Supporting Information

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SI Text

MO Specificity Assays. To establish specificity of our MOs, we used an in vitro translation system. *X. laevis* embryos were coinjected with the gene-specific MO (10 ng) and either a WT mRNA encoding the corresponding gene, fused to N-terminal Myc tag (~100 pg), or a mutated mRNA (also Myc-tagged), bearing 5 mismatched base pairs within the MO target sequence. The embryos were also coinjected with WT mRNAs and control MOs, in a different set of control experiments. Embryos were injected at the 1- or 2-cell stage and allowed to develop to gastrula stages, when they were collected and lysed in protein extraction buffer (50 mM Tris, 100 mM NaCl, 5 mM EDTA, and 1% Nonidet P-40) in the presence of protease inhibitors (Complete Protease Inhibitors; Roche). Lysates were extracted with an equal volume of Freon (1,1,2-Trichloro 1,2,2 trifluoroethane; Spectrum Laboratories) to remove excess yolk and resolved on a 12% SDS/PAGE gel. Immunoblots of Myc-tagged proteins were performed by using anti-myc 9E10 antibody (Santa Cruz Biotechnology), visualized with HRP-conjugated secondary antibodies, and enhanced by chemiluminescence (GE Healthcare).

The results show that each gene-specific MO specifically blocked translation of its corresponding mRNA but not of a mutated message, carrying 5 mismatches in the MO target site (Fig. S3). Similarly, all MOs efficiently inhibited translation of their cognate mRNAs, whereas control MO, even at high concentrations, had no effect (data not shown). These results suggest a high degree of specificity. Furthermore, the same set of MO antisense oligos was used in our previous study, to analyze the knockdown effects of the NC-GRN members on the markers of the bona fide neural crest, *SoxE1* and *FoxD-A*, and their specificity of action confirmed in a separate set of rescue experiments (1).

RNA and MO Injections. FITC-labeled MO that inhibit translation of lamprey Pax3/7, ZicA, MsxA, n-Myc, Id, and AP-2 were obtained from Gene Tools and used as described (1). The following amounts of MO were used for injection: 10 ng per cell of Pax3/7 MO, 20 ng/cell of n-Myc, MsxA, and Id MO, and 40 ng/cell of ZicA and AP-2 MO. The injected embryos were allowed to develop for 96 h. Only embryos that exhibited MO incorporation in either the right or left half of the embryo (as assessed by FITC fluorescence) were selected. The embryos were fixed and processed for in situ hybridization. For rescue experiments, 1 blastomere of 2-cell lamprey embryos was injected with a mixture of 50–100 pg of lamprey full-length AP-2 or ZicA transcript (1) and 10–20 ng of AP-2 or ZicA Mo.

In Situ Hybridization. In situ hybridization on lamprey embryos was performed according to the protocol of Sauka-Spengler *et al.* (7) with several modifications. The time of bleaching of 4-day embryos was reduced to 7 min, and proteinase K treatment was reduced to 5 min. After in situ hybridization, MO localization was visualized by anti-FITC antibody staining. Embryos were washed in PBS, blocked in 20% goat serum + 2% BBR (Boehringer Blocking Reagent) for 1 h, incubated overnight in 1/500 dilution of AP-conjugated anti-FITC FAB fragments (Roche), washed extensively, rinsed with NTMT (100 mM NaCl, 100 mM Tris, pH 9.5, 50 mM MgCI2, 0.1% Tween-20) and stained with 5-bromo-4-chloro-3-indolyl phosphate.

RNA Extractions and RT-QPCR. For QPCR assays, both blastomeres of 2-cell lamprey embryo were injected with MO. Embryos were cultures as above, selected for uniform and complete MO integration, and collected at 96 hours after fertilization. For QPCR analysis of the timeline of gene expression, WT embryos from the same batch were collected at 6-h intervals from days 3 to 4.5. Total RNA was extracted with a RNAqueous kit according to the manufacturer's instructions. Contaminating DNA was removed with a TURBO DNA-free kit (Ambion). Total RNA was reverse-transcribed by using random hexamers (Invitrogen) and SuperScript III reverse transcriptase (Invitrogen).

Real-time PCRs were performed in a 96-well plate on an ABI7000 QPCR machine. Reactions were set up by using SybrGreen (BioRad), 450 μM of each (forward and reverse primer) and 7–50 pg cDNA per reaction. To select a suitable endogenous control, we compared the expression of a number of genes between MO-treated and untreated samples and chose RPS9 (ribosomal protein S9) because its expression levels appeared to be unaffected by MO treatment. The standard curve method was used for quantification, and fold change in expression was determined by dividing the amount of the gene of interest, normalized to RPS9 in MO-injected embryos, by the normalized amount in control embryos.

