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Retinoid-related Orphan Receptors (RORs): Roles in Cellular Differentiation and Development

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Abstract

Retinoid-related orphan receptors ROR α , $-\beta$, and $-\gamma$ are transcription factors belonging to the steroid hormone receptor superfamily. During embryonic development RORs are expressed in a spatial and temporal manner and are critical in the regulation of cellular differentiation and the development of several tissues. ROR α plays a key role in the development of the cerebellum particularly in the regulation of the maturation and survival of Purkinje cells. In ROR α -deficient mice, the reduced production of sonic hedgehog by these cells appears to be the major cause of the decreased proliferation of granule cell precursors and the observed cerebellar atrophy. ROR α has been implicated in the regulation of a number of other physiological processes, including bone formation. ROR β expression is largely restricted to several regions of the brain, the retina, and pineal gland. Mice deficient in ROR β develop retinal degeneration that results in blindness. ROR γ is essential for lymph node organogenesis. In the intestine ROR γ is required for the formation of several other lymphoid tissues: Peyer's patches, cryptopatches, and isolated lymphoid follicles. ROR γ plays a key role in the generation of lymphoid tissue inducer (LTi) cells that are essential for the development of these lymphoid tissues. In addition, ROR γ is a critical regulator of thymopoiesis. It controls the differentiation of immature single-positive thymocytes into double-positive thymocytes and promotes the survival of double-positive thymocytes by inducing the expression of the anti-apoptotic gene Bcl-X_L. Interestingly, all three ROR receptors appear to play a role in the control of circadian rhythms. ROR α positively regulates the expression of Bmal1, a transcription factor that is critical in the control of the circadian clock. This review intends to provide an overview of the current status of the functions RORs have in these biological processes.

Keywords

lymph nodes; thymopoiesis; brain; circadian rhythm; Purkinje cell; transcription; lymphoid tissue inducer cells; cryptopatches; nuclear receptor; embryonic development; cerebellum; bone; adipocyte; ubiquitination; Peyer's patch; apoptosis

Introduction

Retinoid-related orphan receptors (RORs) form a subgroup of the nuclear receptor superfamily (Aranda and Pascual, 2001; Desvergne and Wahli, 1999; Evans, 1988; Giguere, 1999; Jetten, 2004; Jetten et al., 2001; Kumar and Thompson, 1999; Novac and Heinzel, 2004; Willy and Mangelsdorf, 1998). This subfamily consists of three members: ROR α (Becker-Andre et al., 1993; Giguere et al., 1995; Giguere et al., 1994), ROR β (Andre et al., 1998b; Carlberg et al., 1994; Schaeren-Wiemers et al., 1997), and ROR γ (He et al., 1998; Hirose et al., 1994; Jetten et al., 2001; Jetten and Ueda, 2002; Medvedev et al., 1996; Ortiz et al., 1995). These isotypes

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are also referred to as NR1F1-3 (Nuclear Receptor Nomenclature Committee) and RORA-C (Human Gene Nomenclature Committee) and have been cloned from many mammalian species, including mouse, rat, and human (Becker-Andre et al., 1993; Carlberg et al., 1994; Giguere et al., 1994; He et al., 1998; Hirose et al., 1994; Jetten, 2004; Jetten et al., 2001; Koibuchi and Chin, 1998; Medvedev et al., 1996; Ortiz et al., 1995). Homologs of RORs have been identified in several lower species. *Drosophila melanogaster* (Carney et al., 1997; Gates et al., 2004; Horner et al., 1995; Koelle et al., 1992; Sullivan and Thummel, 2003; Thummel, 1995), the nematode *Caenorhabditis elegans*, the spruce budworm *Choristoneura fumiferana* (Kostrouch et al., 1995; Palli et al., 1997; Palli et al., 1996), and the tobacco hawkmoth *Manduca sexta* (Hiruma and Riddiford, 2004; Lan et al., 1999; Lan et al., 1997; Langelan et al., 2000; Palli et al., 1992; Riddiford et al., 2003) express an ROR homolog, referred to as DHR3, CHR3, and MHR3, respectively.

The ROR α , β , and γ genes have been mapped to human chromosome 15q22.2, 9q21.13, and 1q21.3, respectively. The ROR α gene, which comprises 12 exons encoding a coding region of about 1.7 kb, spans a relatively large, 730 kb region of the genome. The ROR β gene covers 188 kb, while the ROR γ spans only 24 kb. The ROR α gene is located in the middle of the common fragile site FRA15A (Smith et al., 2006; Zhu et al., 2006). Genomic instability, including DNA breakage and rearrangements, at common fragile sites may result in changes in the expression and function of genes encoded within these regions. Altered expression of genes associated with common fragile sites, such as the fragile histidine triad (FHIT) and WW domain-containing oxidoreductase 1 (WOX1) gene, have been implicated in human disease and particularly in the development of different types of cancer (Smith et al., 2006). The association of the ROR α gene with FRA15A opens the possibility that genomic instability in this region may lead to changes in the expression of ROR α and be a factor in the development of certain cancers.

Each ROR gene produces several variants or isoforms that are generated by a combination of alternative promoter usage and exon splicing (Andre et al., 1998b; Giguere et al., 1994; Hamilton et al., 1996; He et al., 1998; Matysiak-Scholze and Nehls, 1997; Villey et al., 1999). These isoforms differ only in their amino-terminal A/B domain. In humans, four different ROR α isoforms, referred to as ROR α 1-4, have been identified, while only two isoforms, α 1 and α 4, have been reported for mouse. The ROR β and ROR γ genes each generate two different isoforms, 1 and 2 (Andre et al., 1998b; He et al., 1998; Villey et al., 1999). The variants differ in their pattern of tissue-specific expression and can regulate distinct physiological processes and target genes. For example, ROR α 1 and ROR α 4 are co-expressed in mouse cerebellum while other mouse tissues express predominantly ROR α 4 (Chauvet et al., 2002; Matysiak-Scholze and Nehls, 1997). The expression of ROR γ 2, also referred to as ROR γ t, is highly restricted to CD4⁺CD8⁺ thymocytes in the thymus and to lymphoid tissue inducer (LTi) cells, while other tissues express ROR γ 1 (Eberl et al., 2004; He et al., 1998). Expression of ROR β 2 appears to be restricted to the pineal gland and the retina (Andre et al., 1998b).

Regulation of gene transcription by RORs

RORs exhibit a structural architecture that is typical of nuclear receptors. RORs contain four major functional domains: an amino-terminal (A/B) domain, DNA-binding domain (DBD), a hinge domain, and a ligand-binding domain (LBD) (Evans, 1988; Giguere, 1999; Jetten et al., 2001; Moras and Gronemeyer, 1998; Pike et al., 2000; Steinmetz et al., 2001; Willy and Mangelsdorf, 1998). The DBD consists of two highly-conserved zinc finger motifs involved in the recognition of ROR response elements (ROREs) which consist of the consensus motif AGGTCA preceded by an AT-rich sequence (Andre et al., 1998a; Carlberg et al., 1994; Giguere et al., 1994; Greiner et al., 1996; Jetten et al., 2001; Medvedev et al., 1996; Moraitis and

Giguere, 1999; Ortiz et al., 1995; Schrader et al., 1996). The RORs bind ROREs as a monomer (Andre et al., 1998b; Carlberg et al., 1994; Giguere et al., 1994; Greiner et al., 1996; Medvedev et al., 1996; Moraitis and Giguere, 1999; Ortiz et al., 1995; Schrader et al., 1996). This conclusion was supported by crystal structure analyses that indicated the presence of a kink in H10 of the ROR β (LBD) at A⁴¹¹-K⁴¹² and of the ROR α (LBD) at C⁵⁰⁵-K⁵⁰⁶ (Kallen et al., 2002; Stehlin et al., 2001; Stehlin-Gaon et al., 2003). Because H10 plays a critical role in the homo- and heterodimerization of nuclear receptors, the presence of a kink would greatly affect the dimerization capability of RORs. Therefore, it is unlikely that RORs are able to form receptor homo- or heterodimers. The P-box, the loop between the last two cysteins within the first zinc finger, recognizes the core motif in the major groove (Giguere et al., 1995; Jetten et al., 2001; McBroom et al., 1995). Residues just downstream from the two zinc fingers, referred to as C-terminal extension (CTE), play a critical role in determining the DNA binding specificity of RORs (Andre et al., 1998b; Giguere et al., 1995; Giguere et al., 1994; Sundvold and Lien, 2001; Vu-Dac et al., 1997). The CTE makes contact with the 5'-AT-rich segment of the RORE in the adjacent minor groove. The amino-terminus (A/B domain) also influences the binding affinity of RORs. This modulation likely accounts for the distinct binding specificities exhibited by the different ROR variants (Andre et al., 1998b; Giguere et al., 1995; Giguere et al., 1994; Sundvold and Lien, 2001; Vu-Dac et al., 1997).

The nuclear receptors Rev-ErbA α and Rev-Erb β (NR1D1 and D2, respectively) have very similar binding specificities for ROREs as the RORs (Giguere et al., 1995). Since Rev-Erb receptors act as constitutive transcriptional repressors, they are able to inhibit ROR-mediated transcriptional activation by competing with RORs for the same DNA response element (Austin et al., 1998; Bois-Joyeux et al., 2000; Downes et al., 1996; Forman et al., 1994; Medvedev et al., 1997; Retnakaran et al., 1994). Recent studies have indicated the physiological significance of such interplay in the control of circadian rhythm. Rev-Erb and ROR α , respectively, repress and activate transcription of the Bmal1 gene, which encodes a transcription factor that is critical in the control of the circadian clock, by competing for ROREs in the promoter region of the Bmal1 gene (Akashi and Takumi, 2005; Albrecht, 2002; Gachon et al., 2004; Guillaumond et al., 2005; Nakajima et al., 2004; Preitner et al., 2002; Triqueneaux et al., 2004). Such cross-talk may be implicated in the regulation of other physiological processes as well.

The LBDs of nuclear receptors play a role in ligand binding, nuclear localization, receptor dimerization, contain a transactivation function, and provide an interface for the interaction with co-activators and co-repressors. The LBDs of RORs are moderately conserved; the LBD of ROR α exhibits a 63% and 58% identity with that of ROR β and ROR γ , respectively (Jetten, 2004; Jetten et al., 2001). Although the LBD of nuclear receptors do not exhibit a high degree of homology, their secondary structure is very similar and usually contains 12 α -helices (H1-H12). H12, which contains the activation function 2 (AF2) consensus motif $\Phi\Phi XE/D\Phi\Phi$ (where Φ is a hydrophobic amino acid and X is any amino acid), is 100% conserved among RORs. X-ray structural analysis has demonstrated that RORs have two additional helices, H2' and H11' (Kallen et al., 2002; Stehlin et al., 2001; Stehlin-Gaon et al., 2003). In addition, these studies provided evidence indicating that the activity of RORs is controlled by ligands. Cholesterol, 7-dehydrocholesterol, and cholesterol sulfate have been shown to bind ROR α in a reversible manner and to enhance RORE-dependent transcriptional activation by ROR α (Kallen et al., 2004; Kallen et al., 2002). Whether cholesterol, cholesterol sulfate or other (sulfated) lipid metabolites function as physiological ligands for ROR α or whether they are merely structural co-factors that stabilize the conformation of ROR α has yet to be established.

