

Genetic evidence that oxidative derivatives of retinoic acid are not involved in retinoid signaling during mouse development

Karen Niederreither^{1,4}, Suzan Abu-Abed², Brigitte Schuhbaur¹, Martin Petkovich^{2,3*}, Pierre Chambon^{1*} & Pascal Dollé^{1*}

*These authors contributed equally to this work.

Published online: 15 April 2002, DOI: 10.1038/ng876

Retinoic acid, the active derivative of vitamin A (retinol), is a hormonal signaling molecule that acts in developing and adult tissues¹. The *Cyp26a1* (cytochrome p450, 26) protein metabolizes retinoic acid into more polar hydroxylated and oxidized derivatives^{2,3}. Whether some of these derivatives are biologically active metabolites has been debated^{4,5}. *Cyp26a1*^{-/-} mouse fetuses have lethal morphogenetic phenotypes mimicking those generated by excess retinoic acid administration, indicating that human *CYP26A1* may be essential in controlling retinoic acid levels during development^{6,7}. This hypothesis suggests that the *Cyp26a1*^{-/-} phenotype could be rescued under conditions in which embryonic retinoic acid levels are decreased. We show that *Cyp26a1*^{-/-} mice are phenotypically rescued by heterozygous disruption of *Aldh1a2* (also known as

Raldh2), which encodes a retinaldehyde dehydrogenase responsible for the synthesis of retinoic acid during early embryonic development^{8,9}. *Aldh1a2* haploinsufficiency prevents the appearance of spina bifida and rescues the development of posterior structures (sacral/caudal vertebrae, hindgut, urogenital tract), while partly preventing cervical vertebral transformations and hindbrain pattern alterations in *Cyp26a1*^{-/-} mice. Thus, some of these double-mutant mice can reach adulthood. This study is the first report of a mutation acting as a dominant suppressor of a lethal morphogenetic mutation in mammals. We provide genetic evidence that *ALDH1A2* and *CYP26A1* activities concurrently establish local embryonic retinoic acid levels that must be finely tuned to allow posterior organ development and to prevent spina bifida.

Spina bifida (lack of caudal neural tube closure)¹⁰ and caudal regression¹¹ are prevalent congenital defects whose environmental and genetic causes are unclear. Targeted disruption of *Cyp26a1* is homozygous, lethal and leads to spina bifida and caudal regression, as well as to vertebral transformations and abnormal hindbrain patterning^{6,7}. These phenotypes may result from the absence of *Cyp26a1*-generated bioactive metabolites, from teratogenic effects of excess retinoic acid in mutant embryos or from a combination of the two. We used a genetic approach to distinguish between these possibilities. According to the second hypothesis, the severity of *Cyp26a1*^{-/-} phenotypic dysfunction may be attenuated under conditions that decrease embryonic retinoic acid levels.

