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Mechanisms of Development 113 (2002) 73–77

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Gene expression pattern

Cloning and expression of *Ventrhoid*, a novel vertebrate homologue of the *Drosophila* EGF pathway gene *rhomboid*

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Received 5 October 2001; received in revised form 13 December 2001; accepted 13 December 2001

Abstract

In *Drosophila melanogaster*, the seven-pass transmembrane protein Rhomboid (Rho) is a crucial positive modulator of EGF signaling playing a substantial role in patterning of the ventral neuroectoderm and fate specification of neuroblasts. Here, we describe the cloning and expression pattern of *Ventrhoid* (*Vrho*), the novel evolutionarily conserved vertebrate cDNA related to fruit fly *rho*. Most importantly, like *rho* in *Drosophila*, *Vrho* is also expressed in a spatially restricted manner. *Vrho* expression is most prominent along the developing ventral neural tube, and is also detectable in the ventral forebrain, prospective diencephalon, otic vesicles, mandibular arches, cranial sensory placodes, last formed pair of somites and hindgut in midgestational mouse embryos. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *Ventrhoid*; *Vrho*; *rhomboid*; Ventral neural tube; Ventral forebrain; Pretectum; Diencephalon; Otic vesicle; Mandibular arches; Neurogenic placodes; Somitogenesis; Hindgut; Mouse embryo

1. Results and discussion

In *Drosophila*, EGF signaling plays a crucial role in a wide variety of developmental processes (Schweitzer and Shilo, 1997). Patterning activity of EGF signaling in the ventral neuroectoderm is largely dependent on the seven-pass transmembrane protein, Rhomboid. As a positive upstream modulator, Rhomboid is indispensable for the production of a diffusible EGF signal through processing a membrane bound EGF family member, the TGF-alpha homolog Spitz (Wasserman and Freeman, 1997). Most importantly, Rhomboid mediates the spatial specificity of this signaling system, since it is expressed in a spatially restricted manner in the ventral neuroectoderm, as opposed to the widespread expression of downstream EGF signaling components (Bier et al., 1990; Schweitzer et al., 1995; Golembo et al., 1996; Bier, 1998). Though basic elements of dorsoventral neural patterning (e.g. longitudinal columnar organization of neuroblasts expressing homologous transcription factors) and several components of the EGF signaling cascade are evolutionarily conserved in vertebrates, a role for EGF-family members or the existence of Rhomboid-like modulators has not been demonstrated in the context of initial dorsoventral neural tube regionalization and cell fate specification (Moghal and Sternberg, 1999; Cornell and von Ohlen, 2000; Bogdan and Klämbt, 2001; Buonanno and Fischbach, 2001). It is, therefore, of considerable interest to identify and characterize expression of rhomboid-like molecules in vertebrates. Here, we describe the identification and expression pattern of the novel vertebrate rhomboid-related cDNA *Ventrhoid* (*Vrho*) in mouse embryos.

TBlastN2 searches revealed a number of human clones of which two full-length human cDNA sequences were already annotated as rhomboid-related sequences with no apparent indication of involvement in developmental processes (Pascall and Brown, 1998; NEDO human cDNA sequencing project, unpublished). Further distinct clones mapping to human chromosome 17 showed remarkable sequence similarity to *Drosophila rhomboid*, and encode a novel, rhomboid-related, cDNA. We termed this novel vertebrate molecule as *Vrho* because of its characteristic expression pattern.

The putative exon/intron structure of *Vrho* within the human genomic sequence was determined by GENSCAN exon prediction program. Existence of the predicted transcript was confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) on Human Fetal Brain Multiple