Initial studies of the X-ray structure of the ROR β (LBD) identified stearic acid as a fortuitously captured ligand which appeared to act as a filler and stabilizer rather than as a functional ligand (Stehlin et al., 2001). Follow-up studies demonstrated that several retinoids, including all-trans

retinoic acid (ATRA) and the synthetic retinoid ALRT 1550 (ALRT), were able to act as functional ligands for ROR β (Stehlin-Gaon et al., 2003). These retinoids were able to bind ROR β (LBD) with high affinity and in a reversible manner while the RAR-selective antagonist RO 41-5253 was unable to bind. ATRA and ALRT 1550 were also able to bind ROR γ but not ROR α (Stehlin-Gaon et al., 2003). Both ATRA and ALRT inhibited ROR β -mediated transcriptional activation suggesting that these retinoids act as partial antagonists. Future studies are needed to determine whether cholesterol(sulfate) and retinoic acid are genuine physiological ligands for RORs and, if so, what physiological functions and target genes they regulate. Regardless of whether these agents are physiological ligands or not, these studies have demonstrated that the activity of RORs can be modulated by (synthetic) ligands and, therefore, support the concept that RORs might be potential targets for pharmacological intervention of pathological processes.

For many receptors, binding of a ligand functions as a switch that induces a conformational change in the receptor that involves a repositioning of H12 (AF2) (Darimont et al., 1998; Glass and Rosenfeld, 2000; Harding et al., 1997; Heery et al., 2001; Heery et al., 1997; McInerney et al., 1998; Nagy et al., 1999; Nolte et al., 1998; Xu et al., 1999). Binding of an agonist induces a transcriptionally active conformation of the receptor. The activated state allows recruitment of co-activator complexes which, through their histone acetylase activity, induce chromatin remodeling and subsequently an increase in the transcription of target genes (Glass and Rosenfeld, 2000; McKenna and O'Malley, 2002; Xu, 2005). Recruitment of a co-repressor complex induces, through its histone deacetylase activity, compactation of chromatin and repression of gene expression (Horlein et al., 1995; Hu and Lazar, 2000; Nagy et al., 1999). RORs have been reported to be able to interact with co-repressors as well as co-activators suggesting that RORs can function as repressors and activators of gene transcription. Whether the recruitment of co-repressors and co-activators by RORs is dependent on, respectively, the absence and presence of physiological ligands has yet to be determined. However, study of ROR α -mediated transcriptional regulation shows (Gold et al., 2003) that RORs recruit different co-activator complexes when bound to ROREs in the promoter region of different genes. This suggests that the promoter context plays an important role in determining which co-activators are recruited by RORs (Jetten, 2004; Jetten et al., 2001). Table 1, shows a summary of proteins that have been reported to interact with ROR receptors. NCOA1, NCOA2, PGC-1 α , p300, and CBP are among the co-activators reported to mediate transcriptional activation by RORs (Atkins et al., 1999; Gold et al., 2003; Harding et al., 1997; Harris et al., 2002; Jetten, 2004; Jetten et al., 2006; Kurebayashi et al., 2004; Lau et al., 1999; Littman et al., 1999; Xie et al., 2005). RORs have also been demonstrated to interact with a number of co-repressors, including NCOR1, NCOR2, RIP140, and the neuronal interacting factor X (NIX1) (Greiner et al., 2000; Harding et al., 1997; Jetten, 2004; Jetten et al., 2006; Johnson et al., 2004; Littman et al., 1999)(Table 1).

RORs also interact with several proteins that are involved in ubiquitination or are part of the proteasome complex (Atkins et al., 1999; Jetten et al., 2006)(Table 1). The ubiquitin (Ub)-proteasome system is intimately involved in regulating chromatin structure remodeling and in the transcriptional control by a number of nuclear receptors (Dace et al., 2000; Dennis et al., 2001; Ismail and Nawaz, 2005; Kinyamu et al., 2005; Poukka et al., 1999; Wallace and Cidlowski, 2001). Ubiquitination is a multistep process that involves three types of enzymes: ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3) (Weissman, 2001). E3 ligases link ubiquitin covalently to Lys residues in target proteins and then successively attach additional ubiquitins forming a polyubiquitin chain. Polyubiquitin serves as a recognition signal and targets the proteins to the proteasome for rapid degradation (Adams, 2003; Muratani and Tansey, 2003). Several studies have indicated a link between ROR signaling and the ubiquitin/proteasome system. The proteasome subunit β type 6 (PSMB6) and the proteasome 26S ATPase subunit PSMC5 have been shown to interact with

ROR receptors (Atkins et al., 1999; Jetten et al., 2006). In addition, inhibition of the 26S proteasome complex by the proteasome inhibitor MG-132 has been reported to increase the level of ubiquitinated ROR α protein and to inhibit ROR α -mediated transcriptional activation (Moraitis and Giguere, 2003). These studies suggest that the ubiquitin/proteasome system is an integral part of the mechanism by which RORs control transcription. The latter is supported by studies showing that Hairless (*Hr*) (Cachon-Gonzalez et al., 1994) functions as an effective repressor of ROR-induced transcriptional activation (Hsieh et al., 2003; Moraitis and Giguere, 2003; Moraitis et al., 2002). This repressor activity appears to involve protection of RORs from degradation by the ubiquitin (Ub)-proteasome system. The ubiquitin-conjugating enzyme I (UBE2I or UBC9), which catalyzes sumoylation, also interacts with RORs (Jetten et al., 2006). Whether UBE2I plays a role in the sumoylation of RORs has yet to be established.

RORs have been shown to induce RORE-dependent transcriptional activation of a reporter gene in a cell type-dependent manner (Atkins et al., 1999; Austin et al., 1998; Delerive et al., 2001; Gawlas and Stunnenberg, 2001; Giguere et al., 1994; Greiner et al., 1996; Medvedev et al., 1996; Ortiz et al., 1995; Schrader et al., 1996) and functionally active ROREs have been identified in many putative target genes, including apolipoproteins A-I, C-III, and A-V, prosaposin, and I κ B α (Bois-Joyeux et al., 2000; Carlberg and Wiesenberger, 1995; Chu and Zingg, 1999; Delerive et al., 2002; Delerive et al., 2001; Dussault and Giguere, 1997; Genoux et al., 2005; He et al., 1998; Jin et al., 1998; Jin et al., 2001; Lind et al., 2005; Littman et al., 1999; Matsui, 1996; Matsui, 1997; Paravicini et al., 1996; Raspe et al., 2001; Schrader et al., 1996; Steinhilber et al., 1995; Sun et al., 2003; Sundvold and Lien, 2001; Tini et al., 1995; Villey et al., 1999; Vu-Dac et al., 1997). However, further studies are needed to determine whether these genes are genuine physiological targets of RORs. Recent microarray analysis identified a number of authentic ROR α target genes in cerebellar Purkinje cells. These include sonic hedgehog (*Shh*) and Purkinje cell protein 2 (*Pcp-2*) (Gold et al., 2003). This will be discussed below in more detail.

Functions of ROR α in brain development

ROR α is expressed in a number of adult tissues. In testis, ROR α is expressed in the peritubular cells but not in the seminiferous tubules (Steinmayr et al., 1998). In skin, ROR α is expressed in the suprabasal cells of the epidermis, sebaceous glands, and in anagen hair follicles. Spermatogenesis is unaffected in mice deficient in ROR α expression (ROR $\alpha^{-/-}$ mice) and mice are fertile (Steinmayr et al., 1998). In addition, ROR $\alpha^{-/-}$ mice do not display changes in epidermal differentiation; however, these mice have been reported to develop a less dense fur that grows back slowly after shaving, suggesting a regulatory role in hair development.

Several studies have shown that ROR α is most highly expressed in several regions of the brain, particularly the cerebellum and thalamus (Becker-Andre et al., 1993; Carlberg et al., 1994; Dussault et al., 1998; Hamilton et al., 1996; Matysiak-Scholze and Nehls, 1997; Nakagawa et al., 1997; Nakagawa et al., 1998; Vogel et al., 2000). In the thalamus ROR α mRNA is highly expressed in neurons and in the cerebellum it is detected in Purkinje cells but not in the granule cell layer (Ino, 2004; Matsui et al., 1995; Nakagawa et al., 1997; Sashihara et al., 1996; Sotelo and Wassef, 1991). ROR α is expressed at moderate levels in the olfactory bulb and at low levels in layer IV of the neocortex (Michel et al., 2000; Monnier et al., 1999; Nakagawa and O'Leary, 2003). Low levels of expression of ROR α can also be observed in the pituitary, the superficial region of the dorsal cochlear nucleus, the suprachiasmatic nucleus, the superior colliculus, and the spinal trigeminal nucleus. In the retina, the ganglion cells and cells of the inner nuclear layer contain low levels of ROR α protein and mRNA (Ino, 2004; Steinmayr et al., 1998).

During forebrain development, ROR α mRNA is expressed in a spatiotemporal manner (Nakagawa and O'Leary, 2003; Sashihara et al., 1996). At E11.5, ROR α mRNA is not detectable in the dorsal thalamus but by E12.5 is expressed in a narrow ventrolateral region in the mantle zone where the LIM homeobox 9 gene (Lhx9) is not expressed. At E17.5 of mouse development ROR α is expressed strongly throughout the ventroposterior nucleus, the dorsal lateral geniculate nucleus, and the ventral part of the medial geniculate nucleus (Ino, 2004; Nakagawa and O'Leary, 2003). These regions relay somatosensory, visual and auditory information to the neocortex. It was concluded that expression of ROR α in the principle sensory neurons begins shortly after they become postmitotic. In the neocortex, ROR α is first detected at E18.5 in the middle layers of the middle part of the neocortex and at postnatal day (PND) 0 (day of birth) weakly in the somatosensory area (Nakagawa and O'Leary, 2003). By PND5, a moderate level of expression is seen in cortical layer IV.

