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The bHLH transcription factor *hand2* is essential for noradrenergic differentiation of sympathetic neurons

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The basic helix-loop-helix transcription factor *Hand2*, together with *Ascl1*, *Phox2a*, *Phox2b* and *Gata2/Gata3*, is induced by bone morphogenetic proteins in neural crest-derived precursor cells during sympathetic neuron generation. Hand2 overexpression experiments and the analysis of its function at the *Dbh* promotor implicated Hand2 in the control of noradrenergic gene expression. Using the zebrafish *hand2* deletion mutant *hands off*, we have now investigated the physiological role of *hand2* in the development of sympathetic ganglia. In *hands off* mutant embryos, sympathetic precursor cells aggregate to form normal sympathetic ganglion primordia characterized by the expression of *phox2b*, *phox2a* and the achaete-scute family member *zash1a/ascl1*. The expression of the noradrenergic marker genes *th* and *dbh* is strongly reduced, as well as the transcription factors *gata2* and *tfap2a* (*Ap-2α*). By contrast, generic neuronal differentiation seems to be unaffected, as the expression of *elavl3 (HuC)* is not reduced in *hands off* sympathetic ganglia. These results demonstrate in vivo an essential and selective function of *hand2* for the noradrenergic differentiation of sympathetic neurons, and implicates *tfap2a* and *gata2* as downstream effectors.

KEY WORDS: Zebrafish, Hand2, Tyrosine hydroxylase, Dopamine β-hydroxylase, Phox2b, Gata2

INTRODUCTION

The autonomic nervous system controls body function homoeostasis in vertebrates and is essential for adaptation to internal and external changes. Noradrenergic sympathetic neurons play a major role, for instance by regulating vascular tone, exocrine glands and gut function. To understand the molecular control of differentiated functions of noradrenergic neurons, it is useful to study the mechanisms involved in the initiation of sympathetic neuron differentiation. To this end, considerable progress has been made in recent years in the understanding of mechanisms leading to the generation and differentiation of sympathetic neurons from neural crest precursor cells (Goridis and Rohrer, 2002; Rohrer, 2003).

The development of autonomic neurons depends crucially on the presence of bone morphogenetic proteins (BMPs), extrinsic differentiation factors that are produced in the environment of the primordia of autonomic ganglia. In vivo loss-of-function experiments revealed that BMPs are essential for the generation of noradrenergic neurons of the paravertebral sympathetic chain ganglia (Schneider et al., 1999) as well as for parasympathetic ciliary ganglion neurons (Müller and Rohrer, 2002). Conversely, additional sympathetic and parasympathetic neurons are produced in response to local BMP overexpression (Reissmann et al., 1996; Müller and Rohrer, 2002). The activation of BMP receptor signaling leads to the sequential expression of *Ascl1*, a proneural gene expressed by sympathetic and parasympathetic neurons, and of the paired-homeodomain transcription factors *Phox2b* and *Phox2a*, which are expressed throughout the autonomic nervous system. The elimination of *Ascl1* produces a strong impairment of sympathetic ganglion development, in particular in noradrenergic differentiation (Guillemot et al., 1993; Hirsch et al., 1998). The absence of *Phox2b* results in the complete lack of the autonomic nervous system, including neurons of the enteric nervous system (Pattyn et al., 1999). *Phox2a* is expressed downstream of both *Ascl1* and *Phox2b* during sympathetic ganglion development (Lo et al., 1998; Pattyn et al., 1997; Hirsch et al., 1998), but its physiological function in the initial stages of this lineage is unclear in view of the recent observation that *Phox2a* is unable to rescue the effect of the *Phox2b* knockout on sympathetic neuron development (Coppola et al., 2005).

One of the most interesting properties of the Phox2 transcription factors is their ability to directly activate the transcription of *dopamine-β-hydroxylase* (*Dbh*) and *tyrosine hydroxylase* (*Th*) genes (Swanson et al., 1997; Yang et al., 1998; Zellmer et al., 1995; Kim et al., 1998; Lo et al., 1999). Th and Dbh control essential steps in the synthesis of noradrenaline and represent characteristic marker genes for differentiated noradrenergic neurons. The finding that Phox2 factors control cell type specification in autonomic progenitors as well as their differentiation provided a mechanism to explain noradrenergic differentiation in both the peripheral nervous system (PNS) and the central nervous system (CNS). However, as *Phox2a/b* are also expressed in non-noradrenergic neurons in both PNS and CNS (Morin et al., 1997; Pattyn et al., 2000a; Pattyn et al., 2000b; Pattyn et al., 1997), it is evident that the action of Phox2 on the expression of noradrenergic marker genes requires additional co-regulators that are differentially expressed or modified between noradrenergic and non-noradrenergic neurons. Indeed, a number of additional transcriptional regulators have been implicated in the control of *Th* and *Dbh* in sympathetic precursor cells, including Creb (Swanson et al., 1997; Adachi and Lewis, 2002), Ap-2α (Barallo-Gimeno et al., 2004; Holzschuh et al., 2003; Knight et al., 2003; O'Brien et al., 2004) (also known as Tfap2a), Gata2/3 (Lim et al., 2000; Tsarovina et al., 2004) and Hand2 (also known as dHand) (Howard et al., 1999; Howard et al., 2000; Xu et al., 2003; Rychlik et al., 2003).

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Hand2 has been identified as a basic helix-loop-helix (bHLH) transcription factor expressed in heart, autonomic nervous system and neural crest derivatives (Srivastava et al., 1995; Firulli, 2003). In the autonomic nervous system of chick and mouse, *Hand2* is expressed by sympathetic neurons, adrenal chromaffin cells and enteric neurons (Firulli, 2003; Howard and Cserjesi, 1996; Howard et al., 1999). During development, *Hand2* expression is induced by BMPs but is first observed after the onset of *Ascl1* and *Phox2b* expression in chick sympathetic ganglion priomordia (Howard et al., 2000). This finding, together with the lack of *Hand2* expression in the *Phox2b* knockout and the maintained expression in the *Gata3* knockout (Tsarovina et al., 2004), implicates a function of *Hand2* downstream of *Phox2b* and upstream of *Gata2/3* in the group of BMP-induced transcription factors. *Hand2* overexpression was found to induce the generation of catecholaminergic neurons from neural crest precursor cells in vitro and in vivo (Howard et al., 1999; Howard et al., 2000), acting in concert with *Phox2b*, *Phox2a* and *Ascl1*, which are ectopically expressed after *Hand2* overexpression. Also upon forced *Phox2a* expression, *Ascl1* and *Phox2b* are induced, suggesting cross-regulation between these transcription factors, which are thus considered a network rather than a regulatory cascade (Stanke et al., 1999; Stanke et al., 2004). This cross-regulatory action prohibits firm conclusions from being drawn on the epistatic relationships between these transcription factors, based on overexpression experiments. Nor is it possible to address the individual functions of the factors in sympathetic neuron differentiation, i.e. whether *Hand2* may control specific aspects of the sympathetic neuron subtype, such as *Th* and *Dbh* expression.

A function of Hand2 in the control of *Dbh* expression has been suggested by the finding that Hand2 cooperates with Phox2a in activating transcription from *Dbh* promotor reporter constructs (Xu et al., 2003; Rychlik et al., 2003; McFadden et al., 2002; Howard, 2005; Firulli, 2003). There is also in vivo evidence implicating a role for Hand2 in the maintenance of *Th* and *Dbh* expression in autonomic neurons (Müller and Rohrer, 2002). Developing chick ciliary neurons, by contrast to sympathetic neurons, express *Th* and *Dbh* only transiently. As *Hand2* is not expressed by the vast majority of ciliary neuron precursors (Müller and Rohrer, 2002; Lee et al., 2005), and ectopic *Hand2* expression maintains the noradrenergic phenotype of ciliary neurons, Hand2 may be required for the continued expression of noradrenergic properties (Müller and Rohrer, 2002).

