requires external signals (Benouaiche *et al.*, 2008; Couly *et al.*, 2002; Le Douarin *et al.*, 2004; Le Douarin and Dupin, 2003; Vieux-Rochas *et al.*, 2007) and data suggesting that CNCCs are instead endowed with cell-autonomous information to generate craniofacial structures (Schneider and Helms, 2003). As expected, early signals (Edn1, FGF8, others) appear more conserved in different animal classes, while subsequent complex regulations might considerably vary from genome to genome and could contribute to jaw diversification in vertebrates.

METHODS

Mouse Mutants

Animal procedures were approved by National and Institutional ethical committees. Mouse strains were maintained on B6/D2 F1 hybrid genetic background. *Edn1* mutant mice were genotyped as indicated (Kurihara *et al.*, 1994). Mice with targeted disruption of *Dlx5* or *Dlx5;Dlx6* were genotyped as previously reported (Acampora *et al.*, 1999; Beverdam *et al.*, 2002; Merlo *et al.*, 2002b). The genotypes of embryos obtained from mixed *Dlx* heterozygous parents were determined using the *Dlx5-lacZ* or the *Dlx5;Dlx6*-mutant allele-specific forward primers L-proF and G-proF, respectively, and the *lacZ* reverse primer, with the following sequence:

- *L-proF (Dlx5* allele) 5′CGCAGTAGAAGAACAGC CAC
- *G-proF (Dlx5;Dlx6*-mutant allele) 5′GAGCTATGAC AGGAGTGTTTG
- *KO6 RT-R2 (lacZ* reverse) 5′GGCGATTAAGTTGG GTAACG

$Edn1^{+/-}$ animals were crossbred with $Dlx5^{+/-}$ and $Dlx5^{+/-}$;$Dlx6^{+/-}$ to generate double and triple heterozygotes, and from these $Edn1^{+/-}$;$Dlx5^{-/-}$ and $Edn1^{+/-}$; $Dlx5^{-/-}$;$Dlx6^{+/-}$ animals were obtained.

Skeletal Preparations and In Situ Hybridization

Skeletal staining of E14.5 embryos and newborn animals (Alcian Blue for E14.5 embryos, Alizarin Red/Alcian Blue for newborns) was carried out as previously described (Vieux-Rochas *et al.*, 2007). A minimum of 4, with a maximum of 10, embryos/newborns per genotype were analyzed for skeletal phenotypes, per each genotype.

In situ hybridization was done with DIG-labeled RNA probes corresponding to the antisense sequence of murine *Dlx3*, *Dlx5*, *Dlx6*, *Gsc* and *Hand2* (all previously reported: (Charite *et al.*, 2001; Perera *et al.*, 2004; Radoja *et al.*, 2007), using the procedure described by Wilkinson and Nieto (1993). For each probe, at least three normal and three mutant specimens were examined. For semi-quantitative comparisons, all the procedures were carried out in the same vials on littermate embryos; the time of chromogenic reaction was reduced to avoid signal saturation.

Tissue Collection, RNA Extraction, and RT-qPCR

E9 or E10.5 embryos were genotyped by PCR on DNA extracted from extra-embryonic tissues. The PA1s were dissected under stereomicroscope using fine scissors, further separated into a proximal and a distal part (see Fig. 5c). The anatomic hallmark was the bulge formed at the PA1 end. Sections were carried out vertically in a rostro-caudal way. Tissues were collected in RNA later (Ambion), pooled according to the genotype, transferred in Tripure Reagent (Roche) and processed for RNA extraction as indicated by the manufacturer. A minimum of three PA1s per genotype were pooled in one sample, two biological replicates were prepared. Each sample was analyzed in duplicates (technical replicates). RNA quality, primer efficiency and correct product size were verified by RT-PCR and agarose gel electrophoresis. qPCR was performed with LightCycler (Roche) using FastStart DNA MasterPLUS SYBR-Green I (Roche). Five microliter of cDNA were used in each reaction, standard curve were done using WT cDNA with four calibration points: TQ; 1:3; 1:9; 1:27. Specificity and absence of primer dimers was controlled by denaturation curves. *GAPDH* mRNA was used for normalization. Results of mutant tissues are expressed as fold-change relative to the corresponding WT. For each target, the mRNA abundance was calculated relative to GADPH, using the LightCycler Software 3.5.3, based on the general formula $\Delta(\Delta CT)$. Because of the limited sample size (two replicates) and the two steps of normalization, the Student *t*-test could to determine statistical significance could not be done.

- GAPDH Sens 5′TGTCAGCAATGCATCCTGCA
- GAPDH Antisens 5′TGTATGCAGGGATGATGTTC
- Hand2 Sens 5′CCAGCTACATCGCCTACGTC
- Hand2 Antisens 5′TTGCTGCTCACTGTGCTTTT
- Wnt5a Sens 5′AGGAGTTCGTGGACGCTAGA
- Wnt5a Antisens 5′ACTTCTCCTTGAGGGCATCG
- Bmp7 Sens 5′GCGATTTGACAACGAGACCT
- Bmp7 Antisens 5′AGGGTCTCCACAGAGAGCTG
- Dlx3 Sens 5′CGTTTCCAGAAAGCCCAGTA
- Dlx3 Antisens 5′CGTGGAATGGGAAGATGTGT
- Dlx5 Sens 5′CTGGCCGCTTTACAGAGAAG
- Dlx5 Antisens 5′CTGGTGACTGTGGCGAGTTA
- Dlx6-5F Sens 5′CTCAATACCTGGCCCTTCC
- Dlx6-5R Antisens 5′AGAGCGCTTATTCTGAAACCAT
- Meis2 Sens 5′ATCTCAAGGCAAGGGGAAGT
- Meis2 Antisens 5′GAGTAGGGTGTGGGGTCATC
- Pitx1 Sens 5′ATCGTCCGACGCTGATCT
- Pitx1 Antisens 5′CTTAGCTGGGTCCTCTGCAC
- Gsc Sens 5′ACCGATGAGCAGCTCGAA
- Gsc Antisens 5′GCGGTTCTTAAACCAGACCTC
- Edn1 Sens 5′TCCTTGATGGACAAGGAGTGT
- Edn1 Antisens 5′TCGTACCGTATGGACTGGG

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