ROR α null mice, generated by targeted disruption of the ROR α gene, display ataxia which is correlated with severe cerebellar atrophy (Dussault et al., 1998; Steinmayr et al., 1998). The ataxic and cerebellar phenotype is identical to that observed in homozygous *staggerer* mutant mice (ROR $\alpha^{sg/sg}$) which carry a deletion within the ROR α gene that prevents translation of the LBD (Hamilton et al., 1996; Herrup and Mullen, 1981; Landis and Sidman, 1978; Sidman et al., 1962). Moreover, the electrophysiological characteristics are indistinguishable between the two mouse strains. The ataxic phenotype observed in ROR α -deficient mice is at least partially due to the development of cerebellar atrophy (Gold et al., 2006). The cerebellum of ROR α -deficient mice contains significantly fewer Purkinje cells and exhibits a loss of cerebellar granule cells. (Doulazmi et al., 1999; Hamilton et al., 1996; Herrup and Mullen, 1979; Herrup and Mullen, 1981; Landis and Reese, 1977; Landis and Sidman, 1978; Sidman et al., 1962). It has been reported that the cerebellum of adult ROR $\alpha^{sg/sg}$ mice contains 80% fewer calbindin-positive Purkinje cells compared to wild type mice (Doulazmi et al., 2001). Although the morphology of the cerebellar cortex in young heterozygous (ROR $\alpha^{sg/+}$) mice appears normal, upon aging an accelerated loss of Purkinje cells is noticed. This loss occurs earlier in males than in females, suggesting that gender is a factor (Doulazmi et al., 1999; Hadj-Sahraoui et al., 2001; Hadj-Sahraoui et al., 1997).

The reduction in the number of Purkinje cells could be due to either a defect in the control of proliferation or differentiation of Purkinje cell precursors or to a defect in the maturation or survival of Purkinje cells (Boukhtouche et al., 2006; Bouvet et al., 1987; Gold et al., 2006; Herrup and Mullen, 1979; Herrup and Mullen, 1981; Landis and Sidman, 1978; Messer et al., 1990; Sotelo and Wassef, 1991). All cells in the cerebellum are derived from the germinal matrix region of the metencephalon (Goldowitz and Hamre, 1998). The nuclear and Golgi neurons, and Purkinje cells are generated from the ventricular neuroepithelium. The granule precursors arise from the rhombic lip and will form the external granular layer (EGL). During early post-natal development, granule cell precursors in the outer zone of the EGL proliferate, then exit the cell cycle and differentiate. They subsequently migrate through the molecular layer (ML) past the Purkinje cells to their destination, the internal granule cell layer (IGL). During E11-E13 of mouse development Purkinje cell precursors exit the mitotic cycle, leave the ventricular zone and migrate along radial glia to form a temporary cerebellar plate-like structure (Gold et al., 2003; Goldowitz and Hamre, 1998). At E13-14, they begin to express ROR α . In ROR $\alpha^{sg/sg}$ mice, at E17.5 the normal number of Purkinje cells have been generated (Doulazmi et al., 2001; Vogel et al., 2000). However, by PND5 the number of Purkinje cells is dramatically reduced, the external granular layer is significantly thinner, and a dramatic cerebellar hypoplasia is apparent. The surviving Purkinje cells have stunted dendritic arbors lacking distal spiny branchlets and are deficient in the assembly of mature synapses with granule cells suggesting that their dendritic differentiation is impaired (Landis and Sidman, 1978; Shirley and Messer, 2004; Sotelo and Changeux, 1974; Sotelo and Wassef, 1991). In addition, the remaining ROR $\alpha^{sg/sg}$ Purkinje cells do not express a number of genes normally

expressed in mature postnatal Purkinje cells, including Purkinje cell-specific protein (Pcp-2), calmodulin, Zebrin I, and the N-methyl-D-aspartate (NMDA) receptor (Gold et al., 2003; Hamilton et al., 1996; Messer et al., 1990; Nakagawa et al., 1996a; Nakagawa et al., 1996b; Sotelo and Wassef, 1991). These observations indicate that the surviving $ROR\alpha^{sg/sg}$ Purkinje cells do not mature. Because a reduction in $ROR\alpha^{sg/sg}$ Purkinje cells is observed only after E17.5, the lack of $ROR\alpha$ expression does not affect the genesis of Purkinje cells but rather their survival. It seems that the absence of $ROR\alpha$ is not directly responsible for the death of $ROR\alpha^{sg/sg}$ Purkinje cells but a consequence of the block or inhibition of Purkinje cell maturation (Fig. 1). This concept is supported by the reduced expression of genes normally expressed in mature Purkinje cells and the impaired dendritogenesis observed in $ROR\alpha^{sg/sg}$ Purkinje cells. This conclusion was further strengthened by a recent study examining the effects of lentivirus-mediated overexpression of $ROR\alpha$ on dendritic differentiation in $ROR\alpha^{sg/sg}$ Purkinje cells using organotypic cultures (Boukhtouche et al., 2006). This study showed that expressing $ROR\alpha$ in $ROR\alpha^{sg/sg}$ Purkinje cells restores normal dendritogenesis suggesting that $ROR\alpha$ plays a critical role in the control of dendritic differentiation during development.

Several studies have established that the number of Purkinje cells, to a large extent, determines the size of the granule cell population suggesting that these neurons provide a signal that regulates the proliferation of granule cells (Goldowitz and Hamre, 1998; Vogel et al., 2000). Recent studies have demonstrated that interactions of sonic hedgehog (Shh), produced by Purkinje cells, with its receptor Patched (Ptch), present on granule cells, provides an important signal for the proliferation of granule cell precursors (Dahmane and Ruiz-i-Altaba, 1999; Gold et al., 2003; Kenney et al., 2003; Wallace, 1999). Experiments demonstrating that Shh mRNA is reduced 2- to 3-fold in Purkinje cells of $ROR\alpha^{sg/sg}$ mice suggest that inhibition of Shh expression may be a critical factor in the diminished proliferation of granule cell precursors (Gold et al., 2003). This hypothesis was supported by observations showing that treatment with recombinant Shh could enhance proliferation of granule precursor cells in sections of cerebellum from PND4 $ROR\alpha^{sg/sg}$ mice and partially prevent the reduction in granule cells. The importance of the reduced Shh levels in $ROR\alpha^{sg/sg}$ mice was further illustrated by the observed repression of several Shh target genes, including N-Myc, Baf53, and various cyclins, in the cerebellum of $ROR\alpha^{sg/sg}$ mice (Fig. 1). In addition to Shh, a number of other genes have been identified that are down-regulated in Purkinje cells of $ROR\alpha^{sg/sg}$ mice (Gold et al., 2003). These include genes encoding proteins involved in signal-dependent calcium release, such as the calmodulin inhibitor Pcp4, the IP3 receptor (Itpr1) and its interacting partner Cals1, and major intracellular calcium buffer 1 (Calb1). Another group of $ROR\alpha$ -responsive genes identified by this molecular profile microarray analysis are linked to the glutamatergic pathway and include genes encoding the glutamate transporter Slc1a6 and Spnb3, encoding a brain-specific β -spectrin (Gold et al., 2003). The observation that several of these genes are down-regulated in $ROR\alpha^{sg/sg}$ Purkinje cells, is in agreement with the hypothesis that lack of $ROR\alpha$ inhibits the progression of the maturation of these cells.

The repression of these genes in $ROR\alpha^{sg/sg}$ Purkinje cells raised the question of whether they are regulated by $ROR\alpha$ by a direct or indirect mechanism. Analysis of the promoter sequence of a number of these genes indicated the presence of several putative ROREs (Gold et al., 2003). Chromatin immunoprecipitation (CHIP) assays subsequently demonstrated that $ROR\alpha$ antibodies pulled down the promoters of Pcp2, Pcp4, Itpr1, Shh, and Slc1a6 from chromatin isolated from Purkinje cells of wild type but not from that of mutant mice (Gold et al., 2003; Gold et al., 2006). These results support the conclusion that $ROR\alpha$ binds to these ROREs in these promoter regions *in vivo* and suggest that these genes are directly regulated by $ROR\alpha$.

Further analysis of the protein complexes recruited by $ROR\alpha$ to these different ROREs demonstrated that $ROR\alpha$ recruited distinct co-activators and co-factors to different promoter

regions. ROR α recruited β -catenin and p300 as co-activators to the Shh promoter, β -catenin, Tip60, and SRC-1 to the Pcp2 promoter, CBP, Tip60, SRC1, and GRIP-1 to the Slc1a6 promoter, and β -catenin, SRC-1, p300, and Tip60 to the Pcp4 promoter (Gold et al., 2003)(Fig. 1). Microinjection of blocking antibodies against SRC1, TIP60, and β -catenin greatly inhibited ROR α -mediated activation of the Pcp2 promoter while antibodies against CBP and p/CIP did not have any effect. These results confirmed the functional role of these co-activators in ROR α -mediated activation of this promoter. The recruitment of different co-activator complexes by ROR α is an important and intriguing finding and suggests that the sequence and/or the promoter context of the RORE plays a critical role in determining what co-activator complex is assembled by ROR α .

The association of β -catenin with ROR α /co-activator complexes may suggest a possible link between W and ROR signaling. Activation of β -catenin by Wnt signaling might influence the transcriptional activity of ROR α . ROR α may act downstream of Wnt and because Shh is an ROR α target gene, ROR α may function as a mediator between the Wnt and Shh signaling pathways (Fig. 1). This would be consistent with other studies indicating an interaction between Wnt and Shh signaling pathways (Borycki et al., 2000) and a role for Wnt signaling in cerebellar development (Salinas et al., 1994; Wang et al., 2001).

The function of ROR α in brain development has not been studied much beyond the cerebellum. However, several studies have indicated a role for ROR α in the development of the olfactory bulb (Michel et al., 2000; Monnier et al., 1999). ROR α mRNA and protein has been detected at moderate levels in the mitral cell layer and in tufted and periglomerular cells of the olfactory bulb (Ino, 2004; Matsui et al., 1995; Sashihara et al., 1996). Moreover, cytological alterations been observed in the olfactory bulb of ROR $\alpha^{sg/sg}$ mice (Michel et al., 2000; Monnier et al., 1999). The olfactory bulb of ROR $\alpha^{sg/sg}$ mice is structurally disorganized and the number of neurons and periglomerular neurons in particular mitral cells are significantly decreased (Monnier et al., 1999). Moreover, changes in the granular zone are observed typified by an abundance of glial cell process extension and a reduction of the astrocyte network. Such changes might lead to a failure in normal neuronal input processing. The latter is supported by studies showing a significant reduction in interneural interactions in the olfactory bulb of ROR $\alpha^{sg/sg}$ mice after odorant stimulation (Michel et al., 2000). This reduction may be related to a decrease in post-synaptic elements and due to the decline in mitral cells. Moreover, these changes might be responsible for some of the observed behavioral abnormalities, such as impaired inter-individual recognition.