The following QPCR primers were used: Pm-RPS9–1-Q-F, GTGGCGTGTCAAGTTCACC; Pm-RPS9–1-Q-R, CATCTTGGACTCATCCAGCA; Pm-AP2-a-Q-F, CTGCATCAGATGGGTCATCA; Pm-AP2-a-Q-R, GAGCCGATGACGACTTTCTT; Pm-Id-a-Q-F, TGCAGCACGTGATCGACTA; Pm-Id-a-Q-R, CGTTGTTTCCGGTCAAACTT; Pm-Msx-a-Q-F, ATGCACGGAGCTGCCTACT; Pm-Msx-a-Q-R, TACTGCTTCTGCCGGAACTT; Pm-ZicA-Q-F, ACACAAGAGGACGCACACAG; Pm-ZicA-Q-R, TGCAGAAGTAGGGCTTGTCC; Pm-Pax3–7-a-Q-F, GGCACCAGGACAGAACTACC; Pm-Pax3–7-a-Q-R, CCCGTTGATGAACACTCCTC; Pm-myc-c-Q-F, ATTCCGATGATGACGACGAT; Pm-myc-c-Q-R, TGTGTTGTACACGGTGCGTA.

1. Sauka-Spengler T, Meulemans D, Jones M, Bronner-Fraser M (2007) Ancient evolutionary origin of the neural crest gene regulatory network. *Dev Cell* 13:405–420.

