A cell cycle-dependent co-repressor mediates photoreceptor cell-specific nuclear receptor function

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Photoreceptor cell-specific nuclear receptor (PNR) (NR2E3) acts as a sequence-specific repressor that controls neuronal differentiation in the developing retina. We identified a novel PNR co-repressor, Ret-CoR, that is expressed in the developing retina and brain. Biochemical purification of Ret-CoR identified a multiprotein complex that included E2F/Myb-associated proteins, histone deacetylases (HDACs) and NCoR/HDAC complex-related components. Ret-CoR appeared to function as a platform protein for the complex, and interacted with PNR via two CoRNR motifs. Purified Ret-CoR complex exhibited HDAC activity, co-repressed PNR transrepression function *in vitro*, and co-repressed PNR function in PNR target gene promoters, presumably in the retinal progenitor cells. Notably, the appearance of Ret-CoR protein was cell-cycle-stage-dependent (from G1 to S). Therefore, Ret-CoR appears to act as a component of an HDAC co-repressor complex that supports PNR repression function in the developing retina, and may represent a co-regulator class that supports transcriptional regulator function via cell-cycle-dependent expression.

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Introduction

Members of the nuclear receptor (NR) gene superfamily serve as sequence-specific regulators in the promoters of their

cognate target genes (Mangelsdorf *et al*, 1995). Reflecting the spatiotemporal expression patterns of NRs in animals, a wide variety of biological events are under the control of NR-mediated transcriptional regulation (McKenna and O'Malley, 2002; Rosenfeld *et al*, 2006). Structurally, NR proteins can be divided into five domains, A–E. The highly conserved C domain acts as a DNA-binding domain (DBD), which has two Zn-finger motifs that recognize and stably bind to specific target DNA sequences. The moderately conserved ligand-binding domain (LBD) is mapped to the C-terminal E domain. The N-terminal A/B domain exhibits poor homology among NRs but is responsible for ligand-induced transactivation together with the LBD regions in NRs such as nuclear hormone/vitamin receptors. Unlike hormone/vitamin receptors, several NRs are believed not to require ligand binding, and are therefore classified as orphan receptors that function as ligand-independent regulators (Mangelsdorf *et al*, 1995).

Ligand-dependent and -independent transcriptional control by NRs requires histone modification and chromatin remodeling (Belandia and Parker, 2003; Kitagawa *et al*, 2003). Histone modification coupled with transcriptional control by NRs depends on the input of two types of co-regulators with opposing functions. It appears that most co-regulators exist as multiprotein complexes (McKenna and O'Malley, 2002; Perissi and Rosenfeld, 2005). It is thought that three distinct classes of co-activators support NR transactivation, with two of these classes, CBP/p160 and GCN5/TRAP complexes (Onate *et al*, 1995; Kamei *et al*, 1996; Yanagisawa *et al*, 2002), containing histone acetyltransferase (HAT) enzymes. The other class, DRIP/TRAP complex, is a non-HAT co-activator complex (Fondell *et al*, 1996; Rachez *et al*, 1999). The co-repressor type complexes contain histone deacetylase (HDAC) enzymes, which along with NCoR/SMRT physically interact with NRs via CoRNR motifs, and are thought to be functionally indispensable subunits in NR co-repression complexes (Heinzel *et al*, 1997; Nagy *et al*, 1997). The other histone-modifying enzymes are also likely to co-regulate the NR function (Metzger *et al*, 2005). Although histone modification owing to HAT/HDAC activity in NR co-regulator complexes may in cooperation with chromatin-remodeling complexes explain at least in part the mechanism of NR-mediated transcriptional control via chromatin remodeling (Narlikar *et al*, 2002; Belandia and Parker, 2003; Kitagawa *et al*, 2003), the molecular link between NR-mediated gene regulation and cell cycle control remains elusive.

The photoreceptor-specific orphan receptor PNR (NR2E3) is a pivotal regulator in the developing retina, as it determines cone photoreceptor phenotype (Haider *et al*, 2001; Milam *et al*, 2002; Yanagi *et al*, 2002). Its significance in neuronal differentiation has been established by a number of studies based on spontaneous genetic mutations of the PNR gene in human enhanced S-cone syndrome (ESCS) patients and in

