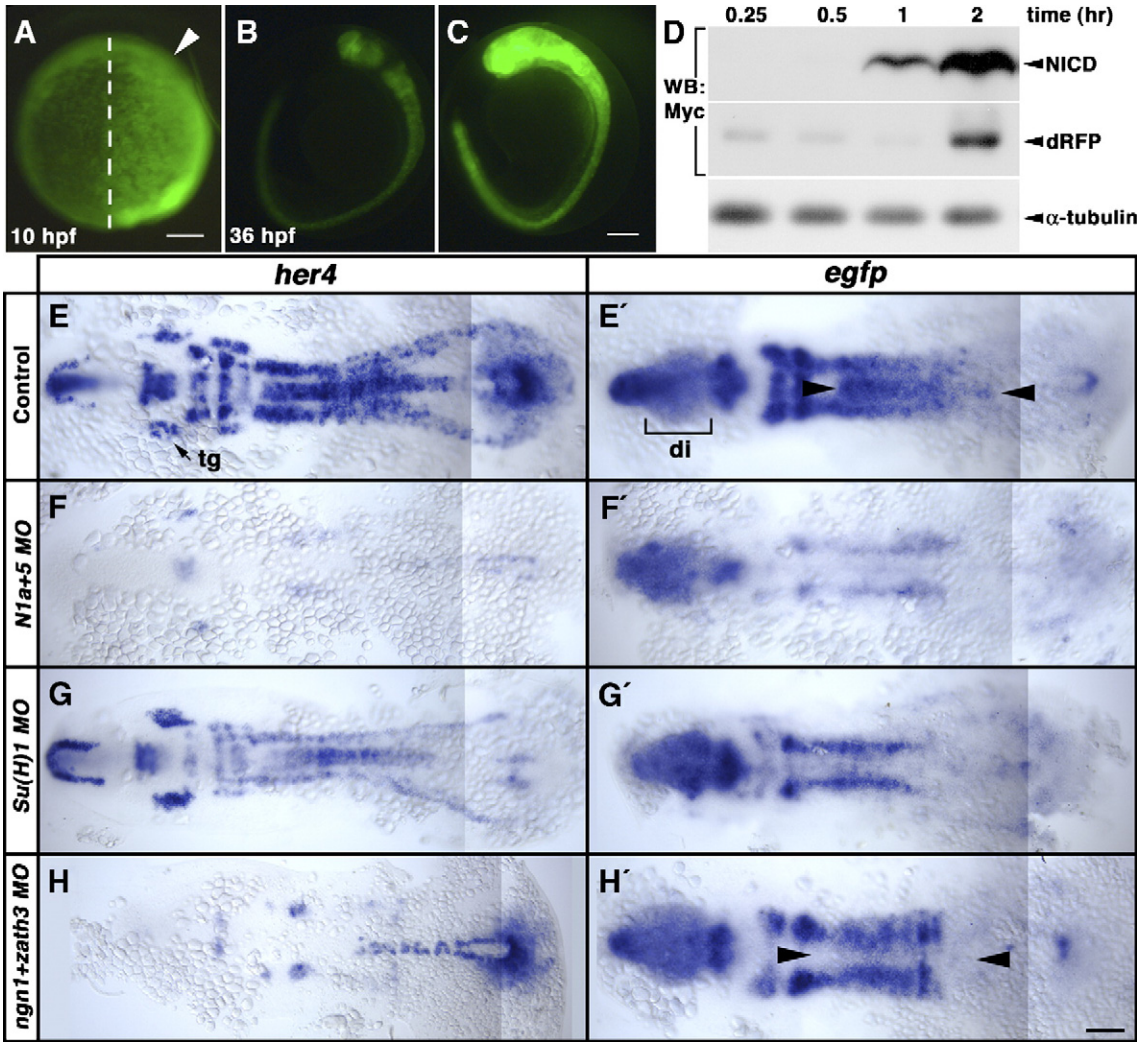

Fig. 4. Responsiveness of the 3.4 kb *her4* promoter to Notch signaling *in vivo*. (A–C) *Tg[her4:EGFP]* embryos injected with mRNA encoding an activated form of Notch5, *N5ICD*, show induction of EGFP expression on the injected side at 10 hpf (A). Dorsal view. The dashed line is the midline and the arrowhead indicates the injected side. Comparison with a *Tg[hsp:GAL4]/+;Tg[her4:EGFP]/+* sibling (B) shows that after heat-shock to induce *N5ICD* at 24 hpf, a *Tg[hsp-GAL4]/+;Tg[UAS:NICD]/+;Tg[her4:EGFP]/+* embryo (C) has exaggerated expression of EGFP in the CNS at 36 hpf. panels B and C are lateral views. (D) Western blots using an anti-Myc antibody showed that Myc-tagged NICD protein was detectable from 1 h after heat-shock induction and Myc-tagged dRFP protein was detectable 2 h after heat-shock in the heterozygous *Tg[hsp:GAL4];Tg[UAS-NICD];Tg[her4:dRFP]* fish. Total protein was prepared from 30 progenies 0.25, 0.5, 1 and 2 h after a 40-min heat-shock at 80% epiboly. The values of total protein were expressed with respect to the α-tubulin control. (E–H) Comparison of *her4* and *egfp* expression following manipulation of Notch signaling and proneural function. Dorsal views. Anterior to the left. Compared to expression of *her4* (E) and *egfp* (E′) in the control embryos, that of both *her4* and *egfp* is reduced in 5-somite embryos following knock-down of *notch1a* and *notch5* with morpholinos (*N1a+5*MOs) (F, F′). *Su(H)1* morpholinos (*Su(H)1 MO*s) also reduce *her4* and *egfp* but they are less effective at reducing expression (G, G′). *her4* expression is reduced by morpholinos against *ngn1*- and *zath3*, however, apart from reduced expression in the midline (between arrow heads), *egfp* expression is not reduced (H, H′). At this stage, ectopic *egfp* expression induced by the *her4* promoter in the prospective diencephalic region (di, bracket) was not affected by any of the manipulations. As before, no *egfp* expression was observed in the trigeminal ganglion (tg) (E′–H′). Knock-down of *notch1a* and *notch5* (F) and *ngn1* and *zath3* (H) by morpholinos led to a reduction of endogenous *her4* expression in the trigeminal ganglion (arrow), however, trigeminal expression was not reduced following *Su(H)1* knock-down (G). Scale bar: 100 μm.

most prominently reduced when Notch function or Su(H) was knocked-down (Fig. 4H′).

Differences in her4 and egfp expression in the trigeminal ganglia at 26 hpf

The comparison of endogenous *her4* and *egfp* expression driven by the 3.4 kb promoter in the transgenic embryos at the 5-somite stage revealed that, while *her4* is expressed in the trigeminal sensory ganglia, *egfp* and *drfp* are not expressed in this domain. Examination of transgenic embryos at 26 hpf, however, showed that EGFP and dRFP is eventually expressed in some cells of the trigeminal sensory ganglia at this stage (Fig. 5E, and data not shown for EGFP expression). For these studies *Tg[her4:dRFP];Tg[huC:EGFP]* embryos were used where the *huC* (*elavr3*) promoter drives expression of EGFP in all neurons, and sensory neurons can be recognized by their green fluorescence (Fig. 5E′).