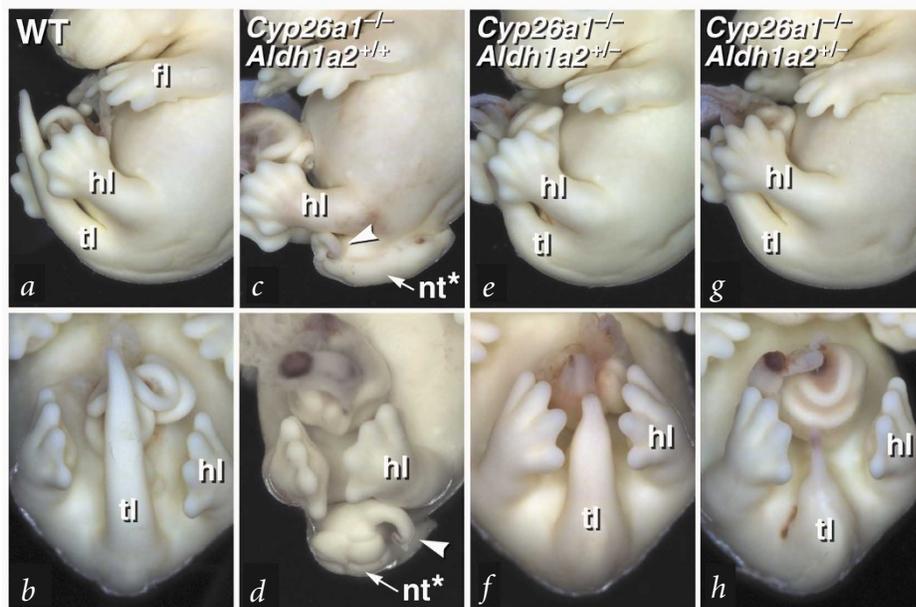


Fig. 1 Rescue of spina bifida and tail development in *Cyp26a1*^{-/-}*Aldh1a2*^{+/-} compound mutants. Fetuses were collected at E14.5 from *Cyp26a1*^{-/-}*Aldh1a2*^{+/-} intercrosses. **a–h**, Profile (upper panels) and caudal (lower panels) views of wildtype (WT, **a, b**), *Cyp26a1*^{-/-} (**c, d**) and *Cyp26a1*^{-/-}*Aldh1a2*^{+/-} (**e–h**) fetuses. fl, forelimb; hl, hindlimb; nt*, open neural tube (spina bifida); tl, tail. The arrowheads in **c** and **d** point to the tail rudiment in the *Cyp26a1*^{-/-} mutant.

¹Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP/Collège de France, BP 163, 67404 Illkirch Cedex, CU de Strasbourg, France. ²Department of Pathology, Cancer Research Laboratories, Queen's University, Kingston, Ontario, Canada. ³Department of Biochemistry, Cancer Research Laboratories, Queen's University, Kingston, Ontario, Canada. ⁴Present address: Departments of Medicine and Molecular and Cellular Biology, Center for Cardiovascular Development, Baylor College of Medicine, Houston, Texas, USA. Correspondence should be addressed to P.D. (e-mail: dolle@igbmc.u-strasbg.fr).

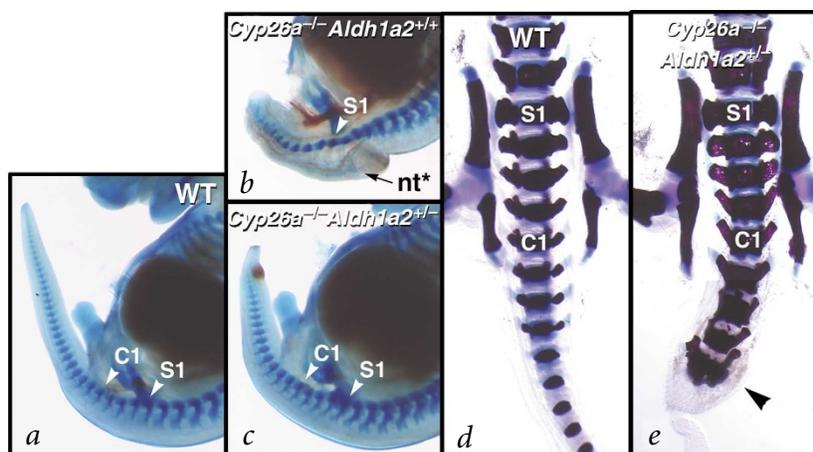


Fig. 2 Caudal vertebral development in *Cyp26a1^{-/-}* and *Cyp26a1^{-/-}Aldh1a2^{+/-}* mutants. **a–c**, Profile views of the sacral and tail region of fetuses at E14.5 (genotypes as indicated) with Alcian blue cartilage staining. **d,e**, Frontal views of the sacral and caudal vertebrae of newborn mice. Alizarin red (bone) and alcian blue (cartilage) staining. C1, first caudal vertebra; S1, first sacral vertebra; nt*, open neural tube (spina bifida). The arrowhead in **e** indicates the last caudal vertebra in the mutant.

During early post-implantation stages, most of the embryonic retinoic acid is synthesized by the aldehyde dehydrogenase *Aldh1a2* (refs 8,9). *Aldh1a2^{-/-}* embryos die at embryonic day (E) 9.5–10.5 and fail to activate retinoic acid-responsive transgenes (except in developing retina)⁹. We therefore intercrossed *Cyp26a1* and *Aldh1a2* mouse mutants, to see whether mutation of *Aldh1a2* might suppress the *Cyp26a1^{-/-}* phenotype.

Compound heterozygotes (*Cyp26a1^{+/-}Aldh1a2^{+/-}*) were healthy and fertile. We intercrossed these mutants and collected fetuses at E14.5 to see whether the *Cyp26a1^{-/-}* phenotype is modified in an *Aldh1a2^{+/-}* genetic background. *Aldh1a2* heterozygosity rescued the spina bifida and lack of caudal development in *Cyp26a1^{-/-}* mutants (Fig. 1). In contrast to the minute tail rudiment (Fig. 1c,d) or sirenornelia (fusion between both hindlimbs; data not shown)^{6,7} observed in *Cyp26a1^{-/-}* animals, compound mutants developed a tail, which was somewhat shorter than that of wildtype littermates (compare Fig. 1a,b with e–h). Skeletal stainings confirmed the rescue of the *Cyp26a1^{-/-}* caudal phenotype. Whereas the prevertebral cartilages of *Cyp26a1^{-/-}* fetuses were disorganized from the lower lumbar levels, those of *Cyp26a1^{-/-}Aldh1a2^{+/-}* compound mutants were normally patterned (compare Fig. 2a,b and c).