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PII: S0925-4773(01)00655-4

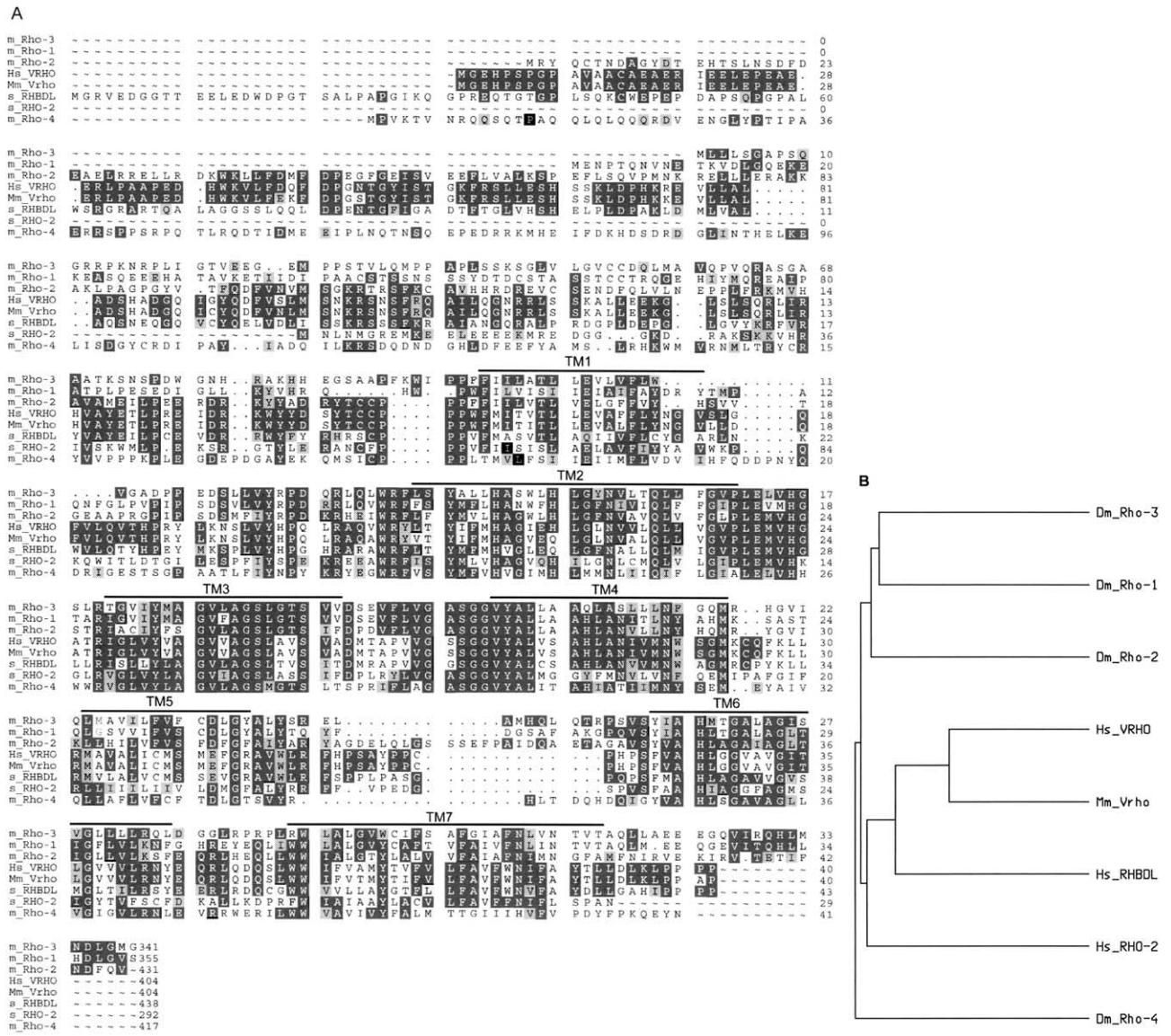

Fig. 1. (A) Multiple sequence alignment (PileUP) of human and mouse Vrho peptide sequences with other Rhomboid-related proteins. Identities are boxed in black, similar residues marked by gray shading. The predicted position of transmembrane domains (TM1–TM7) is indicated by black lines. The loops are located between the transmembrane domains. The alignment reveals that the newly cloned Vrho sequences are Rhomboid-related proteins, and the C-terminal half of the aligned vertebrate and fruit fly Rhomboid-related protein sequences displays a high grade of sequence conservation. The N-terminal half of the sequences seems to be less conserved. (B) Cladogram of Rho-related proteins in vertebrates and *Drosophila*, derived from PileUP alignment. The tree shows the clustering relationship used to create the alignment. Prefixes before the protein names indicate species: Dm, *Drosophila*; Hs, human; Mm, mouse.

