

The basic helix–loop–helix *olig3* establishes the neural plate boundary of the trunk and is necessary for development of the dorsal spinal cord

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olig genes encode a previously unrecognized group of vertebrate-specific basic helix–loop–helix transcription factors. As shown in mice, chickens, and zebrafish, two members of this group, *olig1* and *olig2*, are involved in the differentiation of motoneurons and oligodendrocytes, but nothing is known about the role of the third member, *olig3*. Here, we show that *olig3* plays an essential role in the establishment of the neural crest–lateral neural plate boundary. In zebrafish embryos, morpholino-induced *olig3* inactivation dramatically increases the number of neural crest cells, but lateral neural plate fates (interneurons and astrocytes) are missing. Zebrafish *swirl* mutants that have impaired bone morphogenetic protein signaling and lack neural crest cells display an expanded *olig3* expression domain. Moreover, *olig3* is up-regulated in *mind-bomb* mutants lacking the neural crest because of an impaired *notch* signaling, and *olig3* repression in such mutants rescues the neural crest. In addition, *olig3* regulates *ngn1* and *deltaA* expression in interneuron precursors. Our results indicate that *olig3* has an essential proneural activity in the dorsal spinal cord and cooperates with the Delta/Notch regulatory loop to establish the boundary between the neural crest and the lateral neural plate. Thus, a proper regulation of the *olig* gene family is essential for the formation of three cell types (oligodendrocytes, astrocytes, and neural crest) that are unique to vertebrates.

interneurons | neural crest | *olig3* | zebrafish | glia

The spinal cord of vertebrates is originated by remodeling of the neural plate. Its general plan comprises motoneurons located ventrally and interneurons in a more dorsal position. In zebrafish, Rohon–Beard (RB) cells (primary sensory neurons) are generated at the outer border of the neural plate (within the neural crest domain) and migrate in the dorsal spinal cord, whereas cells of the dorsal root ganglia (also originated in the neural crest) migrate ventrally without entering the spinal cord. Two main signaling pathways are thought to establish the dorsoventral patterning of the embryonic neural tissue: bone morphogenetic protein (BMP) and Hedgehog (Hh). Hh signaling regionalizes the ventral neural tube and restricts the expression of some genes to dorsal regions (1). In parallel, BMP signaling determines a gradient of positional information throughout the entire neural plate that defines the establishment of dorsal and intermediate neuronal cell types of the spinal cord (2). The current view is that BMP and Hh morphogenetic activities generate zones of competence within which other factors will subsequently establish different cell fates. In this regard, components of the Delta/Notch signaling pathway are involved in the generation of different neuronal and glial subtypes with a mechanism called lateral specification. Components of the Delta/Notch genetic loops are often members of the basic helix–loop–helix (bHLH) family of transcription factors. The Neurogenin (Ngn) subfamily of bHLH is critical to establish the neurogenic program and maintain the Delta/Notch regulatory feedback that creates differences within the nervous system (3–5). Recently, a previously unrecognized vertebrate-specific group of bHLH has been shown to participate in neural fate

decisions mediated by Delta/Notch signaling: the known members of this family are *olig1*, *olig2*, and *olig3* (6–9). The discovery that, in mice, chickens, and zebrafish, *olig2* is necessary for the differentiation of motoneurons and oligodendrocytes from a ventral population of multipotent neural precursors raised the crucial issue of whether other neuroepithelial domains in the ventral or dorsal spinal cord can switch from neuron to glial cell production: for example, from neurons to astrocytes (6–8). In mice, the expression domains of *olig1* and *olig2* are partially overlapped and distinct from that of *olig3* (9). Because the function of *olig3* has not been determined yet, we have isolated and analyzed the zebrafish *olig3* gene. Our results show that (i) *olig3* is expressed in lateral domains of the neural plate and (ii) its proneural activity is necessary for the development of neural populations derived from the dorsal neuroepithelium, such as interneurons and astrocytes. Moreover, *olig3* activity is required to establish the boundary between the neural plate and the neural crest, indicating that the three *oligs* collectively participate in the formation of astrocytes, oligodendrocytes, and the neural crest.