We therefore investigated whether the *Cyp26a1^{-/-}Aldh1a2^{+/-}* compound mutants were viable. We obtained *Cyp26a1^{-/-}Aldh1a2^{+/-}* mice at the expected mendelian ratio in newborn litters (see Web Table A online); these mice had truncated tails that varied in length (Fig. 3b,c). Skeletal analysis showed normal

patterning of their sacral and first caudal vertebrae, whereas the most distal caudal vertebrae failed to differentiate (Fig. 2d,e). About two-thirds of the double-mutants (12/17) were still alive and healthy at the age of weaning (see Web Table A online). The remaining double-mutants were found dead (2) or were killed for analysis, as they showed abnormal external genitalia (Fig. 3c) and abdominal distension. Histological analysis of a healthy newborn *Cyp26a1^{-/-}Aldh1a2^{+/-}* mouse revealed no abnormalities in kidney (Fig. 3d), anorectal (Fig. 3e), vaginal (Fig. 3f) and urethral (Fig. 3g) tissues. By contrast, other

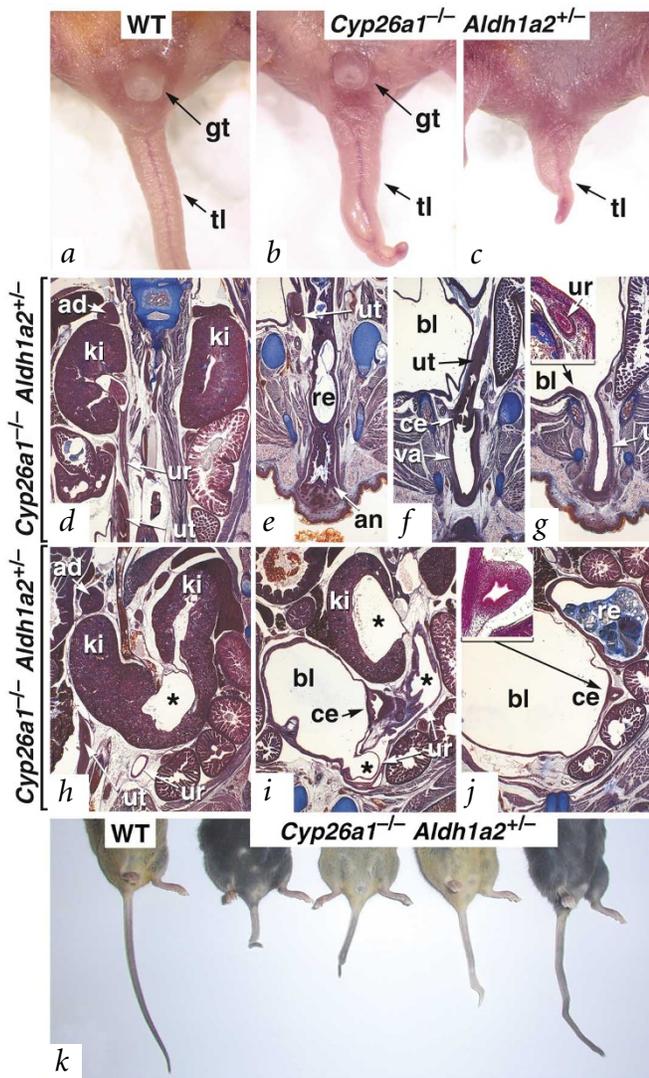
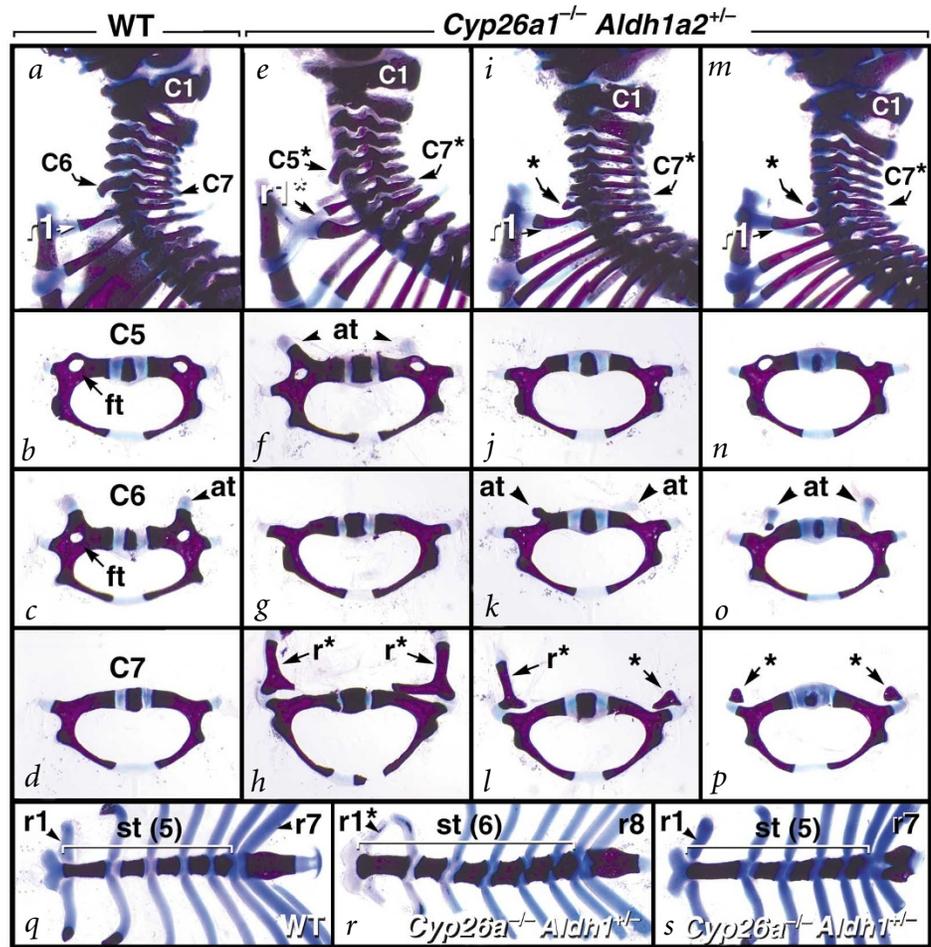


