*Pure Appl. Chem.*, Vol. 75, Nos. 11–12, pp. 1709–1732, 2003.


# Topic 1.6

# Functions of RARs and RXRs in vivo: Genetic dissection of the retinoid signaling pathway*

Manuel Mark and Pierre Chambon‡

*Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC) (CNRS/INSERM/ULP/Collège de France), Institut Clinique de la Souris (ICS) BP 10142, 67404 Illkirch Cedex, France*

*Abstract*: Retinoids, the active metabolites of vitamin A, regulate complex gene networks involved in vertebrate morphogenesis, growth, cellular differentiation, and homeostasis. They are used for the treatment of skin disorders and as chemopreventive agents for certain cancers. Molecular biology and genetic studies performed during the last 15 years in vitro, using either acellular systems or transfected cells, have shown that retinoid actions are mediated through heterodimers between the 8 major RARα, β, and γ isoforms and the 6 major RXRα, β, and γ isoforms that belong to the nuclear receptor (NR) superfamily, and act as ligand-dependent transcriptional regulators. Furthermore, RXRs not only heterodimerize with RARs, but also with numerous other members of the NR superfamily. As in vitro studies are carried out under nonphysiological conditions, they only indicate what is possible, but not necessarily what is actually occurring in vivo. Therefore, mutations have been introduced by homologous recombination (HR) in F9 embryonal carcinoma (EC) cells, a cell-autonomous system that differentiates in the presence of RA, in order to disrupt RAR and RXR genes and establish their cellular and molecular functions in RA-induced differentiation. However, genetic approaches in the animal should be used to determine the function of retinoid receptors under truly physiological conditions. HR in embryonic stem (ES) cells, has therefore been used to generate null mutations of the various RARs and RXRs in the mouse germline. As reviewed here, the generation of such RAR and RXR germline mutations, combined with pharmacological approaches to block the RA signaling pathway, has provided many valuable insights on the developmental functions of RA receptors. However, due to (i) the complexity in "hormonal" signaling through transduction by the multiple RARs and RXRs, (ii) the functional redundancies (possibly artefactually generated by the mutations) within receptor isotypes belonging to a given gene family, and (iii) in utero or postnatal lethality of certain germline null mutations, these genetic studies through germline mutagenesis have failed to reveal many of the physiological functions of RARs and RXRs, notably in adults. We conclude that spatiotemporally controlled somatic mutations generated in animal models in given cell-types/tissues and at chosen times during pre- and postnatal life, are required to reveal the physiological and pathophysiological functions of the receptor genes involved in the retinoid signaling pathway throughout the life of the mouse.

*Report from a SCOPE/IUPAC project: Implication of Endocrine Active Substances for Human and Wildlife (J. Miyamoto and J. Burger, editors). Other reports are published in this issue, *Pure Appl. Chem.* **75**, 1617–2615 (2003).

‡Corresponding author: Tel.: 33 3 88653213, Fax: 33 3 88 653203, E-mail: chambon@igbmc.u-strasbg.fr.

## INTRODUCTION: THE BASICS OF RETINOID SIGNALING

Both clinical and experimental approaches have revealed that vitamin A (retinol) and its biologically active derivatives (collectively referred as to retinoids), notably retinoic acids, exert a wide variety of profound effects on vertebrate embryonic morphogenesis and organogenesis, cell proliferation, differentiation and apoptosis, homeostasis, as well as in their disorders (reviewed in refs. [1–6]). In the 1930s, Hale initially showed that vitamin A deficiency (VAD) induces ocular malformations in newborn pigs [7]. Subsequently, the group headed by Warkany demonstrated that fetuses from VAD rats exhibit a large array of congenital malformations affecting the eyes, the myocardium and heart outflow tract, the diaphragm, as well as the respiratory and urogenital systems (the fetal VAD syndrome, see Tables 1 and 2; reviewed in ref. [8]). Dietary deficiency studies also showed that vitamin A is indispensable throughout postnatal development and adult life for growth, survival, reproduction, vision, and also for the homeostasis of numerous tissues. Indeed, widespread squamous metaplasia of glandular and transitional epithelia, degeneration of the seminiferous tubules and of the retina, are hallmarks of the postnatal VAD syndrome [9]. Retinoic acid (RA), the most biologically active naturally occurring retinoid, can both prevent and rescue the defects caused by a VAD diet in adult animals with the exceptions of night-blindness and retinal degeneration [3,10]. It was also shown recently that RA could replace vitamin A during embryogenesis, at least at certain stages and in certain organs [11,12].

How these structurally simple molecules can exert such pleiotropic effects was a long-standing question which found its solution with the discovery of two classes of nuclear ligand-dependent transcriptional regulators that belong to the superfamily of nuclear receptors (NRs), the three retinoic acid receptors isotypes (RARα, β, and γ, that bind all-trans and 9-cis RAs) and the three retinoid X receptor isotypes (RXRα, β, and γ, that selectively bind 9-cis RA), (for refs. see [13]). RARs and RXRs exhibit the conserved modular structure of NRs (reviewed in refs. [14,15], which can be divided into six variably conserved homology regions A to F. In vitro studies, performed with either cell-free systems or cultured cells co-transfected with vectors overexpressing the different RARs and RXRs together with recombinant reporter genes, demonstrated that RARs and RXRs bind as RAR/RXR heterodimers to DNA response elements of RA-responsive genes, and also allowed the determination of the functions of the A to F regions [13]. It was notably shown that the highly conserved centrally located region C corresponds to the core of the DNA-binding domain (DBD), while region E is functionally complex, as it contains the ligand-binding domain (LBD), a surface for RAR/RXR heterodimerization and, in the case of RARs, a transcriptional silencing domain that binds corepressors. The LBD also contains the ligand-dependent transactivation function AF-2 that requires the integrity of a highly conserved amphipathic helix, the AF-2AD core that corresponds to helix 12 located at the C-terminal end of the LBD [13]. Agonistic ligand binding induces a major structural change in the conformation of helix 12, which creates a new LBD surface for binding of coactivators, while corepressors are released [13,16–19 and refs. therein]. In addition, the amino-terminal A/B region contains the ligand-independent transcriptional activation function AF-1.

For each RAR isotype, there are several isoforms that differ from one another in their N-terminal region A. These isoforms arise from the differential usage of two promoters (of which the downstream one, P2, is RA-inducible) and alternative splicing. There are two major isoforms for RARα (α1 and α2) and for RARγ (γ1 and γ2), and four major isoforms for RARβ (β1 and β3 initiated at the P1 promoter, and β2 and β4 initiated at the P2 promoter) [13,14, and refs. therein]. Similarly, several major isoforms differing from another in their N-amino terminal region have been identified for RXRα (α1 and α2), RXRβ (β1 and β2). and RXRγ (γ1 and γ2) [for refs., see 13]. For a given receptor isoform, the AF-1 and AF-2 activities synergize and exhibit some specificity that is dependent on both the cell-type and the promoter-context of RA-responsive genes [20,21]. Synergistic transcriptional activation has also been observed between RAR and RXR partners, indicating that RXRs are not a priori transcriptionally silent partners in RAR/RXR heterodimers [13,22, and refs. therein]. However, at least in some instances the ligand-dependent activity of RXR is "subordinated" to ligand binding to its RAR partner [23]. Such

an RXR subordination may also apply to other NRs that use RXRs promiscuously as heterodimerization partners, in order to avoid confusion between retinoid and other signaling pathways. Interestingly, this appear to be the case for the thyroid hormone and vitamin D3 signaling pathways, as ligand binding to TR and VDR is a prerequisite for the RXR partner to respond to its agonistic ligand. However, in the case of other NRs for which RXRs act as heterodimeric partners (e.g., FXR, LXRs, and PPARs), the RXR ligand-induced activity appears to be "permissive", i.e., RXR agonists can autonomously activate transcription through the corresponding heterodimers [see 13,15].

The high degree of conservation of the various RAR or RXR isoforms across vertebrate evolution, as well as their selective spatio-temporally expression patterns in developing embryos and adult tissues (for refs., see [14,24,25]), led to the initial suggestion that each RAR and RXR isoform may perform unique functions, thus accounting for the highly diverse effect of retinoic acid throughout vertebrate life [14]. Results of cell-free and cellular in vitro studies led to the further proposal that the highly pleiotropic effects of RA reflect a highly combinatorial mechanism in which the multiple actors (the heterodimers and their coregulators) differentially transduce retinoid signals to selectively control the expression of numerous sets of RA target genes [13,14].

A genetic dissection of the retinoid signaling pathway was obviously required to investigate the in vivo relevance of the above in vitro-characterized molecular mechanisms, and to determine the physiological functions of the multiple retinoid receptors. As genetic analyses in the mouse are tedious, and also because their interpretation at the molecular level could be equivocal due to difficulties in discriminating between cell-autonomous and non-cell-autonomous events in the intact animal, we first chose the RA-responsive F9 murine embryonal carcinoma (EC) cell line as a cell-autonomous model system for analyzing RA signaling under in vitro conditions that mimic, at least to some extent, physiological processes occurring during early embryogenesis (reviewed in ref. [26]). Combining a genetic strategy (targeted mutagenesis of RARs and RXRs through homologous recombination, followed by re-expression of wild-type or mutant receptors in rescued lines), and a pharmacological strategy using RAR isotype- and panRXR-selective synthetic retinoids, established that (i) RXR/RAR heterodimers are the functional units that selectively mediate RA-induced differentiation, growth arrest, and target gene expression in F9 cells; (ii) the AF-1 and AF-2 activation functions of RARs and RXRs act synergistically and selectively to transcriptionally control the physiological and molecular responses of F9 cells to RA; (iii) the AF-2 ligand-dependent transcriptional activity of RXRs is subordinated to ligand binding to their RAR heterodimeric partners; (iv) RAR and RXR gene knock-out may generate artefactual conditions unmasking potential functional redundancies between RAR or RXR isotypes, which do not exist under wild-type conditions. In other words, in these cases, suppression or mutation of a given RAR or RXR can be functionally compensated by another RAR(s) or RXR(s), respectively. In addition, it was found that phosphorylation within the AF-1 activation domain of RAR$\gamma$ is required for primitive endodermal differentiation of F9 EC cells, while phosphorylation of RAR$\alpha$ in the LBD is required for parietal endodermal differentiation, leading to the conclusion that, through binding of cognate ligands and phosphorylation of their activation domains, retinoid receptors act as highly sophisticated signal transducers, integrating signals belonging to distinct signaling pathways involving both membrane and nuclear receptors.

In the present review, we summarize and discuss what has been learnt from the genetic dissection of the retinoid signaling pathway under truly physiological conditions, i.e., at the organismal level, in the mouse, during development, and postnatally. We focus on two main questions: (i) Where and when are RARs and RXRs involved in the transduction of the retinoid signals in the mouse? To that end, we review the phenotypes of mice in which the various RARs and RXRs have been ablated, and compare them to those of mice which have been under a VAD diet, in order to determine in which developmental or homeostatic events the various RARs and RXRs are instrumental. (ii) To what extent do the results of the genetic dissection of the retinoid signaling pathways in the mouse support the molecular mechanisms underlying the transduction of the RA signal by retinoid receptors, as they have been deduced from in vitro acellular and cellular studies?