Materials and Methods

Cloning of Zebrafish *olig3* Gene. The *olig3* sequence was initially retrieved from zebrafish genomic DNA by using combinations of degenerate primer pairs targeting the bHLH domain: bHLH-F1, AAGAAGGCNAAYGAYCGNGA; bHLH-R1, CADATGTARTTRTGNGCGAA; bHLH-F2, CGNGARCGNAAYCGNATGCA; and bHLH-R2, GTNAGNGCCCADATGTARTT. (*n* = A, G, C, and T; Y = C and T; R = A and G; and D = A, G, and T.)

The full-length coding sequence was determined and amplified from a zebrafish shield-stage cDNA library (GeneFinder library 567, RZPD) by using the following specific primers: for *olig3*HLH-F, GCACGACCTCAACCAGACTA, and for *olig3*HLH-R, ATGTAGTTTCTGGCGAGCAG, combined with vector oligos. For the antisense riboprobe, the *olig3* partial CDS/3' UTR was cloned into pCRII-TOPO cut with *Kpn*I and transcribed with T7 RNA polymerase.

Embryo Manipulations. The following morpholinos (MOs) (Gene Tools, Philomath, OR) were used: *olig3*^{AUG}MO, TCTGAATC-

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Abbreviations: bHLH, basic helix–loop–helix; BMP, bone morphogenetic protein; RB, Rohon–Beard; Ngn, Neurogenin; MO, morpholino; *olig3*MO, *olig3*^{AUG}MO; DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-5-phenylglycine t-butyl ester; hpf, hours postfertilization; *mib*, *mindbomb*; *swr*, *swirl*/*bmp2b*; *din*, *dino*/*chordin*.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AJ488292 (*olig2*) and AJ488293 (*olig3*)].

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CATCTTTGAAAGTCTTC (spanning the *olig3* AUG region); *olig3^{UTR}*MO, GAAACAATCCGCTTGAAGTGAGGCG (targeting the *olig3* 5' UTR); *olig3^{mism}*MO, GAAAGaATaCGaTTGAAGTcAGcCG (mismatched MO; mutations are lowercase); and *olig2*MO, TCCATGGCGTTCAGTGCCTCTCAG (spanning the *olig2* AUG region).

*olig3^{AUG}*MO and *olig3^{UTR}*MO acted synergistically, and, when used independently at similar concentrations, they elicited the same phenotype. Unless indicated, the experiments were performed comparing the activity of *olig3^{UTR}*MO (*olig3*MO) with that of *olig3^{mism}*MO. The phenotype of embryos injected with the *olig3^{mism}*MO was normal in shape and pigmentation.

Capped *olig3*, *Xnotch-ICD* (3), β -catenin, *dnTCF* (10), and *GFP* messengers were synthesized by linearization of the expression vectors and transcription with SP6 RNA polymerase (mMessage mMachine *in vitro* transcription kit, Ambion, Austin, TX).

Notch signaling was inactivated *in vivo* by *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester (DAPT) treatment (11).

Embryo Staining and Whole-Mount *In Situ* Hybridization. Whole-mount *in situ* hybridization was performed according to Thisse *et al.* (12). Whenever possible, MO- or mRNA-injected embryos were stained in the same tube with control embryos (which had the tip of the tail cut). RB cells have been revealed at the two-somite stage in *ngn1*:GFP-transgenic embryos (13) by using a GFP antisense probe.

