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Combinatorial Roles of the Nuclear Receptor Corepressor in Transcription and Development

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Summary

Transcriptional repression plays crucial roles in diverse aspects of metazoan development, implying critical regulatory roles for corepressors such as N-CoR and SMRT. Altered patterns of transcription in tissues and cells derived from *N-CoR* gene–deleted mice and the resulting block at specific points in CNS, erythrocyte, and thymocyte development indicated that N-CoR was a required component of short-term active repression by nuclear receptors and MAD and of a subset of long-term repression events mediated by REST/NRSF. Unexpectedly, N-CoR and a specific deacetylase were also required for transcriptional activation of one class of retinoic acid response element. Together, these findings suggest that specific combinations of corepressors and histone deacetylases mediate the gene-specific actions of DNA-bound repressors in development of multiple organ systems.

Introduction

Active repression of gene expression by sequence-specific transcription factors plays a critical role in the regulation of diverse biological processes, including cell proliferation, development, and homeostasis (Mannervik et al., 1999). Nuclear receptors, including the retinoic acid (RAR) and thyroid hormone receptors (T_3R), are essential for the regulation of development and homeostasis both through ligand-dependent activation and through active repression by unliganded nuclear receptors (reviewed in McKenna et al., 1999; Glass and Rosenfeld, 2000; Hu and Lazar, 2000). Investigation of active repression by T_3R and RAR led to the identification of the nuclear receptor corepressor (*N-CoR)* (Hörlein et al., 1995) and the closely related factor, silencing mediator for retinoic acid and thyroid hormone receptors (*SMRT)* (Chen and Evans, 1995). N-CoR and SMRT both contain a conserved bipartite nuclear receptor interaction domain (Seol et al., 1996; Zamir et al., 1996) and three independent repressor domains that are capable of transferring their active repression function to a heterologous DNA binding domain.

While steroid hormone receptors do not appear to interact with N-CoR or SMRT in the presence or absence of agonists, both the estrogen receptor (ER) and the progesterone receptor (PR) can interact with these corepressors in the presence of their respective antagonists (Xu et al., 1996; Jackson et al., 1997; Smith et al., 1997; Lavinsky et al., 1998). The ability of N-CoR or SMRT to serve as corepressors has also been suggested for several other members of the nuclear receptor superfamily (reviewed in Glass and Rosenfeld, 2000). In addition, N-CoR and SMRT have been implicated as corepressors for a variety of unrelated transcription factors, including MAD (Heinzel et al., 1997), CBF1/RBP-Jkappa/Su(H) (Kao et al., 1998), and homeodomain factors (Xu et al., 1998).

Although N-CoR and SMRT can interact with mSin3 (Alland et al., 1997; Heinzel et al., 1997; Nagy et al., 1997) and with certain HDAC proteins, including HDAC 4, 5, and 7 (Huang et al., 2000; Kao et al., 2000), neither N-CoR nor SMRT are components of the mSin3 complex (Hassig et al., 1997; Laherty et al., 1998) or of the HDAC-containing NuRD complex (Xue et al., 1998). In fact, purification of a SMRT complex (Guenther et al., 2000) or HDAC3 complex (Wen et al., 2000) reveals that N-CoR and SMRT form a stable complex with HDAC3 and at least one additional protein, TBL1 (Guenther et al., 2000). These data imply either a redundancy or combinatorial usage of N-CoR-associated deacetylases in active repression.

In this manuscript, we investigated the biological role of the corepressor N-CoR in vivo and found that *N-CoR*$^{-/-}$ embryos exhibit defects in developmental progression of specific erythrocyte, thymocyte, and neural events. N-CoR proves to be required for repression by T_3R and RAR, for the function of ER antagonists, and for the repressive actions of several other classes of DNA binding repressors. We have also established a role for N-CoR in long-term repression mediated by repressor element silencing transcription factor/neuron-restrictive silencer factor (REST/NRSF). Additionally, our data suggest that in particular contexts, N-CoR, with HDAC3, has a role in activation of gene expression. Our findings provide evidence that N-CoR is a required developmental regulator, linking short- and long-term repression events on a subset of gene targets for nuclear receptors and other classes of transcription factors to specific combinations of corepressors.

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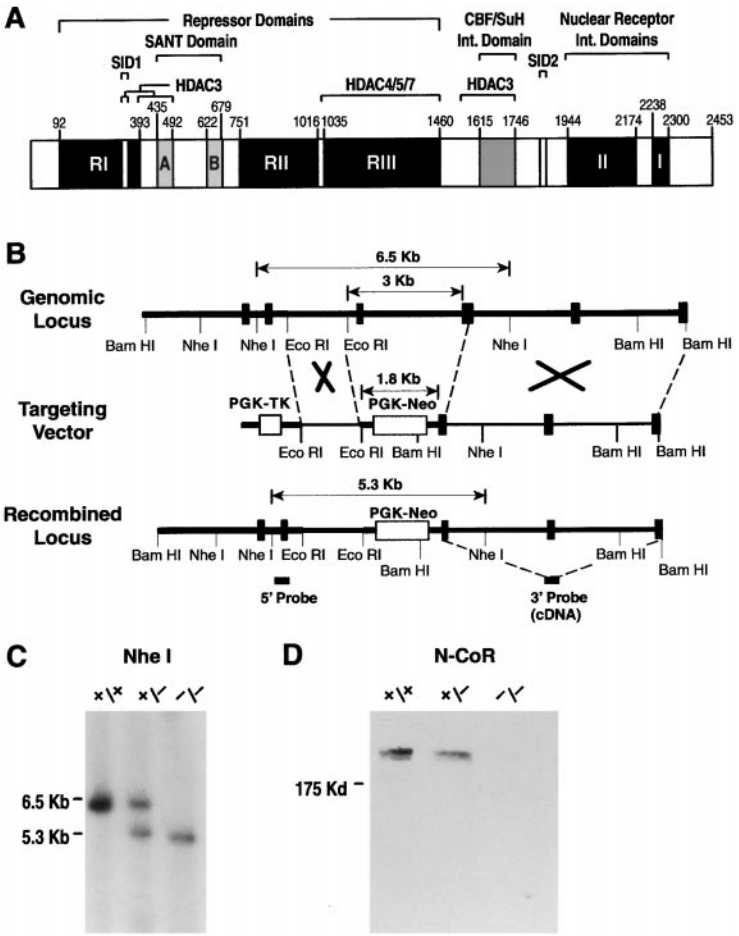

Figure 1. Targeted Deletion of the *N-CoR* Genomic Locus

(A) Schematic of interaction domains of N-CoR. The sites of HDAC, mSin3 (SID), CBF/SuH, and nuclear receptor interaction domains (I and II) are indicated, as are the repression domains (RI, RII, RIII).

(B) Homologous recombination replaced the 5′ SID with the PGKneopA cassette. Location of 5′ and 3′ probes are shown.

(C) After NheI digestion of genomic DNA from mice of each genotype, a 6.5 kb wild-type allele or a 5.3 kb targeted allele was detected by Southern blot analysis with the 5′ probe.

(D) Western blot analysis of protein from MEFs of each genotype probed with anti-N-CoR antibody shows the absence of detectable protein in cells from the *N-CoR*$^{-/-}$ embryo.