## GENETIC EVIDENCE THAT RARs TRANSDUCE RETINOID SIGNALS IN VIVO: RAR (α, β, AND γ) NULL MUTANT MICE DISPLAY SEVERAL ASPECTS OF THE POSTNATAL VAD SYNDROME AS WELL AS SOME CONGENITAL ABNORMALITIES

### *RARα inactivation results in a spermatogenetic impairment and reappearance of an atavistic trait*

While RARα1 and RARα2 null mutants are apparently normal, RARα null mutants males are sterile due to a low production of spermatozoa [27,28, and our unpublished results] (Table 1). The Sertoli cell is the main target of the RARα null mutation, as the RARα protein is normally readily detectable in these cells, but not in germ cells [28,29]. Spermatogenesis is complete in adult mutants testes, which nevertheless display a degeneration characterized by the presence of large and empty vacuoles located between Sertoli cells. These vacuoles probably appear as a consequence of a chronic loss of round spermatids, which are prematurely released into the lumen of the mutant seminiferous tubules, before the completion of their maturation phase (spermiogenesis). The nature of the seminiferous epithelium lesions suggests that a signaling pathway mediated by RARα favors cell cohesiveness within the seminiferous epithelium. Interestingly, a RA-inducible gene, *Stra6*, encodes an integral membrane protein of Sertoli cells whose spermatogenetic cycle-dependent expression is lost in testes of RARα null mutants [30]. The onset of the testicular degeneration is variable: in some RARα null testes, numerous vacuoles are already present at P10 [postnatal day 10, i.e., before completion of puberty (around P35)], whereas other mutant testes still appear histologically normal at six weeks of age (our unpublished results). A second intriguing feature of adult RARα null testes is the patchy distribution of the lesions: seminiferous tubules containing a full complement of germ cells are often found adjacent to tubules composed almost exclusively of Sertoli cells [28]. This variability suggests that RARβ and RARγ, which are also detected in Sertoli cells [31,32], partially compensates for the lack of RARα, and that their activity varies stochastically among RARα null mutant males, and also between the seminiferous tubules of a given RARα null testis. Functional compensation by RARβ and RARγ probably also accounts for the observation that the RARα null testicular phenotype mimics a state of mild VAD, as opposed to a severe and prolonged VAD, which yields testes devoid of germ cells [33].

Aside from this completely penetrant testis degeneration, some RARα null mutants show some congenital defects (Table 1), which include interdigital webbing and a supernumerary skeletal element connecting the incus (a middle ear ossicle) with the alisphenoid bone (a braincase bone). This latter congenital abnormality corresponds to an atavistic trait, defined as the reappearance of a character that was lost during evolution, namely the upper jaw (or pterygoquadrate) cartilage present in reptilian ancestors of mammals. The occurrence of several other atavistic features in RAR single and double null mutants strongly support the view that modulation of RA signaling has been employed during vertebrate evolution as a mean to modify skull shapes and functions (see refs. [34–37]).

### *RARγ inactivation causes epithelial defects and some congenital malformations*

RARγ males are sterile but, contrary to RARα null mice, their spermatogenesis is normal [38] (Table 1). This sterility results from the transformation of the glandular epithelia of the seminal vesicle and prostate (which normally produce essential fractions of the seminal fluid) into epithelia resembling epidermis. Aside from sterility, this aberrant cell differentiation process known as "squamous metaplasia" is responsible for severe genito-urinary tract infections, and extends, upon aging, to other epithelia, namely those of the epididymis, urinary bladder, urethra, and salivary glands [33]. As squamous metaplasia is one of the hallmarks of the postnatal VAD syndrome (see Introduction), RARγ probably plays a crucial role in the maintenance of all epithelia that require RA in the mouse. Along these lines, it is noteworthy that keratinization of the tracheal and conjunctival epithelia, which are hallmarks of rat VAD, are not seen in models of VAD mice, such as CRBPI (cellular retinol binding protein one) and RBP (retinol binding protein) null mutants raised on VAD diets, possibly reflecting species-specific differences in RA tissue requirements [33, and our unpublished results]. Likewise, RARγ null mice and VAD mice, surprisingly, do not develop the corneal ulcerations found in the human VAD [39].

**Table 1** Postnatal manifestations of RAR and RXR knock-outs. CD: congenital defects; PnVAD: abnormalities present in postnatal VAD.

| Genotypes | Abnormalities | Refs. |
|---|---|---|
| RARα1$^{-/-}$ | None | [27,28] |
| RARα2$^{-/-}$ | None | our unpublished results |
| RARα$^{-/-}$ | Growth retardation (PnVAD); male sterility (testis degeneration[a]; PnVAD); impaired alveolar formation. CD: webbed digits; homeotic transformations and malformations of cervical vertebrae; pterygoquadrate cartilage; malformation of the squamosal bone; malformed laryngeal (i.e., cricoid) cartilage | [28,35,38,42,101] |
| RARβ1$^{-/-}$/RARβ3$^{-/-}$ | None | [44] |
| RARβ2$^{-/-}$/RARβ4$^{-/-}$ | CD: persistence and hyperplasia of the primary vitreous body (fetal VAD) | [43] |
| RARβ$^{-/-}$ | Growth retardation (PnVAD); behavioral defects; altered alveolar formation. CD: homeotic transformations and malformations of cervical vertebrae; persistence and hyperplasia of the primary vitreous body (fetal VAD) | [44–46,81,98,99] |
| RARγ1$^{-/-}$ | Growth deficiency (PnVAD). CD: malformations of cervical vertebrae[a]; malformed laryngeal (i.e., cricoid) cartilage; abnormal differentiation of granular keratinocytes[a] | [42], our unpublished results |
| RARγ2$^{-/-}$ | None | [38] |
| RARγ$^{-/-}$ | Growth deficiency (PnVAD); male sterility (squamous metaplasia of the seminal vesicle and prostate gland epithelia[a] (PnVAD); squamous metaplasia of other epithelia (PnVAD); impaired alveolar formation. CD: webbed digits; homeotic transformations and malformations of cervical vertebrae[a]; malformed laryngeal cartilages and tracheal rings[a]; agenesis of the Harderian glands; agenesis of the metoptic pillar of the skull; abnormal differentiation of granular keratinocytes[a] | [35,38,41,100], our unpublished results |
| RARβ/RARγ1$^{-/-}$ | Hydronephrosis | [42] |
| RARβ2$^{-/-}$/RARγ2$^{-/-}$ | Dysplasia and degeneration[a] of the retina. CD: persistence and hyperplasia of the primary vitreous body (fetal VAD); blepharophimosis[a]; partial agenesis of the sclera and choroid[a] | [43] |
| RARβ1$^{-/-}$/RARβ3$^{-/-}$ /RARγ$^{-/-}$ | Degeneration of the retina. CD: persistence and hyperplasia of the primary vitreous body (fetal VAD); partial agenesis of the sclera and choroid[a] | [44] |
| RXRα$^{+/-}$ | Growth retardation (PnVAD). CD: webbed digits | [24,47] |
| RXRβ$^{-/-}$ | Male sterility[a] (defective spermatogenesis); abnormal lipid metabolism in Sertoli cells; behavioral defects | [45,83] |
| RXRγ$^{-/-}$ | Metabolic and behavioral defects | [45,46,81,84] |

[a]These abnormalities are completely penetrant. RARα1$^{-/-}$, RARα2$^{-/-}$, RARβ1$^{-/-}$, RARβ2$^{-/-}$, RARβ3$^{-/-}$, RARβ4$^{-/-}$, RARγ1$^{-/-}$, and RARγ2$^{-/-}$ refer to isoform-specific knock-outs.

RARγ null newborns are readily distinguishable from their littermates by the glossy appearance of their skin [40]. Their flat epidermal surface, accounting for this characteristic external aspect, likely reflects a deficit in corneodesmosomes which normally provide cell cohesion within the stratum

corneum. Moreover, lamellar bodies in the mutant epidermal granular layer are morphologically abnormal and fail to be properly exocytosed, resulting in an accumulation of abnormal vesicles in extracellular spaces and uneven deposition of lipids in the stratum corneum (our unpublished results). These defects persist in adults, and are reminiscent of those observed in cases of human congenital ichthyoses and in elderly skin xerosis. RA is a widely used therapeutic agent for skin diseases. In this context, it is noteworthy that the subtle role of RARγ in the differentiation of granular keratinocytes, which is revealed only by careful electron microscopic analysis of the RARγ null mutants, probably represents the only physiological functions of the whole RAR family in the homeostasis of the mouse resting epidermis [41].

RARγ null newborns also display laryngeal and tracheal cartilage malformations, as well as interdigital webbing (Table 1). The Harderian glands, which provide for lubrification of the eyelids are often missing in RARγ null mutants. Moreover, all of these mutants (as well as some RARα and RARβ mutants) display homeotic transformations of cervical vertebrae, indicating that signaling through RARs is required for the patterning of the antero-posterior body axis during somite formation [35,38] (Table 1).

Interestingly, mice lacking specifically the RARγ1 isoform display the congenital skin defect, as well as vertebral and laryngeal malformations characteristic of the RARγ null mutation (all isoforms disrupted), but not the squamous metaplasia, interdigital webbing, and Harderian gland agenesis of RARγ null mutants [38,42]. In contrast, RARγ2 null mutants are apparently normal [38,42] (Table 1). Thus, RARγ1 is the main RARγ isoform involved in several developmental processes, postnatal growth and skin homeostasis, whereas any of the two isoforms can perform the RARγ functions required for the maintenance of genito-urinary tract and glandular epithelia, and for the involution of the interdigital mesenchyme .

### *RARβ null mutants display the most frequent congenital VAD abnormality and a locomotor deficiency*

Almost all RARβ null mutants display a persistent hyperplastic primary vitreous body (PHPV or retrolenticular membrane), consisting in an abnormal mass of fibro-vascular tissue which is interposed between the lens and the retina, and representing the most frequent malformation of the fetal VAD syndrome [8,35] (Table 1). The RARβ null mutants PHPV can be ascribed to a lack of RARβ2/β4 isoform activity, as it is also observed in almost all RARβ2/β4 null mutants [43]. In contrast, specific inactivations of RARβ1 and RARβ1/β3 isoforms do not yield morphological defects [44].

Abundant RARβ protein is detected in the adult central nervous system, notably in the striatum which is involved in the control of voluntary movements. Despite the apparent normality of this structure at the morphological level, all RARβ mutants display severely impaired abilities in locomotion and motor coordination tests [45]. Performance deficits in spatial learning, possibly unrelated to the visual impairment caused by the PHPV, and alterations of hippocampal synaptic plasticity have also been reported in RARβ null mutants [46].

## SIGNALING THROUGH RARs IS INDISPENSIBLE, AT MANY DISTINCT DEVELOPMENTAL STEPS, FOR EMBRYONIC PATTERNING AND SHAPING, AND ORGANOGENESIS

As reviewed above, RARα, RARβ, and RARγ null mutant mice altogether display some aspects of the postnatal and fetal VAD syndromes (Table 1). However, RAR (α, β, or γ) single null mutant mice abnormalities are confined to a small subset of the tissues normally expressing these receptors during embryogenesis and adulthood, probably reflecting the existence of functional redundancies between RARs [discussed in 3,22,47,48]. To test this hypothesis, mutants lacking a pair of RAR isotypes (RARαβ, RARαγ and RARβγ double null mutants) or two or more isoforms belonging to distinct isotypes were generated. For the sake of clarity, only abnormalities displayed by double null mutants lacking a cou-

ple of RAR isotypes (all isoforms deleted) are listed in Tables 2 and 3. Similar abnormalities, albeit often less penetrant, which are displayed by "isoform-specific" double null mutants are listed in [3] (RARα1/RARβ2/4, RARα1/RARγ, RARα1α2$^{+/-}$/RARγ and RARβ2/4/RARγ mutants), in [42] (RARα/RARγ1 and RARα/RARγ2 mutants), in [49] (RARα1/RARβ mutants), [44] (RARα/RARβ1/3 and RARβ1/3/RARγ mutants), and in [43] (RARβ2/RARγ2 mutants).