Physiological Functions of ROR

ROR β exhibits a rather restricted pattern of expression and is particularly highly expressed in certain regions of the brain and in the retina (Andre et al., 1998a; Andre et al., 1998b; Azadi et al., 2002; Carlberg et al., 1994; Schaeren-Wiemers et al., 1997). In the retina, ROR β expression is highly dynamic during embryonic development. At E15, all cells in the retina appear to express ROR β while at E17.5 and PND5, ROR β is detected only in the inner and outer nuclear layers (Schaeren-Wiemers et al., 1997). At PND9, ROR β is preferentially expressed in the inner nuclear layer. This expression is greatly reduced at PND16 correlating with the eye-opening. With maturation, the adult retina shows ROR β expression to be highest in the outermost part of the outer nuclear layer where the photoreceptor cones reside, with weaker expression in the inner nuclear layer and ganglion cells (Schaeren-Wiemers et al., 1997). In adult brain ROR β mRNA is most highly expressed in the cortex and the thalamus. Expression is also found in the hypothalamus, suprachiasmatic nucleus (SCN), and the pineal gland, while little expression was detected in the cerebellum or hippocampus.

Nakagawa and O'Leary extensively examined the expression of ROR β in the developing mouse neocortex and dorsal thalamus from E12.5 to PND5 (Nakagawa and O'Leary, 2003). These studies indicated that during brain development ROR β expression is highly dynamic and that ROR β mRNA is expressed in a spatial and temporal manner. ROR β expression was undetectable in the neocortical ventricular zone at any of the embryonic stages examined. At E12.5, ROR β is not detectable in the dorsal telencephalon but by E14.5 high levels of expression are evident in the lateral and rostral parts of the neocortex. By E16.5, levels of ROR β mRNA are increased and expressed in a graded fashion: from strong high lateral to low medial and from high rostral to low caudal expression. At birth, high levels of expression were observed in the somatosensory area and putative auditory and visual areas in the caudal neocortex. At PND2, ROR β mRNA is highly expressed in the primary visual, auditory, and somatosensory regions in layer IV and sporadically in layer V (Nakagawa and O'Leary, 2003). In mice at PND5, ROR β is highly expressed in layers IV and V of the primary sensory areas.

During the development of the dorsal thalamus, ROR β mRNA was undetectable at E11.5 and E12.5. At E17.5, ROR β mRNA was expressed at low to moderate levels in the ventroposterior nucleus and the ventral part of the medial geniculate nucleus, respectively, but was undetectable in the dorsal lateral geniculate nucleus (Nakagawa and O'Leary, 2003). A high level of expression was also observed in the centromedial nucleus. By PND2, ROR β is highly expressed in the ventroposterior nucleus and the ventral part of the medial geniculate nucleus and minimally expressed in the dorsal lateral geniculate nucleus.

Disruption of ROR β gene expression in mice causes a number of abnormalities both structural and functional in nature (Andre et al., 1998a). ROR β null mice exhibit a 'duck-like' gait but maintain normal reflexes and balancing responses. No gross anatomical changes were observed in the brain and spinal cord of adult ROR $\beta^{-/-}$ mice. Although at birth, the retina of ROR β null mice appears morphologically very similar to that of wild type mice, the retina in adult ROR $\beta^{-/-}$ mice is disorganized and lacks the normal layer structure. Retinal degeneration occurs during the first weeks after birth and finally results in blindness. The precise molecular mechanism underlying this retinal degeneration is not yet understood.

Role of RORs in circadian rhythm

Circadian rhythms are daily cycles of behavioral and physiological changes that are driven by an endogenous oscillator within a one-day period (Isojima et al., 2003; Schibler and Naef, 2005; Schibler and Sassone-Corsi, 2002). Circadian rhythm impacts behavior, a variety of physiological functions, drug metabolism, and has been implicated in disease, including sleep disorders and cancer. In mammals, light is the principal signal received by the non-cone, non-rod system in the retina. This signal is transmitted through the retinohypothalamic tract to the SCN. Mammalian circadian rhythms are governed by a central circadian clock that resides in the SCN of the anterior hypothalamus (Isojima et al., 2003; Ripperger and Schibler, 2001). This master oscillator can maintain circadian rhythms in the absence of light input. Many physiological processes in most, if not all, tissues are controlled by circadian oscillators, the phase of which is regulated by signals originating from the SCN.

Although their precise role is still far from clear, several studies have implicated RORs in the regulation of circadian behavior (Akashi and Takumi, 2005; Guillaumond et al., 2005; Jetten et al., 2006; Preitner et al., 2002; Schaeren-Wiemers et al., 1997; Ueda et al., 2002b). This is indicated an aberrant circadian behavior observed in mice deficient in ROR α or ROR β (Akashi and Takumi, 2005; Sato et al., 2004; Schaeren-Wiemers et al., 1997). Although examination of circadian-related behavior in ROR α -deficient (ROR $\alpha^{sg/sg}$) mice has been difficult given the locomotor difficulties related to their *staggerer* phenotype, reports indicate that the time period

of circadian-dependent locomotor activity is decreased in both the $ROR\alpha^{sg/+}$ and in $ROR\alpha^{sg/sg}$ mice which may be related to altered circadian rhythm or just altered motor function in $ROR\alpha$ -deficient mice (Akashi and Takumi, 2005). $ROR\alpha^{sg/sg}$ mice display anomalous free-running locomotor activity rhythms and an altered feeding pattern during the circadian cycle (Akashi and Takumi, 2005; Guastavino et al., 1991; Sato et al., 2004).

In several tissues expression of RORs shows an oscillating pattern during the circadian cycle. $ROR\beta$ is particularly highly expressed in tissues that play a key role in the generation and maintenance of circadian rhythms, including the SCN, the pineal gland, and the retina (Andre et al., 1998a; Andre et al., 1998b; Schaeren-Wiemers et al., 1997; Sumi et al., 2002). The expression of $ROR\beta$ mRNA in the pineal gland and retina reaches a maximum during the night period at circadian time CT18 (Andre et al., 1998a; Ueda et al., 2002b). In the SCN, the expression of $ROR\alpha$ and $ROR\beta$ display a similar circadian profile with peaks during the day and troughs during the night, while $ROR\gamma$ is not expressed in the SCN. $ROR\gamma$ mRNA shows a circadian expression pattern in both liver and kidney but not in thymus (Jetten et al., 2006; Ueda et al., 2002b; Ueda et al., 2005). $ROR\gamma$ is expressed at low levels during the day and at optimum levels at CT18 trailing the peak expression of *Rev-ErbA α* (at CT8) and *Per2* (at CT13). $ROR\alpha$ mRNA expression shows little circadian oscillation in liver, kidney, and lung (Akashi and Takumi, 2005; Ueda et al., 2002b).

Although many aspects of the regulation of the circadian oscillator still have to be elucidated, recent studies have made great advances in understanding the control mechanism of the circadian clock (Piggins and Loudon, 2005; Ripperger and Schibler, 2001; Roenneberg and Mellow, 2005; Schibler and Naef, 2005). The main feature of this mechanism is that the circadian oscillators consist of several interconnected molecular feedback loops. The positive loop consists of the basic helix-loop-helix/PAS-type transcription factors *Bmal1* and *CLOCK*, while two cryptochrome (*Cry*) and three period proteins (*Per*) are involved in the negative control of the oscillator (Albrecht, 2002; Isojima et al., 2003; Reppert and Weaver, 2002; Schibler and Naef, 2005; Schibler and Sassone-Corsi, 2002). Heterodimers of *Bmal1* and *Clock* enhance the transcription of *Per* and *Cry* genes through their interaction with E-box enhancers (5'-CACGTG) in the 5'-upstream regulatory regions of these genes. After being synthesized in the cytoplasm, *Per1* and *Per2* become phosphorylated by casein kinase I ϵ/δ . This promotes their dimerization with *Cry1* and allows transfer of the complex into the nucleus where they repress the transcription of *Bmal1*. It has been proposed that *Cry1* represses the activation of their own expression by binding to the *Bmal1*:*CLOCK* complex. The turnover of these proteins then leads to a new cycle of activation of *Bmal1* and *CLOCK*, while in turn increasing levels of *Per2* and *Cry1* suppress the expression of *Bmal1* and *CLOCK*. The repression of *Bmal1* appears to involve the nuclear orphan receptors *Rev-ErbA α* *Rev-Erb β* , which function as transcriptional repressors. The periodic expression of *Rev-ErbA α* is regulated negatively by *Per*:*Cry* complexes through E-box elements in its promoter while *CLOCK*:*Bmal1* stimulates *Rev-ErbA α* promoter activity. In turn, an increase in *Rev-ErbA α* results in the repression *Bmal1* transcription. This repression is mediated through the binding of *Rev-Erb* to several RORE response elements (AAAGTAGGTCA) in the promoter of the *Bmal1* gene (Guillaumond et al., 2005; Nakajima et al., 2004; Preitner et al., 2002; Sato et al., 2004; Ueda et al., 2002b). Mutation of these ROREs abolishes the circadian rhythmicity of the *Bmal1* promoter indicating the importance of this response element in *Bmal1* transcriptional regulation.

The ROREs in the *Bmal1* promoter also bind ROR receptors. Overexpression of *ROR α 1* and *ROR α 4* induces *Bmal1*-promoter activity by interacting with these ROREs, this induction can be inhibited by co-expression of *Rev-ErbA α* (Akashi and Takumi, 2005; Guillaumond et al., 2005; Nakajima et al., 2004; Sato et al., 2004; Ueda et al., 2002b; Ueda et al., 2005). $ROR\beta$ and $ROR\gamma$ are also able to induce *Bmal1* activity; however, *ROR α 4* appears the most effective

in inducing this activity. Recent studies examining the activation of a luciferase reporter, under the control of the Bmal1(−3465 to +57) promoter region, in NIH3T3 cells after serum shock revealed an oscillating profile of transcriptional activation. Co-expression of a dominant-negative ROR α lacking its LBD caused a severe weakening of this oscillation. In addition, a knockdown of ROR α expression using siRNA also attenuated the amplitude of the oscillation. The transcriptional oscillation of Bmal1 promoter activity was muted in mouse embryo fibroblasts isolated from ROR $\alpha^{sg/sg}$ mice compared to wild type fibroblasts. Mutation of one of the RORE sites in the Bmal1 promoter abolished the transcriptional oscillation suggesting the importance of these sites in the regulation of Bmal1 by ROR α (Akashi and Takumi, 2005; Ueda et al., 2002b). This is further supported by findings showing that at CT18 the expression of Bmal1 in the SCN of ROR $\alpha^{sg/sg}$ mice was significantly lower compared to that of wild type mice (Sato et al., 2004). These observations demonstrate that ROR α functions as a positive regulator of Bmal1 and that cross-talk between ROR α and Rev-ErbA α nuclear receptors plays a critical role in the control of Bmal1 expression. At high levels, Rev-ErbA α competes with ROR α for binding to these ROREs and represses Bmal1 expression while at low levels it allows ROR α to bind and activate Bmal1 transcription (Akashi and Takumi, 2005; Guillaumond et al., 2005; Isojima et al., 2003; Preitner et al., 2002; Sato et al., 2004; Triqueneaux et al., 2004). In addition, the circadian regulation of ROR α itself may be a factor in the control of Bmal1 expression as well. Recent studies have shown that a loss of ROR α reduces the expression of several other genes in the SCN, including tubulin alpha 8 (Tuba8) and Rasd1 (RAS, dexamethasone-induced 1) (Sato et al., 2004; Ueda et al., 2002b). These genes contain ROREs in their promoter regulatory region suggesting that they might be regulated by ROR α directly. Observations showing that in the absence of ROR α these genes still retain a certain degree of an oscillating pattern of expression suggests that other factors play a role in the control of their circadian expression.