Results

Generation of *N-CoR* Gene–Deleted Mice

The murine N-CoR protein is encoded by a 7.4 kb mRNA and is highly homologous to the related factor SMRT (Figure 1A). In situ hybridization revealed that both genes were widely expressed but that gene-specific differences in their developmental expression patterns did exist (data not shown). In order to characterize the biological role of N-CoR, we designed a targeting construct to delete a portion of the *N-CoR* genomic locus. The strategy employed replaced 3 kb of genomic sequence encoding the 5′ Sin3 interaction domain (SID1) with the neomycin resistance gene (Figure 1B). Two clonal ES cell lines heterozygous for the mutant allele were used to generate chimeric mouse strains from which we generated mice homozygous for the *N-CoR* mutation (Figure 1C). Disruption of the *N-CoR* gene was confirmed by the absence of detectable transcripts and protein in cells derived from *N-CoR*$^{-/-}$ embryos (Figure 1D and data not shown). In situ hybridization revealed no difference in levels of *SMRT* transcripts between wild-type and *N-CoR*$^{-/-}$ embryos (data not shown). The majority of *N-CoR*$^{-/-}$ embryos died by day 15.5 of gestation (E15.5), with occasional embryos surviving 1–2 days longer.

Definitive Erythropoiesis Was Impaired in *N-CoR*$^{-/-}$ Embryos

N-CoR$^{-/-}$ embryos were markedly pale from E13.5 (Figure 2A), suggesting a severe anemia, and approximately half of the *N-CoR*$^{-/-}$ embryos exhibited secondary edema. Additionally, *N-CoR*$^{-/-}$ embryos were on average 80% the size of their wild-type and heterozygote littermates. The severity of the anemia and edema increased with age and appeared to be the ultimate cause of death. At E14.5, the hematocrit of the *N-CoR*$^{-/-}$ embryos was severely reduced (14.5 $\pm$ 0.83; mean $\pm$ SEM) relative to heterozygote or wild-type littermates (44 $\pm$ 0.88) (Figure 2B). Peripheral blood smears from *N-CoR*$^{-/-}$ embryos showed fewer nonnucleated erythrocytes but many more nucleated erythroblasts than wild-type controls (Figure 2C).

Primitive erythropoiesis is initiated in yolk sac blood islands on E7 and results in the production of nucleated red blood cells. Definitive erythropoiesis is characterized by the production of mature, nonnucleated erythrocytes, and normally occurs in the fetal liver beginning on E12. *N-CoR*$^{-/-}$ embryos survived until E15.5 and anemia was observed only after E13.5, suggesting that these embryos have a defect in definitive rather than primitive erythropoiesis. Indeed, fetal livers of *N-CoR*$^{-/-}$ embryos were approximately one half the size of their wild-type and heterozygous littermates (Figure 2D). *N-CoR* transcripts were highly expressed in E14.5 fetal liver, while expression of *SMRT* transcripts was relatively low (Figure 2E), suggesting that definitive erythropoiesis might be sensitive to ablation of the *N-CoR* gene.

To determine the ability of *N-CoR*$^{-/-}$ fetal liver progenitors to give rise to red cell colonies in vitro, an equal

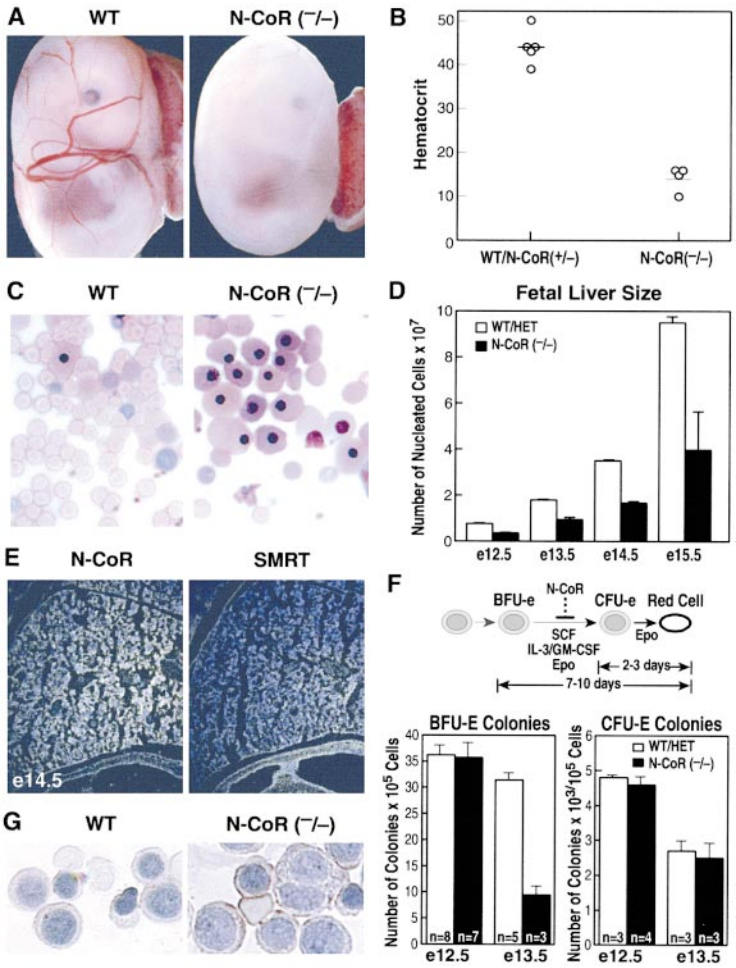

Figure 2. Block in Definitive Erythropoiesis in *N-CoR*$^{-/-}$ Embryos

(A) Micrographs of wild-type and *N-CoR*$^{-/-}$ E14.5 embryos with intact visceral yolk sacs, illustrating the severe anemia of *N-CoR*$^{-/-}$ embryos.

(B) Mean hematocrit of E14.5 wild-type and heterozygote animals (n = 5) was 44.5, as compared to a mean of 14.5 for *N-CoR*$^{-/-}$ embryos (n = 4).

(C) Giemsa staining of E14.5 wild-type blood showed predominantly nonnucleated red cells, with occasional nucleated erythroblasts and yolk sac erythrocytes. *N-CoR*$^{-/-}$ blood contained fewer nonnucleated red cells, yolk sac erythrocytes, and a large number of nucleated erythroblasts.

(D) The average number of nucleated cells in wild-type or heterozygous fetal livers was ~2-fold greater than in *N-CoR*$^{-/-}$ fetal liver.

(E) In situ hybridization of wild-type E14.5 fetal liver using ^{35}S-labeled *N-CoR* and *SMRT* probes showed that *N-CoR* transcripts were highly expressed compared to *SMRT* transcripts.

(F) In vitro differentiation of wild-type or heterozygote and *N-CoR*$^{-/-}$ E12.5 and E13.5 fetal liver cells. BFU-E cells give rise to red cell colonies within 7–10 days and require Epo, stem cell factor, and IL-3 and/or GM-CSF. CFU-E cells require only Epo and give rise to red cell colonies within 2–3 days. *N-CoR*$^{-/-}$ embryos showed a defect in BFU-E colony formation.

(G) Immunohistochemistry with anti-CA II antibody indicated increased CAII immunoreactivity in *N-CoR*$^{-/-}$ fetal liver-derived cells.

number of fetal liver cells from embryos of each genotype were plated in methylcellulose media containing growth factors appropriate for the development of either burst-forming unit-erythroid (BFU-E) or colony-forming unit-erythroid (CFU-E) colonies (Figure 2F) (Gregory and Eaves, 1978). As shown in Figure 2F, there was no apparent difference in BFU-E colony formation by fetal liver cells derived from E12.5 *N-CoR*$^{-/-}$, heterozygous, or wild-type embryos. However, the ability of cells derived from *N-CoR*$^{-/-}$ fetal livers to form BFU-E colonies was reduced 3-fold at E13.5, and this difference was even more pronounced by E14.5 (Figure 2F and data not shown).