**Table 2** Abnormalities of the fetal VAD syndrome present in RARβ null mutants (β), RXRα null mutants, and RAR(α, β, and γ) double null mutants (α/β, α/γ, and β/γ).

| Abnormalities of the fetal VAD syndrome | Genotypes of RAR null mutants showing similar abnormalities | Abnormalities observed in RXRα null mutants |
|---|---|---|
| **Respiratory system defects** | | |
| • Agenesis or hypoplasia of the left lung | α/β[a] | No |
| • Hypoplasia of the right lung | α/β[a] | No |
| • Agenesis of the oesophago-tracheal septum | α/β[a] | |
| • Diaphagmatic hernia | α/β | No |
| **Hypoplasia of the ventricular myocardium** | α/γ | Yes[a] |
| **Heart outflow tract defects** | | |
| • Persistent truncus arteriosus | α/β[a], α/γ[a] | No |
| • High interventricular septal defect or double outlet right ventricle (E18.5)/conotruncal septum defect (E14.5) | α/β[a], α/γ[a], β/γ | Yes |
| • Abnormal great arteries derived from aortic arches | α/β[a], α/γ[a], β/γ | No |
| **Kidney hypoplasia** | α/β[a], α/γ[a] | No |
| **Ureteral defects** | | |
| •Agenesis | α/β, α/γ[a] | No |
| •Ectopia | α/β, β/γ | No |
| **Genital tract defects** | | |
| – **Female** • Agenesis of the oviduct and uterus (E18.5)/agenesis of the Müllerian duct (E14.5) | | |
| – Complete | α/β[a] | No |
| – Partial | α/γ[a] | No |
| • Agenesis of the cranial vagina | α/β[a], α/γ[a] | NA |
| – **Male** • Agenesis or dysplasia of the vas deferens | α/γ[a] | NA |
| • Agenesis of the seminal vesicles | α/γ[a] | NA |
| **Ocular defects** | | |
| • Coloboma of the retina | α/γ[a] | No |
| • Coloboma of the optic disc | β/γ, α/γ[a] | Yes |
| • Persistence et hyperplasia of the primary vitreous body (PHPV) | β, α/β[a], β/γ[a], α/γ[a] | Yes[a] |
| • Hypoplasia of the conjunctival sac | β/γ[a], α/γ[a] | Yes[a] |
| • Thickening of the corneal stroma | β/γ[a], α/γ | Yes[a] |
| • Ventral rotation of the lens | β/γ[a] | Yes[a] |
| • Shortening of the ventral retina | β/γ[a] | Yes[a] |

[a]This abnormality is completely penetrant. NA not applicable as the corresponding structure is normally not found at E14.5, the time around which RXRα null mutants die. From refs. [24,34,35]. Note that most of the abnormalities seen in RARα/RARβ double null mutants occur at similar frequencies in RARα/RARβ2 mutants [50].

**Table 3** Abnormalities absent from the fetal VAD syndrome are found in RAR(α, β, or γ) single null mutants (α, β, γ), and RAR(α, β, and γ) double null mutants (α/β, α/γ, and β/γ).

| Congenital abnormalities not associated with the fetal VAD syndrome | Genotypes of RAR null fetuses showing these defects |
|---|---|
| **Nervous system defects** | |
| • Exencephaly | α/γ |
| • Agenesis of the corpus callosum | $\alpha/\gamma^a$ |
| **Skeletal defects** | |
| • Agenesis or multiple malformations of cranial skeletal elements | $\alpha/\gamma^a$ |
| • Homeotic transformation and malformations of cervical vertebrae | α, β, γ, $\alpha/\gamma^a$, $\alpha/\beta^a$, $\beta/\gamma^a$ |
| • Agenesis and malformations of limb bones | $\alpha/\gamma^a$ |
| • Reappearence of atavistic skeletal elements | |
| – Antotic pillar | $\alpha/\gamma^a$ |
| – Ptrerygoquadrate cartilage | α, $\alpha/\gamma^a$, α/β |
| **Eye defects** | |
| • Corneal-lenticular stalk | α/γ |
| • Agenesis of the lens | α/γ |
| **Glandular defects** | |
| • Agenesis or dysplasia, of the sub-maxillary and sub-lingual glands and/or of their excretory ducts | $\alpha/\gamma^a$, $\beta/\gamma^a$ |
| • Agenesis of the Harderian gland | γ, $\alpha/\gamma^a$, $\beta/\gamma^a$ |
| • Agenesis or ectopia of the thyroid, thymus and parathyroid glands | α/β, α/γ |
| **Other defects** | |
| • Webbed digits | α, γ, $\beta^{+/-}/\gamma^a$, $\beta/\gamma^a$ |
| • Abnormal laryngeal cartilages and tracheal rings | α, $\gamma^a$, $\alpha/\gamma^a$, $\alpha/\beta^a$, $\beta/\gamma^a$ |
| • Kidney agenesis | α/γ |
| • Agenesis of the anal canal | $\alpha/\beta^a$ |

[a]This abnormality is completely penetrant. From refs. [34,35,38,42]. Note that most of the abnormalities seen in RARα/RARβ double null mutants occur at similar frequencies in RARα/RARβ2 mutants [50].

RARαβ, RARαγ and RARβγ double null mutants, as well as double mutants for RAR isoforms belonging to different isotypes (with the exception of RARβ2/RARγ2) die in utero or at birth because of severe developmental defects that altogether include the complete spectrum of malformations corresponding to the classical fetal VAD-induced syndrome reported by Warkany's group 50 years ago [8] (Table 2). As RAR single mutants (see Table 1 and above), RAR double null mutants (Table 3) also exhibit congenital abnormalities that were not described in the classical fetal VAD studies, ranging from ageneses of the Harderian glands to skeletal defects of the skull, face, vertebrae and limbs [34,35,38,42,49,50]. The occurrence of these "non-VAD" defects in RAR double null mutant mice is most probably accounted by the difficulty to achieve, by dietary deprivation, a state of profound VAD compatible with pregnancy (see above). In fact, almost all these "non-VAD" defects have been subsequently produced in rodent embryos (i) deficient in vitamin A, but supplemented with RA [11,12,51]; (ii) lacking the RA synthesising enzymes RALDH2 (retinaldehyde dehydrogenase 2) or RALDH3 [52–56; and our unpublished results] or (iii) treated with synthetic retinoids possessing panRAR antagonistic activities [57–59].

The comparison of the RAR double null mutants phenotypes with those of rodents carrying the aforementioned blocks in RA signal transduction, demonstrate that liganded RARs play crucial roles at

many distinct stages of the development of numerous organs [3, and refs. therein]. For example, the severe malformations found in RARαγ double null embryos are similar to those of RALDH2 null embryos, and reflect early roles of RAR signaling in axial rotation, mesoderm segmentation and closure of the hindbrain, formation of otocysts, pharyngeal arches, and forelimb buds, as well as in closure of the primitive gut [58]. RARs are also indispensable for the ontogenesis of (almost) all the anatomical structures that are derived from mesectodermal cells, i.e., the cranial neural crest cells (NCCs) that give rise to mesenchymal derivatives (reviewed in refs. [3,36,37], and see below). RARs are involved in antero-posterior patterning of the somitic mesoderm and hindbrain neurectoderm [34,35,38,58,60] through controlling homeobox gene expression [60–63], as well as in the establishment of the antero-posterior axis of the limbs [22,34,64]. RARs are required for the development of a large number of eye structures (Tables 2 and 3) and for retinal histogenesis [34,35,43], cardiomyocyte differentiation [24,65], as well as for the control of apotosis in the retina [43], the frontonasal and interdigital mesenchymes [34,35,64], and conotruncal segment of the embryonic heart [44]. In the embryonic urogenital tract, RARs control epithelial-mesenchymal interactions in the kidney through expression of the receptor tyrosine kinase Ret [66–68], as well as the formation of the genital ducts and ureters [35,50,68]. RARs also regulate distinct steps of lung morphogenesis and are required for the partitioning of the primitive foregut into oesophagus and trachea [69, and see below].

Due to the apparent functional redundancy between RARs in RAR knocked-out mice (discussed in refs. [3,22,47,48]), the number of organs that require RA for their development might be underestimated from the panel of malformations displayed by RARαβ, RARαγ, and RARβγ isotype or isoform double mutants (Tables 2 and 3). In this respect, it is noteworthy that the developing tooth, one of the favorite models for the study of morphogenetic epithelial-mesenchymal interactions, expresses a specific RA-metabolizing enzyme (P. Dollé, personal communication) and critically requires RA in organ cultures [70,71], although it is not obviously altered in any of the RAR double null embryos.

## RXRα IS ESSENTIAL FOR TRANSDUCING RA SIGNALS NECESSARY FOR THE DEVELOPMENT OF THE MYOCARDIUM AND EYE STRUCTURES

All RXRα null mutants display an hypoplasia of the compact layer of the ventricular myocardium manifested on histological sections by thin and spongy ventricular walls. This defect appears to be the main cause of mutant death, occurring by cardiac failure around E14.5 [24,65,72] (Table 2). A similar hypoplasia of the myocardium is observed in VAD fetuses, and in some RAR double null fetuses [8,50], suggesting that RXRα is involved in the transduction of an RA signal required for myocardial growth. Additionally, as early as E8.5, the most peripheral ventricular cardiomyocytes in the heart of RXRα null mutants show precocious features of differentiation, such as myofibril striation, indicating that RXRα normally plays an early role in the differentiation of these cells. However, the aforementioned cell differentiation defect, which is also exhibited by RARα null mutants, is not sufficient to account for the hypoplasia of the myocardium [65]. The requirement of RXRα for myocardial growth is unlikely to be cell-autonomous. Indeed, breeding of a transgenic mouse line specifically overexpressing an RXRα protein in cardiomyocytes onto an RXRα null genetic background does not prevent the hypoplasia of the ventricular myocardium and fetal lethality associated with the RXRα null genotype, even though the transgene is expressed in the ventricles as early as E10.5 [73]. Recent data suggest that RXRα located in the adjacent epicardium may act on myocardial growth through a paracrine mechanism [74]. In addition to the abnormal histogenesis of the ventricular myocardium, downregulation of genes involved in general metabolism in RXRα null embryos might participate in their death by cardiac failure [75].

About one-third of the RXRα null mutants lack the conotruncal septum, which normally divides the embryonic heart outflow tract (or conotruncus) into the intracardiac portions of the aorta and pulmonary trunk [24]. Interestingly, deficiencies of this septum represent both a classical VAD defect in rodents and a leading cause of human congenital heart defects, ranging from high interventricular sep-

tal defects to double outlet right ventricle (DORV). The agenesis of the conotruncal septum in RXRα null mutants appears secondary to an enhanced rate of cell death in both the mesenchymal cells of the conotruncal ridges and the parietal conotruncal cardiomyocytes, therefore indicating that RXRα is required for the transduction of the RA signal that controls apoptosis in the conotruncal segment of the embryonic heart [44].

In addition to heart defects, all fetuses lacking RXRα show a characteristic ocular syndrome associating a PHPV, closer eyelid folds, thickened ventral portion of the corneal stroma, ventral rotation of the lens, agenesis of the sclera, and a shorter ventral retina [24] (Table 2). As similar defects are present in VAD fetuses and in RARβγ double null mutants [8,35] (Table 2), RXRα appears essential to transduce the RA signals required for several ocular morphogenetic processes, notably the formation of the ventral retinal field. Interestingly, this later event critically requires the activity of a specific retinaldehyde dehydrogenase, RALDH3 [76, and our unpublished results].

That all the congenital defects exhibited by RXRα null fetuses are also observed in RAR single or double null mutants provided the first genetic evidence of a convergence between RAR and RXR signaling pathways, and also gave the first clue that RXRα/RAR heterodimers are the functional units that transduce RA signals in vivo (see below).

## RXRs CAN BE TRANSCRIPTIONALLY ACTIVE, AND THE AF-1-CONTAINING A/B REGION AND THE AF-2 TRANSCRIPTIONAL ACTIVATION FUNCTION OF RXRα ARE DIFFERENTIALLY INVOLVED IN OCULAR MORPHOGENESIS AND IN MYOCARDIAL GROWTH

Whether RXRs are transcriptionally active within RAR/RXR heterodimers has been a controversial issue in in vitro studies (see Introduction). To determine the roles played by RXRα AF-1 and AF-2 activities in vivo, mouse mutants were engineered that express truncated RXRα proteins lacking either (i) most of the RXRα N-terminal A/B region that includes AF-1 (RXRα*af1*$^o$ mutants [77]), or (ii) the C-terminal 18 amino acid-long sequence of the RXRα protein that includes the core of the activating domain of the activation function 2 (AF-2AD core) (RXRα*af2*$^o$ mutants [22]), or (iii) both AF-1 and AF-2 activities (RXRα*af*$^o$ mutants; Mascrez et al., unpublished results).