So far, the proposed effects of *Hand2* on sympathetic neuron development could not be analysed by in vivo loss-of-function approaches. *Hand2*$^{-/-}$ mouse embryos die too early to be analysed for sympathetic ganglion development (Yamagishi et al., 1999). We have now studied the *hand2*$^{-/-}$ zebrafish mutant *hands off* (Yelon et al., 2000), as embryos in this species survive for a sufficient number of days to analyse noradrenergic differentiation in cervical sympathetic ganglia (CSG) and the CNS. We observed a strong decrease in the expression of *th*, *dbh*, *gata2* and *tfap2a*, with no effect on *phox2b*- and *zash1a*-expressing sympathetic precursor cells. As the *zash1a* gene encodes a protein very similar to the mammalian achaete scute homolog *Mash-1* (Allende and Weinberg, 1994), we refer to *zash1a* as *ascl1*. Interestingly, the expression of the early neuronal marker *elavl3* (also known as *HuC*) (Kim et al., 1996) was not affected in *hands off* embryos. Together, these results demonstrate for the first time an essential and selective function for *hand2* in the expression of noradrenergic marker genes *dbh* and *th* in the autonomic nervous system. The similar selective effect on noradrenergic but not generic neuronal differentiation in *hand2* and *tfap2a* zebrafish mutants (Holzschuh et al., 2003), together with the reduced *tfap2a* expression in the *hands off* mutant, implies that *tfap2a* is a downstream effector of *hand2* in sympathetic neurons.

The development of central noradrenergic neurons of the locus coeruleus is initated by a similar sequence of inducing signals, i.e. BMP-dependent *ascl1* and *phox2a/2b* expression. The absence of effects on *th* and *dbh* expression in the locus coeruleus of *hands off* embryos confirms previous conclusions that after common initial development later steps of noradrenergic maturation are controlled differentially in the PNS and CNS.

MATERIALS AND METHODS

Animals

Zebrafish adults and embryos were reared at the Ohio State University zebrafish facility under standard conditions at 28.5°C. Embryos were staged according to morphological criteria (Kimmel et al., 1995) and hours post-fertilization (hpf). The *han*^{s6} *hand2* mutant was kindly provided by Dr Deborah Yelon, Skirball Institute, NYU. The *han*^{s6} mutant results from the deletion of the entire *hand2* locus (Yelon et al., 2000). To better visualize internal structures such as sympathetic ganglia without interference from superficial melanophores, embryos were typically incubated in 0.2 mmol/l 1-phenyl-2-thiourea (PTU; Sigma) to prevent pigment formation.

Whole-mount RNA in situ hybridization

Analysis of mRNA expression for *hand2* (Yelon et al., 2000), *dbh*, *tfap2a* (Holzschuh et al., 2003), *th* (T. Look, Dana Farber Cancer Institute), *phox2a* (S. Guo, USCF, San Francisco), *phox2b* (full-length construct by Jochen Holzschuh, University of Freiburg, Germany, unpublished), *zash-1a* (T. Look, Dana Farber Cancer Institute), *gata2* (T. Look, Dana Farber Cancer Institute) and *elavl3* (*HuC*) (Kim et al., 1996) was performed in whole-mount preparations according to Thisse et al. (Thisse et al., 1993), with minor modifications. A detailed protocol will be provided upon request.

Embryo dissection and planimetric analysis

After in situ hybridization, the stained embryos were transferred to 100% glycerol. After equilibration for at least 2 hours, the yolk and the tail were removed and used for genotyping. To take pictures at the microscope, the neural tube and parts of the head overlying the cervical sympathetic ganglion (CSG) were removed. In addition, the dorsal part of the somites (about one-third) was removed, using feather scalpels. In embryos stained for *ascl1* the gut was also removed. Pictures were taken with a Zeiss Axiophot 2 with a SPOT digital camera. Of each specifically stained cell cluster in the CSG, one representative focal plane was imaged at 20× magnification. The image was processed and analysed using Metamorph 6.0 Software (Visitron). For planimetric analysis of the pictures, the area to be measured was manually thresholded. The size of CSG was determined as stained areas in μm^2, combining area measurements of all cell groups that belong to the ganglion. This morphometric method has been used before to study sympathetic ganglion development on sections of chick embryos (e.g. Schneider et al., 1999; Howard et al., 2000; Tsarovina et al., 2004). The variation between repeated quantitation of the same ganglion is below 10%. Student's *t*-tests were performed with at least six (6-29) embryos for 2, 3 or 4 dpf embryos and each in situ probe, respectively. As the area measurements from these whole-mount in situ hybridizations depend crucially on probe- and stage-specific parameters affecting penetration, background and staining intensity, they represent relative rather than absolute values. Thus, the data have been normalized for each age and gene analysed, comparing the data of *hands off* mutants to data obtained with the same probe in control wild-type or heterozygous animals.

Genotyping of *hands off* zebrafish

Embryos were genotyped by PCR after Proteinase K digest of the yolk, tail and parts of the removed CNS for 10 hours at 55°C. The primers used were *fgf14*-exon1-F (ACATGGCAGCGGCGATTGCC), *fgf14*-exon1-R (AGCCCAACGCCACAAGTCCC), which gave a band of 324 bp, and *hand2*-UTR-F (AATTTCCCACTACGGACATTGGA) and *hand2*-UTR-R (AGAGACAGAAATAGATAATGAACGT), which gave a band of 225 bp.

PCR conditions were as follows: 94°C for 5 minutes, 40 × 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 1 minute and 72°C for 5 minutes. We could not distinguish between wild-type and heterozygous fishes.

RNA rescue of the *hands off* effect on sympathetic ganglion development

Capped synthetic *hand2* mRNA was generated using a pCS2: *hand2* construct (Yelon et al., 2000) and the mMessage machine system (Ambion) using standard procedures. Embryos from crosses of identified *hand2* mutant heterozygotes were injected with 50-250 pg *hand2* mRNA at the one- to four-cell stage. Injected embryos were raised for 2-4 dpf, fixed and processed for in situ hybridization. All experimental embryos were subsequently genotyped.

RESULTS

Noradrenergic gene expression is strongly reduced in sympathetic ganglia of the zebrafish *hand2* mutant *hands off*

Mutations in the zebrafish *hands off* locus were discovered in a screen for genes affecting heart induction and patterning (Alexander et al., 1998). This locus was later shown to affect differentiation, patterning and morphogenesis of myocardium, jaw and pectoral fin (Yelon et al., 2000). Molecular analysis of two alleles indicated that the *hands off* locus encodes the bHLH transcription factor *hand2* (Yelon et al., 2000). To study the role of *hand2* in sympathetic neuron development, the *han*^{s6} mutation is used, in which the entire *hand2* locus is deleted (Yelon et al., 2000). Although mutant embryos display a severe heart phenotype, they are able to survive up to 4 dpf, the latest stage analysed in the present study. As sympathetic ganglion development in the rostral trunk is initiated just before 2 dpf, the *hands off* mutation provided the opportunity to investigate the role of *hand2* in sympathetic ganglion development. We focus mainly on the initial stages of ganglion development in 2 and 3 dpf embryos, involving the specification and differentiation of sympathetic neurons (Guo et al., 1999; Holzschuh et al., 2001; An et al., 2002; Stewart et al., 2004).