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Figure 1 Identification of Ret-CoR as a novel co-repressor of an orphan nuclear receptor PNR. (A) Schematic diagram of Ret-CoR protein. The two CoRNR motifs, DEVH box motifs and the region corresponding to the isolated cDNA clone (arrows) by yeast two-hybrid screening are illustrated. (B) Expression of Ret-CoR transcripts in the retina. *In situ* RT-PCR of adult mouse retinal sections performed with [RT(+)] or without [RT(-)] reverse transcriptase (RT). PNR (left) and Ret-CoR (right) transcripts as detected by *in situ* RT-PCR are shown. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer nuclear layer; OS, outer segment; PE, pigment epithelium. (C) Expression of Ret-CoR and PNR transcripts in mouse tissue (left panel) and human culture cells (right panel) as determined by Northern blotting. (D) Interaction between PNR and Ret-CoR in each cell line. Whole cell extracts from Y79 cells, 293F cells, and untransfected/PNR-transfected 293F Ret-CoR stable cells were subjected to immunoprecipitation (IP) with anti-PNR antibody. Each immunoprecipitant was then analyzed by Western blot (IB) with anti-PNR or anti-Ret-CoR antibody. (E) The CoRNR motif of Ret-CoR mediates interaction with PNR. *In vitro*-translated PNR proteins were applied for a GST pull-down assay. (F) Ret-CoR mediates the co-repressive function of PNR. Luciferase assays were performed in 293F cells transfected with a GAL4-DBD-binding site $\times$ 4 containing luciferase reporter plasmid (MH100-CMX-luc) (400 ng), GAL-fused expression vectors (200 ng), Ret-CoR expression vectors (100 ng), NCoR expression vectors (100 ng) and 10^{-9} M Trichostatin A. (G) Ret-CoR has a transrepressive activity itself. Luciferase assays were performed as illustrated in (F) using GAL-fused Ret-CoR expression vectors (200 ng (+) or 500 ng (++)).

rd7/rd7 mice, which suffer retinal degeneration and lack color perception due to imbalanced ratio between S- and M-cone cell numbers (Akhmedov *et al*, 2000; Gerber *et al*, 2000; Haider *et al*, 2000). PNR appears to attenuate cell proliferation of S-cone cells from retinal progenitor cells, but is unlikely to control of differentiation of S into M-cone cells.

Given that the well-known NR co-repressor NCoR/SMRT was not potent to co-repress PNR (see Figure 1F), we identified a novel PNR co-repressor, designated as Ret-CoR. By a biochemical approach, Ret-CoR was shown to form an HDAC complex to co-repress PNR. Most notably, appearance of Ret-CoR protein was dependent on cell cycle. Thus, Ret-CoR may represent a novel class of co-regulators that support transcriptional function of sequence-specific regulators via cell cycle-dependent expression.

Results

Identification of Ret-CoR as a novel PNR co-repressor

We used the yeast two-hybrid system to screen a human brain cDNA library for putative co-repressors that supported the constitutive transrepression function of PNR. We identified a specific PNR-interacting clone that encoded the N-terminal

region of the KIAA0890 protein (Nagase *et al*, 1998). Full-length KIAA0890 sequence encoded a 1194-amino acid-protein that contained a DEVH box motif (ATPase/helicase domain) and two CoRNR motifs (Figure 1A) that are thought to function as a physical interface for nuclear receptors and are found in co-repressors such as NCoR and SMRT (Hu and Lazar, 1999; McKenna and O'Malley, 2002). A mouse ortholog was identified from the NCBI database that showed 98% amino-acid sequence identity to human homologue. *In-situ* RT-PCR performed on slices of mouse retina revealed specific signals of this clone located at specific layers. Like expression pattern of the PNR transcript (Kobayashi *et al*, 1999), the transcript of KIAA0890 appeared to express abundantly in the outer nuclear layer (ONL) and to some extents in inner nuclear layer (INC) of the mouse retina (Figure 1B). whereas significant KIAA0890 mRNA expression was detected in mouse retina and brain tissues on postnatal day 0, levels were significantly decreased in 12-week-old mice, as determined by Northern-blot analysis (Figure 1C). The KIAA0890 gene expressed in the Y79 retinoblastoma cell line, as well as in the human embryonic renal cell line 293F, but PNR transcript was detected only in Y79 (Figure 1C, right panel). Endogenous Ret-CoR protein expression was detectable in Y79 and 293F (Figure 1C), in which nuclear localization of

Ret-CoR protein was observed (data not shown). From the expression pattern, the clone was designated as retina co-repressor (Ret-CoR) hereafter.

To investigate for interactions between PNR and Ret-CoR, we performed co-immunoprecipitation experiments on endogenous PNR and Ret-CoR proteins. Association between the proteins was observed in Y79 cells (Figure 1D), and by *in vitro* GST pull-down assay (data not shown). To map the interacting domain in Ret-CoR, *in vitro*-translated PNR and mutant Ret-CoR GST-fusion proteins were assayed. As expected from previous reports (Hu and Lazar, 1999), disruption of either CoRNR motif (V60A or V252A) in Ret-CoR significantly reduced the interaction with PNR (Figure 1E), which suggested that Ret-CoR interacts with PNR via the two CoRNR motifs.