RXRα*af2*$^o$ mutant mice occasionally display the hypoplasia of the myocardium and the ocular syndrome characteristic of the RXRα null syndrome, whereas RXRαaf1$^o$ mutants never display RXRα-like developmental defects apart from a small and weakly penetrant PHPV [22,77]. The low frequency in RXRα*af2*$^o$ mutants, and near-absence in RXRα*af1*$^o$ mutants, of defects that are fully penetrant in RXRα null mutants could reflect a functional compensation by RXRβ and/or RXRγ (note that RXRβ null, RXRγ null, as well as RXRβγ double null mutants develop normally; see below). This seems, indeed, to be the case for RXRα*af2*$^o$ mutants as (i) the frequency of their ocular syndrome increases from less than 15 to 100 % upon further inactivation of RXRβ (which has no effect on its own), and (ii) the frequency of their myocardial defect increases from 5 to about 50 % upon additional inactivation of either the RXRβ gene, or of both the RXRβ and the RXRγ genes, yielding RXRα*af2*$^o$/RXRβ null and RXRα*af2*$^o$/RXRβ null/RXRγ null fetuses, respectively [22]. On the other hand, PHPV is the only defect of the RXRα null syndrome whose frequency is increased in RXRα*af1*$^o$/RXRβ null/RXRγ null mutants, when compared to RXRα*af1*$^o$ mutants [77].

Even though it can be achieved only in an impaired genetic background, the full penetrance of the RXRα null ocular phenotype that is obtained in RXRα*af2*$^o$/RXRβ null mutants (and also in RXRα*af*$^o$ mutants; [22] and unpublished results), supports the view that the RXRα AF-2 activity (and thus possibly 9-cis RA) is indispensible for ocular morphogenesis. In contrast, involution of the primary vitreous body, the developmental process which is likely to require the highest concentration of RA-liganded receptor (as it is the event the most sensitive to VAD) [8], requires the additional integrity of the RXRα A/B domain, while in a wild-type this latter domain is dispensible for the other RA-dependent ocular

morphogenesis event. These interpretations are supported by the observations that RXRα null-like ocular abnormalities: (i) affect 100 % of RXRα*af1*$^o$/RAR(β null or γ null) double mutants, whereas (with the exception of the PHPV) they are all absent in the corresponding single null mutants and (ii) are rare in RXRα*af2*$^o$ mutants, but fully penetrant in RXRα*af2*$^o$ mutants, which additionally lack the AF-1 containing RXRα A/B region. Altogether, these data strongly support the view that the activation functions of RXRα are required for normal eye development, and also that, due to functional redundancy, the role played by RARα AF-1 and AF-2 can be revealed only in certain impaired genetic backgrounds (or RA insufficiency conditions).

On the other hand, the rare occurrence of hypoplasia of the myocardium in RXRα*af2*$^o$ and RXRα*af1*$^o$/RXRβ null/RXRγ null fetuses, and only in half of the RXRα*af2*$^o$/RXRβ null/RXRγ null fetuses, suggests that the transcriptional activity of RXRα is necessary for myocardial growth only in "unfavorable" genetic backgrounds. Along the same lines, we recently found that the vast majority (80 %) of RXRα*af*$^o$ fetuses display a normal heart histology (our unpublished results), thereby demonstrating that a transcriptionally "silent" RXRα can efficiently promote myocardial growth and ruling out the requirement for an RXR ligand in this developmental process.

Interestingly, RXRα*af2*$^o$ and RXRα*af*$^o$ mutants die at birth even though only few of them display a hypoplasia of the myocardium. Thus, both their lethality and severe growth retardation may be at least in part secondary to metabolic problems arising from placental defects (see below).

## SPECIFIC FUNCTION OF THE RXRα AF-1 DOMAIN-CONTAINING A/B REGION IN INVOLUTION OF INTERDIGITAL MESENCHYME

AF-2 appears to be more important than AF-1 for the function of RXR during embryonic development: RXRα*af2*$^o$/RXRβ null/RXRγ null fetuses all die in utero and display a large array of congenital defects, whereas RXRα*af1*$^o$/RXRβ null/RXRγ null mutants are often viable and display few congenital defects. Moreover, AF-2 but not AF-1 of RXRα is crucial for transcription of a RA responsive *lacZ* reporter transgene in the mouse [22,77]. Note, however, that all RXRα*af1*$^o$/RAR (α, β, or γ) compound mutants die in utero and exhibit a large array of malformations that nearly recapitulate the full spectrum of the fetal VAD syndrome, indicating that RXR AF-1 could nevertheless be instrumental to the transcriptional activity of RAR/RXR heterodimers, particularly under conditions of limiting concentrations of RA and/or RA receptors [77]. In this respect, it is noteworthy that RXRα*af2*$^o$/RXRβ double mutants die in utero at a late fetal stage (E14.5 to birth), whereas RXRα*af*$^o$/RXRβ mutants, that additionally lack AF-1, die at midgestation (E10.5) (our unpublished results). Interestingly, the RXRα A/B region, which supports the AF-1 activity, has also a unique role in the RA-dependent disappearance of the interdigital mesenchyme.

The first evidence implicating RA in the involution of the interdigital mesenchyme was provided by whole limb cultured in a RA-deprived medium [78]. Subsequently, it was shown that mice lacking both alleles of either the RARα or the RARγ genes, as well as mice heterozygous for the RXRα null mutation occasionally exhibit mild forms of interdigital webbing (soft tissue syndactyly) ([24,28,35,38]; Table 1). Surprisingly, this defect was absent in RARβ null mutants, even though RARβ is strongly and specifically expressed in interdigital necrotic zones (INZs) ([35]; and references therein). However, interdigital webbing is severe and completely penetrant upon disruption of one (or both) allele(s) of the RARβ gene in a RARγ null genetic background [35]. The persistence of the fetal interdigital mesenchyme is caused by marked decrease in programmed cell death, as well as by an increase of cell proliferation in the mutant INZs [64]. As RARβ and RARγ are not co-expressed in the INZs, involution of the interdigital mesenchyme must involve paracrine interactions between the interdigital mesenchyme (the site of RARβ expression), and either the cartilaginous blastema of the digits or the surface epidermis, which both express RARγ. RARβ/RARγ (RARβγ) compound mutants also display a specific downregulation of tissue transglutaminase (tTG) promoter activity and of stromelysin 3 expression in the interdigital mesenchyme [64]. The presence of putative retinoic acid response ele-

ments in the promoter regions of both tTG and stromelysin 3 genes suggests that RA might promote cell death in the INZs through a direct increase of tTG expression, and could also contributes to the tissue remodeling, which accompanies cell death through an increase of stromelysin 3 expression [64]. It has also been shown that the expression of the anti-death gene *BAG-1*, which is normally downregulated upon initiation of interdigital apoptosis, remains unaltered in the limbs of RARβγ double null mutants [79].

The RXRα A/B region is indispensable for the function of RXRα/RARβ and/or RXRα/RARγ heterodimers in the involution of the interdigital mesenchyme, as the majority of RXRα*af1*$^o$ mutants and all RXRα*af1*$^o$/RXRβ null/RXRγ null mutants display soft tissue syndactyly [77]. Moreover, this function selectively requires the RXRα A/B region, as RXRα*af2*$^o$ and RXRα*af2*$^o$/RXRβ null/RXRγ null mutants never display this defect [22]. Interestingly, phosphorylation of RXRα at a specific serine residue located in the A domain is necessary for the antiproliferative response of F9 teratocarcinoma cells to RA [26,80]. Therefore, within RXRα/RAR (β and γ) heterodimers, phosphorylation of the RXRα A domain may play an important function in the cascade of molecular events that, in vivo, leads to the normal disappearance of the interdigital mesenchyme.

## RETINOIC ACID SIGNALS ARE TRANSDUCED DURING DEVELOPMENT BY SPECIFIC RXRα/RAR(α, β, OR γ) HETERODIMERS

Compound mutants in which a null mutation of a given RAR isotype (α, β, or γ) is associated either with (i) a RXRα null mutation, (ii) a RXRα*af1*$^o$ mutation, or (iii) a RXRα*af2*$^o$ mutation, altogether recapitulate the abnormalities exhibited by RAR double null mutants [22,24,47,77] (Table 4, and see examples below). This synergism between RAR and RXRα loss-of-function mutations support the conclusion that RXRα/RAR heterodimers are the functional units that transduce RA signals during embryonic development. Moreover, RXRα is the functionally most important RXR during development, as the development of RXRβ/RXRγ double null mutants appears to be normal [81]. This last conclusion is further supported by a lack of synergism during development between RAR (either α, β, or γ) and RXRβ or RXRγ inactivations [47].

The analysis of the various RXRα/RAR compound mutants led to the identification of the heterodimers, which, in a given developmental process, are preferentially involved in transducing RA signals. For instance, hypoplasia of the myocardium is never seen in RAR(α, β, and γ) single null mutants nor in RXRα*af1*$^o$ single mutants, while it is found in less than 5 % of RXRα*af2*$^o$ single mutants. On the other hand, 45 % of RXRα*af1*$^o$/RARα null mutants (but none of the RXRα*af1*$^o$/RARβ null or RARγ null mutants) and 80 % of RXRα*af2*$^o$/RARα null mutants (but only 20 % of RXRα*af2*$^o$/RARβ null or RARγ null mutants) display this defect [22,77]. Thus, these genetic analyses indicate that RXRα acts on myocardial growth preferentially in the form of heterodimers with RARα.

Similarly, several lines of evidence indicate that, although all three RARs are expressed in developing ocular structures [82], RXRα acts on eye morphogenesis in the form of heterodimers with either RARβ or RARγ, but not with RARα. Firstly, there is a strong synergism between RXRα and RARβ or RARγ inactivations, which is manifested by a marked increase in the severity of the RXRα null ocular defects in RXRα/RARβ and RXRα/RARγ double null fetuses (Table 4) [47]. Secondly, there is also a strong synergism for the generation of RXRα null-like ocular defects between RARβ or RARγ inactivations and ablations of either RXRα AF-1 or RXRα AF-2. Indeed, apart from the PHPV, the RXRα null ocular syndrome is never present in RAR(α, β, and γ) single null mutants nor in RXRα*af1*$^o$ single mutants, and is found in only a minority (less than 15 %) of RXRα*af2*$^o$ single mutants. On the other hand, this ocular syndrome is observed in 100 % of RXRα*af1*$^o$/RAR(β null or γ null) and RXRα*af2*$^o$/RAR(β null or γ null) double mutants, whereas it is absent in RXRα*af1*$^o$/RARα null mutants, as well as in RXRα*af2*$^o$/RARα null mutants [22,77]. These results indicate that RXRα/RARβ and RXRα/RARγ heterodimers are instrumental in ocular morphogenesis.