Whole-mount in situ hybridization for *dbh* demonstrates the early generation of cervical sympathetic ganglia (CSG) (Fig. 1A,B, arrowheads) in wild-type embryos (Guo et al., 1999; Holzschuh et al., 2001; An et al., 2002) and a massive reduction of *dbh*-expressing cells in *hands off* mutant embryos (Fig. 1C,D, arrowheads). By contrast, the *dbh*-expressing cells of the medulla oblongata/area postrema (Holzschuh et al., 2001; Holzschuh et al., 2003) are not affected (asterisks). To be able to analyse CSG development in wild-type and *hands off* mutant embryos in more detail, yolk sac and dorsal neural tube were dissected after in situ hybridization. Sympathetic ganglia were viewed and quantified morphometrically from dorsal, combining area measurements of all cell groups that belong to the ganglion (Figs 2-6; see Fig. S1 in the supplementary material).

In normal wild-type embryos, *hand2*, *th* and *dbh* are expressed in the CSG by 2 dpf (Fig. 2), as shown by in situ hybridization analysis. CSG are located ventrally to the notochord between somites 1 and 4 in somewhat variable arrangements. Whereas at 2 dpf several smaller cell aggregates are present (Fig. 2A-C; see Fig. S1 in the supplementary material for a higher magnification image), larger cell groups are commonly found in 3 dpf embryos (Fig. 2G-I). In addition, single cells or small cell groups were detected more caudally up to somite 6. The differentiation of sympathetic precursors at somites 3 to 4 seems to precede the onset of differentiation of more rostral cell groups, as individual embryos with preferential *th/dbh* expression in the caudal group were observed, whereas embryos with normal rostral expression always

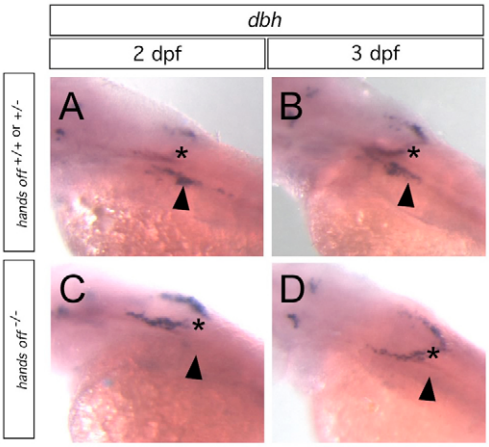

Fig. 1. The development of *dbh*-positive cervical sympathetic ganglia (CSG) is severely affected in *hands off* embryos. Whole-mount in situ hybridization for *dbh* in wild-type (**A**,**B**) and *hands off* mutant embryos (**C**,**D**) at 2 dpf (A,C) and 3 dpf (B,D). *dbh*-positive CSG cells (arrowheads) are missing in *hands off* embryos, whereas *dbh*-positive cells in the dorsally located medulla oblongata are not affected [asterisks; noradrenergic neurons of the medulla oblongata/area postrema form a semicircle (Holzschuh et al., 2003), giving the impression of two stripes in the oblique view from lateral shown].

displayed expression in the caudal group. *hand2*, by contrast to *th* and *dbh,* was also expressed in enteric neurons that are located ventrolaterally and posteriorly to sympathetic ganglia. These cells seem to further migrate between the second and third day of development, as their posterior and ventral location became more evident in 3 dpf embryos (Fig. 2A,G). Enteric neurons formed a continuum of cells throughout the length of the gut (see Fig. S2 in the supplementary material) that could be clearly distinguished from the more dorsally located cells of the CSG, which were restricted to somites 1-4.

In the *hands off* mutant, *hand2* expression was completely absent from sympathetic ganglia (Fig. 2D,J), as well as enteric ganglia, heart, branchial arches and fin primordia (Yelon et al., 2000) (data not shown). The mutant also demonstrated a very dramatic reduction in the expression of *dbh* at both 2 and 3 dpf (Fig. 2E,K). *th* expression was also reduced to very low levels at 2 dpf in *hands off* mutant embryos but was expressed in a slightly larger proportion of cervical sympathetic neurons at 3 dpf (Fig. 2F,L). It was apparent that, by contrast to the complete absence of *hand2* expression, *th* and *dbh* remained detectable in a small number of cervical sympathetic neurons. Quantification of the area of *dbh*-positive cells revealed a more than 90% reduction in the expression for both ages (Fig. 2M). *th*-expression was reduced at 2 and 3 dpf to <5% and 30% of controls, respectively (Fig. 2N).

To demonstrate that the effect observed in *hands off* embryos is caused by the lack of *hand2*, mutant embryos were injected with *hand2* mRNA. Injection of synthetic *hand2* mRNA enhanced the number of *th*-expressing sympathetic ganglion cells in a considerable fraction of injected *hands off* mutant embryos (see Fig. S3 in the supplementary material). Quantitative analysis revealed a highly significant >3-fold increase in *th*-positive cells in rescued *hand2*$^{-/-}$ mutants compared with uninjected mutant embryos (Fig. 2N). Similar rescue of *dbh*-expressing cells was also observed (data not shown). *hand2* injection in zebrafish embryos (n>100) did not result in ectopic noradrenergic neurons, by contrast to previous *Hand2* overexpression in the chick embryo (Howard et al., 2000).

To address the question of whether noradrenergic differentiation, and *th*-expression in particular, is delayed rather than prevented in the absence of *hand2*, sympathetic ganglia were also analysed in 4 dpf embryos. At 4 dpf, in addition to the CSG neurons, a group of strongly *th/dbh*-positive cells that probably represent chromaffin cells (An et al., 2002) are observed ventrolaterally to the caudal CSG (Fig. 3A,C arrowheads). These cells are devoid of *elavl3 (HuC)* (Fig. 6) (An et al., 2002). In the *hands off* mutant, *th* and *dbh* expression was strongly reduced both in CSG neurons and in the chromaffin cells (Fig. 3B,D). The total number of *th*- and *dbh*-expressing cells was reduced to 5-10% compared with wild-type embryos (Fig. 3E; *dbh* 9% of controls), demonstrating a continued reduction of *th* and *dbh* expression in the absence of *hand2*. A similar reduction was observed when *dbh* was analysed specifically in the rostral CSG (up to somite 3), where chromaffin cells are absent (*dbh* 23% of controls). In conclusion, these findings demonstrate an essential function for *Hand2* in noradrenergic differentiation during sympathoadrenergic development.