Fig. 3 Tail and posterior organ development in *Cyp26a1^{-/-}Aldh1a2^{+/-}* compound mutants. **a–c**, Ventral views of the tail and external genitalia of wildtype (**a**) and *Cyp26a1^{-/-}Aldh1a2^{+/-}* (**b,c**) newborn mice. **d–g**, Histological sections through the kidneys (**d**), anorectal region (**e**), cervix and vagina (**f**) and urethra (**g**) of a 'normal' *Cyp26a1^{-/-}Aldh1a2^{+/-}* newborn mutant. These structures are histologically indistinguishable from those of wildtype littermates. **h–j**, Abnormal development of posterior structures in another *Cyp26a1^{-/-}Aldh1a2^{+/-}* newborn. This mutant shows partial fusion between kidneys (**h**), dilation of the kidney and ureter cavities (**h,i**, *; compare with the normal size of the ureter near its junction with the bladder: magnified in **g**), absence of the vagina, the uterine cervix ending blindly along the bladder wall (**i,j**, magnified in inset), and dilation of the rectum (**j**) due to the lack of anal development. **k**, Adult tail phenotypes of *Cyp26a1^{-/-}Aldh1a2^{+/-}* mice in comparison with that of a wildtype mouse. ad, adrenal gland; an, anus; bl, urinary bladder; ce, cervix; gt, genital tubercle; ki, kidney; re, rectum; ur, ureter; ut, uterus; va, vagina.

Fig. 4 Variable rescue of cervical vertebral transformations in *Cyp26a1^{-/-}Aldh1a2^{+/-}* double-mutants. **a–d**, Profile view of the cervical column (a) and dissected C5–C7 vertebrae (b–d) of a wildtype mouse at E18.5. **e–p**, Comparative views of the cervical column and the C5–C7 vertebrae from three *Cyp26a1^{-/-}Aldh1a2^{+/-}* mutants with complete (e–h), intermediate (i–l) or minor (m–p) signs of posteriorization. The presence of anterior tuberculi (at) on C5 instead of C6, and of supernumerary ribs (r*) on C7 indicates a posterior transformation. Two of the mutants show only partial (l) or minute rib rudiments (p, *) on C7. **q–s**, Ventral views of the sternum of a wildtype (q) and two *Cyp26a1^{-/-}Aldh1a2^{+/-}* mice at E18.5, which show, respectively, a super numerary rib (r*) and six (instead of five) sternebrae, or a phenotypically wildtype sternum. at, anterior tuberculum; C1–C7, cervical vertebrae (transformed vertebrae are indicated by *); ft, foramina transversaria; r1–r8, ribs; st, sternebrae.