**Table 4** Evidence that RXRα and RAR act synergistically on embryonic development: similar congenital defects absent (or very rare) in RXRα null, RARα*af2*$^{o}$ RXRα*af1*$^{o}$ and RAR (α, β, or γ ) null single mutants are observed in RXR (α null, α*af2*$^{o}$, or α*af1*$^{o}$)/RAR (α null, β null or γ null) and in RAR/RAR double null mutants.

| Abnormalities | Genotypes of RXR/RAR and RAR/RAR compound mutants showing similar defects | | |
|---|---|---|---|
| | RXRα/RAR (X/A) double null mutants | RAR/RAR (A/A) double null mutants | RXRα*af1*$^{o}$or RXRα*af2*$^{o}$/RAR(α, β, and γ) mutants |
| **Ocular defects (VAD)** | | | |
| Severe shortening or agenesis of the ventral retina | Xα/Aβ[b]; Xα/Aγ$^{+/-}$ [b]; Xα/Aγ[b] | Aβ/Aγ[b] | Xα*af2*$^{o}$/Aβ[b]; Xα*af2*$^{o}$/Aγ; Xα*af1*$^{o}$/Aβ; XX*af1*$^{o}$/Aγ[b] |
| **Respiratory system defects (VAD)** | | | |
| • Lung hypoplasia | Xα/Aα[a] | Aα/Aβ[b] | Xα*af2*$^{o}$/Aα[b]; Xα*af2*$^{o}$/Aβ; Xα*af1*$^{o}$/Aα[a] |
| • Agenesis of the esophagotracheal septum | Xα/Aα[a] | Aα/Aβ[b] | Xα*af2*$^{o}$/Aα; Xα*af2*$^{o}$/Aβ; Xα*af1*$^{o}$/Aα |
| **Heart outflow tract defects (VAD)** | | | |
| • Persistent truncus arteriosus | Xα/Aα$^{+/-}$; Xα/Aα[b], Xα/Aβ; Xα/Aγ | Aα/Aβ[b]; Aα/Aγ[b] | Xα*af2*$^{o}$/Aα; Xα*af1*$^{o}$/Aα |
| • Abnormal arteries derived from aortic arches | Xα/Aα$^{+/-}$; Xα/Aα[a], Xα/Aβ; Xα/Aγ | Aα/Aβ[b]; Aα/Aγ[b] | Xα*af2*$^{o}$/Aα; Xα*af2*$^{o}$/Aβ; Xα*af1*$^{o}$/Aα |
| **Urogenital system defects (VAD)** | | | |
| • Kidney hypoplasia | Xα/Aα[b] | Aα/Aβ[b] | Xα*af2*$^{o}$/Aα; Xα*af1*$^{o}$/Aα |
| • Complete agenesis of Müllerian ducts | Xα/Aα[b] | Aα/Aβ[b] | Xα*af1*$^{o}$/Aα |
| **Hypoplasia of the sub-maxillary gland** | Xα/Aγ[b] | Aα/Aγ[b] | Xα*af2*$^{o}$/Aγ[b] |
| **Skeletal defects** | | | |
| • Multiple cranio-facial defects | Xα/Aγ | Aα/Aγ[b] | Xα*af2*$^{o}$/Aγ[b] |
| • Limb defects | Xα/Aγ | Aα/Aγ[b] | Xα*af2*$^{o}$/Aγ |

[a]This abnormality is present in a majority of the mutants.
[b]This abnormality is completely penetrant. VAD, these abnormalities belong to the fetal vitamin A deficiency syndrome. From refs. [22,24,47,77].

## SELECTIVE FUNCTIONS OF RXRβ AND RXRγ IN THE CONTROL OF FERTILITY, METABOLIC PROCESSES, AND BEHAVIOR

RXRβ null mutant males are sterile due to abnormal spermatid maturation and release, leading to a severe number reduction, decreased mobility and high percentage of abnormalities of spermatozoa. As the RXRβ protein is only detectable in Sertoli cells, a dysfunction of these cells most probably accounts for the spermiogenetic defects in RXRβ null mutants. Moreover, large lipid droplets accumulate in the cytoplasm of RXRβ null Sertoli cells, suggesting functional interactions between RXRβ and nuclear receptor signaling pathways controlling lipid metabolism [83]. Interestingly, males lacking only the AF-2 activity of RXRβ (RXRβ*af2*$^{o}$) are fertile, but display a Sertoli cell-restricted lipid metabolic defect identical to that of the RXRβ null mutant males (our unpublished results). Altogether, these data demonstrate that within the Sertoli cell, RXRβ exert independent functions in spermiogenesis and lipid metabolism and, as lipid accumulations in tissues are never observed under VAD conditions, they also provide the first in vivo evidence that the AF-2 activation function of an RXR can be important for the transcriptional activity of heterodimers other than RXR/RAR.

RXRγ null mutants are fertile and morphologically indistinguishable from wild-type littermates. They exhibit a mild thyroid hormone resistance and an increased metabolic rate [84], and also show defects in cognitive functions [46]. Moreover, the recent observation that growth plate development is more severely impaired in RXRγ/VDR double null mutants than in VDR single null mutants, has sug-

gested that RXRγ/VDR heterodimers may be involved in the differentiation of hypertrophic chondrocytes [85].

Similarly to RARβ, RXRβ and RXRγ are expressed at high levels in the striatum, the main dopaminergic signaling organ controlling coordination of movements. Abnormal locomotor behaviors in RXRβ/RARβ, RXRγ/RARβ, and RXRβ/RXRγ double null mutant mice, and to a lesser extent in single null mutants of these receptors, are correlated with dysfunction of the mesolimbic dopaminergic signaling pathway. Indeed, expression of dopamine receptors types 1 and 2 (DR1 and DR2) in the ventromedial regions of the striatum of these double mutants are significantly reduced, and their response to cocaine, a modulator of dopamine signaling, is blunted [45]. The expression of D2R in the striatum of the mutant animals may be altered at the transcriptional level, as a functional RA response element has been characterized in the promoter of the corresponding gene [86]. That RXRβ/RARβ and RXRγ/RARβ heterodimers mediate retinoid signals required for the function of the mesolimbic dopaminergic system suggests that RA signaling defects may contribute to pathologies such as Parkinson's disease and schizophrenia.

## PLACENTATION FIRST REQUIRES A TRANSCRIPTIONALLY "SILENT" RXR, THEN AN ACTIVE RXRα

Embryos carrying null mutations of both RXRα and RXRβ (RXRαβ) double null mutants display a wide range of abnormalities resembling those of embryos carrying blocks in RA signaling, e.g., RALDH2 null and RARα/RARγ double null mutants (see above). However, they exhibit a unique lethality that occurs at midgestation and appears to be caused by a labyrinthine agenesis, i.e., the lack of formation of the labyrinthine zone of the chorioallantoic placenta [87]. In the normal placenta, this zone represents the main site of exchanges between mother and embryo. Labyrinthine agenesis is never associated with RAR single or double null mutant embryos, but a similar, although less severe abnormality (i.e., a labyrinthine hypoplasia) is seen in embryos lacking either PPARβ or PPARγ [88,89]. At later, fetal stages of gestation, the placenta of RXRα single null mutant displays a thickening of the labyrinthine trabeculae, which are interposed between maternal blood sinuses and fetal capillaries, and represent the placental barrier across which nutrient and gas exchanges between the maternal and fetal blood occur [90].

Altogether, these results indicate that RXRs are involved in placentation at two distinct steps. At E8.5, RXRα and/or RXRβ are required for the initial formation of the placental labyrinthine trabeculae from the chorionic plate. Between E14.5 and the term of pregnancy (E19.0), RXRα is required for the proper differentiation of the trophoblast cells forming the labyrinthine trabeculae. The early RXR-dependent step of placentation (formation of the labyrinth), does not involve RXR/RAR heterodimers, and as RXRα/RAR (α, β, or γ) double null fetuses do not die earlier than RXRα single null fetuses, this probably also applies to the later step [47]. Moreover, the early step is taking place normally in RXRα*af*$^{o}$/RXRβ null placentas, as well as in placentas lacking all RXR AF-1 activities (RXRα*af1*$^{o}$/RXRβ null/RXRγ null placentas) or all AF-2 activities (RXRα*af2*$^{o}$/RXRβ null/RXRγ null placentas) ([77], and unpublished results). Therefore, the heterodimers involved in the initial stages of labyrinthine formation, presumably RXR(α and/or β)/PPAR(β and/or γ) heterodimers (see above), do not require a transcriptionally active RXR. In contrast, as RXRα*af2*$^{o}$ placentas reproduce with a complete penetrance the histological defects of RXRα null placentas, the later RXRα-dependent step of placentation (differentiation of the labyrinthine trophoblast), depends critically on the AF-2 ligand binding-dependent transactivation function of RXRα [77].

## COMBINING GENETIC AND PHARMACOLOGICAL APPROACHES PROVIDE CLUES ON RAR-DEPENDENT CELLULAR MECHANISMS OPERATING DURING EMBRYOGENESIS

### *The endoderm of branchial arches is a major target of RA action mediated by RARα and/or RARβ*

Mutants carrying targeted inactivations of both the RARα and RARβ genes (RARαβ mutants), analyzed at fetal stages of gestation, display the complete set of defects generated in the chick by surgical ablation of large portions of the post-otic neural crest, namely thymus and parathyroid gland ageneses or ectopias, aberrant pattern of the great cephalic arteries, absence of the pulmonary arteries, and aorticopulmonary septum [35,37,50, and refs. therein]. These defects are also present in the DiGeorge syndrome, which is an archetype of human neurocristopathy, i.e., "a condition arising from aberrations of the early migration, growth, and differentiation of neural crest cells (NCC)" [91]. These and other observations led to the proposal that cranial NCC fated to give rise to mesenchymal derivatives (i.e., the mesectodermal cells) are major targets of RA action [37]. Unexpectedly, RARαβ double null mutants analyzed at embryonic stages of gestation do not show NCC alterations, but their caudal branchial arches (BAs) are very small [60]. BAs are transient bulges of the embryonic head and neck, partially filled with NCC and separated from one another by evaginations of the endoderm, the pharyngeal pouches. Caudal BA and pouches give rise to the adult organs affected in the aforementioned NCC ablation experiments. As BA defects of RARαβ embryos are less severe than those of RALDH2 null embryos, which are devoid of RA [52], they do not reflect a complete block in RA signal transduction. To analyze NCC migration and formation of BA and pharyngeal pouches in a situation where the degree of the block in RA signal transduction could be modulated and its timing precisely controlled, a culture system was designed in which wild-type embryos are exposed to a panRAR antagonist, BMS493 [57].

Treatment with the panRAR antagonist induces a complete lack of caudal BA and pharyngeal pouches, and disturbs the paths of post-otic NCC migration, however, without affecting the amount of NCC. Moreover, and most interestingly, this treatment inhibits caudal BA development only during a narrow window of time which does not correspond to the period of post-otic NCC migration. Both the nature and time of appearance of the defects in panRAR antagonist-treated embryos indicate that, contrary to what was expected from the set of abnormalities displayed by RARαβ double null fetuses, migrating NCC destined to the caudal BA do not represent primary targets of RA action. On the other hand, the antagonist-induced alterations in endodermal expression of "patterning" genes (e.g., *Hoxa1, Hoxb1, Pax1*, *Pax9*) and of genes encoding signal peptides (*Fgf3* and *Fgf8*), indicate that RA signaling (i) is required to specify the pharyngeal endoderm, and (ii) may provide a permissive environment for NCC migration through endodermal secretion of specific paracrine factors [57]. These data also raise the possibility that genes deleted in the human DiGeorge syndrome are actually expressed in the endoderm under the control of RA as early as the fourth week of gestation.

### *RARs act on top of a genetic cascade controlling hindbrain segmentation*

The hindbrain of vertebrate embryos is transiently divided into segments (rhombomeres), of which seven (R1 to R7) are visible in mammals. Although early and transient, hindbrain segmentation is instrumental in organizing adult structures, such as cranial nerves. The coordinated expression of several transcriptional regulators is required to pattern the embryonic hindbrain from an initially unsegmented "naïve" neural plate. These transcriptional regulators: (i) control the segmentation process, through which the cells located within the nascent rhombomeres acquire distinct adhesive properties preventing them to mix together, and/or (ii) impart segmental identity, i.e., the acquisition by these cells for specific molecular addresses that will determine their definitive fates. These transregulators also commonly serve as molecular markers of specific rhombomeres in in situ hybridization assays [58, and refs. therein]

The hindbrain of RARα/RARγ double null mutant (RARαγ) embryos shows a posterior expansion of R3 and R4 markers, but fails to express *kreisler*, a specific marker of R5 and R6. In contrast, the

neurectodermal territory corresponding to R5 and R6 is markedly enlarged in RARα/RARβ double null mutant (RARαβ) embryos. Treating E7.0 wild-type embryos with the panRAR antagonist BMS493 produces a phenocopy of the RARαγ hindbrain abnormal phenotype, whereas this treatment started at E8.0 results in a RARαβ-like phenotype. Thus, distinct hindbrain phenotypes in RARαβ and RARαγ null embryos are related to different time windows of RA action: at E7.5 (the time at which embryonic RA synthesis begins), RARγ (and RARα) transduce a signal required to specify the R5/R6 territory; at E8.0, a RARβ- (and RARα)-mediated local increase in RA signaling in the posterior portion of the hindbrain controls the position of the R6 caudal boundary, thus allowing the next caudal rhombomere, R7, to be specified.