Role of ROR γ in the development of secondary lymphoid tissues

Study of ROR γ null mice have revealed several important clues about the function of ROR γ in the development of secondary lymphoid tissues (Eberl and Littman, 2003; He, 2002; He et al., 2000; Jetten et al., 2001; Jetten and Ueda, 2002; Lipp and Muller, 2004). These studies have demonstrated that mice deficient in ROR γ lack lymph nodes and Peyer's patches, suggesting a critical role for ROR γ (ROR γ 2) in lymph node organogenesis (Eberl and Littman, 2003; Eberl et al., 2004; He, 2002; Jetten et al., 2001; Jetten and Ueda, 2002; Kurebayashi et al., 2000; Lipp and Muller, 2004; Littman et al., 1999; Sun et al., 2000).

In recent years, our understanding of lymph node development has been greatly enhanced (Cupedo and Mebius, 2005; Cyster, 2003; Eberl, 2005; Eberl and Littman, 2003; Eberl et al., 2004; Fu and Chaplin, 1999; Mebius et al., 2001; Mebius et al., 1997; Muller et al., 2003; Nishikawa et al., 2003; Rennert et al., 1998). In mice, lymph node organogenesis is initiated around E10.5 with the invagination of endothelial cells resulting in the development of lymphoid sacs. This process involves the conversion of endothelial cells into cells with a lymphatic phenotype. The homeobox protein Prox-1 has been demonstrated to play a critical role in this conversion (Wigle et al., 2002). From these early lymph sacs, lymphatic vessels grow out and eventually form the complete lymphatic network by E15.5. The formation of the lymphatic network is not affected in ROR $\gamma^{-/-}$ mice, suggesting that lack of lymph nodes appears not to be due to defects in the formation of lymph sacs and lymphatic vessels (Sun et al., 2000). The earliest anlagen of lymph nodes are formed after infiltration of organizer cells. It is believed that these specialized cells are derived from differentiating mesenchymal cells; however, much about this differentiation process is still poorly understood (Cupedo and Mebius, 2005; Eberl, 2005). During ontogeny lymphoid tissue inducer (LTi) cells, characterized as CD45⁺CD4⁺CD3[−]IL-7R α ⁺ cells, are among the earliest hematopoietic cells to colonize sites destined to develop into secondary lymphoid organs. Although the precise

origin of LT α cells has not yet been established, it has been suggested that LT α cells may originate from IL-7R α ⁺Sca-1^{low}c-Kit^{low} hematopoietic precursor cells in the fetal liver (Cupedo and Mebius, 2005; Eberl, 2005). By E12.5, LT α cells are detected in spleen, fetal blood, and lymph node anlagen, and by E16 in Peyer's patch anlagen. LT α cells are present in very low numbers after birth. The migration of LT α cells into the anlagen and the crosstalk between LT α and mesenchymal organizer cells play a key role in the development of lymph nodes (Cupedo et al., 2002; Cupedo and Mebius, 2005; Eberl, 2005; Eberl and Littman, 2003; Lipp and Muller, 2004)(Fig. 2). The interaction between these two cell types is mediated by a variety of NF family ligands, chemokines, adhesion proteins, and their corresponding receptors and involves several positive feedback loops and amplifying mechanisms. Binding of membrane-bound lymphotoxin (LT) α 1 β 2 heterotrimers on the LT α cells to lymphotoxin β receptors (LT β R) on mesenchymal organizer cells is critical in the formation of lymph nodes and Peyer's patches (Cupedo and Mebius, 2005; De Togni et al., 1994; Fu and Chaplin, 1999; Futterer et al., 1998; Koni et al., 1997; Rennert et al., 1998; Ruddle, 1999). This interaction results in the activation of the NF- κ B1 pathway through the phosphorylation and destruction of I κ B- α and activation of the NF- κ B-inducing kinase (NIK) in the NF- κ B2 pathway. Mice deficient in the expression of lymphotoxin LT α , the lymphotoxin receptor LT β R, or proteins acting downstream of LT β R signaling, such as NIK, are deficient in lymph nodes and Peyer's patches due to the inability of the LT α and organizer cells to interact. Binding of LT α 1 β 2 to LT β R on mesenchymal organizer cells leads subsequently to the induction of several proteins, including vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), mucosal addressin cellular adhesion molecule-1 (MAdCAM-1), and several chemokines, including CXCL13, CCL19 and CCL21. CXCL13 interacts with CXCR5, its receptor on LT α cells, and promotes the clustering of LT α cells (Cupedo and Mebius, 2005; Muller et al., 2003). The LT α cells, which express integrin α 4 β 7 and α 4 β 1, interact with mesenchymal organizer cells by binding to MAdCAM-1 and VCAM-1, respectively, which function as receptors for these integrins (Cupedo et al., 2002; Cupedo and Mebius, 2005; Eberl, 2005; Eberl and Littman, 2003)(Fig. 2). These proteins further enhance the interaction of LT α cells with organizer cells and amplify the recruitment of LT α cells from the circulation to the anlagen. In addition, these interactions promote the recruitment of other cells from the circulation, including monocytes, and T and B lymphocytes (Cupedo and Mebius, 2005; Muller et al., 2003).

Although the formation of lymph nodes and Peyer's patches have much in common, there are a number of differences. Lymph node development requires the RANKL (TRANCE or TNFSF11) signaling pathway but not that of IL-7R α , while the development of Peyer's patches is dependent on IL-7R α but not RANKL signaling (Cupedo et al., 2002; Cupedo and Mebius, 2005; Yoshida et al., 2002). Activation of either signaling pathway up-regulate surface LT α 1 β 2 expression on LT α cells, thereby enhancing the recruitment of LT α cells to the lymph node and Peyer's patch anlagen.

Characterization of ROR γ ^{-/-} mice showed that LT α cells are absent from spleen, mesentery, and intestine of ROR γ ^{-/-} E18.5 embryos (Sun et al., 2000). These observations indicated that ROR γ t (ROR γ 2) plays a critical role in the generation or in the survival of LT α cells. Creation of ROR γ t^{EGFP/+} and ROR γ t^{EGFP/EGFP} knockin mice, expressing the enhanced green fluorescent protein (EGFP) under the control of the ROR γ t regulatory elements, allowed tracing of cells expressing ROR γ t (Eberl, 2005; Eberl and Littman, 2003; Eberl et al., 2004). These studies have provided valuable new insights into the roles of ROR γ t and LT α cells in lymph node development (Fig. 2). Visualization of EGFP in ROR γ t^{EGFP/+} mice showed that at E16.5 of fetal development ROR γ t is exclusively expressed in LT α cells present in the lymph node anlagen, the submucosal region of the intestine, and around large vessels in spleen. No EGFP-positive cells were detectable in E16.5 ROR γ t-deficient ROR γ t^{EGFP/EGFP} fetuses indicating the absence of LT α cells (Eberl and Littman, 2003; Eberl et al., 2004). In addition, adult ROR γ t-deficient

$ROR\gamma^{tgfp/gfp}$ mice lacked lymph nodes and Peyer's patches confirming that $ROR\gamma$ is essential for the development of lymph nodes and Peyer's patches (Kurebayashi et al., 2000; Sun et al., 2000). From these studies several conclusions were drawn: 1. LTi cells are defined by the expression of $ROR\gamma$; 2. LTi cells are essential for the development of lymph nodes and Peyer's patches; 3. the lack of lymph nodes and Peyer's patches in $ROR\gamma$ - or $ROR\gamma$ -deficient mice is due to the absence of LTi cells (Fig. 2).

The basic helix-loop-helix transcription factor Id2 also plays a critical role in lymph node organogenesis (Yokota et al., 1999). As demonstrated for $ROR\gamma^{-/-}$ mice, Id2 null mice do not develop lymph nodes and Peyer's patches (Eberl and Littman, 2003; Sun et al., 2000; Yokota et al., 1999). In addition, Id2 has been reported to be expressed in LTi cells and these cells were shown to be absent in Id2 null mice. These findings suggest a critical role for Id2 in the generation and/or survival of LTi cells (Eberl, 2005; Yokota et al., 1999; Yokota et al., 2001). In contrast to $ROR\gamma^{-/-}$ mice, Id2 null mice do not contain nasal associated lymphoid tissue (NALT) and have a greatly reduced number of natural killer cells and splenic $CD8\alpha^{+}$ dendritic cells (Fukuyama et al., 2002; Harmsen et al., 2002). These observations indicate that Id2 is involved in the regulation of several other differentiation programs. Moreover, since NALT is formed in $ROR\gamma^{-/-}$ mice in the absence of LTi cells, the organogenesis of this lymphoid tissue seems to involve a different mechanism.

Besides Peyer's patches and mesenteric lymph nodes, the immune system in the gut contains several other lymphoid structures: cryptopatches and isolated lymphoid follicles (ILFs) (Eberl, 2005; Taylor and Williams, 2005). In addition, the gut contains intraepithelial T lymphocytes (IELs), a rather unique group of T lymphocytes that contains a high percentage of $CD8\alpha^{+}TCR\alpha\beta$ and $TCR\gamma\delta$ T-cells. Cryptopatches consist of clusters of hematopoietic cells (Lineage (Lin) $^{-}$ Kit $^{+}$ IL-7R α^{+} CD44 $^{+}$) that appear about one week after birth along the gut epithelium between the crypts in the lamina propria (Eberl, 2005). IL-7R α plays an important role in the development of cryptopatches since these structures are absent in mice deficient in this receptor (Kanamori et al., 1996). Recent studies have demonstrated that cells in cryptopatches and in ILFs express $ROR\gamma$ in conjunction with the helix-loop-helix transcription factor Id2, c-Kit, CD44, and IL-7R α (Eberl, 2005; Eberl and Littman, 2004). Moreover, it was shown that cryptopatches and ILFs were absent in mice deficient in either $ROR\gamma$ or Id2. Targeted expression of Bcl-X_L under the control of the $ROR\gamma$ promoter in $ROR\gamma$ -deficient mice has been reported to rescue $ROR\gamma^{-/-}$ DP thymocytes from undergoing apoptosis but failed to restore the development of Lin $^{-}$ Kit $^{+}$ IL-7R α^{+} , cryptopatches, and ILFs. These studies demonstrated that adult intestinal Lin $^{-}$ Kit $^{+}$ IL-7R α^{+} CD44 $^{+}$ cells share many similarities with LTi cells; both express $ROR\gamma$ and Id2 and require these transcription factors for their development. Based on these observations it was concluded that the Lin $^{-}$ Kit $^{+}$ IL-7R α^{+} CD44 $^{+}$ cells constitute the adult counterpart of LTi cells (Eberl, 2005; Eberl and Littman, 2004)(Fig. 2). It was further proposed that ILFs develop from cryptopatches in response to inflammatory innate immune signals generated by the colonization of the intestine by bacteria.