Cyp26a1^{-/-}Aldh1a2^{+/-} mice (Fig. 3c) had horseshoe-shaped kidneys (Fig. 3h), dilation of the kidney and ureteric cavities (Fig. 3h,i) and lack of formation of the urethra, vagina and terminal part of the rectum (Fig. 3i,j), indicating incomplete phenotypic rescue. Only half of the expected *Cyp26a1^{-/-}* mice were present at birth (see Web Table A online); if not dead (3/4), these were killed owing to their severe spina bifida or sirenomelia phenotype. The surviving *Cyp26a1^{-/-}Aldh1a2^{+/-}* mutants reached adulthood without any phenotypic defect apart from their abnormal tails (Fig. 3k); both males and females were fertile.

We also investigated whether the other phenotypic features of *Cyp26a1^{-/-}* mice—the posterior cervical vertebral transformations and the hindbrain patterning defects—could be rescued by *Aldh1a2* haploinsufficiency. Cervical transformations could be reverted in *Cyp26a1^{-/-}Aldh1a2^{+/-}* mutants, albeit with variable

expressivity: some animals showed transformation of cervical vertebrae (C) 5–7, with supernumerary ribs on C7 (Fig. 4e,h), anterior tuberculi on C5 instead of C6 (Fig. 4f) and six instead of five sternebrae (Fig. 4r). Other double-mutants (Fig. 4i–p) showed

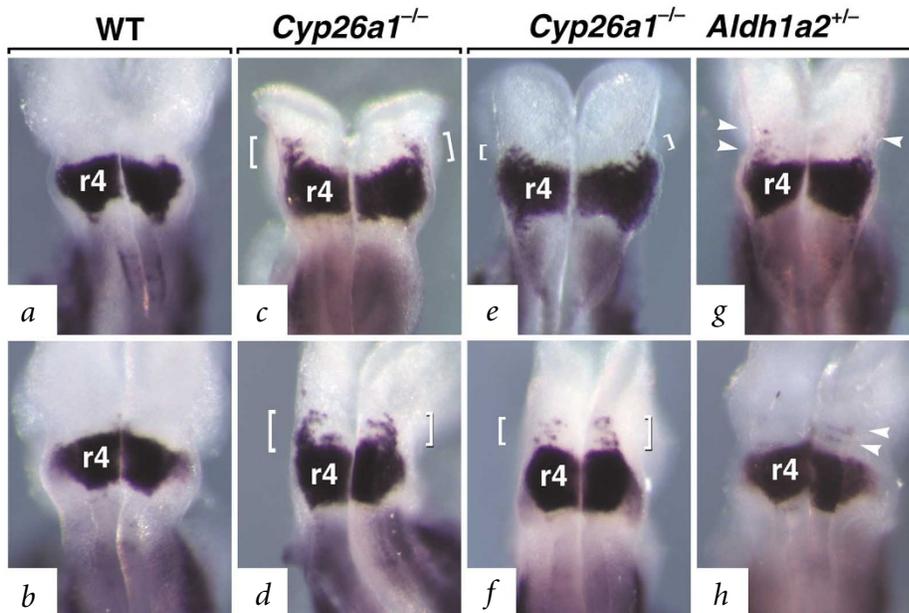


Fig. 5 Partial rescue of abnormal anterior rhombomere patterning in *Cyp26a1^{-/-}Aldh1a2^{+/-}* double-mutants. **a–h**, *Hoxb1* expression in hindbrain was analyzed in wildtype (a,b), *Cyp26a1^{-/-}* (c,d) and *Cyp26a1^{-/-}Aldh1a2^{+/-}* (e–h) embryos. Whole-mount *in situ* hybridization was carried out on embryos at E8.5 (a,c,e,g) or at E8.75 (b,d,f,h) during the process of hindbrain neural tube closure. Fewer cells ectopically expressing *Hoxb1* (indicated by brackets or arrowheads) are detected rostrally to rhombomere 4 (r4) in *Cyp26a1^{-/-}Aldh1a2^{+/-}* embryos than in *Cyp26a1^{-/-}* embryos.