That the expression domains of several important hindbrain patterning genes are altered in RARαβ and RARαγ mutant embryos [58] provides evidence that RA acts on top of the genetic hierarchy controlling hindbrain patterning. Moreover, generation of a graded embryonic block in RA signal transduction through varying the concentrations of the panRAR antagonist in the culture, demonstrates that individual rhombomeres are specified by distinct thresholds of RA signaling, and support the view that RA acts as a posteriorizing signal for the patterning of the embryonic hindbrain [58,92, and refs. therein]. Thresholds levels of RA signaling could be set up through modulations of RAR levels, and/or of RA-synthesizing and metabolizing enzyme expression domains [56,93–96, and refs. therein].

### *RA signals mediated by RXRα/RARα and RXRα/RARβ heterodimers have opposite effects on lung-branching morphogenesis*

The discovery that VAD rat fetuses often display bilateral lung hypoplasia as well as oesophagotracheal septum agenesis provided the first indication that RA signaling is important for the development of the respiratory system [8]. VAD-related lung and tracheal malformations are absent in RAR (α, β, and γ) single null mutants, but are seen in all RARαβ double null mutants [35,50]. Although RARβ can apparently efficiently compensate for the loss of RARα in RARα single null mutants, the functions of these two RARs in prenatal lung growth are not equivalent. Indeed, RARα/RXRα double null mutants constantly display severe lung hypoplasia and oesophagotracheal septum agenesis, whereas RXRα/RARβ double null mutants never show these defects. This observation confirms that functional compensation within the RAR family is much less efficient when RXRα, the main heterodimeric partner of RARs during embryonic development, is absent [see ref. 47]. As mentioned above, the genetic dissection of the retinoid signaling pathway strongly suggests that the functional units involved in the primary lung bud and trachea formation are RXRα/RARα heterodimers [47]. Thus, formation of the primary lung buds provides another example of developmental processes that seemingly involve specific heterodimers.

To investigate the role of RA signaling pathways during primary lung bud formation and subsequently during branching morphogenesis, wild-type embryos or lung explants were cultured in the presence of RA and of the specific panRAR antagonist, BMS493 [69]. PanRAR antagonist treatment of embryos at E8.0, prior to the first appearance of the primary lung buds, inhibits their outgrowth and causes a failure of oesophagotracheal fold formation, indicating that (i) a RA signaling is required for the formation of the primary lung buds from the primitive foregut and (ii) the severe lung and tracheal defects observed at fetal stages of gestation in RARαβ and RXRα/RARα double null mutant mice, as well as in VAD rats, are determined prior or at the onset of lung development [69, and refs. therein].

PanRAR antagonist treatments of explants collected at a later stage of lung-branching morphogenesis, increase the number of distal buds, the sites of lung branching, whereas RA administered during this period has an inverse effect [69,97]. This RA-induced inhibition of lung branching is apparently mediated by RARβ. RARβ transcripts are strictly confined to the morphogenetically stable proximal bronchi during in vivo lung development [44]. However, the branching inhibition induced by RA correlates with an ectopic expression of RARβ in distal buds, whereas stimulation of lung branching caused by the panRAR antagonist correlates with a decrease of RARβ expression in proximal bronchi. Additionally, RA treatments that decrease the number of distal buds in wild-type explants do not affect

explants from RARβ null mutants. Collectively, these findings support the view that activation of RARβ by RA favors morphogenetic stabilization of the developing pulmonary tree [69]. Such a negative control of embryonic lung growth by a RARβ signaling pathway might be disturbed in the congenital cystic adenomatoid malformation, a human pathology characterized by an overgrowth of bronchial tissue at the expenses of the alveolar tissue.

Interestingly, RARβ-transduced RA signals, in addition to negatively regulate lung-branching morphogenesis in utero, also negatively regulate, after birth, the process of alveolar septation [98,99]. Suppressing perinatal RARβ signaling by selective antagonists may thus offer a novel mean of preventing, or curing, failed septation in prematurely born children suffering from bronchopulmonary dysplasia. In contrast, RXRα/RARγ heterodimers appear to be positively involved in alveolar morphogenesis during the perinatal period [100], while RARα may regulate alveolar formation after the perinatal period [99,101].

## COMBINING GENETIC AND PHARMACOLOGICAL APPROACHES PROVIDE CLUES ON RAR AND RXRα-MEDIATED TERATOGENIC EFFECTS

RA is a potent teratogen which, at pharmacological concentrations, can induce congenital defects in all vertebrate species as well as in certain invertebrates [102–104]. RA treatments of animals overexpressing, or carrying null mutations of retinoid receptors, have provided evidence that teratogenic effects of retinoids are receptor-mediated. For instance, it was shown that RARγ null embryos are resistant to RA-induced caudal truncations, whereas RXRα null embryos are resistant to RA-induced limb defects and cleft palate [38,105–107].

In humans, oral intake of Accutane (13-cis RA) during gastrulation and early organogenesis (gestational weeks 2–5) results in a spectrum of congenital malformations collectively referred to as the retinoic acid embryopathy (RAE) [108]. It has been assumed that the branchial arch (BA) defects observed in RA-exposed embryos at the equivalent of E8.0 and E9.0 in the mouse, can account for alterations displayed in newborns, and that neural crest is the primary target tissue of RA-induced teratogenesis in the BA region of the embryo [109,110].

Fusion and hypoplasia of the first two BA, a hallmark of RAE, is generated in E8.0 cultured mouse embryos upon treatment with BMS453, a synthetic compound exhibiting RARβ agonistic properties in transfected cells [111]. In contrast, no BA defects are observed following treatment with synthetic retinoids exhibiting RARα or RARγ agonistic properties. These BMS453-induced BA defects are enhanced in the presence of a panRXR agonist, that is not teratogenic on its own, and they are accompanied by ectopic expression of RARβ and of several other direct RA target genes in the morphologically altered region. On the other hand, BA defects and ectopic expression of RA target genes cannot be induced in RARβ null embryos upon BMS453 treatment. Altogether, these data indicate that craniofacial abnormalities characteristic of RAE are mediated through ectopic activation of RXR/RARβ heterodimers, in which the ligand-dependent activity of RXR is subordinated to that of RARβ.

RAE apparently meets the criteria for a neurocristopathy [91; and see above]. However, NCC do not appear to be primary targets of RA-induced teratogenicity as: (i) retinoid-induced fusion of the first and second BA occurs without alterations of NCC migration or apoptosis [110] and (ii) contrary to other embryonic tissues, NCC do not express a RA-responsive transgene upon treatment with BMS453. In contrast, treatment with BMS453 triggers a RARβ-dependent RA signaling in the endoderm lining the first two BA, manifested by rostral shifts of the expression domains of RA-responsive patterning genes, such as *Hoxa1* and *Hoxb1*. *Hox* gene expression is thought to play an important role in antero-posterior regionalization of the pharyngeal endoderm [57; and refs. therein], while pharyngeal endoderm plays a seminal role in the formation of BA, through imparting patterning information to NCC [112]. Thus, the current data support the view that many RAE defects can result from an abnormal function of the pharyngeal endoderm [111,113].

## CONCLUSIONS AND PERSPECTIVES

We have reviewed here the results of phenotypic analyses of single and compound germline mutants lacking RAR and RXR isotypes and/or isoforms, and of mutants lacking the RXRα transactivation functions. These analyses have provided the first compelling evidence that RA is actually the active metabolite of vitamin A during embryonic development, which was subsequently confirmed by the demonstration that RA synthesized by the retinal deydrogenases 2 and 3 (RALDH2 and RALDH3) acts as an indispensable developmental hormone [52–54,56,114, and our unpublished results]. These genetic studies have also led to equally important conclusions concerning the physiological functions played by the multiple RAR and RXR receptors in vivo, notably during early embryogenesis and organogenesis, but also postnatally. Moreover, they strongly supports the conclusion that the molecular mechanisms underlying the transduction of the RA signal by retinoid receptors, as they have been deduced from in vitro studies in acellular and cellular systems (see Introduction), are also instrumental in RA signaling under truly physiological conditions, i.e., at the organismal level.

Several lines of evidence lead to the conclusion that RXR/RAR, notably RXRα/RAR heterodimers are the main functional units that transduce RA signals during development, and that specific RXR/RAR pairs are involved in given developmental processes. This strongly supports the initial proposal [14] that the highly pleiotropic effects of RA reflect sophisticated combinatorial mechanisms through which multiple RXR/RAR heterodimers differentially transduce retinoid signals to selectively control the expression of numerous sets of RA target genes controlling the shaping and axial patterning of the early embryo, and subsequently multiple aspects of organogenesis.

The mouse genetic studies also demonstrate that within RXR/RAR heterodimers, the RXR partner can be either transcriptionally active (and thus synergistically acting with its RAR partners) or inactive, depending on the developmental event under consideration. However, as previously demonstrated in vitro (see Introduction), it appears that the transcriptional activity of the RXR partner is subordinated to ligand binding to the RAR partner [111,115]. Moreover, when RXRα is transcriptionally active, either one (AF-1 or AF-2) or both activation functions can be instrumental, and their activity also depends on the nature of the RA-controlled event. The frequent requirement of RXRα AF-2, whose transcriptional activity is known to be dependent on 9-cis RA from studies in vitro, strongly suggests that this retinoid or a similar ligand could be instrumental in transactivation by RXR heterodimers in vivo (for further discussions, see ref. [22]). Note that, as the role of RXRα AF-1 has been inferred from deletion of the whole A/B region [77], and as phosphorylation of the RXRα A region has been shown to be required for AF-1 activity in studies in vitro [80], it will be interesting to genetically investigate the function of this phosphorylation in the mouse.

The genetic study of the physiological roles of RAR and RXR has revealed an extensive functional redundancy within the members of each family (RARs or RXRs), although in all cases each of these members appears to individually exert at least one specific physiological function. Even though this functional redundancy is not surprising, as the members of each family share a common ancestor, it raises the question as to whether it is physiologically relevant or artefactually generated when a given RAR or RXR is knocked out, as it has been shown to be the case in cellular studies in vitro [26,116]. It is not unlikely that, in most instances, the functional redundancy does not exist under wild-type conditions. In fact, redundancy is frequent within the RAR or the RXR families, i.e., a given defect is very frequently or exclusively seen in either RAR/RAR or RXRα/RXR double null mutants, while it is weakly penetrant or absent in the corresponding single null mutants). In striking contrast, redundancy is less frequent in the case of the RXRα/RAR heterodimer knock-out which generates this defect in a fully penetrant manner. As discussed extensively elsewhere [22,47,77], assuming that RXRα/RAR heterodimers are indeed the functional transducing units, the easiest way to interpret these observations is to postulate that redundancy can occur when only one of the two partners of the physiological heterodimer is ablated. In other words, the activity of a given heterodimer selectively involved in the control of a given event may still be above a physiological threshold level when either one of the two partners

(RXR or RAR) is ablated, but not when both are missing. Therefore, the selective involvement of a given RAR or RXR could be revealed only under conditions where the threshold level is not reached, which would also account for the observations that single isoform knock-outs are often phenotypically normal, and that the role of the RXRα AF-1 or AF-2 functions cannot be fully revealed unless the activity of the heterodimer is altered by the additional mutation of the RAR partner, or by knock-out of one or both of the potentially redundant RXR isotypes. Thus, any conditions (e.g., a decrease availability of intracellular RA) that would lower the activity of RXR/RAR heterodimers and bring it close to physiological threshold levels, may reduce or abrogate functional redundancy. As the actual intracellular concentration of RA could be more limiting in the wild than in animal facilities, functional redundancy may be less prominent in natural environments. In this respect, it is worth mentioning that, even though phosphorylation of either RAR AF-1 or AF-2 domains can be required for RA-induced differentiation of F9 EC cells in vitro [26], mouse mutants bearing mutations in the phosphorylated amino acid residues are apparently normal (our unpublished results). Therefore, it will be worth investigating whether the function of these phosphorylations can be revealed under conditions of limited RA supply. Similar conditions could also reveal selective functions of the various RAR and RXR isoforms.