We then tested the coregulatory function of Ret-CoR by generating a chimeric protein of PNR fused to the yeast GAL4 DNA-binding domain (DBD). A transrepressive activity was detected using the PNR-fusion protein by a transient expression assay in 293F cells (Figure 1F) and Y79 cells (data not shown). As an HDAC inhibitor trichostatin A (TSA) abrogated the transrepression by PNR (see lane 13 in Figure 1F), the HDAC activity was presumed to be required for the PNR-mediated transrepression. Ret-CoR clearly co-repressed PNR transrepression function, whereas NCoR was not so potent in 293F cells (Figure 1F) and the other cell lines (data not shown). Expectedly, TLX (NR2E1), an NR highly homologous to PNR, was also transrepressed by Ret-CoR (data not shown). Ret-CoR on its own exhibited transrepressive activity when fused with GAL4-DBD (Figure 1G). In contrast, this potentiation of transrepression was abrogated (Figure 1F) by point mutations in the Ret-CoR CoRNR motifs (see Figure 1E for the V60A/V252A mutant). Thus, our results indicated that Ret-CoR appears to serve as a co-repressor for PNR.

Ret-CoR forms an HDAC complex

To explore the molecular basis of Ret-CoR co-repressor function, we biochemically purified (Yanagisawa *et al*, 2002; Kitagawa *et al*, 2003) a Ret-CoR-containing complex. We generated stable 293F cell transformants (Ret-CoR-293F) that expressed human Ret-CoR tagged with FLAG and His epitopes at the N- and C-termini, respectively (Figure 2A). Nuclear extracts were then subjected to sequential affinity column purification using an anti-FLAG M2 affinity resin column and then a Protino Ni-affinity column. After concentrating the complexes using glycerol density gradients (Figure 2C), tagged Ret-CoR was detected in fractions that contained a complex with a molecular weight of about 1 MDa (Figure 2C). As shown in Figure 2B, middle panel, Ret-CoR appeared to form a multiprotein complex with 11 polypeptides, which were then identified by a mass spectrometry. The two background proteins appeared Hsc70 and β -actin. The complex formation and subunit identification by MALDI-TOF/MS was further confirmed by immunoprecipitation of the purified complex by an RbAp46-specific antibody (Figure 2B, right panel). Identities of these components in the glycerol density gradient functions could be further confirmed by Western blotting (see Figure 2C). The same Ret-CoR complex components were detected when the endogenous complex was immunoprecipitated of either tagged PNR or Ret-CoR from parent 293F cells by a FLAG antibody (Figure 2D). Similarly, association of endogenous PNR with

endogenous complex components in Y79 could be detected by immunoprecipitation with endogenous PNR in Y79 (see Figure 3C).

The Ret-CoR complex components could be grouped into three classes based on putative function: transcription factor E2F-associated co-repressors (Sin3A, p107, HDAC1/2, RbAp48 and RbAp46 (Luo *et al*, 1998)); transcription factor Myb-associated co-repressors (NCoR (Li and McDonnell, 2002), Mybbp1A (Tavner *et al*, 1998; Fan *et al*, 2004) and CDK9 (De Falco *et al*, 2000)); and NCoR/SMRT-associated proteins (HDAC3 (Guenther *et al*, 2000)). Although TBL3 (Weinstat-Saslow *et al*, 1993) and TBL1 (Perissi *et al*, 2004) appear to share similar motif organization, the function of TBL3 has not yet been reported. Using Far Western blotting, we found that PNR interacted with Ret-CoR and CDK9, and that Ret-CoR associated with p107 and TBL3 through its N-terminal domain and with p107 and CDK9 through its C-terminal domain (Figure 2E). These interactions were further confirmed by GST-pull-down assays (Figure 2F). From these findings, we presume that Ret-CoR served as a platform protein capable of promoting assembly of various components into a Ret-CoR complex (as illustrated in Figure 7).

Appearance of the Ret-CoR protein is cell cycle-dependent

Identification of cell-cycle transcription factor-related components related to E2F (Ohtani *et al*, 1995) and Myb (Oh and Reddy, 1999) in the Ret-CoR complex led us to consider a putative role for Ret-CoR in the cell cycle. Expression of Ret-CoR through the cell cycle was investigated by Western blotting using Y79 cells synchronized by thymidine at G1/S stage and demecolcine at G2/M phase (Figure 3A). We found that like endogenous cyclin E, a marker that accumulated mostly during the G1/S transition, appearance of endogenous Ret-CoR protein was dependent on the specific cell-cycle stage from the G1 phase to the S phase (Figure 3B, left panel) when the cells were synchronized by thymidine at G1/S phase (Figure 3A, upper panel). Likewise, when the Y79 cells were synchronized by demecolcine at G2/M phase, Ret-CoR protein appearance was also cell cycle-dependent (Figure 3B, right panel). From these drug studies, the highest Ret-CoR expression (see lower panel in Figure 3B for protein levels of Ret-CoR versus Sin3A) appeared around at G1-S transition (Figure 3A, right panel). A synchronous Y79 cells also expressed Ret-CoR protein, but at low levels (Figure 3B). In the contrast, expression levels of other endogenous Ret-CoR complex components (e.g. Sin3A) and PNR proteins looked unchanged (Figure 3B). As Ret-CoR mRNA levels remained constant during the cell cycle (data not shown), the cell cycle-dependent appearance of Ret-CoR protein appears to be under post-transcriptional control, such as through protein turnover and/or translation rate. In fact, Ret-CoR protein appeared susceptible to ubiquitination for rapid degradation (Figure 3C). Consistent with the Ret-CoR protein appearance during cell cycle, association of endogenous PNR with endogenous Ret-CoR complex components was also dependent on cell cycle and most evident 4 h after G1/S arrest by thymidine (Figure 3D). These findings raised the possibility that the Ret-CoR co-repressor functions as a cell cycle-dependent repressor as well as supporting PNR transrepressive activity. This idea was further supported by the