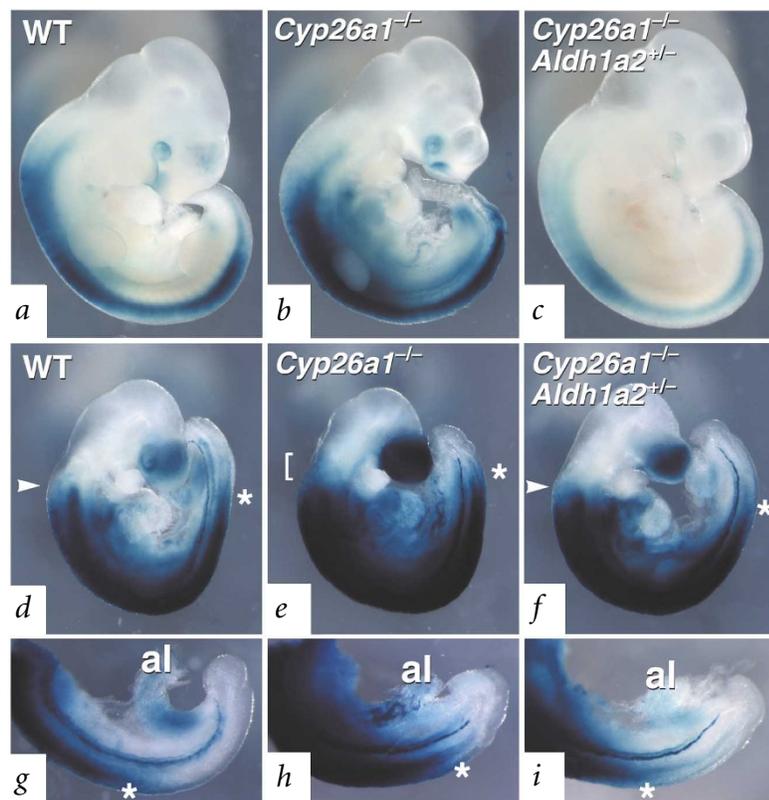


Fig. 6 Retinoic acid–reporter transgene activity in embryos with single or compound mutations of *Cyp26a1* and *Aldh1a2*. **a–c**, Embryos at E10.5 from a *Cyp26a1*^{+/–}/*Aldh1a2*^{+/–} intercross, whose father harbored the *RARE-hsp68-lacZ* transgene, were briefly stained (1 h) with X-gal. *Cyp26a1*^{–/–} mutants (**b**) show elevated *lacZ* activity in comparison with control littermates (**a**). *Aldh1a2*^{+/–} mutants show reduced activity, even in the case of *Cyp26a1*^{–/–} compound inactivation (**c**). **d–i**, Embryos at E9.5 from the same type of cross were overstained (5 h) to compare the extent of transgene activity. Profile views (**d–f**) and close-up views of the tail region (**g–i**) are shown. In *Cyp26a1*^{–/–} embryos (**e, h**), transgene activity extends rostrally within the hindbrain (bracket) and caudally (*) beyond the level of the allantoic bud (al). In *Cyp26a1*^{–/–}/*Aldh1a2*^{+/–} embryos (**f, i**), the rostral (arrowhead) and caudal (*) expression limits of transgene activity were the same as those of control embryos (**d, g**).

by exogenous excess retinoic acid¹⁴. If one assumes that the *Cyp26a1*^{–/–} phenotype (or part of it) results from impaired 4-oxo-RA signaling, then *Aldh1a2* haploinsufficiency, by decreasing the level of the substrate for oxidizing enzymes, should lead to lower amounts of 4-oxo-RA. Thus, no phenotypic improvement would be expected in the double-mutants and, if anything, an exacerbation rather than improvement of this phenotype would be expected. *Cyp26a1* and *Aldh1a2* are expressed in juxtaposed domains during early mouse development (for example: the anterior epiblast versus the posterior mesoderm during gastrulation; the tail bud versus the adjacent

more subtle (or even minute) signs of posteriorization: C6 had anterior tuberculi, but lacked foramina transversaria (Fig. 4k,o); C7 harbored incomplete ribs (Fig. 4l) or minute rib rudiments (Fig. 4p) and five normal sternbrae were present (Fig. 4s).

Two retinoic acid–metabolizing enzymes, *Cyp26a1* and *Cyp26b1* (ref. 12), are expressed in specific segments (rhombomeres) of the developing hindbrain. *Cyp26a1*^{–/–} mice have abnormal patterning of the anterior hindbrain region, as seen by ectopic expression of *Hoxb1*, a rhombomere (r) 4-specific marker, in the r3/r2 territory (compare Fig. 5a,b and c,d)^{6,7}. *Hoxb1* ectopic expression was detected in *Cyp26a1*^{–/–}/*Aldh1a2*^{+/–} mutants (Fig. 5e–h), but did not extend as far rostrally as in *Cyp26a1*^{–/–} embryos (Fig. 5e), or was limited to a few single cells (Fig. 5f–h).