As described below, comparison of *her4* and dRFP expression in 26-hpf embryos following manipulations that

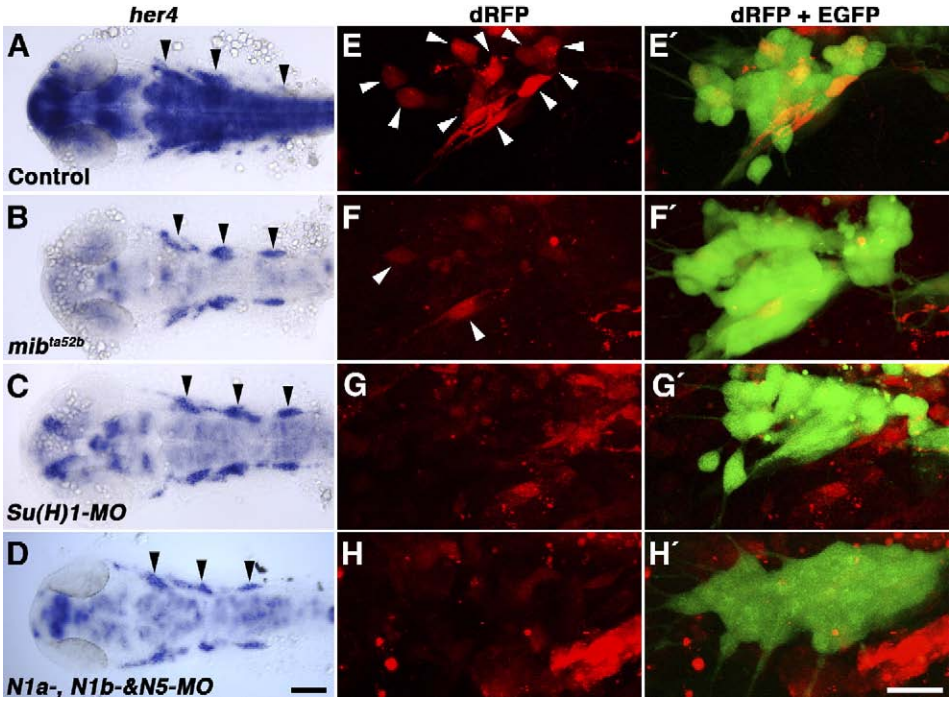

Fig. 5. Dependence of *her4* and fluorescent reporter expression in the cranial ganglia. (A–D) Expression of *her4* in embryos with impaired Notch signaling. Dorsal views. Anterior to the left. Expression of *her4* in control (A), *mib*^{ta52b} mutant (B), *Su(H)1-MO* (C), and *N1a, N1b* and *N5-MO* injected embryos (D). At this stage, *her4* CNS expression is reduced following all these manipulations that reduce Notch signaling, however, expression in cranial ganglia, including trigeminal sensory neurons, is not significantly reduced (arrowheads). (E–H) Reporter expression in trigeminal ganglia following reduction of Notch signaling. Lateral views. Confocal images of *Tg[her4:dRFP];Tg[huC:EGFP]* embryos identified *her4* promoter-driven dRFP-expressing cells (white arrowheads) (E–H) and EGFP-expressing trigeminal sensory neurons, E′–H′ are merged images of the Red and Green channels showing reporter expression the trigeminal ganglia. dRFP reporter expression was dramatically reduced in the trigeminal ganglia of *mib*^{ta52b} mutant (F, F′), *Su(H)1-MO* (G, G′), and *N1a-, N1b-* and *N5-MO* injected embryos (H, H′). Consistent with reduced Notch signaling producing a neurogenic phenotype, these manipulations were accompanied by an increase in the number of EGFP expressing cells in the trigeminal ganglia (E′–H′). Scale Bar, 100 μm (A–D), 50 μm (E–H and E′–H′).