Development from the BFU-E to the rapidly dividing CFU-E is a continuous process (Figure 2F). CFU-E colony number was unaffected in cultures from both E12.5 and E13.5 *N-CoR*$^{-/-}$ fetal livers. These results suggested that the BFU-E progenitors present in E12.5 fetal livers of *N-CoR*$^{-/-}$ embryos were able to continue their development into CFU-E. Colony forming unit-granulocyte macrophage (CFU-GM) colony formation from *N-CoR*$^{-/-}$ fetal livers was also unaffected, and the number of megakaryocytes present in *N-CoR*$^{-/-}$ fetal liver smears was similar to that of wild-type, suggesting that erythropoiesis was specifically affected (data not shown). We did not detect any increase in apoptosis in fetal liver cells from *N-CoR*$^{-/-}$ embryos, as assessed by cell surface expression of Annexin V, nor was there any detectable difference in Epo receptor (*EpoR)* transcripts (data not shown).

To assess a transcriptional role for N-CoR in erythrocytes, we examined expression of carbonic anhydrase II (CA II), which is upregulated in the presence of T_3 and repressed in erythrocytes transformed by the constitutive repressor v-ErbA (Pain et al., 1990). An increase in CA II immunoreactivity was observed in erythroid cells in *N-CoR*$^{-/-}$ mice (Figure 2G), suggesting that in the absence of N-CoR, the ability of T_3R to mediate repression of specific target genes was lost.

T Cell Development in N-CoR-Deficient Embryos Was Arrested at the Double-Negative Stage

The thymus was markedly smaller in E14.5 *N-CoR*$^{-/-}$ embryos (Figure 3A). While both *N-CoR* and *SMRT* transcripts were highly expressed in embryonic thymus (Figure 3B), Western blot analysis revealed that N-CoR protein was detected in whole thymus, thymocytes, and, at slightly lower levels, in thymic stroma, while SMRT protein was detected mainly in whole thymus and in thymic stroma, with lower levels in thymocytes (Figure 3C). This complementary pattern of N-CoR and SMRT expression suggested that the reduction in thymus size in *N-CoR*$^{-/-}$ embryos may be associated with a specific defect in thymocyte development.

T cell development begins with the migration of precursor cells from the bone marrow or fetal liver to the

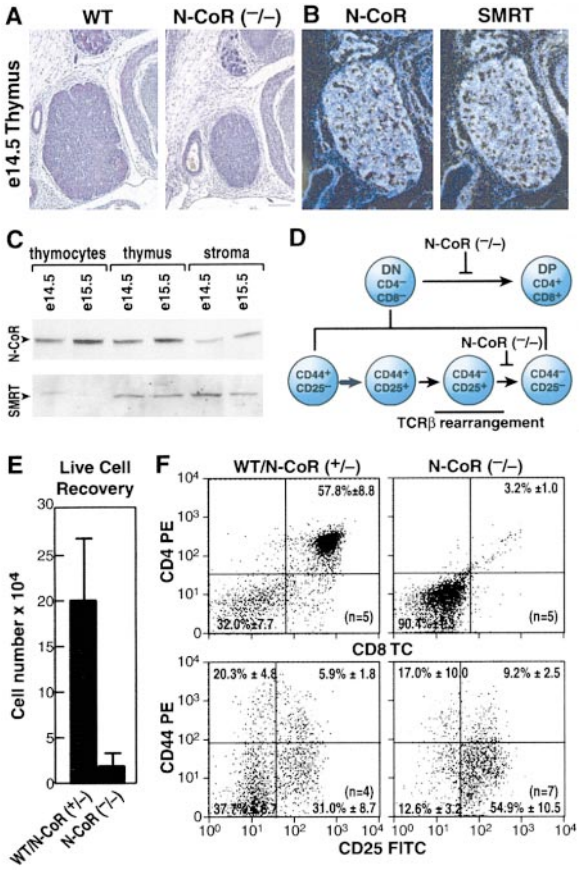

Figure 3. Defect in T Cell Development in *N-CoR*$^{-/-}$ Mice

(A) Hematoxylin and eosin (H&E)–stained sagittal sections of wild-type and *N-CoR*$^{-/-}$ thymii on E14.5. Thymii of *N-CoR*$^{-/-}$ embryos were consistently 25%–30% the size of their wild-type and heterozygote littermates. Size marker = 100 μm.

(B) In situ hybridization of wild-type E14.5 thymus sections using ^{35}S-labeled *N-CoR* and *SMRT* probes. Both *N-CoR* and *SMRT* transcripts were present in E14.5 thymus.

(C) Western blot analysis of whole thymus, isolated thymocytes, and thymic stroma. N-CoR protein was expressed in thymocytes, whole thymus, and at slightly lower levels in thymic stroma; in contrast, SMRT protein was expressed in whole thymus, thymic stroma, and at lower levels in thymocytes.

(D) Schematic diagram of the progression from double-negative (DN) to double-positive (DP) T cells, with stages affected in *N-CoR*$^{-/-}$ embryos indicated.

(E) Recovery of live T cells after fetal thymic organ culture.

(F) Flow cytometric analysis of viable cells from E14.5 fetal thymic organ cultures after 3 days in culture. In the top panels, antibodies against mouse CD4 and CD8 were used and showed a block from DN to DP T cells in thymii from *N-CoR*$^{-/-}$ embryos. In the bottom panel, CD25 and CD44 expression was analyzed in DN cells and showed a block at the CD25$^+$CD44$^-$ stage in thymii from *N-CoR*$^{-/-}$ embryos.

thymus, where maturation is characterized by a "double-negative" stage (DN) where CD4 and CD8 coreceptors are absent, a "double-positive" stage (DP) where both coreceptors are expressed, and finally a "single-positive" mature stage (SP) where either CD4 or CD8 coreceptor is expressed (Figure 3D). *N-CoR*$^{-/-}$ thymii were analyzed by fetal thymic organ cultures (FTOC) (Ramsdell, 1992). E14.5 fetal thymus lobes were cultured for 72 hours to allow for differentiation from CD4$^-$/CD8$^-$ (DN) to CD4$^+$/CD8$^+$ (DP) and then analyzed by flow cytometry. The total number of live thymocytes, as assessed by forward scatter versus side scatter profiles, was reduced ~8-fold in the cultured *N-CoR*$^{-/-}$ thymii (Figure 3E). An increased rate of cell death, as evidenced by detection of a large number of dead cells in cultures from *N-CoR*$^{-/-}$ thymii, may account for this reduction. Additionally, in contrast to wild-type and heterozygote littermates, in which ~58% of thymocytes were CD4$^+$/CD8$^+$ after 3 days of culture, thymii from *N-CoR*$^{-/-}$ embryos were largely blocked in the CD4$^-$/CD8$^-$ stage, with only 3% of thymocytes expressing both CD4 and CD8 (Figure 3F). To determine whether this small number of CD4$^+$/CD8$^+$ cells could progress to the single-positive stage, thymii from E15.5 *N-CoR*$^{-/-}$ embryos were analyzed after 5 days of FTOC, during which time differentiation to single-positive cells occurs. Thymocytes from E15.5 *N-CoR*$^{-/-}$ embryos were able to progress to the single-positive stage; however, thymocyte numbers were drastically reduced (data not shown).