Critical functions of $ROR\gamma$ in thymopoiesis

Initial reports showed that $ROR\gamma$ is highly expressed in the thymus suggesting that $ROR\gamma$ might have a role in the regulation of thymopoiesis (Guo et al., 2002; He, 2000; He et al., 1998; Hirose et al., 1994; Medvedev et al., 1996; Ortiz et al., 1995). This was supported by studies demonstrating that the size of the thymus and number of thymocytes in $ROR\gamma$ null mice was greatly reduced compared to those of wild type mice (Jetten et al., 2001; Jetten and Ueda, 2002; Kurebayashi et al., 2000; Sun et al., 2000). This reduction was due to a dramatic decrease in the number of double positive (DP) $CD4^{+}CD8^{+}$ and mature single positive (SP) $CD4^{+}CD8^{+}$ and $CD4^{+}CD8^{-}$ thymocytes. Mice deficient in the expression of the $ROR\gamma$

(ROR γ 2) isoform exhibited the same thymic phenotype as ROR γ null mice that are deficient in both ROR γ 1 and ROR γ t (Eberl and Littman, 2003), suggesting that the observed thymic phenotype is due to the loss of ROR γ t. These observations indicated that ROR γ plays a critical role in thymocyte homeostasis by regulating differentiation, proliferation, and/or apoptosis of thymocytes (He, 2002; Jetten, 2004; Jetten et al., 2001; Winoto and Littman, 2002).

Pluripotent lymphocyte progenitors, after their migration from fetal liver or adult bone marrow to the thymus, become committed to the T cell lineage, predominantly T cell receptor (TCR) $\alpha\beta^+$ T cells (SP cells, CD4⁺CD25⁺T_{reg} and NK1.1⁺ natural T cells). This program of differentiation proceeds via a highly coordinated series of steps and involves differentiation, expansion of subpopulations through cell proliferation, and gene recombination (Anderson et al., 1996; Baird et al., 1999; Ellmeier et al., 1999; Fehling and von Boehmer, 1997; Jameson et al., 1995; Kisielow and von Boehmer, 1995; Sebzda et al., 1999). In addition, it involves a number of checkpoints to either select thymocytes or eliminate them by apoptosis (Starr et al., 2003; Zhang et al., 2005). Thymopoiesis can be divided into several major stages based on the presence of cell surface antigens, including CD44, CD25, CD3, CD8, and CD4. Components of the TCR complex and several cytokine signaling pathways play a critical role in controlling the transition between the various stages. A number of transcription factors, including several nuclear receptors, have been identified as key regulators of different steps of thymopoiesis (Eberl and Littman, 2003; He, 2000; He, 2002; Jetten et al., 2001; Jetten and Ueda, 2002; Winoto and Littman, 2002; Yokota et al., 2001). Double negative (DN) CD4[−]CD8[−] thymocytes constitute 3–5% of the total thymocyte population which can be divided into four subsets, DN1-4, based on the presence of CD25 and CD44. When committed to the T-cell lineage, the T cell precursor CD25[−]CD44⁺ cells (DN1) differentiate successively via two intermediate stages, CD25⁺CD44⁺ (DN2) and CD25⁺CD44[−] (DN3), into CD44[−]CD25[−] (DN4) thymocytes (Fig. 3). During these stages TCR γ , δ , and β gene rearrangements, and β -selection occurs (Anderson et al., 1996; Baird et al., 1999; Ellmeier et al., 1999; Fehling and von Boehmer, 1997; Jameson et al., 1995; Kisielow and von Boehmer, 1995). A pre-TCR signal induces the DN thymocytes to undergo proliferation and differentiation into a distinct, intermediate-stage cell type, referred to as immature single positive (ISP), CD3[−]CD4[−]CD8^{low} cells (Miyazaki, 1997). Without a proper pre-TCR signal DN cells undergo apoptosis. The Bcl-2 family member Bfl-1 plays an important role in protecting DN cells from undergoing apoptosis (Chen et al., 2000; Zhang et al., 2005). The ISP cells subsequently differentiate into CD4⁺CD8⁺ (DP) thymocytes. DP thymocytes constitute the majority of thymocytes (75%) in the thymus. After successful TCR α gene rearrangement, DP cells expressing TCR $\alpha\beta$ receptor undergo a careful selection process to eliminate thymocytes expressing nonfunctional or autoreactive TCR (Starr et al., 2003; Zhang et al., 2005). DP thymocytes with low affinity for self-peptide-major histocompatibility antigen complex (MHC) undergo apoptosis by a process referred to as death by neglect, while DP thymocytes that express potentially self-reactive T-cell antigen receptors are eliminated by apoptotic process referred to as negative selection. It is estimated that the majority (90%) of thymocytes are eliminated by death by neglect and only a small fraction of thymocytes (about 5%), exhibiting intermediate affinities for self-peptide-MHC complexes, undergo positive selection (Sebzda et al., 1999). The molecular mechanisms underlying these apoptotic processes are not yet fully understood. The positive selected DP thymocytes mature into single positive (SP) CD4⁺CD8[−] helper and CD4[−]CD8⁺ cytotoxic lineages. These mature T cells then leave the thymus to colonize the secondary lymphoid organs, including the spleen, lymph nodes, and Peyer's patches.

The expression of ROR γ t during thymopoiesis is tightly regulated (Guo et al., 2002; He et al., 2000; He et al., 1998; Jetten, 2004; Jetten et al., 2001; Sun et al., 2000). ROR γ t is undetectable in DN thymocytes and expressed at low levels in ISP cells. ROR γ t is highly induced when ISP cells differentiate into DP thymocytes. Subsequently, when DP thymocytes differentiate into mature SP T lymphocytes, the expression of ROR γ t is again down-regulated (Fig. 3). Recent

studies analyzing $ROR\gamma^{tgfp/+}$ mice, in which EGFP expression is under the control of the $ROR\gamma$ promoter, confirmed that $ROR\gamma$ expression is restricted to DP thymocytes (Eberl et al., 2004). Analysis of different thymocyte subpopulations in $ROR\gamma^{-/-}$ mice showed that the percentage of ISP thymocytes is greatly increased. The accumulation of ISP cells in $ROR\gamma^{-/-}$ mice appears to be due to a delay in the differentiation of ISP into DP cells suggesting a role for $ROR\gamma$ in the regulation of the ISP-DP transition (Guo et al., 2002). In addition to $ROR\gamma$, the HMG-box transcription factors TCF-1 and LEF-1, and the IL-7R α signaling pathway have been implicated in the control of the transition of ISP to DP thymocytes (He et al., 2000; Verbeek et al., 1995; Yu et al., 2004). IL-7R α is highly expressed in DN and SP thymocytes, and absent in DP thymocytes. Activation of the IL-7R α signaling pathway in DN thymocytes positively regulates the levels of the anti-apoptotic protein Bcl2 which is needed for the survival and proliferation of these cells. In contrast, IL-7R α activation suppresses the expression of TCF-1, LEF-1, and $ROR\gamma$. Turning off the activation of IL-7R α signaling appears to be a prerequisite for the induction of TCF-1, LEF-1, and $ROR\gamma$, and for the differentiation of ISP cells into DP thymocytes to proceed (Yu et al., 2004)(Fig. 3).

In addition to the increase in ISP thymocytes, the number of DP and SP thymocytes in $ROR\gamma^{-/-}$ mice was dramatically reduced while the percentages of DN cells were significantly enhanced (Guo et al., 2002; He, 2000; Jetten, 2004; Jetten et al., 2001; Jetten and Ueda, 2002; Kurebayashi et al., 2000; Sun et al., 2000; Winoto and Littman, 2002). Cell cycle analysis indicated that the number of thymocytes in S phase was dramatically increased in the thymus of $ROR\gamma^{-/-}$ mice (Kurebayashi et al., 2000; Sun et al., 2000). The latter may partially reflect the observed increase in the percentage of DN thymocytes; however, it might also involve an aberrant regulation of the proliferation of $ROR\gamma^{-/-}$ thymocytes.

TUNEL Staining provided the first indication that apoptosis was significantly increased in sections from thymus of $ROR\gamma^{-/-}$ mice (Jetten, 2004; Jetten et al., 2001; Kurebayashi et al., 2000; Sun et al., 2000). Flow cytometric analysis demonstrated that the cells undergoing apoptosis were DP thymocytes. These observations indicated that the reduction in DP thymocytes in $ROR\gamma^{-/-}$ mice was in part related to increased apoptosis. In addition to enhanced apoptosis *in vivo*, $ROR\gamma^{-/-}$ thymocytes placed in culture undergo accelerated apoptosis as indicated by the rapid loss of mitochondrial membrane potential ($\Delta\Psi_m$), an increase in annexin IV binding, and the activation of several caspases (Jetten and Ueda, 2002; Kurebayashi et al., 2000; Sun et al., 2000; Ueda et al., 2002a). The decreased life-span of DP thymocytes was shown to be related to a dramatic reduction in the expression of the anti-apoptotic gene Bcl-X_L. Little change was observed in the expression of Bax and Bak mRNAs. The expression pattern of Bcl-X_L during thymopoiesis is very similar to that of $ROR\gamma$; expression of both genes is induced during the ISP to DP transition and again down-regulated during the DP to SP transition. Based on their co-expression and the repression of Bcl-X_L expression in $ROR\gamma^{-/-}$ DP thymocytes it was concluded that $ROR\gamma$ acts as a positive regulator of Bcl-X_L expression and consequently promotes survival of these cells (Eberl et al., 2004; He et al., 1998; Jetten, 2004; Jetten et al., 2001; Jetten and Ueda, 2002; Kurebayashi et al., 2000; Ma et al., 1995) (Fig. 3). This is supported by findings demonstrating that a transgene encoding $ROR\gamma$ restored the expression of Bcl-X_L in DP thymocytes of $ROR\gamma^{-/-}$ mice as well as the survival of these cells (Xie et al., 2005). In addition, studies showing that overexpression of $ROR\gamma$ in T cell hybridomas protects cells from activation-induced apoptosis, are in agreement with this concept (He et al., 1998). Whether $ROR\gamma$ regulates Bcl-X_L directly, by binding to a specific RORE in the regulatory region of the Bcl-X_L gene, or indirectly has yet to be established.