reduce Notch signaling revealed additional differences in the response of endogenous *her4* and 3.4 kb *her4* promoter. Mind bomb is a protein that is essential for effective Notch signaling (Itoh et al., 2003). Examination of *her4* in *mind bomb* (*mib*^{ta52b}) mutant embryos at 26 hpf revealed a dramatic reduction in *her4* expression in the CNS, however, expression of *her4* persisted in the cranial sensory ganglia including the trigeminal ganglion (Fig. 5B). A similar reduction of *her4* expression in the CNS and persistence of *her4* expression in adjacent cranial sensory ganglia of the PNS was observed when Su(H)1 was knocked-down (Fig. 5C). Knock-down of *notch1a* and *notch5* alone did not effectively reduce *her4* expression (data not shown). However, at 26 hpf an additional *notch* homologue, *notch1b*, is expressed in the CNS. Simultaneous knock-down of *notch1a*, *notch1b* and *notch5* resulted in reduced CNS *her4* expression and persistent *her4* expression in cranial ganglia, as observed in *mib* mutants and Su(H) morphants . In contrast to the persistence of *her4* in cranial ganglia, expression of dRFP was reduced or lost in the trigeminal sensory ganglia of *mib* mutants (Figs. 5F and F′) and in morphants with knock-down of *Su(H)1* (Figs. 5G and G′) and *notch* genes (Figs. 5H and H′). Together these observations show that while active Notch signaling is not essential for endogenous *her4* expression in the trigeminal ganglia, expression of dRFP in the trigeminal ganglia driven by the 3.4 kb *her4* promoter does depend on Notch signaling.

Fluorescent protein reporter expression in early and late differentiating neurons

Analysis of fluorescent protein reporter expression directed by the 3.4 kb promoter of *her4* showed that though there are important differences between expression driven by the endogenous *her4* and that by the 3.4 kb *her4* promoter, reporter expression in the transgenic fish was fairly responsive to changes in Notch activity. This suggested that the transgenic fish could be used to examine how the pattern of Notch activation correlates with adoption of different cell fates, especially in the ventral spinal cord/medial neural plate where reporter expression is most sensitive to changes in Notch signaling. Previous studies have shown that Notch activation prevents a subset of cells in the ventral spinal cord from becoming primary motor neurons and this allows some of them to become secondary motor neurons instead (Appel et al., 2001). This distinction predicts that expression of reporter fluorescent protein in the transgenic embryos should be excluded from the primary motor neurons and seen in later differentiating cells like secondary motor neurons.

To validate the transgenic lines as tools for determining how Notch activation determines cell fate, we examined if reporter fluorescent proteins are differentially expressed in primary and secondary motor neurons. Early differentiating primary motor neurons can be recognized by the stereotyped position of their