The double-negative stage of thymocyte development can be further subdivided on the basis of surface expression of CD25 and CD44, in which maturation proceeds through the CD25$^-$CD44$^+$ → CD25$^+$CD44$^+$ → CD25$^+$CD44$^-$ → CD25$^-$CD44$^-$ stages (Figure 3D). To further analyze the CD4$^-$/CD8$^-$ compartment, surface expression of CD25 and CD44 was determined. After 72 hr in culture, there was an ~2-fold increase (from 31% to 54%) in the CD25$^+$/CD44$^-$ population, and an ~3-fold decrease (from 38% to 13%) in the CD25$^-$/CD44$^-$ population in the thymocytes derived from *N-CoR*$^{-/-}$ embryos versus those from wild-type littermates (Figure 3F). These data indicated a block in thymocyte development beyond the CD25$^+$/CD44$^-$ stage.

Studies in a number of mutant mouse strains have led to the proposal of a cascade of events that are required for progression in and beyond the CD25$^+$/CD44$^-$ stage. Briefly, cells must undergo productive V(D)J recombination of the T cell receptor (TCR) β locus, express TCRβ polypeptides, assemble a TCRβ-pTα/CD3 complex, and activate signaling cascades thought to be dependent on protein tyrosine kinases of the Src (Lck; Fyn) and Syk (ZAP-70; Syk) families (reviewed in Fischer and Malissen, 1998). The expression of TCRβ on the surface of CD25$^+$/CD44$^-$ cells did not differ between *N-CoR*$^{-/-}$ embryos and wild-type or heterozygous littermates, suggesting productive recombination of the TCRβ locus (data not shown). CD3 expression was also unaltered, further suggesting that *N-CoR*$^{-/-}$ embryos were capable of assembling a pre-TCR complex (data not shown). Antibodies to CD3ε can induce CD4$^-$/CD8$^-$ thymocytes to differentiate into CD4$^+$/CD8$^+$ cells in a manner that is independent of TCRβ expression (Levelt et al., 1993) but dependent on signal transduction by Lck and Ras (Swat et al., 1996). Following a 72 hr treatment with anti-CD3ε antibody, *N-CoR*$^{-/-}$ thymocytes progressed to the CD4$^+$/CD8$^+$ stage, and the number of CD25$^+$/CD44$^+$ thymocytes was similar to that of heterozygote and wild-type littermates (comparable to Figure 3F, left-hand panels), indicating that events downstream of pre-TCR assembly were unaffected. The recovery of live cells from *N-CoR*$^{-/-}$ thymii after FTOC with anti-CD3ε antibody was also increased compared to cultures without anti-CD3ε treatment (data not shown). This is consistent with the hypothesis that a specific block in progression underlies the decreased number of cells found in cultured thymii from *N-CoR*$^{-/-}$

embryos. Therefore, N-CoR expression was required for progression of thymocyte development from the $CD25^{+}$/$CD44^{-}$ stage to the double-positive stage.

Role of N-CoR in CNS Development

While *N-CoR* and *SMRT* transcripts were expressed throughout the developing nervous system in a grossly similar pattern, a detailed analysis revealed several regions with differential expression. One such region was the developing dorsal thalamus, where *N-CoR* mRNA was highly expressed and *SMRT* transcripts were barely detectable (Figure 4A). The relative size of the developing thalamus was reduced in *N-CoR*$^{-/-}$ embryos at E12.5. This difference became increasingly pronounced and by E15 there was a general increase in the size of the third ventricle in *N-CoR*$^{-/-}$ embryos (Figure 4B). Nissl-staining of coronal brain sections revealed that specific thalamic nuclei, such as the lateral geniculate nucleus, were markedly smaller in *N-CoR*$^{-/-}$ embryos (Figure 4B). No evidence of apoptosis was detected (data not shown). These results suggested a role for N-CoR in the proliferation and/or differentiation events required for normal diencephalic development.

Differences in *N-CoR* and *SMRT* expression were also observed in the outer layers of the developing neocortex (Figures 4A and 4C). At E14.5, *N-CoR* and *SMRT* mRNA were expressed at similar levels in the cortical ventricular zone, which consists of undifferentiated, proliferating neural cells. However, in the outer cortical layers, which consist of more differentiated cells, there was differential expression of *N-CoR* and *SMRT*. As shown at E14.5 (Figure 4C), the marginal zone and cortical plate expressed high levels of *N-CoR* transcripts, while *SMRT* was expressed only minimally. To address the possibility that N-CoR might function in the differentiation of the outer cortical layers, we investigated the expression of microtubule-associated protein 2 (MAP2), a widely used marker for late neuronal differentiation. We found that MAP2 expression was reproducibly enhanced in the outer cortical layers in the *N-CoR*$^{-/-}$ embryos at E14.5, as evidenced by a higher number of cells displaying strong MAP2 immunoreactivity (Figure 4C).

To further investigate early neural development in the *N-CoR*$^{-/-}$ embryo, the expression of the intermediate neurofilament nestin was examined. Nestin is expressed both in undifferentiated cells committed to neural differentiation and in radial glial cells that are important for correct neuronal migration (Josephson et al., 1998). No difference in nestin expression was observed at E10.5. However, a decrease in the labeling intensity was observed in the *N-CoR*$^{-/-}$ embryo beginning on E12.5, and this difference was more pronounced by E14.5 (Figure 4D). Equivalent staining of radial glial cells by glial fibrillary acidic protein (GFAP) in both *N-CoR*$^{-/-}$ and wild-type littermates suggested that there was no change in the number of radial glial cells (Figure 4D). No unequivocal evidence for variations in migration or regionalization could be detected in the brains of *N-CoR*$^{-/-}$ embryos compared to heterozygote or wild-type littermates (data not shown). These changes in gene expression indicated a role for N-CoR in the developing central nervous system.

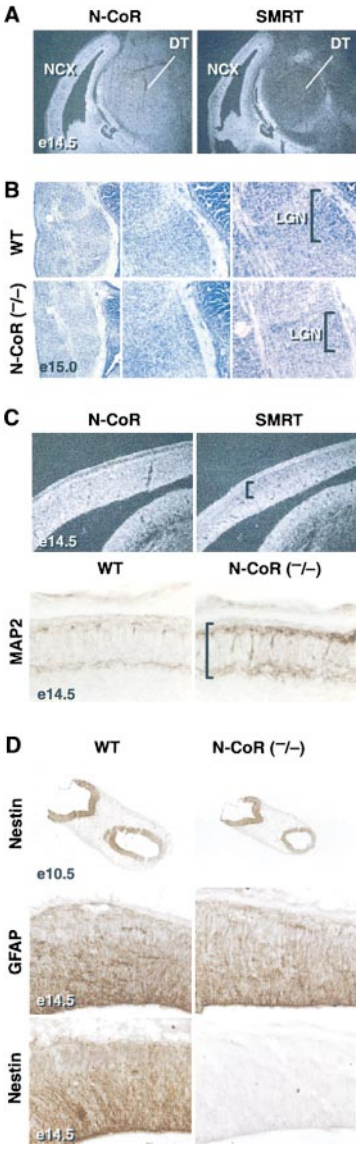

Figure 4. N-CoR Effects on CNS Development

(A) *N-CoR* was highly expressed at E14.5 in the developing dorsal thalamus (DT), while *SMRT* expression was virtually absent. NCX, neocortex.

(B) Thionin stained sections revealed a smaller thalamus in *N-CoR*$^{-/-}$ embryos and a decrease in size of the lateral geniculate nucleus (LGN; bracket) as compared to littermates.