Genetic analysis of RAR and RXR functions have also demonstrated that the teratogenic effects resulting from administration of exogenous RA to embryos do not reflect a physiological role of endogenous RA in the corresponding developmental processes. Indeed, in two instances in which an involvement of a given RAR or RXR in the mediation of a teratogenic event was demonstrated, the same receptor was clearly not required for the development of the corresponding structure during embryogenesis. This is the case for the RA excess-induced lumbosacral truncation that is mediated by RARγ [38] and the RA excess-induced limb truncations that do not occur in RXRα mutants [106].

Clearly, the generation of RAR and RXR germline mutations, combined with pharmacological approaches to block the RA signaling pathway, have provided many valuable insights on the developmental functions of RA receptors. However, this strategy has intrinsic limitations that are mostly due to the introduction of the mutation in the germline. First, the effect of a germline mutation may be functionally compensated during development, thus precluding the appearance of a defect in the adult animal. On the other hand, the mutation can be lethal in utero or postnatally, thus preventing analysis of the functions of the gene at later developmental or postnatal stages. This is the case for RXRα knock-out. Along the same lines, germline mutations can arrest the development of a given organ at an early stage, thus preventing further analysis of the gene functions at later stages of organogenesis. For instance, the function of RARβ in lung-branching morphogenesis is not revealed in RARβ single mutants, and therefore remains cryptic in RARαβ double null mutants in which a very early step of embryonic lung formation is impaired. Moreover, introducing mutations in the germline often makes it very difficult to distinguish cell-autonomous from non-cell-autonomous functions of a gene belonging to a family involved in highly pleiotropic signaling pathways. Thus, in many instances, these limitations of germline mutations prevent the determination of the function of a given gene product in a defined tissue at a given time of the animal life. This is obviously the case for RARs and RXRs.

To overcome these limitations, strategies for targeted spatio-temporally controlled somatic mutagenesis of RARs and RXRs in the mouse have been designed, which are based on cell-type-specific expression and inducible activity of the bacteriophage P1 Cre recombinase. To that end, conditional tamoxifen-inducible Cre recombinases (called Cre-ER$^{T}$ and Cre-ER$^{T2}$) have been generated by fusing Cre with a mutated ligand-binding domain of the estrogen receptor ERα which binds tamoxifen, but not estrogens [117,118]. Selective ablation of the RXRα and RARγ genes in keratinocytes indicate that RXRα has key roles in hair cycling, most probably through RXR/VDR heterodimers, as well as in homeostasis of proliferation/differentiation of epidermal keratinocytes and of the skin immune system though non-RXRα/RAR(α, β, or γ) heterodimers [41,119,120]. The same approach applied to adipocytes and hepatocytes has demonstrated that RXRα is involved both in preadipocyte differentiation, adipogenesis, and lipolysis, probably in the form of RXRα/PPARγ heterodimers [121], and plays important cell-autonomous functions in mechanisms that control the lifespan of hepatocytes and are in-

volved in liver regeneration [122]. These examples show that the combined use of transgenic mouse lines expressing tamoxifen-inducible chimeric Cre recombinases in specific cell-types and of mouse lines harboring "floxed" receptor genes will provide invaluable mouse models to further elucidate the in vivo functions of retinoid receptors.

## ACKNOWLEDGMENTS

We thank all past and present members of the retinoid groups of the IGBMC for useful discussions. Our work was supported by funds from the Centre National de la Recherche Scientifique (CNRS), the Institut National de la Santé et de la Recherche Médicale (INSERM), the Hôpital Universitaire de Strasbourg, the Collège de France, the Institut Universitaire de France, the Association pour la Recherche sur le Cancer (ARC), the Fondation pour la Recherche Medicale (FRM), the Ligue Nationale Contre le Cancer, the Human Frontier Science Program, and Bristol Myers Squibb.

## REFERENCES

1. R. Blomhoff. *Nutr. Rev*. **52**, S13–S23 (1994).
2. M. B. Sporn, A. B. Roberts, D. S. Goodman. *The Retinoids: Biology, Chemistry and Medicine*, Raven Press, New York (1994).
3. P. Kastner, M. Mark, P. Chambon. *Cell* **83**, 859–869 (1995).
4. A. Agadir et al. *Retinoids*: *The Biochemical and Molecular Basis of Vitamin A and Retinoid Action*, Springer-Verlag, Berlin (1999).
5. M. A. Livrea. *Vitamin A and Retinoids: An Update of Biological Aspects and Clinical Applications,* Birkhäuser Verlag, Basel (2000).
6. G. M. Morriss-Kay and S. J. Ward. *Int. Rev. Cytol.* **188**, 73–131 (1999).
7. F. Hale. *J. Hered*. **24**, 105–106 (1933).
8. J. G. Wilson, C. B. Roth, J. Warkany. *Am. J. Anat*. **92**, 189–217 (1953).
9. S. B. Wolbach and P. R. Howe. *J. Exp. Med*. **42**, 753–777 (1925).
10. J. N. Thompson, J. McC. Howell, G. A. J. Pitt. *Proc. Royal Soc*. **159**, 510–535 (1964).
11. E. D. Dickman, C. Thaller, S. M. Smith. *Development* **124**, 3111–3121 (1997).
12. J. C. White, V. N. Shankar, M. Highland, M. L. Epstein, H. F. DeLuca, M. Clagett-Dame. *Proc. Natl. Acad. Sci. USA* **95**, 13459–13464 (1998).
13. P. Chambon. *FASEB J.* **10**, 940–954 (1996).
14. M. Leid, P. Kastner, P. Chambon. *Trends Biochem. Sci.* **17**, 427–433 (1992).
15. P. Germain, J. Iyer, C. Zechel, H. Gronemeyer. *Nature* **15**, 187–192 (2002).
16. D. Moras and H. Gronemeyer. *Curr. Opin. Cell. Biol.* **10**, 384–391 (1998).
17. C. K. Glass and M. G. Rosenfeld. *Genes Dev*. **14**, 121–141 (2000).
18. P. F. Egea, B. P. Klaholz, D. Moras. *FEBS Lett.* **476**, 62–67 (2000).
19. H. Gronemeyer and V. Laudet. *Protein Profile* **2**, 1173–1308 (1995).
20. S. Nagpal, M. Saunders, P. Kastner, B. Durand, H. Nakshatri, P. Chambon. *Cell* **70**, 1007–1019 (1992).
21. S. Nagpal, S. Friant, H. Nakshatri, P. Chambon. *EMBO J.* **12**, 2349–2360 (1993).
22. B. Mascrez, M. Mark, A. Dierich, N. Ghyselinck, P. Kastner, P. Chambon. *Development* **125**, 4691–4707 (1998).
23. B. Durand, M. Saunders, C. Gaudon, B. Roy, R. Losson, P. Chambon. *EMBO J.* **13**, 5370–5382 (1994).
24. P. Kastner, J. M. Grondona, M. Mark, A. Gansmuller, M. LeMeur, D. Décimo, J. L. Vonesch, P. Dollé, P. Chambon. *Cell* **78**, 987–1003 (1994).
25. R. Mollard, S. Viville, S. J. Ward, D. Decimo, P. Chambon, P. Dollé. *Mech. Dev.* **94**, 223–232 (2000).

26. C. Rochette-Egly and P. Chambon. *Histol. Histopathol*. **16**, 909–922 (2001).
27. E. Li, H. M. Sucov, K. F. Lee, R. M. Evans, R. Jaenisch. *Proc. Natl. Acad. Sci. USA* **90**, 1590–1594 (1993).
28. T. Lufkin, D. Lohnes, M. Mark, A. Dierich, P. Gorry, M. P. Gaub, M. LeMeur, P. Chambon. *Proc. Natl. Acad. Sci. USA* **90**, 7225–7229 (1993).
29. B. Chapellier, M. Mark, J. M. Garnier, M. LeMeur, P. Chambon, N. B. Ghyselinck. *Genesis* **32**, 87–90 (2002).
30. P. Bouillet, V. Sapin, C. Chazaud, N. Messaddeq, D. Decimo, P. Dollé, P. Chambon. *Mech. Dev.* **63**, 173–186 (1997).
31. J. M. Dufour and K. H. Kim. *Biol. Reprod.* **61**, 1300–1308 (1999).
32. B. Boulogne, C. Levacher, P. Durand, R. Habert. *Biol. Reprod.* **61**, 1548–1557 (1999).
33. N. B. Ghyselinck, C. Bavik, V. Sapin, M. Mark, D. Bonnier, C. Hindelang, A. Dierich, C. B. Nilsson, H. Hakansson, P. Sauvant, V. Azais-Braesco, M. Frasson, S. Picaud, P. Chambon. *EMBO J*. **18**, 4903–4914 (1999).
34. D. Lohnes, M. Mark, C. Mendelsohn, P. Dollé, A. Dierich, P. Gorry, A. Gansmuller, P. Chambon. *Development* **120**, 2723–2748 (1994).
35. N. B. Ghyselinck, V. Dupé, A. Dierich, N. Messaddeq, J. M. Garnier, C. Rochette-Egly, P. Chambon, M. Mark. *Int. J. Dev. Biol.* **41**, 425–447 (1997).
36. M. Mark, D. Lohnes, C. Mendelsohn, V. Dupé, J. L. Vonesch, P. Kastner, F. Rijli, A. Bloch-Zupan, P. Chambon. *Int. J. Dev. Biol*. **39**, 111–121 (1995).
37. M. Mark, N. Ghyselinck, P. Kastner, V. Dupé, O. Wendling, W. Krezel, B. Mascrez, P. Chambon. *Eur. J. Oral Sci.* **106** (suppl. 1), 24–31 (1998).
38. D. Lohnes, P. Kastner, A. Dierich, M. Mark, M. LeMeur, P. Chambon. *Cell* **73**, 643–658 (1993).
39. B. A. Underwood and P. Arthur. *FASEB J*. **10**, 1040–1048. (1996).
40. N. B. Ghyselinck, B. Chapellier, C. Calleja, A. Kumar Indra, M. Li, N. Messaddeq, M. Mark, D. Metzger, P. Chambon. *Ann. Dermatol. Venereol.* **129**, 793–799 (2002).
41. B. Chapellier, M. Mark, N. Messaddeq, C. Calleja, X. Warot, J. Brocard, C. Gerard, M. Li, D. Metzger, N. B. Ghyselinck, P. Chambon. *EMBO J*. **21**, 3402–3413 (2002).
42. V. Subbarayan, P. Kastner, M. Mark, A. Dierich, P. Gorry, P. Chambon. *Mech. Dev*. **66**, 131–142 (1997).
43. J. M. Grondona, P. Kastner, A. Gansmuller, D. Décimo, P. Chambon, M. Mark. *Development* **122**, 2173–2188 (1996).
44. N. B. Ghyselinck, O. Wendling, N. Messaddeq, A. Dierich, C. Lampron, D., Décimo, S. Viville, P. Chambon, M. Mark. *Dev. Biol.* **198**, 303–318 (1998).
45. W. Krezel, N. Ghyselinck, T. A. Samad, V. Dupé, P. Kastner, E. Borrelli, P. Chambon. *Science* **279**, 863–867 (1998).
46. M. Y. Chiang, D. Misner, G. Kempermann, T. Schikorski, V. Giguere, H. M. Sucov, F. H. Gage, C. F. Stevens, R. M. Evans. *Neuron*. **21**, 1353–1361 (1998).
47. P. Kastner, M. Mark, N. B. Ghyselinck, W. Krezel, V. Dupé, J. M. Grondona, P. Chambon. *Development* **124**, 313–326 (1997).
48. M. Mark, P. Kastner, N. B. Ghyselinck, W. Krezel, V. Dupé, P. Chambon. *C.R. Soc. Biol.* **191**, 77–90 (1997).
49. J. Luo, H. M. Sucov, J. A. Bader, R. M. Evans, V. Giguere. *Mech. Dev*. **55**, 33–44 (1996).
50. C. Mendelsohn, D. Lohnes, D. Décimo, T. Lufkin, M. LeMeur, P. Chambon, M. Mark. *Development* **120**, 2749–2771 (1994).
51. J. C. White, M. Highland, M. Kaiser, M. Clagett-Dame. *Dev. Biol.* **220**, 263–284 (2000).
52. K. Niederreither, V. Subbarayan, P. Dollé, P. Chambon. *Nat. Genet.* **21**, 444–448 (1999).
53. K. Niederreither, J. Vermot, B. Schuhbaur, P. Chambon, P. Dollé. *Development* **127**, 75–85 (2000).