Figure 2 Identification of a novel protein complex containing Ret-CoR. **(A)** Schematic diagram of the purification for Ret-CoR-containing complex. **(B)** Mass spectrometric analysis of the purified Ret-CoR complex components. MALDI-TOF/MS analysis of the complex subunits is shown on the middle. Asterisks indicate background proteins. Silver stain of the indicated fractions of glycerol density gradient were shown at middle panel. Right panel displayed the immunoprecipitant of the purified Ret-CoR complex (middle panel) by anti-RbAp46 antibody (Pre-IP experiment). Left panel displayed immunoprecipitant of Mock cells (wild type 293F cells) precipitated by anti-FLAG M2 resin followed by the same procedure as performed with Ret-CoR complex purification. **(C)** Fractions separated by glycerol density gradient were assayed by Western blot using the indicated antibodies. **(D)** Endogenous components of Ret-CoR complex interact with tagged PNR and Ret-CoR in 293F cells. Immunoprecipitants of tagged PNR and Ret-CoR by a FLAG antibody were applied for Western blotting. **(E)** Far Western blotting of Ret-CoR complexes. Labeled probes were used, as indicated at the top of the panel. The detected bands corresponded to the proteins indicated on the right side. Asterisk indicates a background peptide. **(F)** The Ret-CoR complex components p107, TBL3 and CDK9 directly interact with PNR and Ret-CoR. *In vitro*-translated p107, TBL3 and CDK9 proteins were captured by GST-PNR, Ret-CoR N-terminal and Ret-CoR C-terminal proteins in a GST-pull down assay.

findings that co-transrepression of PNR by Ret-CoR was dependent on cell cycle stage. The PNR-mediated transcription activity was fluctuated during cell cycle and was lowered at the G1/S phase (Figure 3E), when Ret-CoR expression was high. However, by knocking down of Ret-CoR by RNAi (Supplementary Figure 1A) and an HDAC inhibitor Trichostatin A (TSA) treatment, such potent transrepressive activity of PNR at G1–S stages was abrogated (Figure 3E). Likewise, knockdown of combination of CDK9/TBL3, but not single component (only the results of several major components are displayed), resulted in clear impairment of the transrepressive PNR function even when Ret-CoR protein level was presumed high at G1–S stages through synchronization by demecolcine (Figure 3E). These findings supported again the idea that Ret-CoR serves as a co-repressor through forming an HDAC complex.

A purified Ret-CoR complex co-represses the transrepression function of PNR *in vitro*

To address the co-regulator function of the Ret-CoR complex on PNR, the purified Ret-CoR complex was applied to an *in vitro* transcription assay with a GAL4-DBD-fused PNR LBD chimeric protein (GAL-PNR) and a DNA template chromatinized using purified HeLa histone octamers (Kitagawa *et al*, 2003; Fujiki *et al*, 2005). A basal transcriptional activity was observed from the reporter promoter upon the binding of GAL4-DBD (GAL) alone. However, in the presence of GAL-PNR LBD, the basal activity was drastically reduced (Figure 4A, lane 2), and the addition of the purified Ret-CoR complex potently co-repressed this PNR repressor function (Figure 4A, lane 4). Reflecting the putative role of HDACs in the Ret-CoR complex in co-repression of PNR transrepression function, TSA potently attenuated the

Figure 3 Function of Ret-CoR containing complex is regulated by cell cycle-dependent expression of Ret-CoR. (A) FACS analysis of synchronized Y79 cells. Y79 cells were arrested at G1/S phase with thymidine or at G2/M phase with demecolcine. DNA contents of the cells and % populations in each synchronized culture were analyzed by FACS analysis at an indicated time after drug release. (B) Expression profiles of PNR and Ret-CoR through the cell cycle. Western blots shows the protein levels indicated in the panel (upper panel). Cyclin E1 was used as a G1/S transition maker. The level in the each step was quantified, normalized in asynchronous cells (lower panel; open circle, Ret-CoR; closed circle, Sin3A). (C) Cell cycle-dependent ubiquitination of endogenous Ret-CoR protein. Endogenous Ret-CoR was ubiquitinated in unsynchronized Y79 cells (U) and synchronized Y79 cells treated with MG132 for 3 h, and samples were taken at the indicated times after drug release. Whole cell extracts were immunoprecipitated using anti-Ret-CoR antibody and subjected to Western blotting with antibodies against indicated proteins. (D) Ret-CoR is required for the cell cycle-dependent interaction between endogenous Ret-CoR complex components and endogenous PNR in Y79 cells. Proteins were extracted from Y79 cells 4 h after releasing from thymidine treatment. (E) Cell cycle-dependent transcriptional repressive function of the Ret-CoR containing complex in Y79 cells. Luciferase assays were performed in asynchronous/synchronized Y79 cells. Relative transcriptional activity means the ratio of the GAL-PNR activity versus activity of GAL alone (GAL-PNR/GAL).