(C) High power micrographs of neocortex show *N-CoR* and *SMRT* were expressed at similar levels in the ventricular zone, while SMRT expression was lower in the outer cortical layers (bracket). Increased MAP2 immunoreactivity could be detected in the outer cortical layers (bracket) in *N-CoR*$^{-/-}$ mice as compared to littermates.

(D) While nestin immunoreactivity was equivalent in wild-type and *N-CoR*$^{-/-}$ neural tube at E10.5, at E14.5 it was decreased in *N-CoR*$^{-/-}$ mice compared to wild-type or heterozygous littermates. There was no difference in GFAP immunoreactivity at E14.5.

N-CoR Was Required for Nuclear Receptor-Mediated Repression Events

Mouse embryo fibroblasts (MEFs) isolated from wild-type, heterozygous, or *N-CoR*$^{-/-}$ embryos grew at equivalent rates and equivalently expressed LacZ from a CMV promoter and an Sp-1-dependent promoter (Figure 5A and data not shown). Additionally, SMRT protein expression was unchanged in *N-CoR*$^{-/-}$ MEFs as compared to MEFs derived from littermates (data not shown). Single-cell nuclear microinjection revealed that ligand-independent reporter activity of a minimal promoter under the

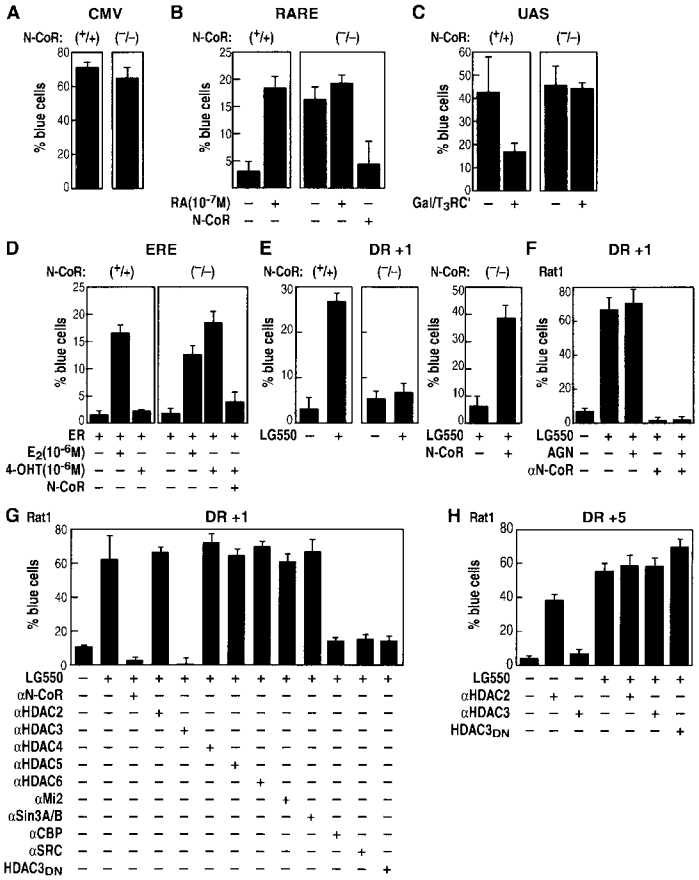

Figure 5. N-CoR Was Required for Transcriptional Regulation by Nuclear Receptors in MEFs

(A) Microinjection of CMV-driven LacZ reporters gave similar results in wild-type (+/+) or *N-CoR*$^{-/-}$ MEFs.

(B) In *N-CoR*$^{-/-}$ MEFs, basal expression of a DR+5 RARE-dependent promoter was increased, and this increase was abolished with coinjection of an N-CoR expression vector.

(C) In *N-CoR*$^{-/-}$ MEFs, a Gal/T_3R C-terminal fusion protein failed to repress in the absence of ligand.

(D) 4-Hydroxy-Tamoxifen (4-OHT) acted as an agonist of ER in *N-CoR*$^{-/-}$ MEFs.

(E) RAR-specific ligand (LG550)-dependent activation on the DR+1 site did not occur in *N-CoR*$^{-/-}$ MEFs.

(F) Microinjection of anti-N-CoR showed that N-CoR was also required for LG550-dependent activation in Rat-1 cells. Similar results were obtained following simultaneous addition of both RAR- and RXR-specific ligands (LG550, AGN).

(G) Roles of cofactors in ligand-dependent activation of the DR+1 reporter. Of antibodies microinjected, only anti-HDAC3, anti-CBP, and anti-SRC/p160 IgGs blocked ligand-dependent activation, as did a dominant-negative HDAC3 ($HDAC3_{DN}$).

(H) Evaluation of the roles of HDAC2 and HDAC3 in modulating activity of unliganded RAR on a DR+5 reporter. Anti-HDAC2 IgG caused stimulation of basal activity. Neither anti-HDAC2 IgG, anti-HDAC3 IgG, nor dominant-negative HDAC3 ($HDAC3_{DN}$) altered ligand-dependent activation of a DR+5 reporter.

regulation of a retinoic acid response element with direct repeat core motifs spaced by 5 base pairs (DR+5 RARE) was minimal in wild-type or heterozygous MEFs, while in *N-CoR*$^{-/-}$ MEFs, ligand-independent activation of reporter activity was observed (Figure 5B). Reexpression of N-CoR by an N-CoR expression vector blocked the ligand-independent activation of the DR+5 RARE in *N-CoR*$^{-/-}$ MEFs (Figure 5B). N-CoR was also required for the repressor function of the C-terminal portion of the T_3R (Figure 5C). These experiments confirmed the requirement for N-CoR in ligand-independent repression by RAR and T_3R.

Although steroid hormone receptors do not appear to interact with corepressors in the absence of agonists, recent studies have suggested that antagonists allow N-CoR or SMRT to interact with this class of nuclear receptor (Xu et al., 1996; Jackson et al., 1997; Smith et al., 1997; Lavinsky et al., 1998). The ER antagonist 4-hydroxy-tamoxifen (4-OHT) failed to activate ER-dependent transcription in wild-type MEFs but acted as a full agonist in *N-CoR*$^{-/-}$ MEFs. Introduction of an N-CoR expression vector was sufficient to reverse this effect (Figure 5D). These experiments confirmed that N-CoR was required for the antagonist effect 4-OHT confers to ER and suggested that its ability to function as an agonist in specific settings may require lack of expression or functional inactivation of N-CoR.

Several nuclear receptors, including RAR and retinoid X receptor (RXR), can bind to response elements that are characterized by a spacing of 1 base pair between direct repeat core motifs (DR+1 sites). RAR/RXR heterodimers bind to DR+1 sites with a polarity opposite to that observed on the DR+5 site (Kurokawa et al., 1994; Rastinejad et al., 2000) and exhibit a decreased ability to release N-CoR upon binding of ligand (Kurokawa et al., 1995). In contrast to the observation that the RAR-specific ligand, LG550, activated gene expression from the DR+1 site in MEFs from wild-type or *N-CoR*$^{+/-}$ embryos, activation was never observed in MEFs from *N-CoR*$^{-/-}$ mice (Figure 5E). As microinjection of an N-CoR expression vector restored activation by LG550 in *N-CoR*$^{-/-}$ MEFs, N-CoR was required, directly or indirectly, to permit ligand-dependent activation through a DR+1 site. Similar results were obtained with simultaneous addition of RAR- (LG550) and RXR-specific (AGN) ligands in Rat-1 cells (Figure 5F).