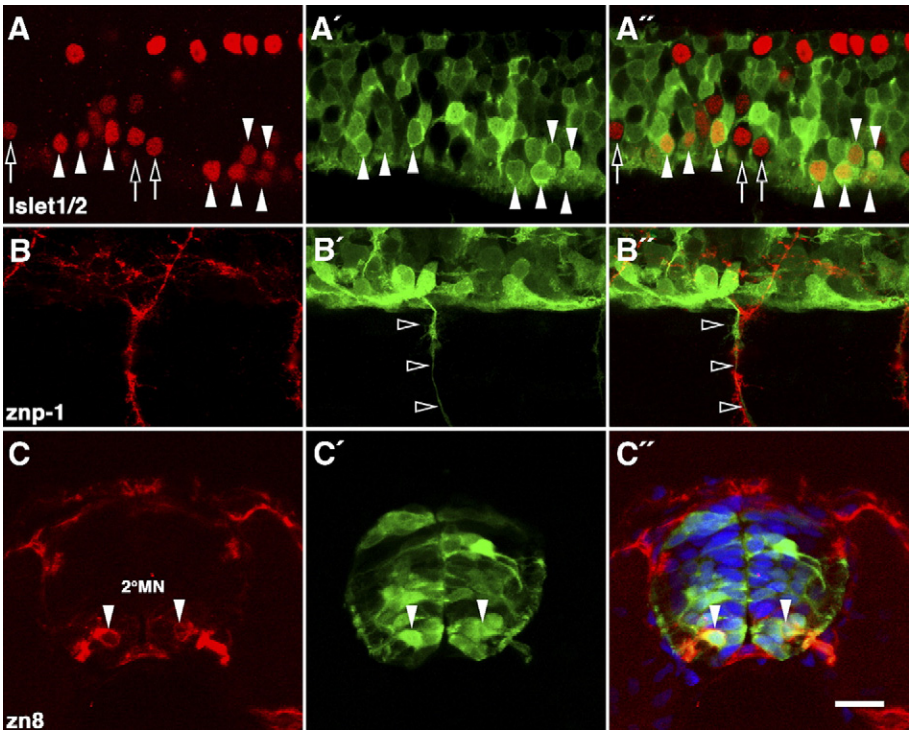

Fig. 6. *Tg[her4:EGFP]* embryos reveal reporter expression in secondary motor neurons. (A, B) Lateral views, anterior to the left and dorsal up. (C) Transverse section, dorsal up. Confocal images of the spinal cord identify Islet1/2-labeled cells (A), znp1-labeled cells (B), zn8-labeled cells (C) in the Red channel, EGFP-expressing cells of *Tg[her4:EGFP]* (A′–C′) in the Green channel and the merged images (A″–C″) at 28 hpf. White arrowheads indicate EGFP-expressing motor neurons and white open arrows indicate Islet1/2-labeled motor neurons (A–A″). Axons of EGFP-expressing motor neurons are distinct from primary motor neuron axons labeled with znp-1 (white open arrowheads) (B–B″). Confocal image of a transverse spinal cord section reveals that zn8-labeled secondary motor neurons express EGFP (white arrowheads) (C–C″). Scale bar: 25 μm.

large cell bodies and by the labeling of their axons by the znp-1 antibody. Later differentiating secondary motor neurons have smaller cell bodies and the zn8 antibody labels their axons.

At 26 hpf, the Islet1/2 antibody labeled RB sensory neurons in the dorsal cord and a number of motor neurons in *Tg[her4:EGFP]* embryos. EGFP expression was excluded from RB neurons in the dorsal cord, while it co-labeled a subset of motor neurons in the ventral cord (Figs. 6A–A″). In some embryos it appeared that EGFP expression was specifically excluded from motor neurons that had especially large cell bodies, consistent with EGFP not being expressed in primary motor neurons. Labeling with the znp-1 antibody, which labels primary motor neurons, showed that EGFP expressing motor axons are distinct from znp-1 labeled axons (Figs. 6B–B″), supporting the possibility that EGFP is expressed in secondary motor neurons, not primary neurons. Further examination of *Tg[her4:EGFP]* embryo by cryosection showed that EGFP is indeed expressed in secondary motor neurons identified by the zn8 antibody (Figs. 6C–C″). The spinal cord sections also showed that that EGFP was expressed in neuroepithelial cells adjacent to the ventricular surface with process that extend to both the pial and ventricular surfaces.

Discussion

We have characterized the *her4* promoter in zebrafish, a gene that is a target of the Notch signaling pathway in the zebrafish neural plate. Luciferase reporter assays showed that the 3.4 kb 5′-flanking region contains *cis*-acting elements that can respond to Notch signaling, and deletions of the promoter identified specific fragments of the promoter that are essential for mediating this response. Furthermore, the luciferase assays showed that without Notch, Su(H) effectively represses transcription regulated by this promoter. Zebrafish transgenic lines were established in which the 3.4 kb *her4* promoter drives expression of various fluorescent proteins in a pattern that mimics key aspects of *her4* expression in the CNS. However, some important differences in expression driven by the endogenous and the 3.4 kb *her4* promoter were defined. This analysis also showed that *her4* may be differently regulated in the PNS, where Notch activity is not essential for *her4* expression and where Su(H) may have an essential role in repressing *her4* expression. Finally, preliminary studies validated the transgenic lines as potentially useful tools for determining how the pattern of Notch activation regulates cell fate in the developing spinal cord.