54. K. Niederreither, J. Vermot, N. Messaddeq, B. Schuhbaur, P. Chambon, P. Dollé. *Development* **128**, 1019–1031 (2001).
55. K. Niederreither, V. Fraulob, J. M. Garnier, P. Chambon, P. Dollé. *Mech. Dev*. **110**, 165–71 (2002).
56. K. Niederreither, J. Vermot, B. Schuhbaur, P. Chambon, P. Dollé. *Development* **129**, 3563–3574 (2002).
57. O. Wendling, C. Dennefeld, P. Chambon, M. Mark. *Development* **127**, 1553–1562 (2000).
58. O. Wendling, N. B. Ghyselinck, P. Chambon, M. Mark. *Development* **128**, 2031–2038 (2001).
59. D. M. Kochhar, H. Jiang, J. D. Penner, A. T. Johnson, R. A. Chandraratna. *Int. J. Dev. Biol*. **42**, 601–608 (1998).
60. V. Dupé, N. B. Ghyselinck, O. Wendling, P. Chambon, M. Mark. *Development* **126**, 5051–5059 (1999).
61. V. Dupé, M. Davenne, J. Brocard, P. Dollé, M. Mark, A. Dierich, P. Chambon, F. M. Rijli. *Development* **124**, 399–410 (1997).
62. M. Houle, P. Prinos, A. Lulianella, N. Bouchard, D. Lohnes. *Mol. Cell. Biol*. **20**, 6579–6586 (2000).
63. D. Allan, M. Houle, N. Bouchard, B. I. Meyer, P. Gruss, D. Lohnes. *Dev Biol.* **240**, 46–60 (2001).
64. V. Dupé, N. B. Ghyselinck, V. Thomazy, L. Nagy, P. J. A. Davies, P. Chambon, M. Mark. *Dev. Biol*. **208**, 30–43 (1999).
65. P. Kastner, N. Messaddeq, M. Mark, O. Wendling, J. M. Grondona, S. Ward, N. Ghyselinck, P. Chambon. *Development* **124**, 4749–4758 (1997).
66. C. Mendelsohn, E. Batourina, S. Fung, T. Gilbert, J. Dodd. *Development* **126**, 1139–1148 (1999).
67. E. Batourina, C. Choi, N. Paragas, N. Bello, T. Hensle, F. D. Costantini, A. Schuchardt, R. L. Bacallao, C. L. Mendelsohn. *Nat. Genet*. **32**, 109–115 (2002).
68. E. Batourina, S. Gim, N. Bello, M. Shy, M. Clagett-Dame, S. Srinivas, F. Costantini, C. Mendelsohn. *Nat. Genet.* **27**, 74–78 (2001).
69. R. Mollard, N. B. Ghyselinck, O. Wendling, P. Chambon, M. Mark. *Int. J. Dev. Biol*. **44**, 457–462 (2000).
70. M. P. Mark, A. Bloch-Zupan, J. V. Ruch. *Int. J. Dev. Biol*. **36**, 517–526 (1992).
71. A. Bloch-Zupan, M. P. Mark, B. Weber, J. V. Ruch. *Arch. Oral Biol*. **39**, 891–900 (1994).
72. H. M. Sucov, E. Dyson, C. L. Gumeringer, J. Price, K. R. Chien, R. M. Evans. *Genes Dev*. **8**, 1007–1018 (1994).
73. V. Subbarayan, M. Mark, N. Messadeq, P. Rustin, P. Chambon, P. Kastner. *J. Clin. Invest.* **105**, 387–394 (2000).
74. T. Chen, T. Chang, J. Kang, B. Choudhary, T. Makita, C. Tran, J. Burch, H. Eid, H. Sucov. *Dev. Biol.* **250**, 198 (2002).
75. P. Ruiz-Lozano, S. M. Smith, G. Perkins, S. W. Kubalak, G. R. Boss, H. M. Sucov R. M. Evans, K. R. Chien. *Development* **125**, 533–544 (1998).
76. F. A. Mic, A. Molotkov, X. Fan, A. E. Cuenca, G. Duester. *Mech. Dev*. **97**, 227–230 (2000).
77. B. Mascrez, M. Mark, W. Krezel, V. Dupe, M. LeMeur, N.B. Ghyselinck, P. Chambon. *Development* **128**, 2049–2062 (2001).
78. M. Lussier, C. Canoun, C. Ma, A. Sank, C. Shuler. *Int. J. Dev. Biol*. **37**, 555–564 (1993).
79. A. Crocoll, U. Herzer, N. B. Ghyselinck, P. Chambon, A. C. Cato. *Mech. Dev.* **111**, 149–152 (2002).
80. J. Bastien, S. Adam-Stitah, J. L. Plassat, P. Chambon, C. Rochette-Egly. *J. Biol. Chem.* **277**, 28683–28689 (2002).
81. W. Krezel, V. Dupé, M. Mark, A. Dierich, P. Kastner, P. Chambon. *Proc. Natl. Acad. Sci. USA* **93**, 9010–9014 (1996).
82. M. Mori, N. B. Ghyselinck, P. Chambon, M. Mark. *Invest. Ophthalmol. Vis. Sci.* **42**,1312–1318 (2001).

83. P. Kastner, M. Mark, M. Leid, A. Gansmuller, J. M. Grondona, D. Décimo, W. Krezel, A. Dierich, P. Chambon. *Genes Dev*. **10**, 80–96 (1996).
84. N. S. Brown, A. Smart, V. Sharma, M. L. Brinkmeier, L. Greenlee, S. A. Camper, D. R. Jensen, R. H. Eckel, W. Krezel, P. Chambon, B. R. Haugen. *J. Clin. Invest.* **106**, 73–79 (2000).
85. N. Yagishita, Y. Yamamoto, T. Yoshizawa, K. Sekine, Y. Uematsu, H. Murayama, Y. Nagai, W. Krezel, P. Chabon, T. Matsumoto, S. Kato. *Endocrinology* **142**, 5332–5341 (2001).
86. T.A. Samad, W. Krezel, P. Chambon, E. Borrelli. *Proc. Natl. Acad. Sci. USA* **94**, 14349–14354 (1997).
87. O. Wendling, P. Chambon, M. Mark. *Proc. Natl. Acad. Sci. USA* **96**, 547–551 (1999).
88. Y. Barak, M. C. Nelson, E. S. Ong, Y. Z. Jones, P. Ruiz-Lozano, K. R. Chien, A. Koder, R. M. Evans. *Mol. Cell* **4**, 585–595 (1999).
89. Y. Barak, D. Liao, W. He, E. S. Ong, M. C. Nelson, J. M. Olefsky, R. Boland, R. M. Evans. *Proc. Natl. Acad. Sci. USA* **99**, 303–308 (2002).
90. V. Sapin, P. Dollé, C. Hindelang, P. Kastner, P. Chambon. *Dev. Biol*. **191**, 29–41 (1997).
91. R. P. Bolande. *Pediatr. Pathol. Lab. Med.* **17**, 1–25 (1997).
92. V. Dupé and A. Lumsden. *Development* **128**, 2199–2208 (2001).
93. H. L. Ang and G. Duester. *Dev. Dyn.* **208**, 536–543 (1997).
94. K. Niederreither, S. Abu-Abed, B. Schuhbaur, M. Petkovich, P. Chambon, P. Dollé. *Nat. Genet*. **31**, 84–88 (2002).
95. G. MacLean, S. Abu-Abed, P. Dollé, A. Tahayato, P. Chambon, M. Petkovich. *Mech. Dev.* **107**, 195–201 (2001).
96. S. Abu-Abed, P. Dollé, D. Metzger, C. Wood, G. MacLean, P. Chambon, M. Petkovich. *Development* **130**, 1449–1459 (2003).
97. S. Malpel, C. Mendelsohn, W. V. Cardoso. *Development* **127**, 3057–3067 (2000).
98. G. D. Massaro, D. Massaro, W. Y. Chan, L. B. Clerch, N. Ghyselinck, P. Chambon, R. A. Chandraratna. *Physiol. Genomics* **4**, 51–57 (2000).
99. D. Massaro and G. D. Massaro. *Am. J. Respir. Cell. Mol. Biol*. **28**, 271–274 (2003).
100. S. Mc Gowan, S. K. Jackson, M. Jenkins-Moore, H. H. Dai, P. Chambon, J. M. M. Snyder. *Am. J. Respir. Cell. Mol. Biol*. **23**, 162–167 (2000).
101. G. D. Massaro, D. Massaro, P. Chambon. *Am. J. Physiol*. **284**, 431–433 (2003).
102. D. R. Soprano and K. J. Soprano. *Annu. Rev. Nutr.* **15**, 111–132 (1995). Retinoids as teratogens.
103. M. D. Collins and G. E. Mao. *Annu. Rev. Pharmacol. Toxicol.* **39**, 399–430 (1999).
104. H. Escriva, N. D. Holland, H. Gronemeyer, V. Laudet, L. Z. Holland. *Development* **129**, 2905–2916 (2002).
105. A. Lulianella and D. Lohnes. *Dev. Dyn.* **209**, 92–104 (1997).
106. H. M. Sucov, J. C. Izpisua-Belmonte, Y. Ganan, R. M. Evans. *Development* **21**, 3997–4003 (1995).
107. P. Nugent, H. M. Sucov, M. Pisano, R. M. Green. *Int. J. Dev. Biol.* **43**, 567–570 (1999).
108. E. J. Lammer, D. T. Chen, R. M. Hoar, N. D. Agnish, P. J. Benke, J. T. Braun, C. J. Curry, P. M. Fernhoff, A. W. Grix, I. T. Lott, J. M. Richard, S. C. Sun. *N. Engl. J. Med.* **313**, 837–841 (1985).
109. R. M. Pratt, E. H. Goulding, B. D. Abbott. *J. Craniofac. Genet. Dev. Biol.* **7**, 205–217 (1987).
110. Y. M. Lee, N. Osumi-Yamashita, Y. Ninomiya, C. K. Moon, U. Eriksson, K. Eto. *Development* **121**, 825–837 (1995).
111. N. Matt, N. B. Ghyselinck, O. Wendling, P. Chambon, M. Mark. *Development* **130**, 2083–2093 (2003).
112. G. Couly, S. Creuzet, S. Bennaceur, C. Vincent, N. M. Le Douarin. *Development* **129**, 1061–1073 (2002).
113. G. B. Mulder, N. Manley, L. Maggio-Price. *Teratology* **58**, 263–275 (1998).
114. J. Vermot, K. Niederreither, J. M. Garnier, P. Chambon, P. Dollé. *Proc. Natl. Acad. Sci. USA* **100**, 1763–1768 (2003).

115. M. M. Elmazar, R. Ruhl, H. Nau. *Toxicol. Appl. Pharmacol*. **170**, 2–9 (2001).
116. R. Taneja, B. Roy, J. L. Plassat, C. F. Zusi, J. Ostrowski, P. R. Reczek, P. Chambon. *Proc. Natl. Acad. Sci. USA* **93**, 6197–6202 (1996).
117. D. Metzger and P. Chambon. *Methods* **24**, 71–80 (2001).
118. D. Metzger, A. Indra Kumar, M. Li, B. Chapellier, C. Calleja, N. B. Ghyselinck, P. Chambon. *Methods Enzymol.* (2003). In press.
119. M. Li, A. K. Indra, X. Warot, J. Brocard, N. Messaddeq, S. Kato, D. Metzger, P. Chambon. *Nature* **407**, 633–636 (2000).
120. M. Li, H. Chiba, X. Warot, N. Messaddeq, C. Gérard, P. Chambon, D. Metzger. *Development* **128**, 675–688 (2001).
121. T. Imai, M. Jiang, P. Chambon, D. Metzger. *Proc. Natl. Acad. Sci. USA* **98**, 224–228 (2001).
122. T. Imai, M. Jiang, P. Kastner, P. Chambon, D. Metzger. *Proc. Natl. Acad. Sci. USA* **98**, 4581–4586 (2001).