We investigated whether N-CoR-dependent activation on a DR+1-containing promoter required the function of other components of proposed corepressor complexes. Microinjection of IgG against HDAC2, 4, 5, and 6 and Mi-2 did not affect ligand-dependent activation of the DR+1 reporter (Figure 5G). In contrast, anti-HDAC3 IgG fully abolished activation of the DR+1 reporter by LG550 (Figure 5G). Addition of a dominant-negative form of HDAC3 (V. Perissi and S. H. Baek, personal communication) fully abolished ligand-dependent activation by retinoic acid receptor–specific ligands on the DR+1 reporter, supporting a specific requirement for deacetylase function in this activation event (Figure 5G). In contrast, microinjection of this dominant-negative HDAC3 with a DR+5-containing promoter, which required HDAC2 but not HDAC3, did not affect ligand-independent or ligand-dependent function (Figure 5H). Therefore, it appears that N-CoR, via its

recruitment of enzymatically active HDAC3, was required to mediate ligand-dependent activation on a DR+1 element. Antibodies against several of the coactivators required for DR+5-dependent activation in this assay also abolished ligand-induced activation on the DR+1 site (Figure 5G), indicating that the same families of coactivators were required for ligand-dependent activation of both DR+1 and DR+5 reporters.

Role of N-CoR in Short- and Long-Term Repression by Other Classes of Transcriptional Repressors

We have proposed that repression by the bHLH protein MAD is at least in part mediated through N-CoR (Heinzel et al., 1997). Microinjection assays in MEFs confirmed that MAD is dependent on N-CoR for its repressive actions (Figure 6A). In contrast, the bHLH family member Hes1 functioned effectively as a repressor in the absence of N-CoR (Figure 6B). Similarly, no role for N-CoR was observed for the repressor Msx1 (Figure 6C). These data were consistent with the hypothesis that the requirement for N-CoR in transcriptional repression was factor specific.

We next assessed a potential role for N-CoR in long-term repression mediated by REST/NRSF, a zinc finger transcription factor proposed to repress expression of neural genes in nonneural tissues (Chong et al., 1995; Schoenherr and Anderson, 1995). Transcriptional repression of a REST/NRSF response element (RE1/NRSE), which is found in the promoter of numerous genes and restricts their expression from nonneural cell types (e.g., Mori et al., 1992; Kallunki et al., 1997), was relieved when Rat-1 cells were microinjected with anti-N-CoR IgG, and microinjection of an N-CoR expression vector restored repression (Figure 6D). Anti-REST affinity-purified antibody and IgG-purified anti-HDAC2 and anti-Sin3A/B antibodies also relieved repression in this assay (Figure 6D), consistent with the proposed role for these factors in REST/NRSF-mediated repression (Huang et al., 1999; Naruse et al., 1999; Grimes et al., 2000; Roopra et al., 2000). Similar data was obtained when a Gal4DBD-REST/NRSF fusion protein was evaluated for its ability to repress a 3XUAS-TK promoter (Figure 6E).

We used the chromatin immunoprecipitation (ChIP) assay to determine if the rat *SCG10* promoter, which contains an RE1/NRSE element (Mori et al., 1992), recruits N-CoR. REST/NRSF contains two transferable repressor domains (Tapia-Ramirez et al., 1997), an N-terminal domain that appears to associate with yeast or mammalian HDACs and Sin3, and a C-terminal domain that interacts with CoREST (Andres et al., 1999). Amplification using primers flanking the region determined to be required for REST/NRSF-mediated repression of the *SCG10* promoter (Mori et al., 1992) revealed that REST/NRSF, N-CoR, and HDAC2, but not CoREST, interacted with this region of the *SCG10* promoter (Figure 6F). In contrast, the collagenase I proximal promoter, which does not have an RE1/NRSE element, was not immunoprecipitated with the REST antibody (Figure 6F).

In wild-type MEFs, transcriptional repression of an RE1/NRSE element was observed and could be partially relieved by microinjection of anti-N-CoR IgG (Figure 6G). In contrast, repression from an RE1/NRSE element was not observed in *N-CoR*$^{-/-}$ MEFs, and microinjection of an N-CoR expression vector restored repression, further supporting a role for N-CoR in REST/NRSF-mediated repression. REST/NRSF and CoREST levels were comparable in wild-type and *N-CoR*$^{-/-}$ MEFs (data not shown).

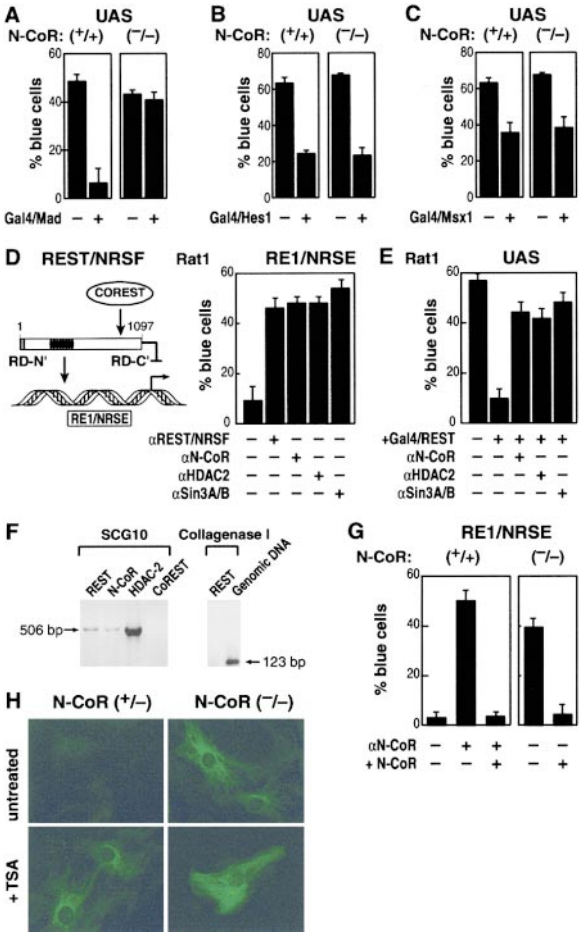

Figure 6. N-CoR Mediates Repression by Other Classes of Transcription Factors

(A) In the absence of N-CoR, a Gal4/MAD fusion protein did not repress a 3XUAS reporter.

(B and C) Inhibitory actions of Gal4/Hes1 or Gal4/Msx1 fusion proteins were not altered in *N-CoR*$^{-/-}$ MEFs.

(D) Repression of a REST/NRSF response element (RE-1/NRSE) in Rat-1 cells was dependent upon REST/NRSF, mSin3 A/B, HDAC2, and N-CoR.

(E) Repression of a 3XUAS reporter by the Gal/REST fusion protein was blocked by microinjection of anti-N-CoR, anti-HDAC2, and anti-mSin3 A/B.

(F) ChIP Assay performed on the murine *SCG10* REST/NRSF regulatory region from Rat-1 cells. Binding of REST, HDAC2, and N-CoR was detected, while CoREST was not. In contrast, the collagenase I promoter does not recruit REST.

(G) Repression of an RE1/NRSE was observed in MEFS from wild-type and heterozygous but not *N-CoR*$^{-/-}$ embryos.

(H) Neuronal-specific class III β-tubulin was detected by immunohistochemistry in *N-CoR*$^{-/-}$ MEFs but not in heterozygous MEFs, while treatment with TSA allowed immunohistochemical detection of class III β-tubulin in both.