Regulation of her4 by Notch signaling in the CNS and PNS

Previous studies had shown expression of *her4* could be induced in the embryo by ectopic expression of an activated Notch receptor (Takke et al., 1999). Our observations extend these studies by showing knock-down of *notch1a* and *notch5* effectively reduces *her4* expression at the 5-somite stage, when

these are the only Notch homologues expressed in the neural plate. Additional knock-down of *notch1b* is required to reduce *her4* expression at 26 hpf when this additional Notch homologue is also expressed in the CNS. These observations underscore the dependence of *her4* expression on Notch signaling in the CNS.

While Notch signaling is clearly required for *her4* expression in the CNS, requirements for *her4* expression in the PNS, specifically the trigeminal sensory ganglion, are more complicated. At the 5-somite stage, Notch knock-down broadly reduces *her4* expression in the nervous system including trigeminal ganglia. However, at 26 hpf persistent expression in the cranial ganglia suggests that *her4* may be expressed in a Notch-independent manner in specific domains of the nervous system. It is unlikely that the manipulations do not effectively reduce Notch signaling since *her4* reporter expression is reduced and the number of trigeminal sensory neurons is increased in the trigeminal ganglia of *Tg[her4:dRFP];Tg[huC:EGFP]* embryos, which is consistent with reduced Notch signaling.

The cis-regulatory logic of E(spl) expression

*C*BF1 and its related CSL family members, *S*u(H) and *L*ag1, are DNA binding factors with a dual role in regulating gene expression. While they facilitate activation of Notch target genes, they can also mediate basal repression of some target genes in the absence of Notch activation. Recent studies have elucidated the *cis*-regulatory logic of *E(spl)* expression in proneural clusters of the *Drosophila* PNS, where expression of proneural genes gives cells the potential to become sensory organ precursors (SOPs), and where lateral inhibition mediated by Notch signaling restricts SOP fate to a single cell within each proneural cluster (Castro et al., 2005). The *Drosophila* studies show that upstream binding sites for bHLH transcription factors and Su(H) allow the proneural transcription factors and activated Notch to synergistically activate *E(spl)* genes, restricting E(spl) expression to cells of a proneural cluster with relatively high levels of Notch activation. Proneural factors alone could potentially induce expression of *E(spl)* genes in the prospective SOP cell, where the low level of Notch activation is compensated by the relatively high level of proneural gene expression. However, *E(spl)* gene expression is prevented by Su(H) in the SOP, where it maintains basal repression of *E(spl)* genes through its association with co-repressors like Groucho and CtBP (Barolo et al., 2002). In cells that surround the prospective SOP, where Notch is activated at relatively high levels, the repressor complex is disassembled and Su(H) drives the expression of *E(spl)* genes by associating instead with NICD in an activator complex. In this manner *E(spl)* gene expression is typically restricted to cells in the proneural cluster that surround the prospective SOP in the *Drosophila* PNS.

Consistent with the regulation of *E(spl)* genes in *Drosophila* described above, this study showed that bHLH proneural genes are also essential for effective *her4* expression in zebrafish embryos. Knock-down of *ngn1* and *zath3* dramatically reduced *her4* expression in the nervous system, while tail bud expression was relatively unaffected. This suggests that *her4* expression in the nervous system is restricted to neuronal progenitors defined by expression of proneural genes like *ngn1* and *zath3*.

Analysis of the 3.4 kb her4 promoter and comparison of expression driven by the endogenous promoter

The comparison between endogenous *her4* and fluorescent gene expression in the transgenic fish indicated that while many aspects of *her4* expression were recapitulated by the 3.4 kb promoter, there were important distinctions. First, the 3.4 kb promoter initiated almost no expression at the 5-somite stage near the edge of the neural plate where *her4* is expressed in association with Rohon–Beard sensory neurons, trigeminal sensory ganglia and unidentified cells at the rostral edge. However, expression was eventually observed in trigeminal ganglia at 26 hpf. This suggests that the 3.4 kb *her4* promoter does not induce high levels of reporter gene expression in these domains and that expression only becomes apparent after it has accumulated for a relatively long time. Alternatively, there may be distinct temporal regulatory elements essential for early expression in some domains like the trigeminal ganglia that are absent in the 3.4 kb *her4* promoter.