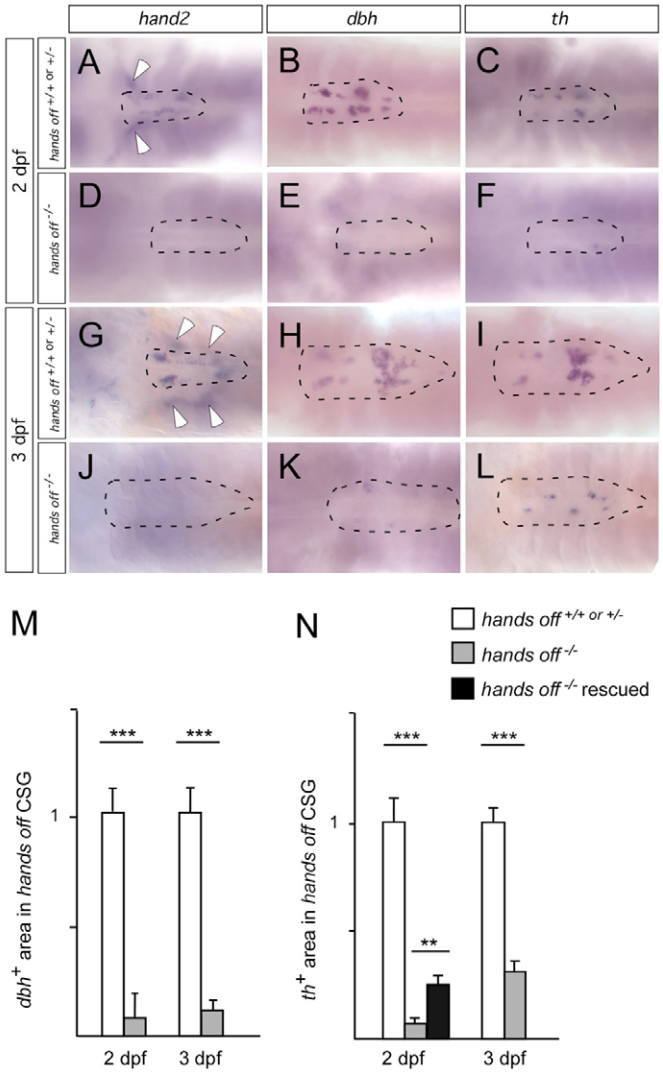

Fig. 2. *dbh* and *th* expression in cervical sympathetic ganglia of wild type and *hands off* mutants. Whole-mount in situ hybridizations for *hand2* (**A**,**D**,**G**,**J**), *dbh* (**B**,**E**,**H**,**K**) and *th* (**C**,**F**,**I**,**L**) viewed from dorsal after dissection of the yolk sac and spinal cord. *hand2* labels sympathetic ganglia (indicated by dashed circle) and more ventrally located enteric neurons (diffuse staining below the focal plane; white arrowheads) that migrate to a more lateral position at 3 dpf (G). *hands off* embryos at 2 dpf (D) and 3 dpf (J) are devoid of *hand2* expression and display only very few dbh^+ (E,K) and th^+ cells (F,L). (**M**) Area of *dbh*-expressing cells at 2 and 3 dpf in *hands off* mutants compared with wild type. (Number of embryos analysed: nine wild type, three mutant for 2 dpf; 11 wild type, seven mutant for 3 dpf.) (**N**) Area of *th*-expressing cells at 2 and 3 dpf in *hands off* mutants compared with wild type. Data are presented as mean ± s.e.m. (Number of embryos analysed: 28 wild type, ten mutant for 2 dpf; 41 wild type, 16 mutant for 3 dpf.) The injection of wild-type *hand2* mRNA resulted in a significant increase in the number of *th*-expressing cells in *hands off* mutants analysed at 2 dpf (n=10). (**P<0.01; ***P<0.001.)

The lack of *hand2* does not affect sympathetic ganglion formation and the expression of *ascl1* and *phox2b*

The observed reduction in the number of *th*- and *dbh*-expressing cells could either reflect a lack of sympathetic ganglion cells or an effect on the expression of these genes. The latter possibility implies that sympathetic neuron precursors would still be present but, in the absence of *hand2*, would be unable to further differentiate. To

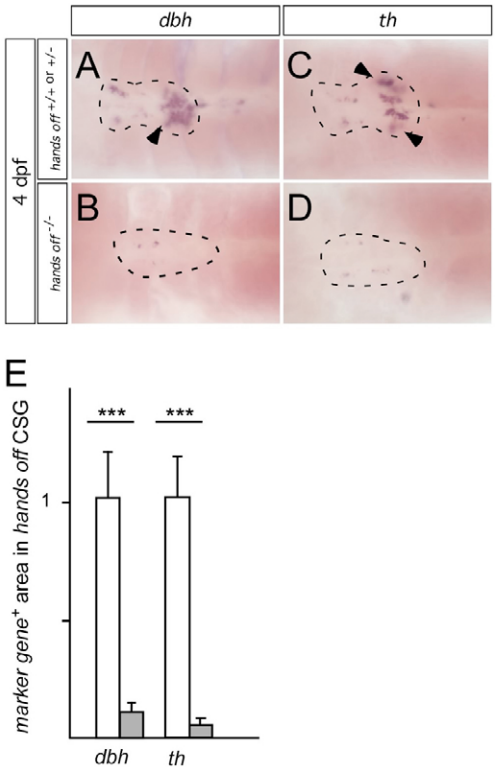

Fig. 3. *dbh* and *th* expression in 4 dpf cervical sympathetic ganglia of wild type and *hands off* mutants. Whole-mount in situ hybridizations for *dbh* (**A**,**B**) and *th* (**C**,**D**) as in Fig. 2. *dbh and th* label sympathetic ganglia. In addition, in the caudal region of the CSG a cluster of more ventrally located chromaffin cells with more intensive staining becomes apparent at 4 dpf (arrowheads). *hands off* embryos show only very few dbh^+ (C) and th^+ cells (D), demonstrating that noradrenergic differentiation is prevented in 4 dpf CSG neurons and chromaffin cells. (**E**) Area of total *dbh*- and *th*-expressing cells in *hands off* mutants compared with wild type. Data are presented as mean ± s.e.m. (Number of embryos analysed: 14 wild type, six mutant for *dbh*; 12 wild type, seven mutant for *th*.) (***P<0.001.)

address this issue, *phox2b* and *ascl1* expression in sympathetic precursor cells was analysed in control and *hands off* mutant embryos.