REST/NRSF gene-deleted mice, which die at E9.5, exhibit ectopic expression of a neuronal-specific class III β-tubulin in a subset of cells in some nonneural tissues (Chen et al., 1998). Expression of a dominant-negative form of REST/NRSF in developing chick embryos also causes misexpression of several RE1/NRSE-containing

neural-specific genes, including *SCG10* and *Ng-Cam* (Chen et al., 1998). To determine if N-CoR could be a component of this repression, expression of class III β-tubulin was assessed in MEFs using the TuJ1 antibody. Class III β-tubulin expression was observed in ~20 percent of *N-CoR*$^{-/-}$ MEFs but not in heterozygous MEFs (Figure 6H). After incubation with TSA for 24–48 hr, ~20% of both heterozygous and *N-CoR*$^{-/-}$ MEFs expressed class III β-tubulin (Figure 6H). This was consistent with effects of TSA on REST/NRSF-repressed genes in NIH 3T3 cells (Naruse et al., 1999). Thus, in addition to its role in mediating short-term repression events, N-CoR may also be involved in long-term repression events mediated by REST/NSRF on a subset of its target genes in specific cell types.

Discussion

N-CoR Was Required for Active Repression by Several Classes of DNA Binding Transcription Factors

Transcriptional activation and repression mediated by sequence-specific DNA binding factors underlie the binary decisions necessary for orchestration of developmental programs in metazoans. Transcriptional activation by different classes of DNA binding proteins is dependent upon recruitment of a series of multisubunit coactivator complexes, exemplified by the alternative requirements for SAGA, ADA, and other complexes by different transcription units in yeast (Grant et al., 1997). The discovery of a large and ever increasing number of putative vertebrate corepressors and of a family of histone deacetylases has raised the question of whether combinations of repressors and corepressors are required to mediate specific cell fate decisions. As homologous proteins are not encoded by the yeast genome, N-CoR and SMRT appear to have arisen during the evolution of metazoans to allow additional factors, including unliganded nuclear receptors, to mediate transcriptional repression.

In this manuscript, we have provided in vivo evidence that the corepressor N-CoR was required for mediation of active repression by specific nuclear receptors and several additional classes of transcription factors on a subset of gene targets, thereby acting as a critical factor for specific developmental events in erythrocytic, thymic, and neural systems. Our data has also validated the hypothesis that antagonist activity of ER-mediated transcription conferred by 4-OHT requires N-CoR. This has clear implications in acquired drug resistance to antagonists that are used in treatment of breast cancer.

We have also provided evidence through several independent types of analyses that N-CoR can act as a required component of long-term gene repression events mediated by the DNA binding repressor REST/NRSF. Interestingly, while N-CoR was present on the promoter of the *SCG10* gene, CoREST, the recruitment of which is required for repression of other REST/NRSF-dependent genes (G. M. et al., unpublished data), was not. There is thus likely to be gene specificity in cofactor recruitment to REST/NRSF response elements. Our data indicated that the REST/NRSF-dependent restriction of genes out of specific cell types may involve independent mechanisms and that for at least a subset of transcription units, N-CoR was both recruited to a REST/NRSF-dependent repression complex and was required for its effective long-term repression.

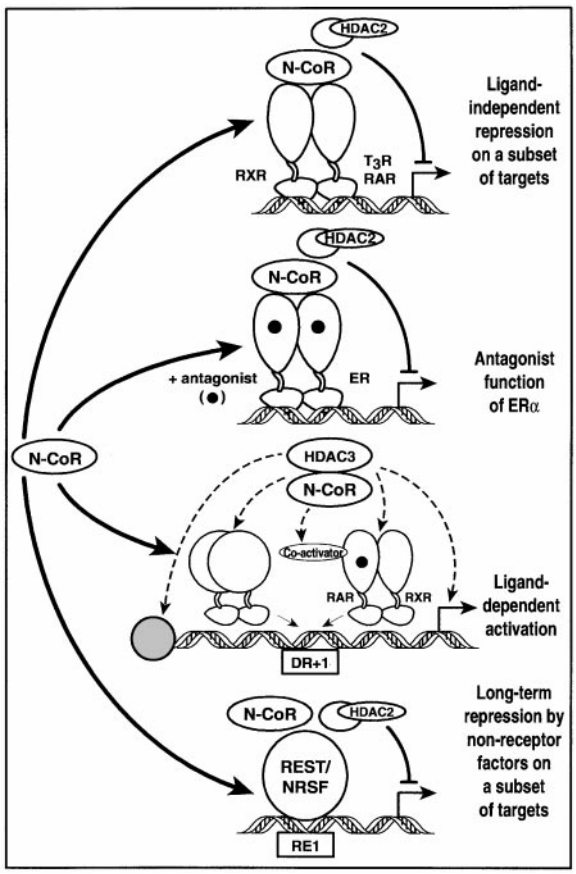

Figure 7. Model of Repressor-Corepressor Integration by N-CoR

N-CoR was a required component for effective ligand-independent repression by RAR and T_3R and prevented agonist functions by the estrogen receptor mixed agonist 4-hydroxy-tamoxifen. On a DR+1 site, N-CoR, with HDAC3, appeared to be required for RAR-specific ligand-dependent activation, either directly or via modulation of site-bound transcription factors. This could reflect several mechanisms, including deacetylation of receptors, coactivators, or chromatin-associated proteins, or components of the core transcriptional machinery. N-CoR was also involved in long-term repression mediated by REST/NRSF on a subset of target genes in specific cell types.

The unexpected requirement for N-CoR in RA-dependent activation of a DR+1 element suggested that, directly or indirectly, N-CoR can repress or activate gene expression, analogous to roles of Sin3 and rpd3 in yeast (Struhl et al., 1998). This activation also required enzymatically active HDAC3, which has been recently identified to be in stable association with an N-CoR/SMRT complex (Guenther et al., 2000; Wen et al., 2000). Based on the complexity of the events on the DR+1 site, activation by N-CoR/HDAC3 could be mediated through effects on receptors, coactivators, or components of chromatin. Indeed, corepressor/HDAC complexes may serve as positive coregulators for other classes of transcription factors. For example, HDAC-containing complexes could antagonize the proposed CBP-mediated repression of dTCF through acetylation and subsequent abolishment of TCF interaction with the coactivator β-catenin/Armadillo (Waltzer and Bienz, 1998).

Role of N-CoR in Development

N-CoR appeared to exert critical roles at specific steps in both erythroid and thymocyte development, linking a

specific corepressor to discrete cell-type specification events. Studies in primary avian erythroblasts showed that overexpression of T_3R in the absence of T_3 resulted in sustained proliferation and tightly arrested differentiation of erythroblasts, while addition of T_3 caused loss of self-renewal capacity and induced terminal differentiation (Bauer et al., 1998). As *N-CoR*$^{-/-}$ erythroblasts had enhanced levels of the CA II, unliganded T_3R appeared to require N-CoR for repression events critical to expansion of erythroblast progenitors. Proliferation in the absence of T_3 appears to require cooperation with c-kit, because in the absence of the c-kit ligand SCF, differentiation occurs (Bauer et al., 1998). This suggests that stages of development that require SCF may also involve T_3R. It is then interesting to note that CFU-E colony formation, which does not require SCF, was normal in *N-CoR*$^{-/-}$ embryos at the ages tested, while SCF-dependent BFU-E colony formation was impaired.