Choice cDNA (CH-1015, OriGene). The PCR results showed that GENSCAN had over-estimated the length of the putative open reading frame (ORF) (data not shown). Next, we amplified a murine *Vrho* cDNA fragment with the help of degenerated PCR primers from E9.75 mouse embryo cDNA. Sequence analysis of the obtained murine cDNA fragment showed that it has an overall identity of 96% to the corresponding segment of the human sequence (data not shown). To obtain full-length murine *Vrho* cDNA, an adult mouse brain cDNA library was screened with PCR. Three independent clones were isolated and sequenced. The missing 5′-end was identified by further BlastN2 searches and confirmed by RT-PCR. Multiple sequence alignment (PileUP) of the newly cloned human and murine Vrho peptide sequences with the above-mentioned human and recently cloned *Drosophila* Rhomboid molecules (Wasserman et al., 2000) reveals a high sequence conservation in the C-terminal half of their sequences (Fig. 1). The most conserved regions of Rhomboid-related proteins are those that form the transmembrane domain 2–6 and the loop between the first two transmembrane domains (Wasserman et al., 2000).

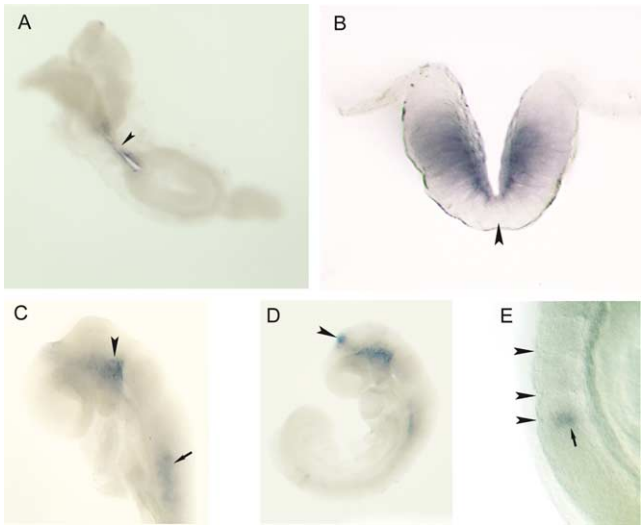

Fig. 2. Expression analysis of *Vrho* around neurulation and somitogenesis using whole-mount ISH. (A) Dorso-lateral view of a 4 somite stage embryo. *Vrho* transcripts are seen in the lower cervical (arrowheads) region where the neural folds are apposed to form the neural tube. (B) A transversal section through the neural folds at the level of the second pair of somites. *Vrho* expression is seen at both sides of the CNS midline floor (arrowhead). (C) Lateral view of an E8.5 embryo showing marked expression of *Vrho* in the cervical neural tube (arrow) and at the base of the developing mandibular arch (arrowhead). At 8.75 (D), before closure of the anterior neuropore *Vrho* is expressed at the prospective pretectum (arrowhead). (C,D) Lateral views. (E) During somitogenesis, *Vrho* is expressed in the last formed pair of somites shown at E9. *Vrho* transcripts are detected at the caudal part of the somite marked by arrow. Somitic furrows are indicated by arrowheads.

To analyze sites of *Vrho* expression in toto, we performed whole-mount in situ hybridization (ISH) with DIG-labeled cRNA probes on a series of mouse embryos (E8, E8.5, E8.75, E9, E9.5, E10). ISH analysis revealed that like *rho* in *Drosophila*, *Vrho* is also expressed in a spatially restricted manner in all stages investigated, and its expression pattern changes dynamically during the course of development. At the beginning of somitogenesis (E8) expression of *Vrho* is restricted to the developing central nervous system (CNS), and precedes the closure of the neural tube. It is expressed in a bilateral domain adjacent to the CNS midline floor in the lower cervical region where the neural folds are apposed to form the neural tube (Fig. 2A, B). Later on (E8.5–E8.75), *Vrho* expression is most prominent in the cervical neural tube, and expression appears also in non-neural tissues. *Vrho* is expressed at the base of the developing mandibular arches (Fig. 2C, D), and according to cross-sections the staining is in the surface ectoderm (data not shown). During somitogenesis, *Vrho* is expressed in the caudal part of the last formed pair of somites (Fig. 2E). By E8.75, *Vrho* can also be detected in the prospective pretectum (Fig. 2D). As development proceeds expression of *Vrho* becomes stronger and more extensive. In the developing nervous system, five major domains can be distinguished at E9 and onwards (Fig. 3A–G). The rostralmost expression site in the developing CNS is the ventral forebrain (Fig. 3A, G). Strong *Vrho* expression can be detected in the prospective pretectum and diencephalon. *Vrho* shows a ring-like expression pattern