Compared to endogenous *her4* expression, fluorescent reporter gene expression was not as dramatically reduced by knock-down of proneural genes as it was with manipulations that inhibit Notch signaling. This suggests that unlike the endogenous *her4* promoter in which proneural genes and Notch signaling both play a critical role in driving expression (compare Fig. 4E with G), expression driven by the 3.4 kb promoter is more dependent on Notch signaling and less on proneural factors (compare Fig. 4E′ with G′). In this context, additional proneural binding sites present in the endogenous *her4* promoter but not in the 3.4 kb fragment might account for reduced responsiveness of the transgenic promoter in the domains where trigeminal and RB sensory neurons are specified.

The 3.4 kb promoter directed broad expression in the diencephalon where there is no corresponding endogenous *her4* expression at the 5-somite stage. Expression of *egfp* in this domain was not significantly altered by manipulation of either Notch, Su(H)1 or Ngn1/Zath3 function suggesting that expression directed by the 3.4 kb promoter in this domain is not so dependent on these factors. Importantly, Su(H)1 knock-down did not induce ectopic *her4* expression in the diencephalon, suggesting that Su(H)-mediated repression does not normally prevent endogenous *her4* expression in this domain. This makes it unlikely that diencephalic fluorescent reporter gene expression in the transgenics is due to absence of additional Su(H) binding sites that are present in the endogenous promoter. Histone deacetylase 1 (HDAC1) is also responsible for silencing Notch target gene expression in specific domains of the zebrafish nervous system (Cunliffe, 2004). However, preliminary experiments suggest (data not shown) that HDAC1 is not required to suppress endogenous *her4* expression in the diencephalon, and hence ectopic diencephalic expression

in the transgenics is also unlikely to be due to absence of HDAC1 binding sites in the 3.4 kb promoter. Interestingly, although *her4* is not normally expressed in the diencephalic region at the 5-somite stage, it is expressed at a later stage in this region and this suggests that the 3.4 kb promoter specifically lacks elements for correct temporal expression of *her4*.

A final difference identified in the behavior of the endogenous *her4* promoter and the 3.4 kb promoter in transgenic embryos was the response in the trigeminal ganglia to reduced Notch signaling. Three independent manipulations that reduce Notch signaling in the embryo reduced *her4* expression in the CNS while its overall pattern of expression was not significantly altered in the cranial sensory ganglia at 26 hpf. In contrast, reporter gene expression was reduced in the trigeminal ganglia showing that in this context expression of the fluorescent reporter is more dependent on Notch signaling than endogenous *her4*. It remains possible that, while loss of Notch signaling does not dramatically alter the number of cells expressing *her4* in the cranial ganglia, the identity of the cells that express *her4* is quite different following the manipulations that reduce Notch signaling. We are currently investigating this possibility.

Transgenic lines for monitoring the dynamic pattern of Notch activation

Although the comparison between endogenous *her4* and fluorescent gene expression in the transgenic fish identified important distinctions, overall the analysis of the transgenic reporter lines demonstrated that they are effective reporters of Notch activity, particularly in the ventral spinal cord. Furthermore, examination of fluorescent reporter gene expression in identified early and late differentiating motor neurons in the spinal cord showed, as expected, that *her4* is preferentially expressed in later differentiating cells. The transgenic lines are now poised for use in cell lineage studies where the patterns of activation will be examined at the single-cell level to determine how the pattern of Notch activation influences cell fate in the spinal cord. We have also begun to use the lines in screens to identify manipulations that alter the pattern of Notch activation during late neurogenesis, a stage at which the role of Notch signaling remains poorly understood.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.10.020.

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