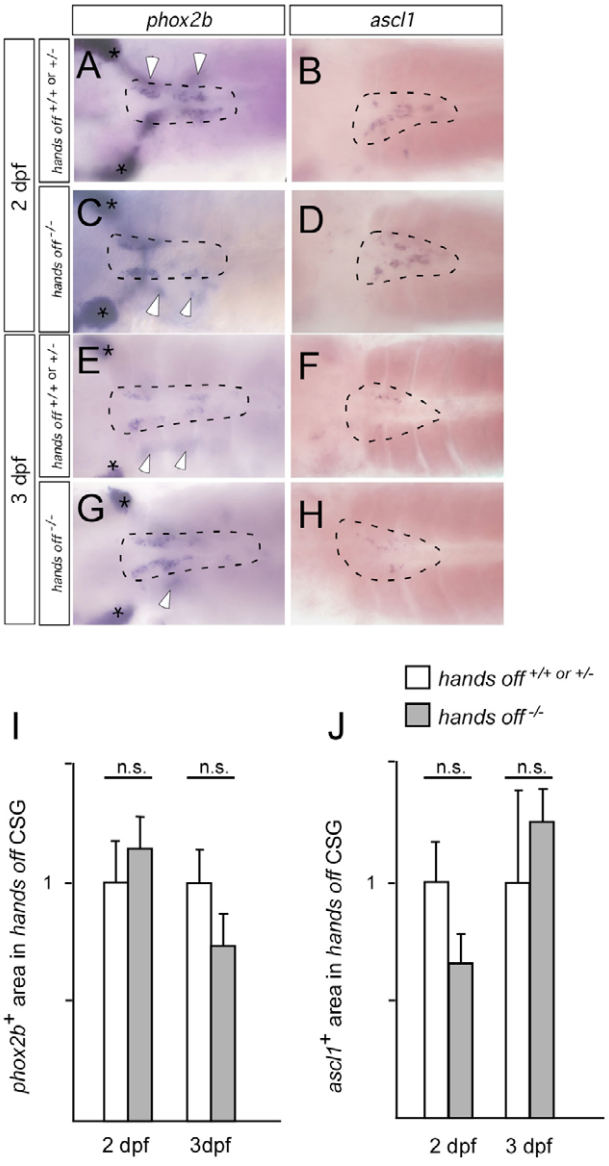

Fig. 4. *phox2b* and *ascl1* expression in cervical sympathetic ganglia of wild type and *hands off* mutants. *phox2b* and *ascl1* whole-mount in situ hybridizations viewed from dorsal. *phox2b* labels sympathetic ganglia (dashed circle) and more ventrally located enteric neurons (diffuse staining from below focus plane; white arrowheads). The gut was removed from *ascl1*-stained embryos. Control and *hands off* embryos show the same expression of *phox2b* at 2 dpf (**A**,**C**) and 3 dpf (**E**,**G**). Also *ascl1* expression is not affected in *hands off* embryos at 2 dpf (**B**,**D**) and 3 dpf (**F**,**H**). The area and intensity of *ascl1* expression is, however, strongly reduced at 3 dpf for both wild-type and hands off embryos. (**I**) Area of *phox2b*-expressing cells at 2 and 3 dpf in *hands off* mutants compared with wild type. (Number of embryos analysed: 15 wild type, five mutant for 2 dpf; 12 wild type, four mutant for 3 dpf.) (**J**) Area of *ascl1*-expressing cells at 2 and 3 dpf in *hands off* mutants compared with wild type. (Number of embryos analysed: ten wild type, seven mutant for 2 dpf; four wild type, four mutant for 3 dpf.) Data are presented as mean ± s.e.m.; n.s. not significant. *phox2b* staining of epibranchial placodes is indicated by asterisks.

phox2b expression in sympathetic ganglion primordia of wild-type embryos was equivalent to the expression of *hand2*, *th* and *dbh* at 2 and 3 dpf (Fig. 4A,E) but decreased to undetectable levels at 4 dpf (not shown). *Ascl1* was expressed only very transiently in sympathetic ganglia (Fig. 4B,F). The *ascl1* signal was already reduced to <10% in 3 dpf embryos, compared with 2 dpf ganglia. Enteric neurons were also strongly positive for *phox2b* (e.g. Fig. 3C; see Fig. S1 in the supplementary material) and *ascl1* (not shown). Due to the very strong *ascl1* expression in enteric neurons, the gut was removed in embryos stained for *ascl1* (Fig. 4B,D,F,H).

In *hands off* embryos, *phox2b*$^{+}$ and *ascl1*$^{+}$ sympathetic precursors were unaffected and were found in their normal rostral position in 2 and 3dpf embryos. Quantification of the areas of *phox2b*-positive and *ascl1*-positive CSG cells revealed no significant difference between *hand2*-deficient and control embryos. Thus, the absence of *hand2* has no immediate effects on the survival of *phox2b*$^{+}$/*ascl1*$^{+}$-sympathetic precursor cells and the level of *phox2b* and *ascl1* expression up to 3 dpf, the latest stage that could be analysed using these markers. Taken together, these results confirm the epistatic relationship between *phox2b*, *ascl1* and *hand2*, with *hand2* as a downstream member in this group of transcription factors.

Expression of *phox2a*, *gata2* and *tfap2a* in *hands off* embryos

The timing of *Hand2* expression in avian sympathetic primordia, together with the results of *Hand2* overexpression experiments suggested that *Hand2* is expressed upstream of *Phox2a* and may control *Phox2a* expression (Howard et al., 2000). We have now observed that *phox2a* expression was not affected in *hands off* mutant embryos up to 3 dpf (Fig. 5A,D,G,J,M). *phox2a* expression was, however, reduced to 34±6% of wild-type controls in 4 dpf embryos (mean±s.e.m.; P<0.007; n=37) (data not shown). These results demonstrate that *hand2* is not required for the initiation of *phox2a* expression. The decrease at 4 dpf may be explained by a loss of ganglion cells and/or a decreased *phox2a* expression. The presence of wild-type numbers of *elavl3*-expressing CSG neurons at 4 dpf (see below) supports the latter possibility.

The Zn-finger transcription factors Gata2 and Gata3 are essential for sympathetic neuron development in chick and mouse, respectively, and belong to the BMP-controlled network of transcription factors in sympathetic precursors (Lim et al., 2000; Tsarovina et al., 2004). *Gata2* expression in chick sympathetic ganglia is detectable after the onset of *Hand2* expression, suggesting a function downstream of *Hand2*. The continued expression of *Hand2* in sympathetic precursor cells of *Gata3*-knockout mice is in agreement with the notion that Gata2/3 are acting downstream of Hand2 (Tsarovina et al., 2004). This is now confirmed by the massive reduction of *gata2* expression in the *hands off* mutant (Fig. 5B,E,H,K,N). Whereas at 3 dpf the reduction of *gata2* expression was virtually complete (about 7% of control; Fig. 5K,N), some *gata2*-expressing cells were detectable in the rostral part of the CSG at earlier stages (Fig. 5E,N).

The Ap-2α transcription factor has been implicated in the control of noradrenergic differentiation, because it binds to the *Th* and *Dbh* promotor and stimulates transcription from *Th*- and *Dbh*-reporter constructs. In addition, the analysis of the zebrafish mutation *mont blanc/tfap2a* provided in vivo evidence for a function of *tfap2a* in noradrenergic differentiation (Holzschuh et al., 2003; O'Brien et al., 2004). However, it was unclear at which point *tfap2a* interacts with members of the BMP-induced genetic cascade. The strong reduction in *tfap2a* expression in *hands off* embryos (Fig. 5C,F,I,L,O)

demonstrates a direct or indirect regulation by *hand2*. Therefore, Ap-2α seems to act, together with Gata2/3, downstream of *hand2* in the control of noradrenergic development.

Effects of *hand2* on generic neuronal differentiation

With the exception of *tfap2a*, the transcription factors involved in the control of autonomic neuron specification display pleiotropic roles, i.e. the elimination of *Ascl1*, *Phox2b* and *Gata-2/3* resulted in the simultaneous impairment of noradrenergic and generic neuronal genes (Guillemot et al., 1993; Hirsch et al., 1998; Pattyn et al., 1999; Tsarovina et al., 2004; Holzschuh et al., 2003). Under these conditions, the resulting immature neuronal cells subsequently died by apoptosis (Pattyn et al., 1999; Tsarovina et al., 2004). To address this issue for *hand2*, the expression of the generic neuronal gene *elav3l (HuC)* was studied in the *hands off* mutant. Interestingly, *elav3l (HuC)* expression was not impaired in the *hands off* mutant up to 4 dpf, and even transiently increased at 3 dpf (Fig. 6A-G). Similar observations were made using whole-mount immunostaining for Hu protein expression (data not shown). The comparison of *elavl3* and *th/dbh* expression in 4 dpf embryos (compare Fig. 3B,D with Fig. 6F) demonstrates the lack of *elavl3* in the chromaffin cell cluster. More importantly, the massive decrease in *th* and *dbh*, by contrast to the maintained *elavl3* expression, supports the notion of a selective function of *hand2* in the development of the noradrenergic neurotransmitter phenotype.