Results

***olig3* Is Expressed in the Dorsal Neural Plate.** By PCR-based screening, we retrieved *olig3* cDNA from libraries of zebrafish at different developmental stages, including a shield-stage cDNA pool. By *in situ* hybridization, *olig3* transcripts are first detected at the shield stage in a stripe of the deep cell layer (not shown). This early and wide expression is peculiar to *olig3* and is not observed for the other members of the *olig* gene family (8, 9). At the tail bud stage (Fig. 6*a*), *olig3*-positive cells are arranged in two bilateral domains located in the prospective lateral neural plate and along the midline. Double-staining experiments performed at the one-somite stage with *sox10* and *ngn1* show that *olig3* is expressed medially to neural crest markers (Fig. 1*a*) and that the lateral (l) *olig3* expression domain comprises the region where *ngn1*-positive interneurons differentiate (Fig. 1*b*). At the same stage, the anterior border of the *olig3* domain is delimited by *krox20* expression in rhombomere-5 (r5) (Fig. 1*c*). At the two-somite stage, *olig3* is expressed at the medial side of *dlx3*-expressing cells, directly adjacent to the prospective RB cells (Fig. 1*d* and *e*). It is noteworthy that, at the two-somite stage, the earliest and anterior RB-cell precursors have already migrated in the neural plate, whereas the more posterior RB-cell precursors (originated later) are still in the neural crest domain (Fig. 1*e*). Because in *ngn1*:GFP transgenics the number of RB/GFP-expressing cells is normal and not increased as expected by a shift of the *ngn1* expression domain (13), in this specific case, it is unlikely that segregation of fates is achieved by changes in gene expression domain (without movement of cells).

olig3 Is Necessary for the Differentiation of the Dorsal Spinal Cord.

The early and strong expression and the lack of information about *olig3* function prompted us to address its role in development by means of loss and gain of function studies. The targeted inactivation of *olig3* was achieved by using antisense MO oligonucleotides (14). The MO-injected embryos (morphants) display a general retardation of development, a curved tail, a scarce motility, and, strikingly, an increased pigmentation at 36 hours postfertilization (hpf) (not shown). Injection of a mismatched MO (Figs. 2 and 3) as well as rescue experiments in which the

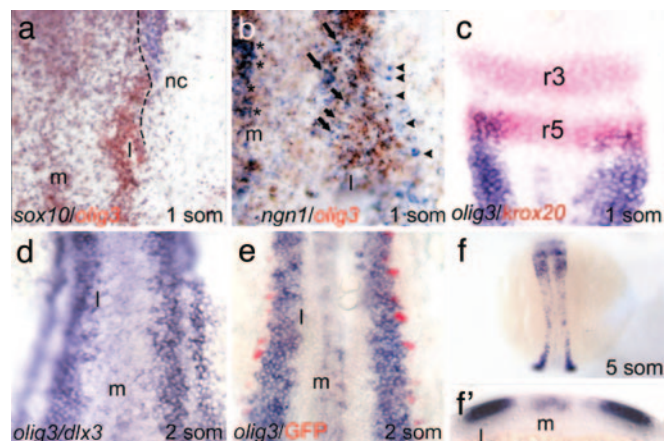


Fig. 1. *olig3* expression pattern. (a) High magnification of the right side of the rhombencephalon region showing that *olig3* (brown) is expressed medially to the neural crest marker *sox10* (blue); the dashed line represents the boundary of expression of the two markers. (b) High magnification of the right side of the trunk region shows that the lateral (l) *olig3* (brown) expression domain comprises the region where *ngn1*-positive interneurons (arrows) differentiate. RB cells (arrowheads) are in a more lateral position and are outside the *olig3* expression domain. (c) The anterior border of the *olig3* domain (blue) is delimited by *krox20* (red) in rhombomere-5 (r5). (d) The *olig3* domain has elongated rostrocaudally, and a two-cell-wide gap separates *olig3* and *dlx3*. (e) *ngn1*:GFP transgenics showing that RB-cell precursors (red) and *olig3*-expressing cells are in close contact. (f) At the five-somite stage, the lateral domain has further elongated. (f') Transverse section at the level of the posterior trunk of the embryo in *f*. The embryos are in dorsal view, anterior to the top.