Bcl-X_L positively regulates the stability of the cyclin-dependent kinase 2 (cdk2) inhibitor p27 by inhibiting its degradation via the proteasome pathway (Gil-Gomez et al., 1998). The reduction in Bcl-X_L expression in $ROR\gamma^{-/-}$ DP thymocytes results in a decrease in the level

of p27 that consequently causes an increase in cdk 2 activity (Jetten and Ueda, 2002; Sun et al., 2000). These results indicate that by regulating the expression of Bcl-X_L, ROR γ t promotes cell survival and inhibits cell division (Fig. 3).

Rearrangements of genes encoding the TCR α chain is a distinctive feature of DP cells that are controlled by the T early α promoter and the TCR α enhancer. Rearrangement of TCR V α and J α gene segments begins with the proximal segments recombining before the distal ones. The shortened lifespan of ROR γ ^{-/-} DP thymocytes has been causally linked to the impaired rearrangements of 3' J α segments and the bias for J α rearrangements to the proximal 5' end of the J α locus observed in these cells (Guo et al., 2002). Constitutive expression of the early growth response gene 3 (Egr3), a zinc finger transcription factor that promotes proliferation when DN thymocytes differentiate into ISP cells, in thymocytes increases apoptosis among DP cells and shortens their life span *in vitro*. This is accompanied by reduced expression of both ROR γ t and Bcl-X_L (Xi and Kersh, 2004). This correlation is in agreement with the concept that ROR γ t and Bcl-X_L act as promoters of cell survival. Future studies will determine whether there is a direct link between Egr3 and the repression of ROR γ t transcription.

Targeted expression of Bcl-X_L to immature thymocytes rescues ROR γ ^{-/-} DP thymocytes from undergoing accelerated apoptosis and restores their normal life-span and levels of p27 protein (Sun et al., 2000). These results indicate that the reduction in Bcl-X_L expression is an early and key event in causing accelerated apoptosis in ROR γ ^{-/-} thymocytes. The expression of Bcl-X_L also restores J α rearrangements of more distal 3' J α segments (Guo et al., 2002). However, expression of Bcl-X_L does not change the delay in the ISP-DP transition, suggesting that this impairment occurs independently of Bcl-X_L (Guo et al., 2002).

Although ROR γ ^{-/-} mice appear initially healthy, by the age of 4 months about 50% of the mice have died of thymic lymphoma (Jetten and Ueda, 2002; Ueda et al., 2002a). The lymphoma cells metastasized to the kidney, liver, and spleen. The thymic lymphomas contained a greatly increased number of mitotic as well as apoptotic cells. Analysis of the CD4/CD8 phenotype showed that the thymic lymphomas are heterogeneous and often contained greatly increased numbers of DN and SP CD8⁺ cells. The molecular mechanism underlying this increased susceptibility to T-cell lymphoma development is not yet understood but may relate to defects in the control of cellular proliferation and differentiation of thymocytes observed in ROR γ ^{-/-} thymocytes. Increased proliferation may result in an enhanced probability to acquire genetic alterations that lead to additional changes in the expression of tumor suppressor and proto-oncogenes (Jetten and Ueda, 2002; Ueda et al., 2002a).

Role of ROR α in lymphocyte development

Both the spleen and the thymus are significantly smaller in ROR α -deficient mice suggesting that ROR α may have a role in the regulation of thymopoiesis and lymphocyte development (Dzhagalov et al., 2004; Trenkner and Hoffmann, 1986). ROR α mRNA is expressed at low levels in DP thymocytes and at high levels in SP thymocytes. ROR α expression is decreased in mature CD8⁺ and increased in mature CD4⁺ T lymphocytes. B220⁺ B lymphocytes express low levels of ROR α . The total number of thymocytes and splenocytes is greatly diminished in ROR α ^{-/-} mice. The DP thymocyte population in the thymus was dramatically reduced whereas the percentages of DN, CD4⁺ and CD8⁺ SP cells were greatly enhanced. Although the total number of CD4⁺ and CD8⁺ T lymphocytes in the spleen was decreased, their percentage was higher than in wild type mice, while the percentage of B cells was reduced. These findings suggest that ROR α plays an important role in lymphocyte development. Reconstitution of lymphocyte development in Rag-2^{-/-} mice with bone marrow cells of ROR α ^{-/-} showed that these cells could completely restore normal T and B cell development (Dzhagalov et al., 2004). The results indicate that the impaired lymphocyte development in ROR α ^{-/-} mice is not

due to intrinsic defects in these cells. It has been suggested that ROR α indirectly regulates T and B lymphocyte development by providing an appropriate microenvironment (Dzhagalov et al., 2004). In addition, it is well-known that defects in innervation can effect the size of tissues. Therefore, reduced innervation of thymus and spleen in ROR $\alpha^{-/-}$ mice due to cerebellar defects may be partially responsible for the reduced size of these tissues.

No differences were observed in the induction of proliferation of CD4⁺ and CD8⁺ T lymphocytes in response to lymphopenic environment or the addition of anti-CD3 or lipopolysaccharide (LPS) between ROR $\alpha^{-/-}$ and wild type mice. Also, there was little difference in the induction of the interleukins IL-2 and IL-6, and of TNF α . This is in contrast to ROR $\alpha^{-/-}$ mast cells and macrophages, in which the production of IL-6 and TNF α was greatly increased after LPS treatment (Dzhagalov et al., 2004; Kopmels et al., 1992). Similarly, CD8⁺ T cells from ROR $\alpha^{-/-}$ mice but not CD4⁺ T cells produce higher levels of interferon γ after LPS stimulation. These results indicate that at least in certain immune cells ROR α functions as a negative regulator of cytokine expression. Although the mechanism of this repression is not fully understood, ROR α has been reported to positively regulate the expression of IKB α , a negative regulator of the NF-KB signaling pathway (Delerive et al., 2001). These observations suggest that ROR α may function as a negative regulator of inflammation. The latter is supported by studies showing that ROR $\alpha^{sg/sg}$ mice exhibit an enhanced susceptibility to LPS-induced lung inflammation (Stapleton et al., 2005).

Role of ROR in mesenchymal differentiation

Although several studies have provided evidence for regulatory roles of RORs in adipocytes, myoblast differentiation, and bone formation, knowledge about their precise molecular and physiological functions is still limited. Both ROR α and ROR γ have been reported to be induced during differentiation of 3T3L1 preadipocytes (Adachi et al., 1996; Austin et al., 1998). This induction is promoted by peroxisome proliferator-activated receptor γ (PPAR γ) and PPAR γ agonists, and inhibited by retinoic acid. The induction of ROR α and ROR γ expression appears to be a late event and unrelated to the commitment of preadipocytes to undergo adipocyte differentiation (Adachi et al., 1996; Austin et al., 1998). In adult mice, ROR γ is expressed in brown adipose tissue and not in white adipose tissue, suggesting that it may regulate a function that is typical for brown adipose tissue. Brown adipose tissue is a major site for adaptive thermogenesis (Lowell and Spiegelman, 2000). However, ROR γ does not appear to play a major role in regulating adaptive thermogenesis since no significant difference was observed between wild type and ROR γ -deficient mice in the induction of uncoupling protein 1 during cold-induced thermogenesis (Jetten, A.M., unpublished observations). Moreover, ROR γ expression in brown adipose tissue did not change in wild type mice during cold-induced thermogenesis.

A role for ROR α in bone metabolism was suggested by studies showing that ROR $\alpha^{sg/sg}$ mice are osteopenic (Meyer et al., 2000). The total mineral content and bone density are significantly reduced in bones of ROR α -deficient mice compared to those of wild type mice. These observations indicate an imbalance between bone formation and bone resorption and suggest a positive regulatory function for ROR α in bone development. This is supported by studies showing that expression of ROR α mRNA is significantly enhanced during differentiation of mesenchymal stem cells into osteoblasts and that ROR α is able to activate the promoter of the bone sialoprotein gene (Meyer et al., 2000). Although these observations are in agreement with the hypothesis that ROR α regulates osteoblast function and activity, further studies are needed to determine the precise role of ROR α in bone metabolism.

ROR α and ROR γ mRNA have been reported to be expressed in skeletal muscle and in mouse myoblast C2C12 cells suggesting a possible regulatory role for these receptors in myogenesis

or muscle function (Becker-Andre et al., 1993; Lau et al., 1999). This was supported by studies showing that ectopic expression of a dominant-negative form of ROR α repressed myogenesis as indicated by an inhibition of the induction of the muscles-specific helix-loop-helix transcription factors MyoD and MYF4, and the cdk inhibitor p21 (Lau et al., 1999). This inhibition appears to involve a direct interaction of ROR α 1 with the co-activator p300 and MyoD. The interaction of ROR α and MyoD is mediated through their DBD and amino-terminal region, respectively. These findings are consistent with the hypothesis that ROR α functions as a positive regulator of myogenesis. Since Rev-ErbA represses myogenesis and is able to compete with RORs for RORE binding, regulation of myogenesis may involve cross-talk between these two receptor pathways (Burke et al., 1996; Downes et al., 1996; Lau et al., 1999).

Recent studies have demonstrated that expression of a dominant-negative form of ROR α in myoblast C2C12 cells attenuates the expression of several genes involved in the regulation of lipid metabolism suggesting a role of ROR α in the control of lipid homeostasis (Lau et al., 2004). Evidence was provided indicating that muscle carnitine palmitoyltransferase-1 and caveolin-3 promoters are regulated directly by ROR α and involve recruitment of the co-activators p300 and PGC-1. Reports showing that ROR α functions as a positive regulator of the expression of several apolipoproteins further support a role of ROR α in lipid metabolism (Genoux et al., 2005; Lind et al., 2005; Mamontova et al., 1998; Rader, 2002; Raspe et al., 2001; Vu-Dac et al., 1997).

Role of the ROR homologs DHR3 and CHR3 in development

DHR3, the ROR homolog in *Drosophila melanogaster* has been reported to play an important role in *Drosophila* development (Carney et al., 1997; Gates et al., 2004; Horner et al., 1995; King-Jones and Thummel, 2005; Koelle et al., 1992; Sullivan and Thummel, 2003; Thummel, 1995). DHR3 plays a critical role during embryogenesis prior to molting and at the onset of metamorphosis. During embryogenesis DHR3 mRNA is detectable between 6–12 hours after egg lay (Sullivan and Thummel, 2003). Through this stage, DHR3 functions as transcriptional repressor and cooperates with other repressors. During larval and prepupal development DHR3 is expressed in the second half of the second instar and in the early prepupa stage (Lam et al., 1999; Lam et al., 1997). During development expression of DHR3 mRNA is in synchrony with the pulses of steroid hormone 20-hydroxyecdysone (20-E) suggesting that its expression is regulated by 20-E. 20-E acts by binding to ecdysteroid receptors (EcRs), nuclear hormone receptors that occur in many isoforms. The ecdysone receptors form a heterodimer with ultraspiracle (USP), the insect homolog of the retinoid $\times$ receptor (RXR). Activation of this heterodimer by 20-E and binding to ecdysone response elements in the promoter region of target genes results in enhanced gene transcription. However, optimal induction of DHR3 is dependent on protein synthesis suggesting that its transcriptional regulation by 20-E occurs at least partially by an indirect mechanism. DHR3 up-regulates the expression of another nuclear receptor β FTZ-F1, the homolog of the mammalian nuclear receptor SF-1 (NR5A3) (Kageyama et al., 1997). This induction is mediated through the binding of DHR3 to DHR3-binding sites in the β FTZ-F1 promoter. The nuclear receptor E75B, a homolog of the mammalian nuclear receptor Rev-Erb (NR1D) that acts as a transcriptional repressor, can repress β FTZ-F1 expression by competing with DHR3 for the same DNA binding sites. It is interesting to note that a similar antagonism, as observed between DHR3 and E75B, has been reported for their mammalian homologs, the ROR and Rev-Erb receptors (Akashi and Takumi, 2005; Austin et al., 1998; Bois-Joyeux et al., 2000; Downes et al., 1996; Forman et al., 1994; Guillaumond et al., 2005; Medvedev et al., 1997).