Hand2 is not required for central noradrenergic neurons

The essential role of *Hand2* in the control of noradrenergic differentiation in sympathetic neurons raised the question of whether *Hand2* would also be required for the generation of central noradrenergic neurons. Although in previous expression studies *Hand2* expression was not detected in the locus coeruleus (Goridis and Rohrer, 2002), the possibility remained that *Hand2* levels below the detection level of the in situ hybridization technique might be sufficient to maintain noradrenergic differentiation. The present study, showing that *th* and *dbh* expression in the locus coeruleus is unchanged and develops to normal size in *hand2*-deficient zebrafish (Fig. 7), excludes this possibility. These results indicate that the molecular control of noradrenergic differentiation differs between the PNS and the CNS, although the initial set of regulatory factors is identical, i.e. BMPs as extrinsic signals and Ascl1/Phox2 as downstream transcription factors.

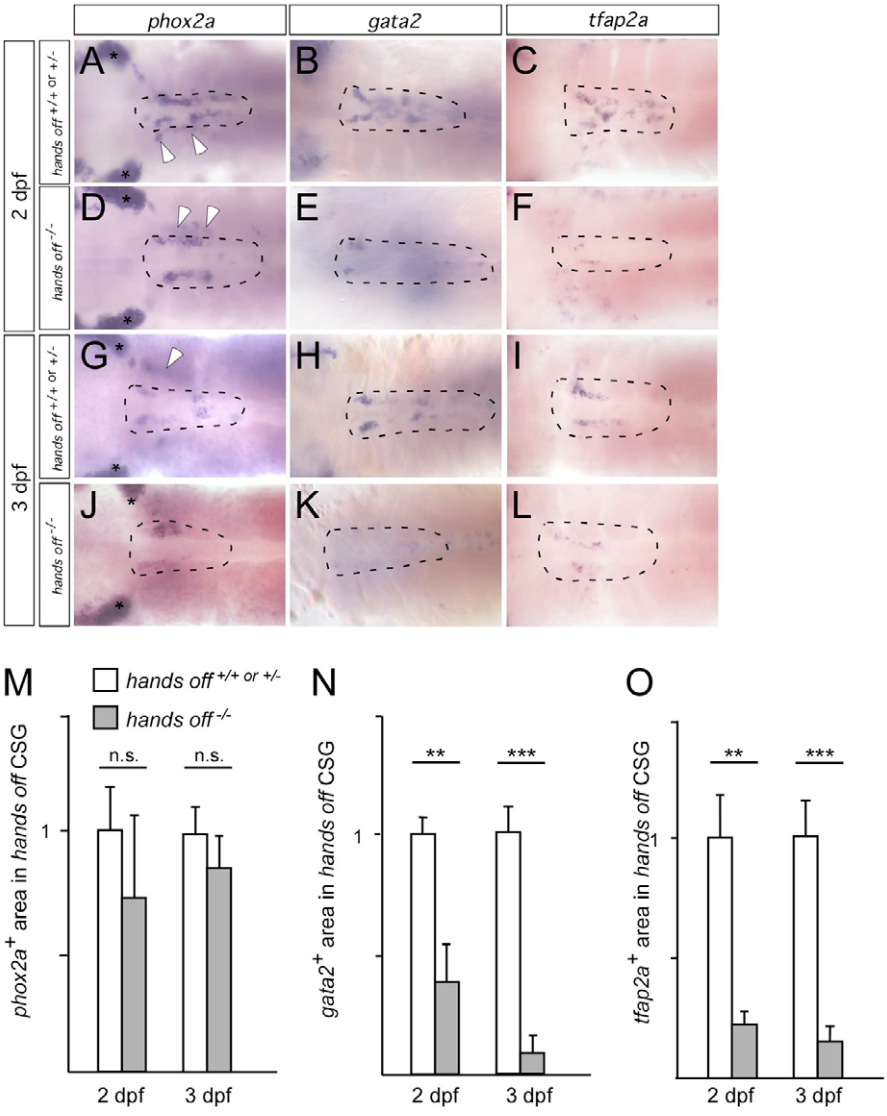

Fig. 5. *phox2a*, *gata2* and *tfap2a* expression in cervical sympathetic ganglia of wild type and *hands off* mutants. Whole-mount in situ hybridizations viewed from dorsal. (**A**,**D**,**G**,**J**) *phox2a*-labeled sympathetic ganglia (dashed circle) and enteric neurons (white arrowheads). Control and *hands off* embryos show the same expression of *phox2a* at 2 dpf (A,D) and 3 dpf (G,J). *gata2* expression is strongly reduced in *hands off* embryos (**E**,**K**) compared with wild-type embryos (**B**,**H**). *tfap2a* expression is massively reduced in *hands off* embryos (**F**,**L**) compared with wild type (**C**,**I**). (**M**) Area of *phox2a*-expressing cells at 2 and 3 dpf in *hands off* mutants compared with wild type. (Number of embryos analysed: seven wild type, three mutant for 2 dpf; ten wild type, five mutant for 3 dpf.) (**N**) Area of *gata2*-expressing cells at 2 and 3 dpf in *hands off* mutants compared with wild type. (Number of embryos analysed: six wild type, five mutant for 2 dpf; 12 wild type, nine mutant for 3 dpf.) (**O**) Area of *tfap2a*-expressing cells at 2 and 3 dpf in *hands off* mutants compared with wild type. (Number of embryos analysed: five wild type, 14 mutant for 2 dpf; 15 wild type, four mutant for 3 dpf.) Data are presented as mean ± s.e.m.; n.s. not significantly different (***P*<0.01, ****P*<0.001). *Phox2a* staining of epibranchial placodes is indicated by asterisks.

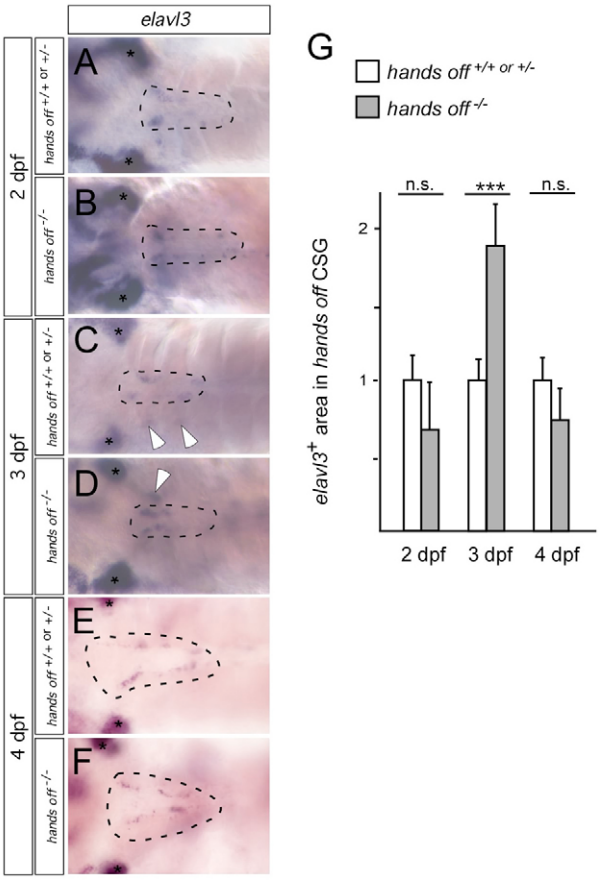

Fig. 6. *elavl3 (HuC)* expression in cervical sympathetic ganglia of wild type and *hands off* mutants. *elavl3* whole-mount in situ hybridizations viewed from dorsal. *elavl3*-labeled sympathetic ganglia (dashed circle) and enteric ganglia (white arrowhead) are indicated. Expression of *elavl3* in control and *hands off* embryos at 2 dpf (**A**,**B**), 3 dpf (**C**,**D**) and 4 dpf (**E**,**F**). (**G**) Area of *elavl3*-expressing cells at 2, 3 and 4 dpf in *hands off* mutants compared with wild type. (Number of embryos analysed: 13 wild type, six mutant for 2 dpf; 36 wild type, 13 mutant for 3 dpf; nine wild type, seven mutant for 4 dpf). Data are presented as mean ± s.e.m.; n.s. not significantly different; ***P<0.001; asterisks label *elavl3* (*HuC*) staining of epibranchial placodes.