*olig3^{UTR}*MO was injected with *olig3* mRNA demonstrate the specificity of the MO used in these analyses (line 6 of Table 1). To investigate whether the increased pigmentation in *olig3* morphants reflects an increment of neural crest cells, we have analyzed the expression pattern of neural crest-expressed markers. It can be observed (Fig. 2 *e–l*) that *foxd3/fkd6*, *crestin*, and *sox10* (15–17) are expressed at considerably higher levels in *olig3* morphants compared with siblings injected with a mismatched *olig3*MO. Moreover, in the morphants, these markers are expressed ectopically and along the dorsal midline (Fig. 2 *e–l*). Conversely, misexpression of *olig3* messengers is associated with decreased or absent pigmentation (lines 4 and 5 of Table 1) and reduced expression of early neural crest markers (Fig. 2 *a–d*). These results show that *olig3* is able to block the specification of trunk neural crest precursors. We next analyzed the neural populations deriving from the lateral neural plate and neural crest: astrocytes, sensory neurons, and different classes of interneurons (D1–D3) (18, 19). Analysis of *olig3* morphants with *isl1* and *isl2* indicates that D2 interneurons are missing; *lim1* reveals that D3 interneurons are fairly normal or slightly decreased (Fig. 3 *a–h*). In addition, the RB cells, deriving from the neural crest, are increased in number in *olig3* morphants compared with control embryos (Figs. 3 *b, d*, and *f* and 4*r*). As shown in Fig. 3*j*, *olig3* inactivation affects the expression of the glial fibrillar acidic protein gene, which is normally expressed in astrocytes (Fig. 3*i* and ref. 20). In summary, *olig3* morphants lack neurons and glial cells derived from the neural plate domain between D3 interneurons and the neural crest. Because levels of cell death and proliferation are comparable in *olig3* morphants and controls, apoptosis and control of cell cycle cannot explain the lack of astrocytes or interneurons in morphants (not shown).

***olig3* Is a Proneural Gene.** Notch signaling, by repressing the expression of its ligands (Delta and Jagged), couples cell-autonomous and non-cell-autonomous regulatory loops of gene

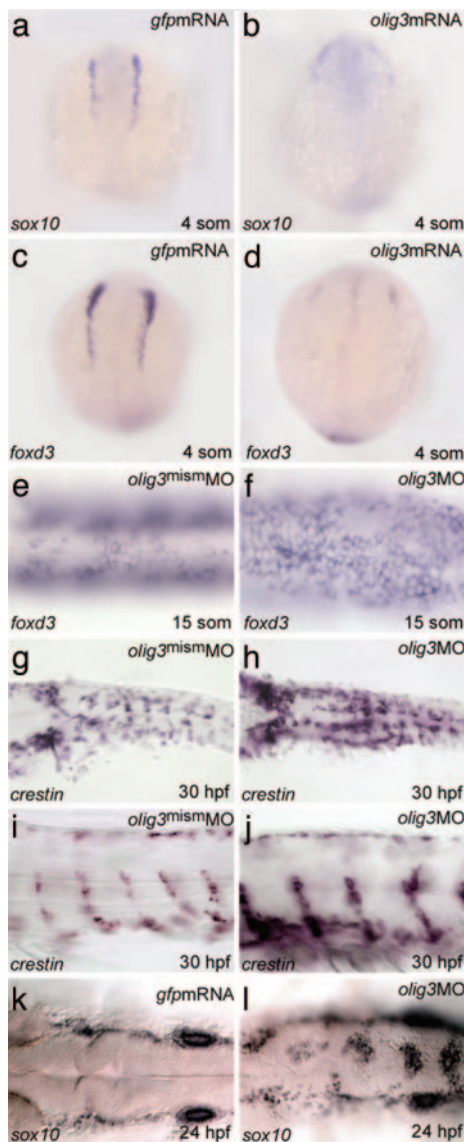


Fig. 2. *olig3* controls specification of the neural crest. (a–d) The levels and the expression domains of *sox10* and *foxd3* are decreased in four-somite embryos injected with *olig3* mRNA (b and d) (phenotype in >90% of injected embryos, *n* = 30). (e and f) The expression of *foxd3* is increased in 15-somite embryos treated with *olig3*MO (f) (morphant phenotype > 90%, *n* = 20). Similarly, the expression of *crestin* is increased in 30-hpf embryos treated with *olig3*MO (h and f) (morphant phenotype > 90%, *n* = 40). Similar results are obtained in 24-hpf embryos hybridized with *sox10* (k and l) (morphant phenotype > 90%, *n* = 30). Embryos are in dorsal (a–h, k, and l) or lateral (i and j) view, anterior to the top (a–d) or to the left (e–l).