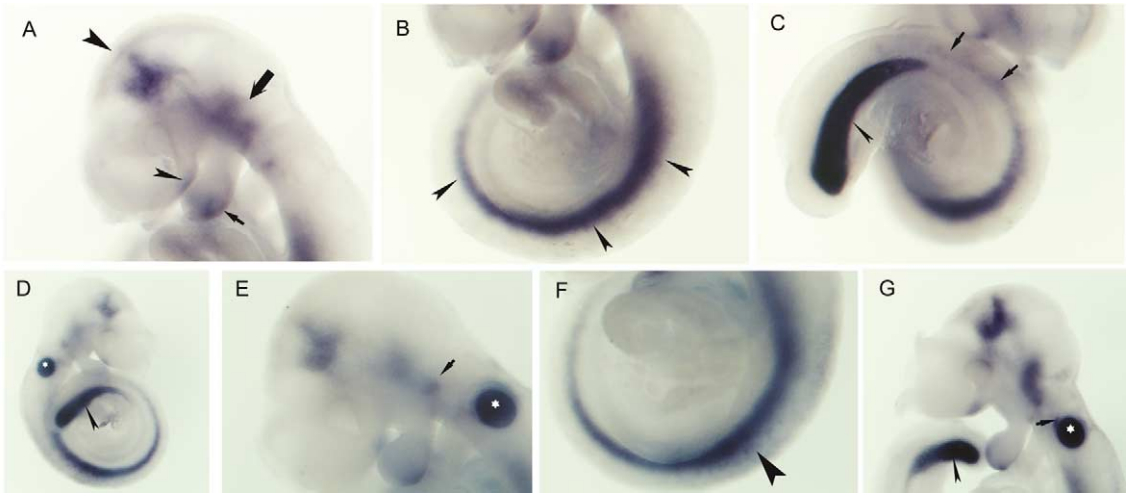

Fig. 3. Expression of *Vrho* during midgestational period (A–G). Expression of *Vrho* is up-regulated in the developing CNS. (A) Lateral view of the cranial half of an E9 embryo. *Vrho* is detected in the ventral forebrain (small arrowhead), pretectum and dorsal diencephalon (fat arrowhead), and in the metencephalon (fat arrow). The mandibular arch also expresses *Vrho* (small arrow). (B,C) Caudal half of the embryo seen on (A). (B) Expression of *Vrho* is greatly expanded in the ventral spinal neural tube (small arrowheads), but (C) it is less prominent in the ontogenetically younger more caudal part of the tube (small arrows). Strong expression can also be seen in the hindgut diverticulum (small arrowhead). (C) Antero-lateral direction. (D) Lateral view of an E9.5 embryo. Expression of *Vrho* in the developing CNS and hindgut diverticulum (small arrowhead) remains unchanged. (E) Higher magnification of the cranial part of the embryo seen on (D). Strong expression of *Vrho* is seen in the otic vesicle (white star) and the trigeminal placode (arrow) also contains *Vrho* transcripts. (F) At higher magnification of the trunk *Vrho* is also seen at more dorsal coordinates (fat arrowhead) in the cervical and thoracic spinal neural tube. (G) By E10 *Vrho* expression in the CNS and hindgut diverticulum (small arrowhead) remains unaltered. The vestibulo-acoustic placode (small arrow) also strongly expresses *Vrho*. White stars on (D,E,G) indicate otic vesicles.