Ecdysone is important in orchestrating the reorganization of neural circuits during metamorphosis (Weeks, 2003). Analysis of *Drosophila* DHR3 mutants showed that they form

a normal larval cuticle but that few survive to hatching (Carney et al., 1997). DHR3 mutants display various defects in the peripheral nervous system that include a general disorganization of the neuronal clusters and an absence or mislocalization of neurons. Since ROR α has been shown to regulate dendritic arborization of Purkinje cells in mice, a similar role has been suggested for the ROR homolog DHR3 in the regulation of remodeling of neurons in *Drosophila* (Boukhoutche et al., 2006).

As has been demonstrated for DHR3, the expression of CHR3 and MHR3, the ROR homologs identified in *C. elegans*, *C. fumiferana* (Palli et al., 1997; Palli et al., 1996), and the tobacco hawkmoth *M. sexta* (Kostrouch et al., 1995), respectively, is also regulated by 20-E (Hiruma and Riddiford, 2004; Langelan et al., 2000). CHR3 regulates molting at all four larval stages of *C. elegans*. Disruption of CHR3 in *C. elegans* results in several developmental changes, including incomplete molting and generation of a short, fat phenotype (Kostrouchova et al., 1998; Kostrouchova et al., 2001). The CHR3 receptor is also required for proper epidermal development. In *M. sexta*, MHR3 is induced by 20-E in the epidermis. Evidence has been provided indicating that this induction is regulated by ecdysone receptor-USP-1 heterodimers (Riddiford et al., 2003). These results demonstrate that as their mammalian homologs DHR3, CHR3, and MHR3 are essential for the development of several tissues.

Summary

As illustrated in this review, ROR receptors are critical regulators of cellular differentiation and the development of several tissues. This includes a role for ROR α in the development of the cerebellum and bone formation, a role for ROR β in the brain and retina, and a role for ROR γ in the development of several secondary lymphoid tissues and in the control of thymopoiesis. In addition, RORs play an important role in the regulation of circadian rhythms. Whether ROR activity *in vivo* and consequently the physiological processes controlled by RORs are regulated by endogenous ligands has yet to be determined. However, evidence has been provided indicating that in cultured cells ROR transcriptional activity can be modulated by ligands. These observations leave open the possibility that synthetic (ant)agonists might be useful in the development of new therapeutic strategies for several human diseases in which RORs are implicated. This overview shows that great insights have been obtained into the physiological function of RORs in several tissues; however, RORs are expressed in many other tissues in which the physiological function of RORs still needs to be uncovered.

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Fig. (1). Functions of $ROR\alpha$ in the development of the cerebellum. **A.** $ROR\alpha$ plays a critical role in the maturation of Purkinje cells. Immature Purkinje cells arise from $ROR\alpha^-$ precursor cells. $ROR\alpha$ becomes highly expressed in postmitotic Purkinje cells at E12.5 of mouse embryonic development. $ROR\alpha$ is required for dendritogenesis to proceed and for the induction of a number of genes normally expressed in mature Purkinje cells. Dendritogenesis and the expression of several genes normally expressed in mature Purkinje cells are inhibited in $ROR\alpha$ -deficient mice. **B.** $ROR\alpha$ regulates expression of several genes, including *Pcp2* and *Shh*,

directly by binding to ROREs in their promoter regions. Interestingly, the composition of the co-activator complexes assembled by ROR α appears to be distinct for each gene, suggesting that the promoter context plays a critical role. These co-activators include β -catenin (β -cat), SRC1, p300, CBP, and Tip60. Activation of β -catenin by Wnt signaling might influence the transcriptional activity of ROR α . ROR α may act downstream of Wnt and because Shh is an ROR α target gene, ROR α may function as a mediator between the Wnt and Shh signaling pathways. Shh released by Purkinje cells interacts with Patched (Ptch) receptors present on granule cell precursors and lead to the activation of GLI transcription factors. Subsequently, this results in the transcriptional activation of several growth regulatory genes, including PCNA and cyclins, and an induction of the proliferation of these cells. The reduced Shh expression in Purkinje cells from ROR α -deficient mice is a major cause of the cerebellar atrophy observed in these mice (Boukhtouche et al., 2006; Gold et al., 2003; Gold et al., 2006; Goldowitz and Hamre, 1998; Hamilton et al., 1996; Herrup and Mullen, 1979; Kenney et al., 2003; Landis and Sidman, 1978; Steinmayr et al., 1998; Wallace, 1999).

Fig. (2).

ROR γ t is essential for the development of secondary lymphoid tissues. **A.** Lymphoid tissue inducer (LTi) cells are derived from hematopoietic stem cells and are essential in the development of lymph nodes, Peyer's patches, and cryptopatches. The isolated lymphoid follicles (ILFs) are thought to be derived from cryptopatches after the colonization of the intestine by bacteria. **ROR γ t** is required for the generation and/or survival of LTi cells. The absence of these lymphoid tissues in **ROR γ -deficient** mice is due to a deficiency of LTi cells in these mice. **B.** Role of **ROR γ t** and LTi cells in lymph node development. The transcription factors **Id2** and **ROR γ t** are both essential for the generation of LTi cells ($CD4^+CD3^-CD45^+IL-7R\alpha^+ROR\gamma^+Id2^+$). LTi cells are recruited to lymph node anlagen through their interaction with mesenchymal organizer cells. Interactions between these two cell types are key in the recruitment of LTi cell from the circulation to the lymph node anlagen. Activation of the **RANK** signaling pathway enhances the expression of lymphotoxins (LT) in LTi cells and promotes lymph node development. Binding of **LT α 1 β 2** on LTi cells to the **LT β R** on mesenchymal organizer cells plays a key role in lymph node development. The latter results in the activation of **NF- κ B** pathways and induction of **VCAM-1**, **ICAM-1**, and **MAdCAM-1** in mesenchymal organizer cells and their subsequent binding to

integrins on LT α i cells further promote their interaction and the recruitment of additional LT α i cells. In addition, induction of various chemokines in mesenchymal organizer cells, including CXCL13 and CCL19, interact with their corresponding receptors on LT α i cells thereby promoting their interaction and allow the recruitment of monocytes, T and B lymphocytes. (Cupedo et al., 2002; Eberl, 2005; Eberl and Littman, 2003; Eberl et al., 2004; Jetten, 2004; Jetten et al., 2001; Kurebayashi et al., 2000; Lipp and Muller, 2004; Sun et al., 2000; Yokota et al., 1999).

Fig. (3).

RORγ exhibits multiple functions in thymopoiesis. CD4⁻CD8⁻CD25⁻CD44⁺ (DN1) cells hematopoietic precursor cells, differentiate via DN2 and DN3 into CD4⁻CD8⁻CD25⁻CD44⁻ (DN4) cells. The DN4 cells then give rise to immature single positive (ISP) cells (CD3⁻CD4⁻CD8^{low}). The ISP cells subsequently differentiate into CD3⁺CD4⁺CD8⁺, DP thymocytes. Expression of RORγt, as well as Bcl-X_L, LEF, and TCF-1, are induced during the ISP-DP transition and again down-regulated during the differentiation of DP into SP cells. RORγt promotes the differentiation of ISP into DP cells and is a positive regulator of Bcl-X_L expression. The latter stabilizes the cdk inhibitor p27, which subsequently inhibits cdk2 activity and increases the lifespan of DP thymocytes. Lack of RORγt expression inhibits the ISP to DP transition. In addition, expression of Bcl-X_L in DP thymocytes is reduced resulting in increased apoptosis, reduced the lifespan of DP thymocytes and consequently impaired TCRα rearrangements (Eberl and Littman, 2003; Guo et al., 2002; He, 2002; He et al., 2000; He et al., 1998; Jetten et al., 2001; Jetten and Ueda, 2002; Kurebayashi et al., 2000; Sun et al., 2000).

Table I

Summary of proteins recruited by ROR receptors.

Protein Symbol	Reference
Co-activators	
CREBBP (CBP)	Gold et al., 2003; Jetten et al., unpublished observations
P300	Gold et al., 2003; Harris et al., 2002; Lau et al., 1999
NCOA1 (SRC1)	Littman et al., 1999; Kurebayashi et al., 2004; Moraitis et al., 2002; Gold et al., 2003; Xie et al., 2005
NCOA2 (GRIP1, TIF2)	Atkins et al., 1999; Moraitis et al., 2002; Harris et al., 2002; Gold et al., 2003; Xie et al., 2005; Jetten et al., unpublished observations
NCOA3 (SRC3)	Gold et al., 2003; Moraitis et al., 2002
NCOA6 (AIB3, PRIB)	Jetten et al., unpublished observations
PPARGC1A (PGC-1 α)	Jetten et al., unpublished observations
CTNNB1 (β -catenin)	Gold et al., 2003
HTATIP (TIP60)	Gold et al., 2003
TRIP11 (TRIP230)	Atkins et al., 1999
PPARBP (TRIP2)	Atkins et al., 1999; Harris et al., 2002
Co-repressors	
NCOR1 (N-CoR)	Harding et al., 1997; Jetten et al., unpublished observations
NCOR2 (SMRT)	Harding et al., 1997
NRIP1 (RIP140)	Littman et al., 1999; Jetten et al., unpublished observations
CDH4 (Mi-2b)	Johnson et al., 2004
NIX1	Greiner et al., 1996
HR (Hairless)	Moraitis and Giguere, 2003; Moraitis et al., 2002
Ubiquitin-proteasome system	
UBE21 (UBC9)	Jetten et al., unpublished observations
PSMB6 (macropain)	Jetten et al., unpublished observations
PSMC5 (TRIP1, SUG1)	Atkins et al., 1999; Jetten et al., unpublished observations
Others	
TRIM24 (TIF1)	Atkins et al., 1999
PNRC-1/2	Jetten et al., unpublished observations
MyoD	Lau et al., 1999