co-repressor function of the purified Ret-CoR complex (Figure 4A, lane 5). Indeed, an HDAC activity was found in the Ret-CoR immunoprecipitant (Figure 4B). When each of the complex components except Ret-CoR was knocked down by RNAi (Supplementary Figure 1A), the Ret-CoR-associated HDAC activity was not altered (Figure 4B). However, knock-down of two components (CDK9 + TBL3) caused a significant reduction in the HDAC activity like the single Ret-CoR RNAi, in accordance with the findings of co-repression of the PNR transrepressive function by the Ret-CoR complex components (Figure 3E). Thus, our findings suggested that the Ret-CoR forms an HDAC complex as a core component to co-repress PNR function.

Ret-CoR co-represses PNR function at the PNR target gene promoter cyclin D1, but not at the TBX2 promoter in Y79 cells

As shown previously in transgenic mice, proliferation in the developing retina requires cyclin D1 (Sicinski *et al*, 1995).

Therefore, we reasoned that the expression of this gene, as well as that of the predicted PNR target gene TBX2 (Qian *et al*, 2005), may be under the control of both PNR and Ret-CoR. To test this hypothesis, we first measured the mRNA expression levels of cyclin D1 and TBX2 in Y79 cells by a semi-quantitative RT-PCR. Although no clear expression of cyclin D1 and TBX2 was detected in Y79 cells untransfected and transfected with a control siRNA, after knockdown of PNR expression by RNAi (Supplementary Figure 1A), significant induction of the endogenous Cyclin D1 and TBX2 genes was observed (Figure 5A, lane 2). Ret-CoR RNAi also effectively induced the Cyclin D1 gene, presumably through release of PNR transrepression, but TBX2 gene expression remained repressed (Figure 5A, lane 3). As it is believed that cyclin D1 gene expresses in proliferating retina neurons, whereas TBX2 expresses in differentiated retina (Sowden *et al*, 2001), Ret-CoR was presumed to function in developing retina.

We then directly tested the co-repressor function of Ret-CoR for PNR on PNR target gene promoters (Figure 5B) using

Figure 4 Characterization of the transcriptional repressive function of Ret-CoR containing complex. (A) Transrepressional ability of PNR and Ret-CoR were measured with the chromatinized histone octamers and the purified Ret-CoR complex (Figure 2B) by *in vitro* transcription assay. Each assay was performed with or without 500 nM Trichostatin A. (B) HDAC activity of a Ret-CoR immunoprecipitants from Y79. Asynchronized whole cell extracts were prepared and immunoprecipitated with either rabbit IgG antibody, anti-Ret-CoR antibody or anti-HDAC1 antibody. Immunoprecipitants were applied on an HDAC assay with or without 10^{-9} M Trichostatin A.

a luciferase reporter and ChIP assays in Y79 cells. The cyclin D1 gene appeared to harbor no putative, or related, PNR-binding sites in its promoter. However, together with a ChIP analysis (Figure 5C and D), functional analysis of promoter deletion mutants (Figure 5E) mapped a putative PNR target region similarly acting in the promoters (L-CycD1-luc and S-CycD1-luc) (see Figure 5B, upper panel). For the TBX2 promoter, recruitment of PNR, but not Ret-CoR, was observed

(Figure 5C), which reflected the results of the repressor function experiments (Supplementary Figure 1C). Although PNR among NRs is closely related to TLX that is recently reported as a critical factor for development of neurons including retina (Zhang *et al*, 2006), TLX recruitment was not detected in the PNR target gene promoters (Figure 5C). Conversely, in a TLX target promoter (PTEN) (Zhang *et al*, 2006), neither PNR nor Ret-CoR was recruited (Figure 5C).