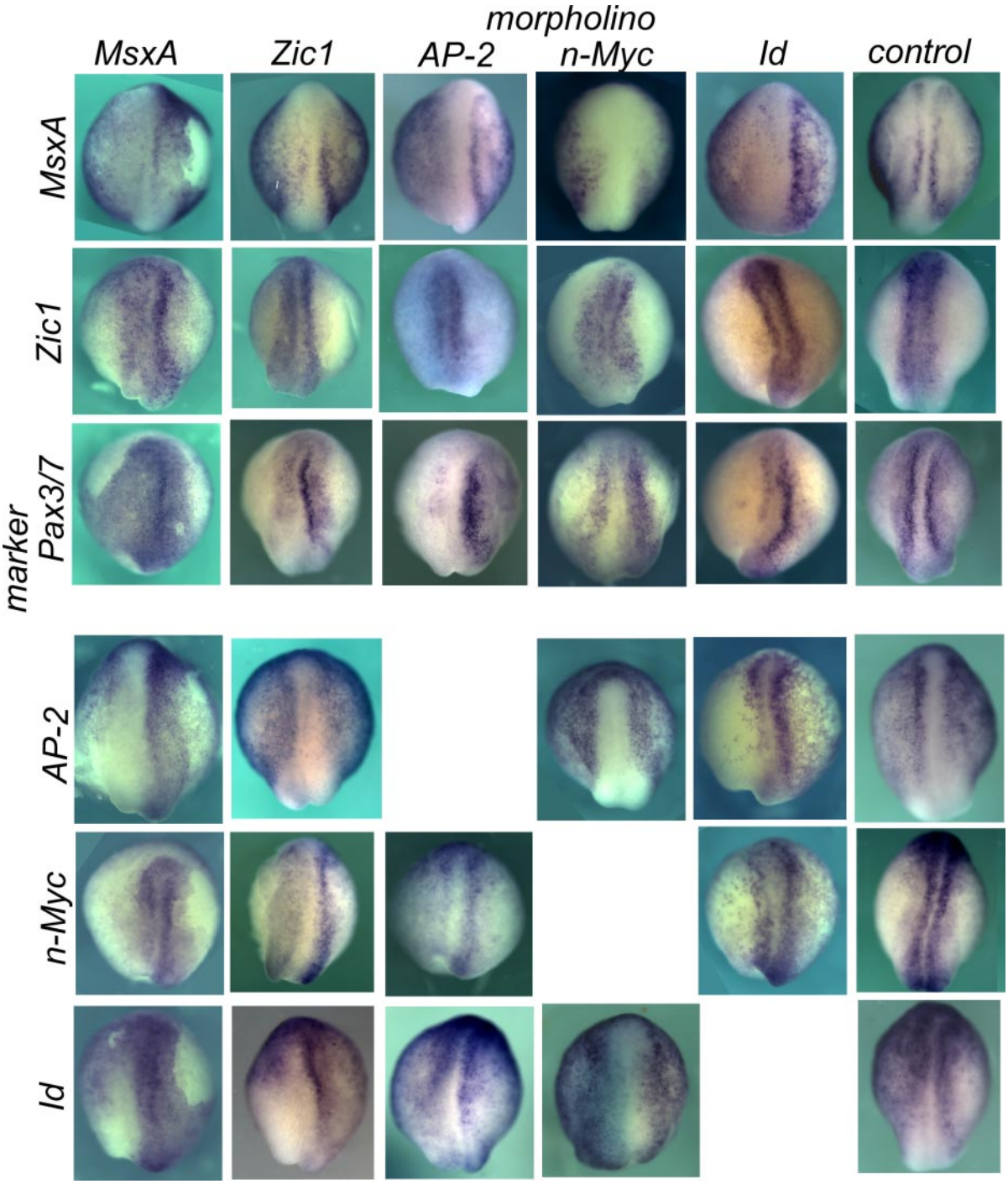

Fig. S1. The effects of MO-mediated knockdown of three neural border specifiers (*MsxA*, *ZicA*, and *Pax3/7*) and three early neural crest specifiers (*AP-2*, *n-Myc*, and *Id*), on each other's expression, visualized by in situ hybridization. Images of the same embryos as in Fig. 2, taken before anti-FITC antibody staining, provide better representation of the phenotypes. (Magnification: 18×.)

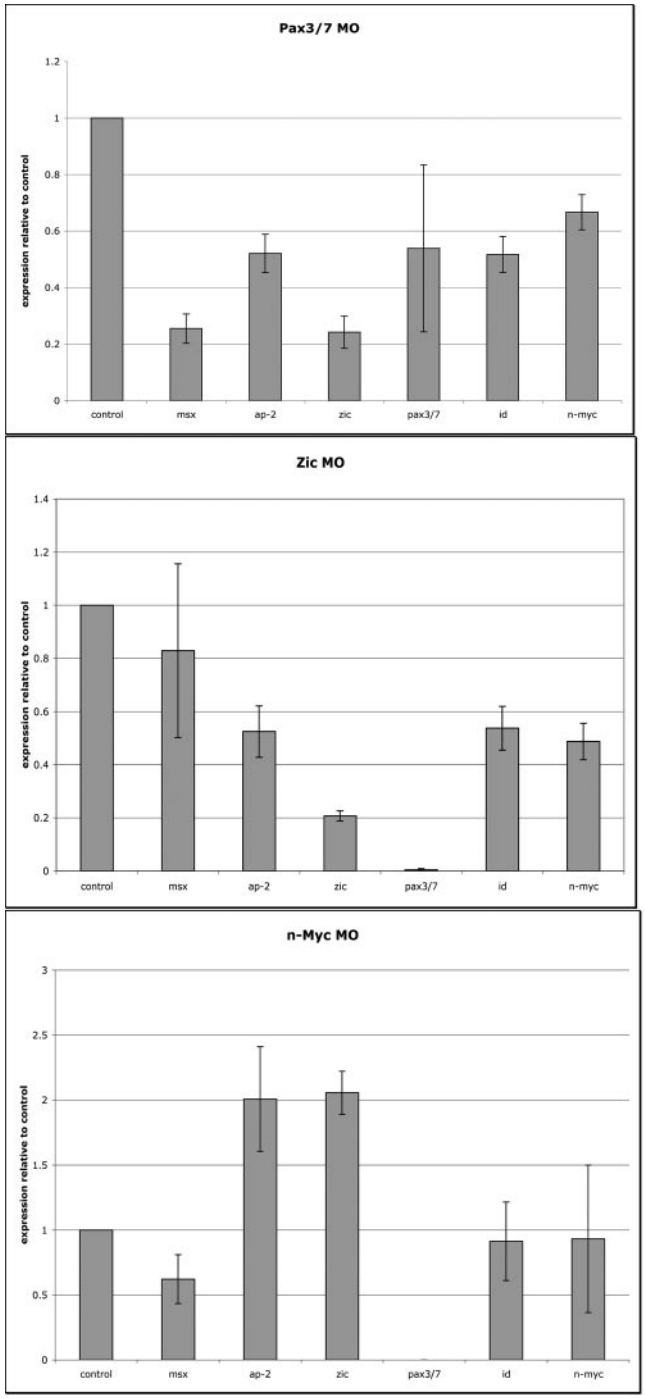

Fig. S2. The effects of MO-mediated knockdown of Pax3/7, ZicA, and n-Myc on gene expression levels of other transcription factors present at the neural plate border, quantified by RT-QPCR.

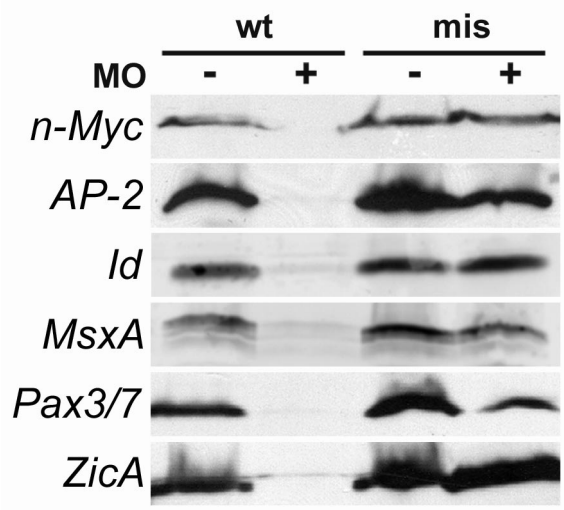

Fig. S3. In vitro assay to demonstrate MO specificity. The binding specificity of each MO was tested by preparing Western blots of lysates from *Xenopus* embryos coinjected with gene-specific MO and either Myc-tagged WT mRNAs (wt) or modified mRNAs containing 5-bp mismatch within the MO target site (mis). In the case of each neural plate border or early neural crest specifier, the MO specifically blocked translation of the corresponding WT mRNA but not that of a mismatched mRNA.