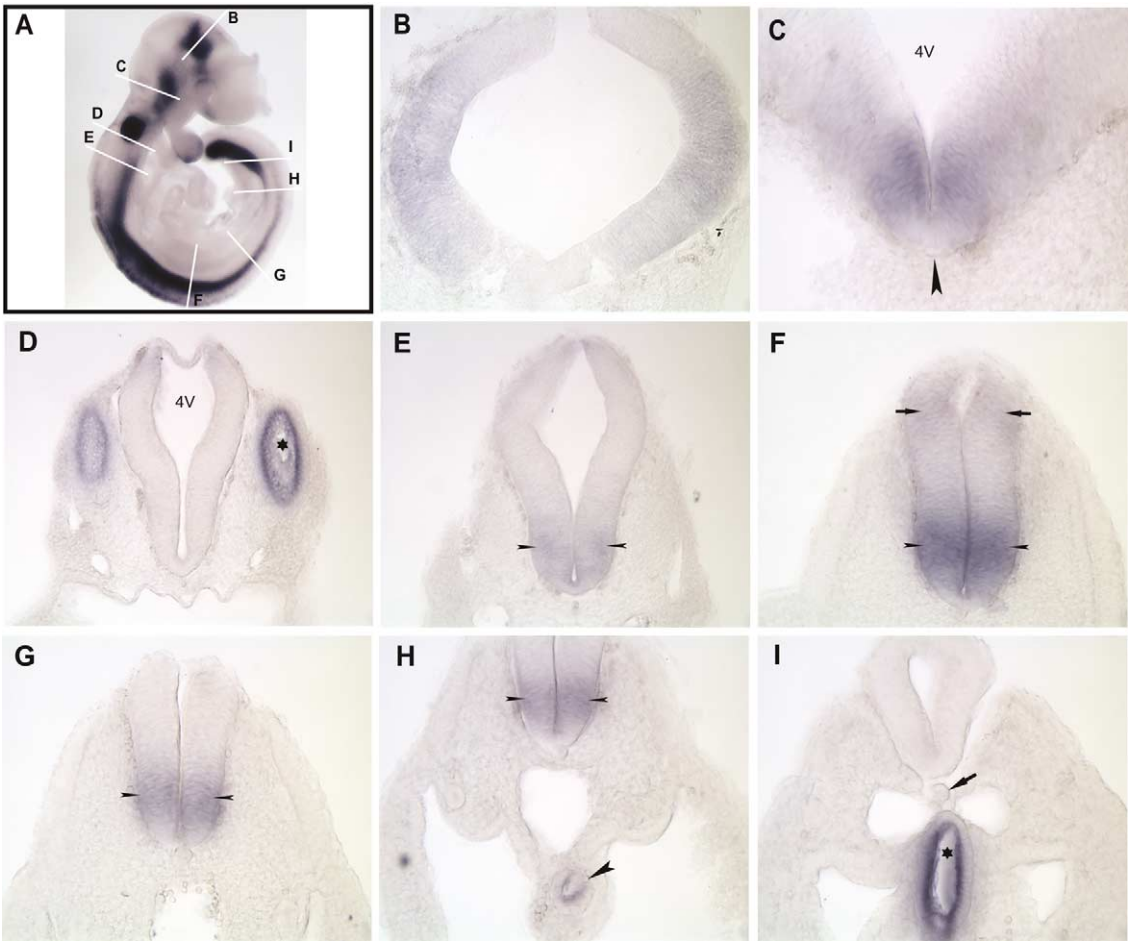

Fig. 4. Analysis of *Vrho* expression on cross-sections at E10. (A) The level and orientation of the sections seen on (B–I). (B) Marked *Vrho* expression can be detected in the pretectum at both sides of the ventral midline extending into the alar plate. (C) In the metencephalon expression of *Vrho* is ventrally restricted. The CNS midline floor is indicated by large arrowhead. (D) Expression of *Vrho* in the otic vesicles (black star). At the level of the otic vesicles the ventral rhombencephalon lacks detectable level of *Vrho* expression. (E) Expression of *Vrho* is relatively weak at the transition of rhombencephalon and spinal neural tube (small arrowheads). (F) At the level of forelimb buds expression of *Vrho* is strong (small arrowheads) and even a second dorsal expression domain is visible (small arrows). (G,H) Expression of *Vrho* (small arrowheads) decreases toward the ontogenetically younger more caudal parts of the spinal neural tube, and (I) the most caudal parts of the neural tube lack *Vrho* expression. The notochord is indicated by arrow. Expression of *Vrho* is also detected in the hindgut diverticulum (H,I) indicated by arrowhead and black star, respectively. 4V indicates position of the fourth ventricle.