We used the retinoic acid–responsive *RARE-hsp68-lacZ* transgene¹³ to monitor embryonic retinoic acid levels in mice with single or double mutations. As previously reported⁷, *Cyp26a1*^{–/–} embryos had higher overall transgene activity and therefore higher retinoic acid levels than those of control littermates (compare Fig. 5a and b). *Cyp26a1*^{–/–}/*Aldh1a2*^{+/–} mutants showed less overall transgene activity than both control and *Cyp26a1*^{–/–} littermates (Fig. 6c). *Cyp26a1*^{–/–} embryos also showed abnormal expansion of transgene expression, both rostrally towards the hindbrain and caudally towards the tail bud (compare Fig. 6d,g and e,h). In *Cyp26a1*^{–/–}/*Aldh1a2*^{+/–} mutants (Fig. 6f,i), the rostral and caudal boundaries of transgene expression were comparable with those seen in control littermates.

These findings provide genetic evidence that the *Cyp26a1*^{–/–} phenotype results from deleterious effects of excess embryonic retinoic acid ('endogenous' retinoic acid teratogenicity) due to a lack of tissue-specific catabolism, rather than a lack of signaling by bioactive retinoic acid metabolites such as 4-oxo-retinoic acid (4-oxo-RA). These data are consistent with the fact that *Cyp26a1* overexpression in *Xenopus* embryos can rescue the developmental defects induced

somitic mesoderm; the anterior hindbrain neuroepithelium versus the posterior hindbrain mesenchyme)^{3,12,15,16}. We therefore propose that, as *Aldh1a2* haploinsufficiency results in a decrease in retinoic acid synthesis, double-mutant embryos are (at least partially) protected against teratogenic effects of embryonic retinoic acid in tissues lacking *Cyp26a1*. Although we cannot rule out the possibility that the remaining abnormalities (such as tail truncation) of the double-mutants could reflect a lack of 4-oxo-RA, the observation that all of these abnormalities correspond to milder phenotypes than those seen in *Cyp26a1*^{–/–} mice is consistent with the idea that they correspond to lesser effects of local embryonic retinoic acid teratogenicity rather than abnormalities due to a lack of 4-oxo-RA. In addition, variations in the residual levels of retinoic acid may account for the variability of the phenotypic rescue. The present data do not exclude any possible role for 4-oxo-retinol^{17,18}.

Our study suggests that imbalance between the synthesis and degradation of retinoic acid during embryogenesis could lead (or contribute) to the appearance of spina bifida and posterior organ abnormalities in humans. Moreover, although partial rescue of a specific morphogenetic mutation by a nonallelic mutation has been reported¹⁹, our study is the first report of a heterozygous mutation rescuing another mutation that on its own is homozygous embryonic lethal in mammals. Thus, the *Aldh1a2* mutation behaves as a dominant suppressor of the *Cyp26a1* phenotype. The search for suppressor mutations has long and successfully been used in systems readily amenable for genetic studies, such as *Drosophila melanogaster* (see ref. 20 for example). So far, genetic intercrosses between mutant mouse lines have mostly been generated to uncover new phenotypic defects, indicative of synergistic or partly redundant functions. Given the ever increasing number of engineered mouse lines, searching for suppressor mutations becomes a realistic means of identifying genes with opposing functions.

Methods

Generation of mice. The generation of mice with targeted disruptions of *Cyp26a1* (ref. 6) and *Aldh1a2* (ref. 9) has been described. The experimental design was approved by the institutional ethics committee in charge of the Institut de Génétique et de Biologie Moléculaire et Cellulaire. As both mutant lines had different genetic backgrounds (129/sv-C57/Bl6 and CD1, respectively), we carried out all phenotypic comparisons and assays between littermate specimens to avoid bias due to background effects.

Genotype analysis. We carried out genotyping^{6,9} by PCR analysis of extra-embryonic or skin DNA. Histology, skeletal staining²¹, *in situ* hybridization²² and X-gal staining¹³ were carried out as previously described.

Note: Supplementary information is available on the Nature Genetics website.

Acknowledgments

We thank J.M. Bornert and V. Fraulob for technical assistance. We are grateful to J. Rossant for providing the RARE-*hsp-lacZ* transgene. This work was supported by funds from the Centre National de la Recherche Scientifique, INSERM, the Collège de France, the Hôpitaux Universitaires de Strasbourg, the Association pour la Recherche sur le Cancer, the Fondation pour la Recherche Médicale and Bristol-Myers Squibb. S.A.A. holds a studentship from the Canadian Institutes of Health Research (CIHR), and M.P. is supported by the CIHR and the National Cancer Institute of Canada.

Competing interests statement

The authors declare that they have no competing financial interests.

Received 15 October 2001; accepted 20 March 2002.