Figure 5 Promoter-specific function of the Ret-CoR containing complex. (A) The expression levels of the indicated genes were measured by RT-PCR with extracted total RNA from Y79, transfected with siPNR-Y79, siRet-CoR-Y79 or siControl. (B) Schematic diagrams of cyclin D1 promoter and TBX2 promoter with a putative PNR-binding element site. (C) ChIP assay was performed to endogenous Cyclin D1 promoter, TBX2 promoter and PTEN promoter with anti-IgG antibody, anti-PNR antibody, anti-Ret-CoR, anti-E2F4 and anti-TLX antibody. (D) ChIP assay was performed to Cyclin D1 promoter PNR responsive region (Cyclin D1-B) with anti-IgG antibody, anti-PNR antibody, anti-Ret-CoR antibody and anti-acetyl-Histone H3 treated with transfected siPNR, siRet-CoR or siControl. (E) Suppressing effects of PNR and Ret-CoR on Cyclin D1 promoter function. The functions of PNR-CyclinD1-luc (–1082 to 53 bp) or ΔCyclinD1 (–586 to 53 bp) luc promoters in a luciferase assay. (F) Co-immunoprecipitation of endogenous E2F4 with endogenous PNR and Ret-CoR. The immunocomplex was precipitated in Y79 cells with anti-PNR or Ret-CoR antibody and Western-blotted with anti-E2F4 antibody. (G) Ret-CoR co-represses transcriptional activity of E2F4 on the 6 × E2F response element-containing reporter. Luciferase assays were performed in Y79 cells transfected with 6 × E2F-luc reporter plasmids (400 ng), E2F4 expression vectors (200 ng), Ret-CoR expression vectors (100 ng) and double-stranded siRet-CoR or siControl (20 μM). (H) Cell cycle-dependent recruitment of Ret-CoR complex components to Cyclin D1 and TBX2 promoters. ChIP analysis was performed and synchronized Y79 cells at M phase and tested at an indicated time after drug release. Efficiency of synchronization of Y79 cells was determined by FACS as shown in Figure 3A.

A PNR mutant that lacked DNA-binding ability (PNR-N65A/G66A) still potently suppressed Cyclin D1 promoter function (Figure 5E). Notably, the repressive effect of PNR was attenuated by a PNR point mutation (R311Q) found in ESCS patients (Gerber *et al*, 2000; Haider *et al*, 2000), and by mutations in the CoRNR motif (V60A/V252A) of Ret-CoR (Figure 5E). No clear DNA binding of PNR was found in the Cyclin D1 gene promoter region by EMSA (data not shown). To explore further the molecular basis of the transrepressive function of PNR/Ret-CoR in the cyclin D1 gene promoter, we examined an idea if PNR/Ret-CoR associates with E2F family members, as the mapped PNR response element compromises DNA-binding sites for E2F family transcriptional regulators. This idea was further supported by the findings that the isolated Ret-CoR complex contains potential binding components for E2Fs (see Figure 2B). By co-immunoprecipitation, association of endogenous PNR and Ret-CoR proteins with endogenous E2F4 was detected (Figure 5F). Co-repression of E2F4 transcriptional activity by Ret-CoR/PNR was

seen in the Cyclin D promoter (L-CycD1-luc) (data not shown) and in the synthetic E2F-binding sites (Figure 5G). Consistent with these findings, recruitment of E2F4 to the promoter Cyclin D, was detectable (Figures 5C, D and H). Moreover, cell cycle-dependent recruitment of PNR and Ret-CoR complex components to regulatory regions in the Cyclin D1 gene promoter was detected by ChIP analysis (Figure 5H). However, for the TBX2 gene promoter, recruitment of PNR, but not Ret-CoR, was observed (Figure 5H). Thus, taken together, our findings suggested that the transrepressive function of PNR in the developing retina requires the co-repressor function of Ret-CoR, presumably as an HDAC complex, with cell cycle-dependent appearance.

Cyclin D1 and TBX2 gene expression is under negative control by PNR in the developing retina

Finally, to address the physiological significance of the observed findings *in vitro*, we measured the expression of PNR and Ret-CoR in the developing retina. Although high PNR and

Figure 6 Expression profiles of PNR, Ret-CoR and their target genes during retinal development. (A) Transcript levels of the mouse retina at embryonic day 16 to postnatal day 4 and adult phase were quantified by real-time PCR. (B) Increased expression of Cyclin D1 and TBX2 as putative PNR/Ret-CoR complex target genes in the retina of wild mice or rd7/rd7 mice quantified by real-time PCR. (C) Cyclin D1 and TBX2 promoter occupancy of PNR and Ret-CoR in the mice retina was showed by *in vivo* ChIP assay. (D) Hyperproliferation of the retinal progenitor cells. The retina of the wild mice and rd7/rd7 mice at postnatal day 5 were stained with hematoxylin and eosin (upper panel). Rosette formation in the rd7/rd7 mice suggests hyperproliferated S-cone cells from retinal progenitor cells. Sections of developing retinas of wild-type and rd7/rd7 mice at P0 were double-immunostained with as 'green' for PCNA, a marker for dividing retinal progenitor cells and as 'red' for S-cone opsin (lower panel). (E) Dissociated retina of the wild mice and rd7/rd7 mice at postnatal day 0- and 12-week old were cultured and incorporated BrdU. Retro virus of control-shRNA (shControl), shPNR or shRet-CoR was infected, respectively.