in this pretecto-diencephalic compartment (Figs. 3A, D, E, G and 4B). *Vrho* expression can also be seen in the developing ventral metencephalon and hindbrain (Fig. 3A, D, E, G) in close proximity to the ventral midline (Fig. 4C). Expression of *Vrho* does not trespass the MHB, and the mesencephalon lacks *Vrho*. *Vrho* is also expressed in the developing otic vesicles (Figs. 3D, E, G and 4D). The most prominent site of *Vrho* expression is the spinal neural tube (Fig. 3B, D, F), where it is expressed in two longitudinal stripes along both sides of the ventral midline adjacent to the floor plate (Fig. 4F–H). *Vrho* expression is less prominent toward the ontogenetically younger, more caudal parts of the spinal neural tube (Figs. 3C and 4H), but it progressively expands caudally with the course of development. Expression of *Vrho* does not show a sharp limit toward the dorsal side of the neural tube, but its expression rapidly declines toward dorsal coordinates as revealed on cross-sections after whole-mount ISH (Fig. 4). In the thoracic spinal neural tube *Vrho* seems to have a second weak dorsal expression domain (Fig. 4F). Outside the developing CNS, marked *Vrho* expression can be seen in the mandibular arches (Fig. 3A, E, G), trigeminal and vestibulo-acoustic neurogenic/sensory ganglion/placodes (Fig. 3E, G, respectively), and in the developing hindgut (Figs. 3C, D, G and 4H, I).

In conclusion, we cloned *Vrho*, a likely vertebrate homolog of the fruit fly *rho* gene. The striking similarities in the ventrally restricted expression patterns of *Vrho* in the mouse neural tube and *rho* in the *Drosophila* neuroectoderm points to an evolutionarily conserved role of EGF signaling in regionalization of the CNS along the D/V axis. It will be interesting to see how the function of *Vrho* is conserved during vertebrate embryonic development, and in particular, in neuronal cell fate specification.

2. Methods

Human *Vrho* was identified by TBlastN2 (gapped version of TBlastN embedded in the HUSAR sequence analysis software package, DKFZ, Heidelberg) algorithm using *Drosophila* Rho peptide sequence as a query. To define putative coding exons GENSCAN was used provided also by HUSAR. The corresponding murine cDNA was obtained by degenerate PCR, and PCR screening of a pooled commercially available cDNA library (Rapid-screen Adult Mouse Brain cDNA library, MAB-1001, OriGene) using a Gold GeneAmp 9700 thermal cycler. Sequencing was performed on an ABI 377 automated sequencer. Details of oligos and PCR used in the cloning procedure of *Vrho* can be obtained from the authors. Murine and human *Vrho* cDNA sequences were deposited in EMBL Nucleotide Sequence Database under AJ313479 and AJ313480 accession numbers, respectively. The position of transmembrane domains in the newly cloned murine and human *Vrho* sequences was predicted by the HMMTOP program version 2.0 developed by Tusnády and Simon (2001). Timed pregnant NMRI mice were sacrificed according to S1 method of humane killing (Animal Scientific Procedures Act, 1986, UK). Embryos were then collected in L-15 medium, washed, fixed in 4% PFA, and dehydrated in 100% methanol and stored at −20°C until use. Whole-mount ISH was performed according to a protocol established by Henrique et al., 1995. DIG-labeled cRNA probes were synthesized with T7 RNA polymerase (Roche) using a 600 bp PCR fragment ligated into pCRII-TOPO vector (Invitrogen). Unincorporated nucleotides, DNaseI were removed by RNeasy Mini Kit (Qiagen GmbH, Helden, Germany). Stained embryos were refixed in 4% PFA with 0.1% glutaraldehyde, and then they were photographed without clearing in glycerol. To analyze expression on cross-sections, embryos after ISH were transversally cut with sharp tungsten needles and mounted, or cryoprotected, embedded in OCT compound (Sakura) and were cut at 70 μm on a Microm cryostat.

Acknowledgements

This work was supported by grants from the European Union (QLG3-CT-2001-02310), and the Max Planck Society to M.B. The authors wish to thank Lilla M. Farkas for helpful discussions and comments on the manuscript, Muriel Rhinn for careful reading of the manuscript, and Glenis Wiebe for DNA sequencing.

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