1. Livrea, M.A. *Vitamin A and Retinoids: An Update of Biological Aspects and Clinical Applications* (Birkhauser, Basel, 2000).
2. White, J.A. *et al.* Identification of the retinoic acid-inducible all-*trans*-retinoic acid 4-hydroxylase. *J. Biol. Chem.* **271**, 29922–29927 (1996).
3. Fujii, H. *et al.* Metabolic inactivation of retinoic acid by a novel P450 differentially expressed in developing mouse embryos. *EMBO J.* **16**, 4163–4173 (1997).

4. Pijnappel, W.W. *et al.* The retinoid ligand 4-oxo-retinoic acid is a highly active modulator of positional specification. *Nature* **366**, 340–344 (1993).
5. Sonneveld, E., van den Brink, C.E., Tertoolen, L.G., van der Burg, B. & van der Saag, P.T. Retinoic acid hydroxylase (*Cyp26a1*) is a key enzyme in neuronal differentiation of embryonal carcinoma cells. *Dev. Biol.* **213**, 390–404 (1999).
6. Abu-Abed, S. *et al.* The retinoic acid-metabolizing enzyme, *CYP26A1*, is essential for normal hindbrain patterning, vertebral identity, and development of posterior structures. *Genes Dev.* **15**, 226–240 (2001).
7. Sakai, Y. *et al.* The retinoic acid-inactivating enzyme *Cyp26a1* is essential for establishing an uneven distribution of retinoic acid along the anterior-posterior axis within the mouse embryo. *Genes Dev.* **15**, 213–225 (2001).
8. Zhao, D. *et al.* Molecular identification of a major retinoic-acid-synthesizing enzyme, a retinaldehyde-specific dehydrogenase. *Eur. J. Biochem.* **240**, 15–22 (1996).
9. Niederreither, K., Subbarayan, V., Dollé, P. & Chambon, P. Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. *Nature Genet.* **21**, 444–448 (1999).
10. Northrup, H. & Volcik, K.A. Spina bifida and other neural tube defects. *Curr. Probl. Pediatr.* **30**, 313–332 (2000).
11. Valenzano, M. *et al.* Pathological features, antenatal ultrasonographic clues, and a review of current embryogenic theories. *Hum. Reprod. Update* **5**, 82–86 (1999).
12. MacLean, G. *et al.* Cloning of a novel retinoic-acid metabolizing cytochrome P450, *Cyp26B1*, and comparative expression analysis with *Cyp26a1* during early murine development. *Mech. Dev.* **107**, 195–201 (2001).
13. Rossant, J., Zirnbigl, R., Cado, D., Shago, M. & Giguère, V. Expression of a retinoic acid response element-*hsp-lacZ* transgene defines specific domains of transcriptional activity during mouse embryogenesis. *Genes Dev.* **5**, 1333–1344 (1991).
14. Hollemann, T., Chen, Y., Grunz, H. & Pieler, T. Regionalized metabolic activity establishes boundaries of retinoic acid signalling. *EMBO J.* **17**, 7361–7372 (1998).
15. Niederreither, K., McCaffery, P., Dräger, U.C., Chambon, P. & Dollé, P. Restricted expression and retinoic acid-induced downregulation of the retinaldehyde type 2 (RALDH-2) gene during mouse development. *Mech. Dev.* **62**, 67–78 (1997).
16. Swindell, E.C. *et al.* Complementary domains of retinoic acid production and degradation in the early chick embryo. *Dev. Biol.* **216**, 282–296 (1999).
17. Achkar, C.C. *et al.* 4-oxoretinol, a new natural ligand and transactivator of the retinoic acid receptors. *Proc. Natl Acad. Sci. USA* **93**, 4879–4884 (1996).
18. Lane, M.A., Chen, A.C., Roman, S.D., Derguini, F. & Gudas, L.J. Removal of LIF (leukemia inhibitory factor) results in increased vitamin A (retinol) metabolism to 4-oxoretinol in embryonic stem cells. *Proc. Natl Acad. Sci. USA* **96**, 13524–13529 (1999).
19. Litingtung, Y. & Chiang, C. Specification of ventral neuron types is mediated by an antagonistic interaction between Shh and Gli3. *Nature Neurosci.* **3**, 979–985 (2000).
20. Haines, N. & van den Heuvel, M. A directed mutagenesis screen in *Drosophila melanogaster* reveals new mutants that influence hedgehog signaling. *Genetics* **156**, 1777–1785 (2000).
21. Dollé, P. *et al.* Disruption of the *Hoxd-13* gene induces localized heterochrony leading to mice with neotenic limbs. *Cell* **75**, 431–441 (1993).
22. Décimo, D., Georges-Labouesse, E. & Dollé, P. *In situ* hybridization to cellular RNA. in *Gene Probes 2, A Practical Approach* (eds Hames, B.D. & Higgins, S.J.) 182–210 (Oxford University Press, New York, 1995).