expression that lead to boundary formation or cell-lineage divergence (21). Because Notch signaling is necessary for neural crest specification (4, 22), we analyzed *olig3* expression in *mindbomb* (*mib*) embryos that bear a mutation in a ubiquitin ligase gene necessary for efficient Delta activation of Notch (23). *mib* embryos are characterized by excess RB cells and interneurons at the expense of pigment cells and other trunk neural crest derivatives (4, 24, 25). In *mib* mutants, expression of *olig3* is increased (Fig. 4 a and b), suggesting that it is down-regulated by Notch signaling. Further evidence that Notch controls *olig3* expression comes from the misexpression of Notch-ICD, a dominant form of Notch intracellular that activates Notch signaling constitutively (3). Embryos injected with Notch-ICD

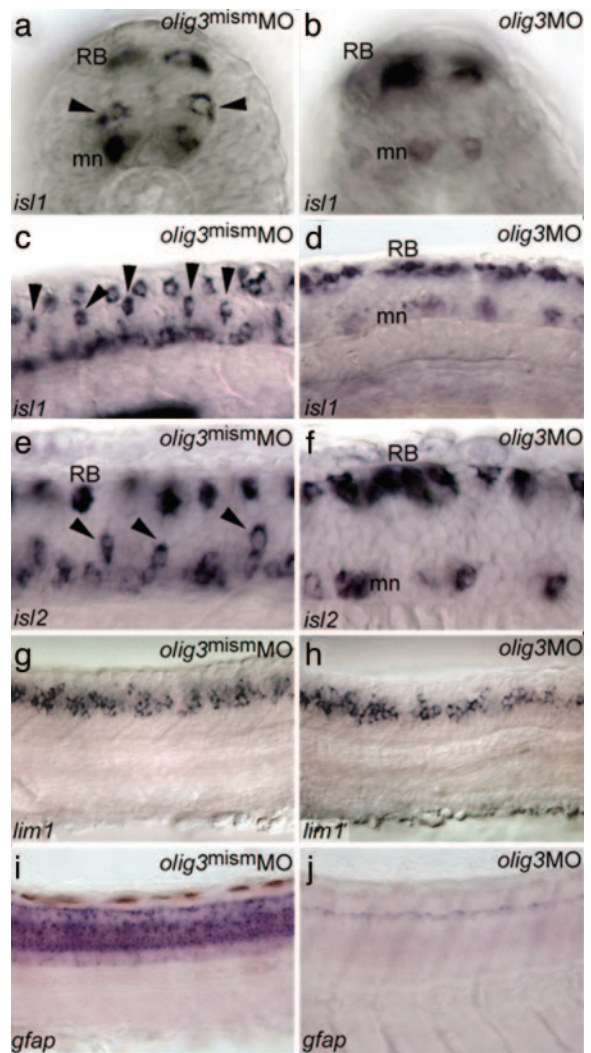


Fig. 3. *olig3* knockdown affects differentiation of lateral neural plate cells. (a–f) *islet1/2*-expressing interneurons (a and b; black arrowheads in a) (18, 19) are absent in 20-somite (b and d) and 24-hpf (f) embryos injected with *olig3*MO. Conversely, RB cells (RB) are increased in number in *olig3* morphants (b, d, and f) (morphant *islet1*-phenotype > 90%, *n* = 40; morphant *islet2*-phenotype > 90%, *n* = 30). mn, motoneurons. (g and h) *lim1* is expressed at 24 hpf in D3 interneurons of the dorsal spinal cord (g). D3 interneurons are slightly affected in embryos injected with *olig3*MO (h) (morphant phenotype > 90%, *n* = 15). (i and j) The glial fibrillar acidic protein gene (*gfap*) is expressed at 3 days postfertilization in astrocytes of the dorsal spinal cord (i). *gfap* expression is absent in embryos injected with *olig3*MO (j) (morphant phenotype > 90%, *n* = 20). Embryos are in transverse sections (a and b) or in lateral view with anterior to the left (c–j).