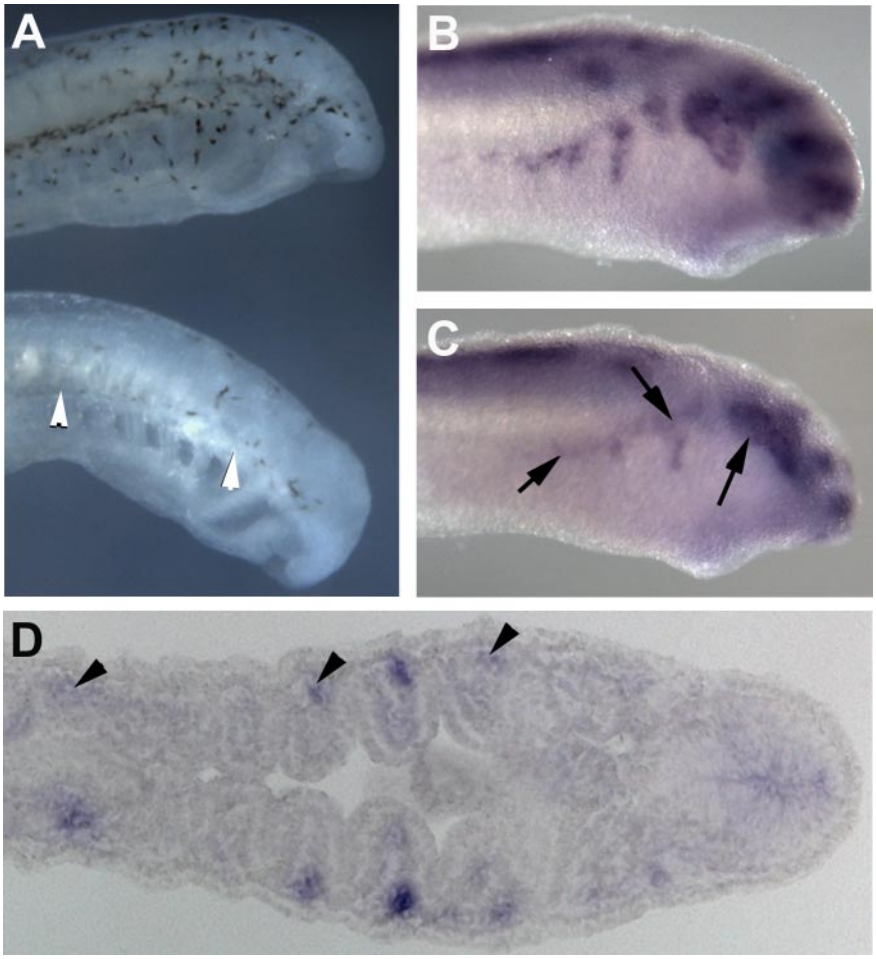

Fig. S4. AP-2 depletion causes loss of neural crest derivatives. (*A*, *C*, and *D*) AP-2 deficient embryos show loss of all neural crest derivatives: pigment cells (*A*; white arrowheads), cranial ganglia (*C*; black arrows), and neural crest-derived cartilage (*D*; black arrowheads). (*B* and *C*) Shown are the uninjected control side (*B*) and the MO-injected side of the same E11 lamprey embryo (*C*), with cranial ganglia visualized by in situ staining for *Neurogenin*. (*D*) Neural crest-derived prospective cartilage cells are labeled with *SoxE1* in situ probe.

Table S1. Summary of morpholino knockdown experiments: Effects on neural plate border marker expression

	Morpholino used				
Morpholino used	MsxA	ZicA	AP-2	n-Myc	Id
MsxA	↓	↓	↓	↓	↓
ZicA		↓ Neural plate expansion	—	Neural plate expansion	—
Pax3/7	↓	↓	↓	↓	↓
AP-2	↓	—	Not determined	—	↓
n-Myc	↓p	↓	↓p	Not determined	—
Id	↓p	↓	↓p	—	Not determined

↓, gene down-regulated in the neural plate border; ↓p,- gene down-regulated in the posterior neural plate border; — expression not affected.