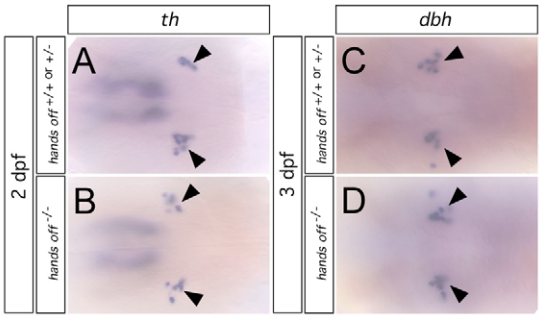

Fig. 7. *th* and *dbh* expression in the locus coeruleus of wild type and *hands off* mutants. *th* and *dbh* whole-mount in situ hybridizations viewed from dorsal. *th* (**A**,**B**) and *dbh* (**C**,**D**) expression in the locus coeruleus is not different between wild type (A,C) and *hands off* mutants (B,D). Two and 3 dpf embryos are shown for *th* and *dbh*, respectively. The locus coeruleus is indicated by arrowheads.

DISCUSSION

The bHLH transcription factor Hand2 belongs to the network of regulatory factors implicated in the specification and differentiation of sympathetic neurons. Using the zebrafish *hand2* mutant *hands off*, we now demonstrate an essential in vivo function of *hand2* in the expression of *dbh* and *th* in sympathetic neurons. *Gata2* and *tfap2a* expression is also strongly impaired in the *hands off* mutant. This suggests that Gata2 and Ap-2α may mediate, at least in part, the effects of Hand2 on noradrenergic differentiation.

Hand2 has no effect on the initial stages of sympathetic neuron development, including the expression of the proneural gene *ascl1* and the pan-autonomic regulatory genes *phox2b* and *phox2a*, nor on generic neuronal differentiation as assessed by the expression of *elavl3*. Therefore, *hand2* seems to be required selectively for initial noradrenergic but not pan-neuronal differentiation.

Hand2 has been considered an important member of the transcriptional network controlling sympathetic neuron development due to its ability to induce the generation of noradrenergic/catecholaminergic neurons in gain-of-function experiments (Howard et al., 1999; Howard et al., 2000). Overexpression is an important tool to identify candidate target genes, but in the sympathetic lineage different effects were observed upon transcription factor overexpression compared with in vivo loss-of-function approaches. *Gata2/3*, for instance, is essential for *Th* expression during normal development of sympathetic neurons but has virtually no effect on *Th* expression in overexpression experiments (Tsarovina et al., 2004). A further complication is the induction of upstream genes by the forced ectopic expression of transcription factors (Stanke et al., 1999; Stanke et al., 2004). Thus, it is essential to confirm the functions of candidate target genes identified from overexpression experiments using loss-of-function approaches.

The role of Hand transcription factors in the development of catecholaminergic cells has been addressed in quail neural crest cultures, using antisense oligonucleotides directed against *Hand2* and *Hand1* (Howard et al., 1999). Inhibitory effects were observed only with the combined interference of both *Hand1* and *Hand2*. As *Hand2,* but not *Hand1*, seems to be involved in sympathetic neuron generation in vivo (Howard et al., 2000), it was unclear to what extent the neural crest cultures reflect the in vivo situation. To address the physiological function of *Hand2* during in vivo development of sympathetic ganglia, *Hand2*-deficient mice were not informative due to the early death of mouse embryos between embryonic day 9.5 and 10.5, caused by impaired heart development (Yamagishi et al., 1999).

Zebrafish mutations in the *hands off* locus display similar malformations as observed for *Hand2*$^{-/-}$ mouse embryos (Yelon et al., 2000; Yamagishi et al., 1999). The dramatic reduction in ventricular precursors is even more severe in *hands off* zebrafish than that observed in *Hand2* knockout mice, possibly because of the lack of compensatory activity of a second *Hand* gene in this organism. However, by contrast to the mouse, the zebrafish embryos survive sufficiently long to permit the analysis of the development of cervical sympathetic ganglia, which differentiate several days before trunk sympathetic neurons (An et al., 2002). Cervical sympathetic neurons are detected in 2 dpf embryos by the expression of *phox2b*, *phox2a*, *ascl1*, *hand2*, *gata2*, *tfap2a*, *th*, *dbh* and *elavl3* (*HuC*) between somites 1 to 4. The location of cells varies from individual to individual but generally becomes organized in two major cell groups with increasing age.

The massive loss of *th* and *dbh* expression in cervical sympathetic ganglia of *hands off* zebrafish embryos demonstrates an essential role for *hand2* in sympathetic neuron development. *hand2* is essential for *th/dbh* expression, not only in sympathetic ganglia but also in cells with the characteristics of chromaffin cells that become apparent in 4 dpf embryos (An et al., 2002). As the number of *ascl1*-, *phox2b*- and *phox2a*-expressing sympathetic precursor cells is initially not affected, a function of Hand2 in the control of *th* and *dbh* expression is the most probable mechanism of action, and indirect effects on cell migration can be excluded. A direct action is supported by the interaction and synergistic function of Phox2a and Hand2 at the *Dbh* promotor (Xu et al., 2003; Rychlik et al., 2003). Although evidence for the function of Hand2 at the *Th* promotor is still lacking, it is tempting to speculate that Hand2 and Phox2a act together to induce both *Dbh* and *Th* in this lineage. However, the results on the transactivation of the *Th* promotor by Phox2a are controversial [cf. Zellmer et al. (Zellmer et al., 1995) and Yang et al. (Yang et al., 1998)] and there is also evidence for differential regulation of *Th* and *Dbh* expression in developing sympathetic ganglia (Tsarovina et al., 2004).

The loss of *th* and *dbh* expression in the *hands off* mutant was significantly reduced by the injection of wild-type *hand2* mRNA. This result is in line with the previously shown rescue of the *hands off* myocardial phenotype (Yelon et al., 2000) and confirms that the defects in the *hands off* (*han*^{s6}) mutant are due to a deficiency of *hand2*. Overexpression of *hand2* in wild-type embryos by injection of *hand2* mRNA did not notably affect the formation of sympathetic ganglia, nor cause the generation of ectopic noradrenergic neurons. By contrast, *Hand2* overexpression in the chick embryo using RCAS-retroviral vectors resulted in ectopic neurons in the peripheral nerve (Howard et al., 2000). In avian and rodent peripheral ganglia and nerves, significant numbers of pluripotent neural crest stem cells have been identified (e.g. Le Lièvre et al., 1980; Duff et al., 1991; Bixby et al., 2002) (Binder, Tsarovina and H.R., unpublished). The lack of ectopic cells in zebrafish may be due to differences in the timing or expression levels of *hand2* mRNA or due to lower numbers/absence of neural crest stem cells in the developing zebrafish PNS. Although it is known that some pre-migratory neural crest cells in zebrafish are multipotent (Raible and Eisen, 1994), it is not currently known whether zebrafish neural crest cells at any embryonic stage possess stem cell properties.