mRNA and analyzed at gastrula stage displayed reduced *olig3* expression compared with controls (Fig. 4 c and d). We reasoned that, if *olig3* is under negative control of Notch signaling and its activity represses neural crest fates, its inactivation in *mib* mutants should restore neural crest development. Consistent with this hypothesis, we found that *olig3* inactivation, induced with MO, restores pigmentation in *mib* mutant embryos (Fig. 4 e–g). Moreover, we tested the rescue of neural crest cells in embryos in which the intracellular release of Notch was blocked with DAPT, a γ -secretase inhibitor (11). Zebrafish embryos treated with DAPT display a phenotype that strictly resembles that of *mib* mutants (11), and their expression of *crestin* is dramatically reduced (Fig. 4i). However, when injected with *olig3*MO, DAPT-treated embryos show complete rescue of

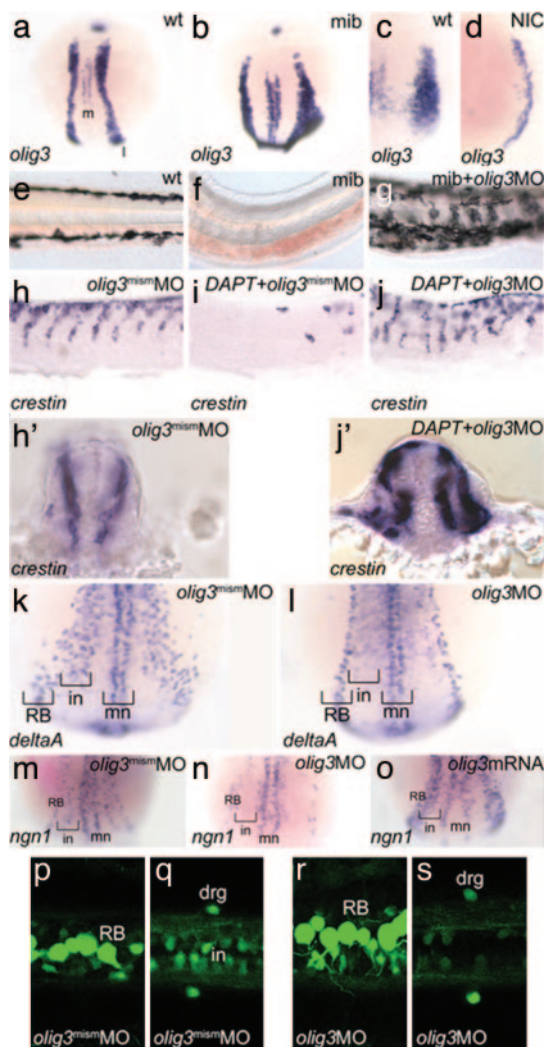


Fig. 4. *olig3* is controlled by Notch and regulates *ngn1* and *deltaA* expression in the lateral neural plate. (a–d) Notch signaling controls *olig3* expression. (a) *olig3* expression at the two-somite stage showing the medial (m) and lateral (l) domains. (b) *mib* embryo at the two-somite stage showing increased and broadened expression of *olig3*. (c and d) Tail bud-stage embryo injected with Notch-ICD (NIC) showing dramatic reduction of *olig3* expression (d) (shown phenotype > 90%, $n = 20$). (e–g) *olig3MO* rescues pigmentation in *mib* mutants. (e) Trunk pigmentation at 30 hpf. (f) *mib* embryo lacks melanocytes. (g) Rescue of the pigmentation in a *mib* mutant injected with *olig3MO* (morphant phenotype 9/10). (h–j, h', and j') *olig3MO* rescues the neural crest in DAPT-treated embryos. (h) *crestin* expression in a normal embryo at 30 hpf. (h') Transverse section of the embryo in h. (i) A DAPT-treated embryo lacks most of the neural crest cells. (j) Injection of *olig3MO* in an embryo treated with DAPT rescues neural crest cells (morphant phenotype 9/10). (j') Transverse section of the embryo in j. (k and l) *olig3* controls the expression of *deltaA* in interneurons. (k) *deltaA* expression pattern in a four-somite-stage control embryo injected with *olig3^{mism}MO*. (l) The stripe of *deltaA*-positive cells corresponding to interneurons (in) is missing in embryos injected with *olig3MO* (morphant phenotype > 90%, $n = 40$). (m–o) *In situ* hybridizations at two-somite stage showing that *olig3* activates *ngn1* expression in the lateral neural plate. (m) Expression of *ngn1* in motoneurons (mn), interneurons (in), and RB cells (RB) in control embryos injected with *olig3^{mism}MO*. (n) In an *olig3MO*-injected embryo, *ngn1* is expressed only in the medialmost (mn) and lateralmost (RB) neuronal populations (morphant phenotype > 90%, $n = 20$). (o) Injection of *olig3* mRNA up-regulates *ngn1* expression (shown phenotype > 90%, $n = 20$). (p–s) RB and dorsal root ganglion (drg) cells are present in *ngn1*:GFP-transgenic embryos (13) injected with *olig3MO* (r and s), as in controls (p and q). Interneurons (in) are reduced or absent (q and s). Embryos are shown in dorsal view with anterior to the top (a–d and k–o), lateral view with anterior to the left (e–j), and dorsal view with anterior to the left (p–s).

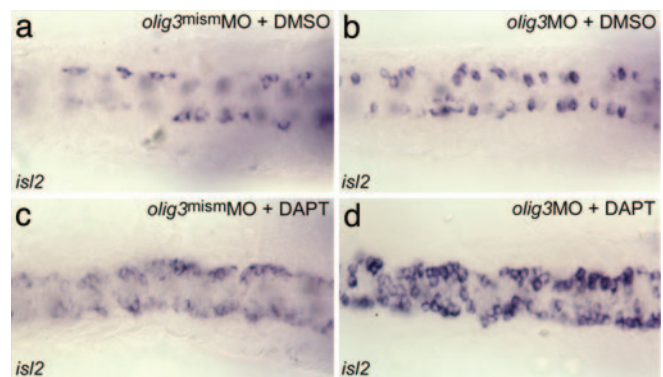


Fig. 5. *olig3* mediates mediolateral specification. *isl2* staining of RB cells is shown. RB cells originating in the neural crest domain are increased in number in *olig3MO*-injected embryos (morphant *isl2* phenotype > 90%, $n = 20$) (b) as well as in DAPT-treated embryos (morphant *isl2* phenotype > 90%, $n = 40$) (c) compared with controls (a). DAPT treatment and *olig3MO* act synergistically (d) (morphant *isl2* phenotype > 90%, $n = 20$). Embryos are shown in dorsal view with anterior to the left.

According to this model, BMP and Notch signals restrict *olig3* transcription. In addition, *swirl/bmp2b* is essential to establish neural crest and lateral neural plate zones of competence, as postulated by Nguyen *et al.* (37). This zone of competence is likely to be generated at the cleavage stage by a Wnt-mediated dorsal repression of Bmp (33).