Ret-CoR expression levels were observed in developing retinas from wild-type mice from stages E18 to P4, only low levels were observed in the mature retina of adult mice (Figure 6A). Further suggestive of the repressor function of PNR, clear upregulation of Cyclin D1 and TBX2 gene expression was observed in the developing retina of mice that lacked functional PNR (rd7/rd7, a spontaneous PNR gene mutant line) (Figure 6B). However, in the retina of adult rd7/rd7 mice, upregulation of the TBX gene, but not the cyclin D1 gene, was observed, which suggested a developmental stage-specific function for PNR through interaction with multiple co-repressor complexes in the retina (see Figure 7). Indeed, by *in vivo* ChIP analysis, a clear Ret-CoR recruitment to the Cyclin D1 gene promoters was detected in the developing retina of P0 mice, rather than in the mature retina of adult mice (Figure 6C). As previously reported in mice and human ESCS patients (Akhmedov *et al*, 2000; Gerber *et al*, 2000; Haider *et al*, 2000), an increased number of S-cone cells with normal M-cone cell number was reported from the retina of rd7/rd7 mice (see upper panel in Figure 6D). In the retina of the wild-type mice at P0, S-cone opsin (stained as red) expression was not detected in the layers of PCNA-positive dividing cells (green), confirming that at P0 stage, no proliferation of the S-cone progenitor cells. However, in the retina of rd7/rd7 mice, S-cone opsin expression was detectable in the layers of PCNA-positive dividing cells (see

arrowheads in lower panels in Figure 6D), suggesting that S-cone progenitor cells are still proliferating at P0. Such PNR function in retina development was then examined in embryonic retina cells (Figure 6E). Knockdown of PNR with a retrovirus (Supplementary Figure 1B) appeared to induce cell proliferation, as seen in hyperproliferative retina cells derived from rd7/rd7 mice at P0, supporting the putative PNR suppressive function for retina cell proliferation. Thus, Ret-CoR co-repressor function, presumably as an HDAC complex, appears to be required for PNR function in retinal progenitor cells, but not in differentiated retinal cells (Figure 7).

Discussion

The role of Ret-CoR function in retina development

PNR is known to serve as a negative regulator for cell proliferation of retina neurons. As established in ESCS patients and rd7/rd7 mice, S-cone cell proliferation appears under a negative control by PNR, whereas differentiation switching of S-cone cells into M-cone cells appears to require the other factors (Haider *et al*, 2001; Milam *et al*, 2002; Yanagi *et al*, 2002). The molecular mechanism of suppression of S-cone cell proliferation by PNR still remains elusive, but PNR-mediated transcriptional repression of genes involving cell-cycle regulation has been already revealed (Yanagi *et al*, 2002). Together with the present findings that the Ret-CoR

Figure 7 Schematic diagrams of PNR and Ret-CoR complex function. In the proliferative stage cells such as retinal progenitor cells, PNR is recruited together with a Ret-CoR containing complex on their target gene promoters at G1/S phase and inhibitory for cell proliferation. After cell differentiation, PNR with the other co-repressor complex(es) may control the target genes at G0 phase.

gene expresses in developing, but not in differentiated retina, Ret-CoR appears physiologically important for the PNR suppressor function for cell cycle regulation in retinal progenitor cells (Figure 7). Through the PNR-mediated transcriptional controls, Ret-CoR may serve as a suppressor for S-cone cell proliferation. Given the facts that Ret-CoR expression was also seen in brain, it is also possible to speculate that Ret-CoR co-represses the function of the other sequence-specific regulators, which determine cell fate of neurons through transcriptional control. In this respect, it is interesting to examine functional interaction of Ret-CoR with TLX and its relevance to physiological events in brain by comparing with the reported co-repressors (Perissi and Rosenfeld, 2005), although in the tested promoters of PNR and TLX target genes, Ret-CoR appeared unlikely to functionally associate with TLX.

Multiple co-repressor complexes facilitate the transrepressive function of PNR?

Transcriptional regulation by sequence-specific regulators upon the target gene promoters is believed to couple with histone modification and chromatin remodeling (McKenna and O'Malley, 2002; Belandia and Parker, 2003; Kitagawa *et al*, 2003; Perissi and Rosenfeld, 2005). Reflecting the biological events on the chromatin, a number of co-regulator complexes for NRs has been recently suggested to support the complicated but sequential cycling events for transcriptional events (Metivier *et al*, 2003). In this regard, it is reasonable to speculate that PNR requires multiple co-repressor complexes for its transrepressive function.