In *N-CoR*$^{-/-}$ embryos, the block in thymocyte development at the CD25$^+$/CD44$^-$ stage during the DN to DP transition was similar to that seen in mice with deletion of particular transcription factors. Mice lacking the transcriptional repressor Hes1 exhibit blocks at both CD25$^-$/CD44$^+$ and CD25$^+$/CD44$^-$ stages of development (Tomita et al., 1999), but preliminary results with a Gal4-Hes1 fusion protein suggested that in MEFs Hes1 maintained its repressive function in the absence of N-CoR. *TCF/LEF* double gene-deleted mice exhibit a block at the CD25$^+$/CD44$^-$ stage, as well as at the immature CD8 single-positive (CD8$^+$ ISP) stage, raising the possibility that N-CoR-associated deacetylase activity may affect *TCF/LEF*-dependent activation events (Waltzer and Bienz, 1998).

The early events of neural induction appeared to be largely maintained as assessed by the normal onset of nestin expression in *N-CoR*$^{-/-}$ embryos. While the mechanism of downregulation of nestin at midgestation remains to be elucidated, it is intriguing that the enhancer that drives nestin gene expression in the CNS harbors binding sites for both POU factors and nuclear receptors (Josephson et al., 1998). The finding that MAP2, which can be induced by retinoic acid and harbors RAREs in its promoter (Neuman et al., 1995), was upregulated in the outer layers of neocortex of *N-CoR*$^{-/-}$ embryos identifies *MAP2* as a putative target gene for N-CoR-mediated repression through unliganded RAR. Experiments in cell lines have suggested that decreased levels of MAP2 inhibit neuronal differentiation and neurite formation (Dinsmore and Solomon, 1991).

In conclusion, N-CoR appeared to exert corepressor roles for subsets of genes under control of specific DNA binding repressors and was involved in repression events mediated by specific nuclear receptors and several other classes of DNA binding transcription factors (Figure 7). Because of the linkage between N-CoR and REST/NRSF-dependent repression, our evidence suggested that N-CoR participates in both transient and long-term repression events. Additionally, N-CoR appeared to serve as a cofactor required, directly or indirectly, for gene activation on certain nuclear receptor response elements (Figure 7). Interestingly, both short- and long-term repressor functions and specific activation functions appeared to require the actions of distinct HDACs, suggesting that there may be DNA site-, promoter-specific usage of N-CoR-associated HDACs. These studies functionally link the corepressor N-CoR to repressor-mediated determination of lineage progression in distinct cell types in mammalian development.

Experimental Procedures

Generation of *N-CoR*$^{-/-}$ Mice

Genomic DNA clones encoding the murine *N-CoR* gene were isolated from a 129Sv λFIXII library (Stratagene, La Jolla, CA). The targeting construct, designed to delete amino acids 282–308 and induce a frameshift mutation if the resulting mRNA were translated, was assembled using a 1.8 kb EcoRI fragment and a 5.0 kb XhoI–SalI PCR fragment. Transfected R1 ES cells (J. D. Marth, University of California, San Diego) were selected in medium containing 0.4 mg/ml G418 and 10 μg/ml gancyclovir. Two clones containing the targeted allele were identified by Southern blotting. Cells were injected into 3.5-day-old C57BL/6 blastocysts, which were transferred into Swiss foster mothers. Chimeric males were crossed with C57BL/6 females.

Immunohistochemistry and In Situ Hybridization Analysis

Immunohistochemistry and in situ hybridization were performed as previously described (Hermanson et al., 1994) using probes of base pairs 2766–3171 of *SMRT* and 3954–4689 of *N-CoR*. The following antisera were used: mouse anti-MAP2 (Sigma), rabbit anti-nestin (R. McKay), mouse anti-nestin (PharMingen), TuJ1 (Babco), and mouse anti-GFAP (ICN).

Erythropoietic Analyses

Hematocrit were determined using heparinized Micro-Hematocrit Capillary Tubes (Fisher). Giemsa staining of blood smears was performed according to manufacturer's instructions using Accustain, Giemsa Stain, Modified (Sigma). For colony-forming assays, individual fetal livers were dissected, disaggregated into single-cell suspensions, and passed through a cell strainer (70 μm, Falcon). Cells were diluted in 2% acetic acid to lyse nonnucleated mature erythrocytes and the remaining cells counted. Cells amounting to 1×10^5 (BFU-E) or 1×10^4 (CFU-E) from each fetal liver were plated in methylcellulose medium containing erythropoietin with (for BFU-E assays) or without (for CFU-E assays) SCF, IL-3, and IL-6 (Stem Cell Technologies, Vancouver, BC). Benzidine-positive BFU-E colonies were counted after 10 days; CFU-E colonies were counted after 3 days. Sheep anti-human Carbonic Anhydrase II antibody (The Binding Site, UK) was used for detection of CA II in fetal liver cells.

Thymocyte Analyses

Thymocytes were released by straining lobes through 70 μm Nylon Cell Strainers (Falcon). Thymic stroma devoid of thymocytes was prepared by culturing E14.5 or E15.5 fetal thymic lobes for 5 days in the presence of 1.35 mM dGUO (Sigma). Western blot analysis was performed as previously described using anti-rabbit antibodies to N-CoR (Heinzel et al., 1997) or anti-guinea pig antibodies to SMRT (Lavinsky et al., 1998). For experiments with anti-CD3ε, thymii were cultured 3 days with 25 μg/ml purified anti-CD3ε (PharMingen). Flow cytometry was performed using 1×10^6 cells that were washed in FACS buffer (PBS/2% fetal calf serum/0.1% NaN_3) and incubated in 100 μl FACS buffer plus antibody for 20 min. The process was repeated with 2° antibodies as necessary. Cells were collected prior to analysis on a FACScan (Becton Dickinson). Collection and analysis were performed using CellQuest software (Becton Dickinson). Directly conjugated antibodies specific for mouse CD4 and CD8 (Caltag) and CD25, CD44, TCRβ, and CD3 (PharMingen) were used.

Single-Cell Nuclear Microinjection

Each experiment was performed on three independent coverslips consisting of ~1000 cells. Where no experimental antibody was used, preimmune rabbit or guinea pig IgG was coinjected. Antibodies to N-CoR, Sin3A/B, and HDAC2 (Heinzel et al., 1997), to CBP and SRC-1 (Torchia et al., 1997), to REST and CoREST (Andres et al., 1999), to HDAC3, 4, 5, and 6, and to Mi-2 (Santa Cruz Biotechnology) were used. The RXR-specific ligand AGN 194204 was a gift from R. Chandraratna (Allergan Pharmaceuticals).

Chromatin Immunoprecipitation (ChIP) Assay

Rat-1 cells were fixed with 1% formaldehyde for 30 min at RT and treated as described (Hecht and Grunstein, 1999). Cross-linked adducts were resuspended and sonicated resulting in DNA fragments

of 500–600 bp. Immunoprecipitation was performed using antibody-coated tosylactivated Dynabeads M-280 (Dynal, Oslo, Norway). Protein-bound, immunoprecipitated DNA was dissolved in TE buffer and treated at 65°C overnight. Digestion buffer (100 mM NaCl;10 mM Tris-HCL, pH 8; 25 mM EDTA, pH 8; 0.5% SDS) was added to the sample and incubated 2 hr at 50°C with 0.1 mg/ml Proteinase K (Sigma). Following extraction and precipitation, 50 cycles of PCR (T_a = 56°C) were performed. Primers for *SCG10* were as described (Mori et al., 1992). ChIP experiments on the collagenase I promoter were performed as above, except Hela cell extract was used. Oligos were as follows: 5′-CAAATAATCTGCTAGGAGTCACCA; 3′-ATATA GAGTCCTTGCCCTTCCAGA.

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