In the prospective lateral neural plate, Chordin, secreted by the organizer, binds to BMP and releases the transcription of *olig3*, which (i) represses the expression of neural crest-specific genes, (ii) non-cell-autonomously activates Notch in prospective neural crest cells, and (iii) promotes some lateral neural plate

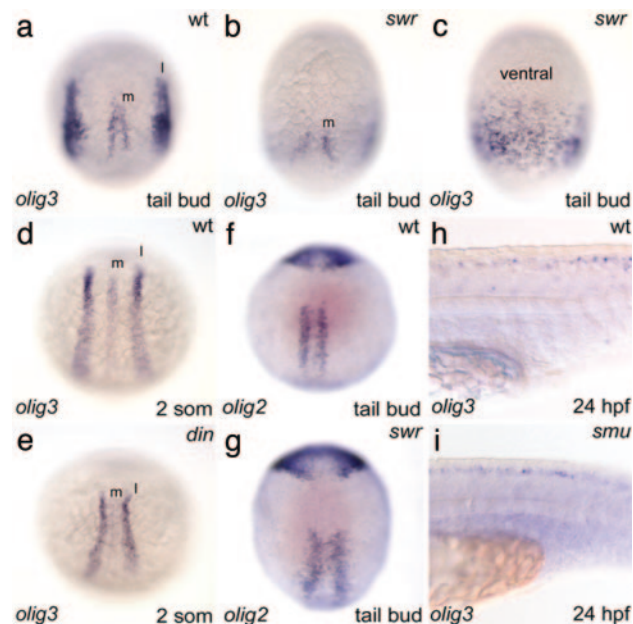


Fig. 6. BMP-dependent restriction of *olig3* expression domains. (a) Normal embryo. (b) *swr* embryo showing a widened space between the two medial domains (m). (c) Ventral view of a *swr* embryo showing that the lateral *olig3* domains are broadened and merge on the ventral side. (d) Normal embryo. (e) *din* embryo with reduced lateral (l) domain. (f and g) Normal (f) and *swr* (g) embryos show a similar *olig2* pattern. (h and i) Normal expression of *olig3* in the dorsal spinal cord of the *smu* embryo. Embryos in a–g are in dorsal view with anterior to the top, except in c (ventral view). Embryos in h and i are in lateral view, anterior to the left.

fates, such as that of astrocytes and *deltaA*- and *ngn1*-positive interneurons. The initial mediolateral unbalance of BMP signaling creates an asymmetry in *olig3* expression that, by means of the Delta/Notch genetic loop, establishes the boundary between the neural crest and the lateral neural plate. This model predicts that, in *mib* mutants, the lack of Notch signaling allows general *olig3* expression, which, in turn, represses neural crest-specific genes, as seen in misexpressed *olig3* embryos. Conversely, when *olig3* is knocked down, the expression of neural crest-specific genes is up-regulated in wild-type embryos or rescued in *mib* mutants. This effect is more dramatic, compared with *ngn1* knockdown (4), suggesting that *ngn1* might be involved in specific cell fate subdecisions downstream of the *olig3* activity. The significant increase of RB cells in *olig3*MO-injected embryos implies that not only the neural crest, but also the RB-cell specification is negatively regulated by *olig3*. The dramatic increase of RB and neural-crest cells, due to the MO-mediated block of *olig3* translation, is associated with reduction of dorsal neural cell types, such as interneurons and astrocytes. Notably, these effects are not accompanied by changes in cell death or proliferation, implying that dorsal neural populations lacking *olig3* activity are fated to neural crest and RB cells. Overall, our finding would suggest that *olig3* promotes the astrocyte/interneuron domain at the expense of the dorsal domain that corresponds to RB/crest cells. Thus, *olig3* appears to act like *olig2*, which promotes the motoneuron/oligodendrocyte domain

at the expense of the dorsal astrocyte/interneuron domain. Notably, the specific function of *olig2* and *olig3* in neuronal development reveals a spatial model of neuron/glia differentiation, with motoneurons/oligodendrocytes and interneurons/astrocytes originating in the ventral (*olig2*⁺) and dorsal (*olig3*⁺) neuroepithelium, respectively. In conclusion, Olig proteins form a vertebrate-specific group of bHLH transcription factors controlling the development of neural crest cells, astrocytes, and oligodendrocytes, three neuroectodermal-derived cell types occurring only in vertebrates. A better knowledge of the genetic pathways that control activation and repression of *olig* genes will help to dissect the mechanisms leading to either differentiation or malignant transformation of these cell lineages.

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