We have found that the expression of *gata2* and *tfap2a* requires *hand2* function and *gata2/3* and *tfap2a* have previously been shown to be essential for sympathetic neuron development (Lim et al., 2000; Tsarovina et al., 2004; Holzschuh et al., 2003). The mechanism of action for Gata2/3 is unclear, although a context-dependent function has been suggested based on the different effects of gain- and loss-of-function approaches (Tsarovina et al., 2004). By contrast, specific AP-2α-binding sites have been identified in the upstream regions of *Dbh* and *Th* genes in different species (Greco et al., 1995; Kim et al., 1998; Kim et al., 2001). Ap-2α transactivates *Th* and *Dbh* promotor activities in non-catecholaminergic cells (Kim et al., 2001). The genetic link between *hand2* and *tfap2a* revealed in the *hands off* mutant, together with the similar sympathetic neuron phenotype of *hands off* and *mont blanc* mutants (Holzschuh et al., 2003), suggests the possibility that Ap-2α functions downstream of *hand2* and may, at least in part, mediate *hand2* effects on *th* and *dbh* expression.

The analysis of the *hands off* mutant confirmed the proposed epigenetic relationship between *hand2* and *gata2* but not for *hand2* and *phox2a*. Our data suggest that the initial *phox2a* expression is not controlled by *hand2*. The previously suggested scheme with *hand2* upstream of *phox2a* was essentially based on the difference between the onset of expression of these genes, deduced from in situ hybridization analysis. The discrepant conclusions can be explained by a comparatively low sensitivity of the *phox2a* in situ hybridization probe, preventing the detection of *phox2a* expression at early developmental stages. The decreased *phox2a* expression in 4 dpf *hands off* embryos may reflect a *hand2* requirement for continued *phox2a* expression or a decrease in cell numbers. To address the question of whether sympathetic neuron number was reduced in 4 dpf mutant embryos, the expression of the generic neuronal marker *elav3l (HuC)* was studied.

The question of to what extent generic neuronal markers would be affected in the *hands off* mutant was also of interest, as Hand2 overexpression in chick neural crest cells results in the expression of both noradrenergic and generic neuronal genes (Howard et al., 2000). The analysis of the *hands off* mutants revealed that neuronal differentiation, i.e. *elavl3* expression, was not impaired up to 4 dpf. The reason for the transiently increased area of *elavl3*-expressing cells in 3 dpf *hands off* embryos is unclear but may be due to effects of *hand2* on the timing of neuronal differentiation or a transient inhibitory effect. By contrast to the maintenance or even increase of generic neuronal differentiation in *hands off* embryos, *th* and *dbh* expression is strongly reduced already at 2 dpf in the absence of *hand2*. This selective loss of noradrenergic properties is very similar to the phenotype of the *tfap2a* zebrafish mutation *mont blanc* in the CSG (Holzschuh et al., 2003). It supports the notion that *hand2*, acting through *tfap2a*, may selectively control the expression of subtype-specific noradrenergic genes in the sympathoadrenal lineage.

To what extent is the function of Hand2 maintained in other lineages of the nervous system? *hand2* is expressed in neurons of all parts of the peripheral autonomic nervous system, in cells of the sympathoadrenal lineage, i.e. sympathetic neurons and adrenal chromaffin cells, parasympathetic ganglia (Dai et al., 2004) (F.M. and H.R., unpublished) and enteric neurons (Wu and Howard, 2002; Dai et al., 2004). As there is a variable extent of noradrenergic differentiation in parasympathetic ganglia during development, *Hand2* expression and *Th/Dbh* expression may also correlate in parasympathetic ganglia, as observed for the chick sphenopalatine ganglion (Tsarovina et al., 2004) (F.M. and H.R., unpublished). Whether *Hand2* is involved in the expression of (transient) adrenergic properties of rodent enteric neurons (Howard and Cserjesi, 1996; Wu and Howard, 2002) is presently unclear. This question could not be addressed in the present study because we did not detect *th* and *dbh* expression in *phox2b*$^{+}$/*phox2a*$^{+}$/*hand2*$^{+}$ zebrafish enteric neurons. The *th/dbh* expressing group of cells in close vicinity of the CSG are considered to be chromaffin cells rather than enteric neurons (An et al., 2002; Holzschuh et al., 2001). It will be interesting to address the role of *hand2* in the enteric nervous system using the *hands off* mutant in future studies.

Central noradrenergic neurons of the locus coeruleus have been shown to depend on similar inducers to those in the PNS. The development of locus coeruleus neurons is dependent on the transcription factors *Ascl1*, *Phox2a* and *Phox2b* (Morin et al., 1997; Hirsch et al., 1998; Pattyn et al., 2000b). The normal development of the locus coeruleus in *hands off* mutants now demonstrates that downstream of *phox2b* noradrenergic differentiation is differentially regulated in PNS and CNS. This has also been shown for *Gata2/3*, which is required in the PNS but not in the locus coeruleus for *Th* and *Dbh* expression (Tsarovina et al., 2004).

Besides the nervous system, *Hand2* is involved in the development of heart, branchial arches and limbs (Srivastava, 1999; Firulli, 2003). Although only a limited number of target genes are

known so far, it is of interest that in several cases a maintenance function has been proposed. In *hands off* mutant embryos, the T-box transcription factor *tbx5* is initiated in the developing heart and fins, but its expression is not maintained (Yelon et al., 2000). Another putative *Hand* downstream target in the heart is *Irx4*, which is initiated in *Hand2*-deficient mice but not maintained during development (Bruneau et al., 2000). In sympathetic neurons, a maintenance function of *hand2* is now suggested for *gata2* and *phox2a*, as their expression is more strongly affected with increasing age of the embryos. Also the presence of a small number of *th/dbh*-positive cells in *hands off* mutants may indicate that noradrenergic differentiation is initiated in newly born neurons but not maintained in the absence of *hand2*. An alternative possibility would be that *hand2* is expressed and/or required for *th/dbh* expression in most but not all CSG cells. However, a definitive decision between an induction or/and maintenance function would require a conditional knockout of *hand2* in differentiated sympathetic neurons.

A further common finding is that the function of Hand2 does not require DNA binding, i.e. Hand2 participates in a transcriptional complex, where the interaction with the promotor is mediated by the binding partner(s) of Hand2 rather than by Hand2 itself (McFadden et al., 2002; Xu et al., 2003; Rychlik et al., 2003). It is not clear whether the co-activation function of Hand2 is linked to the maintenance role, but it is tempting to speculate along this line. An additional characteristic observed in several lineages is the interaction of Hand2 with members of the Gata family of Zn-finger transcription factors. In the heart there is evidence suggesting a common action of Hand2 and Gata4 in the transcriptional control of a number of cardiac specific marker genes (Dai et al., 2002).

In conclusion, we demonstrate that *hand2* is essential for the expression of noradrenergic marker genes *th* and *dbh* in zebrafish cervical sympathetic ganglia. The dramatic loss of *dbh* and *th* expression at early developmental stages is compatible with the notion, derived from previous work in the chick (Howard et al., 1999; Howard et al., 2000; Müller and Rohrer, 2002), that *hand2* is involved in the induction and/or maintenance of noradrenergic characteristics. The effect of the *hand2* loss of function on subtype-specific rather than generic neuronal genes correlates with the phenotype of the *tfap2a* mutant (Holzschuh et al., 2003) and implicates Hand2 and Tfap2a (Ap-2α) as selective regulators of the noradrenergic sympathetic neuronal phenotype.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/20/4015/DC1

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