In retinal progenitor cells, we presume that the Ret-CoR HDAC complex co-represses PNR as a major co-repressor complex. However, from the findings that clear recruitment of Ret-CoR to the TBX2 gene promoter was undetectable, irrespective of the PNR-transrepressive function, in the differentiated retina of mice (Figure 6C), PNR appears to associate with the other HDAC complex(es) in differentiated retina cells. In fact, by a ChIP analysis of the TBX2 gene promoter

in Y79 cells, a recruitment of HDAC1 without Ret-CoR was seen (Figure 5F). Together with the observations that Ret-CoR was absent on the TBX2 promoter in mouse retina (Figure 6C), PNR may associate with the other co-repressor complex(es), at least, in differentiated retina cells (Figure 7).

The cell cycle-dependent appearance of Ret-CoR is regulated through protein degradation?

Our findings suggested that the Ret-CoR complex acts as a negative regulatory complex of the cell cycle through inhibition of cell cycle-related factor gene expression via increased Ret-CoR protein levels during the G1/S transition stage. Supporting the cell cycle-dependent appearance of Ret-CoR protein, the Ret-CoR harbors a destruction box R (1142)-R-A-L-G-R-M-V-E (1150) (compared to the consensus R-X-X-L-X-X-X-X-N/D/E) (Glutzer *et al*, 1991) identified in cyclin B as an interacting domain for E3 ligase and for cell cycle-regulated protein degradation (Wei *et al*, 2004). Consistently, Ret-CoR was highly susceptible to ubiquitination (Figure 3C), perhaps appearance of Ret-CoR protein is, at least in part, attributed to regulated protein degradation through the ubiquitin-dependent proteasomal pathway (Vodermaier, 2004; Peters, 2006). As endogenous Ret-CoR protein expression appears to be tightly regulated via the cell cycle, Ret-CoR function in the developing retina may be specific to late G1 stage or the G1/S transition stage, presumably in retinal progenitor cells. It is possible that through the co-regulatory function of such cell cycle-dependent co-repressor complexes, retina cell differentiation can be tightly regulated by PNR, along with other classes of sequence-specific regulators such as TRβ2 (Ng *et al*, 2001) and Crx (Furukawa *et al*, 1997), which together may constitute a photoreceptor-specific transcription factor cascade (Kobayashi *et al*, 1999; Gerber *et al*, 2000; Haider *et al*, 2000; Yanagi *et al*, 2002). In tissues that do not express PNR, such as the brain, cell cycle-dependent Ret-CoR complexes may also serve as co-repressor complexes for other sequence-specific regulators such as TLX. Although it remains unclear whether the functions of other known

co-regulator complexes are also cell cycle-dependent, our findings suggest a model in which the level of expression of a co-regulator complex is regulated in a cell cycle-dependent manner.

Materials and methods

Purification and characterization of the Ret-CoR complex

Nuclear extracts (Yanagisawa *et al*, 2002; Kitagawa *et al*, 2003) from 293F cells transformed with Ret-CoR and Y79 cells with PNR were loaded onto an anti-FLAG M2 affinity resin column, and washed extensively with washing buffer (20 mM Tris-HCl (pH 8.0), 300 mM KCl, 0.2 mM EDTA, 0.05% NP40, 10% glycerol, 0.5 mM PMSF and 1 mM DTT). Bound proteins were eluted from the column by incubation with 133 µg/ml FLAG peptide in washing buffer for 30 min at room temperature. Next, the eluted solution was applied on the Protino column (MACHEREY-NAGEL) for His-tag binding and washed with a buffer His (40 mM HEPES pH 7.4, 300 mM KCl, 0.05% NP40, 10% glycerol). Ret-CoR complex was eluted by 250 mM Imidazole buffer His. For fractionation on glycerol gradients, elutants were layered on top of 13 ml linear 10–40% glycerol gradients in washing buffer and centrifuged for 16 h at 4°C at 40 000 r.p.m. in a SW-40 rotor (Beckman, CA). After getting each fraction, Re-IP was carried out with anti-RbAp46 antibody for fraction 10–12. Protein standards used were ovalbumin (44 kDa), β-globulin (158 kDa) and thyroglobulin (667 kDa). Each samples were applied on NuPAGE Bis-Tris 4–12% gradient gel (Invitrogen). Identification of each component was performed following our past papers (Kitagawa *et al*, 2002, 2003; Yanagisawa *et al*, 2002) using Voyager DE-STR (Perspective Biosystems).

Cell-cycle analysis

Y79 cells were synchronized at G1/S phase (with thymidine) and at G2/M phase (with demecolcine), essentially as described in our previous report (Kitagawa *et al*, 2003). Briefly, for the G1/S arrest, cells were exposed for 24 h with 2.5 mM thymidine in RPMI medium supplemented with 3% serum. Nine hours after the drug release, cells were cultured again for 16 h in the presence of 2.5 mM thymidine. Cells were yielded approximately 70% G1/S population. For the G2/M phase arrest, cells synchronized at G1/S phase by thymidine were released by incubation for 9 h with RPMI medium with 10% serum, and were treated with 0.015 µg/ml demecolcine for 8 h, which yielded approximately 75% G2/M population.

In vitro transcription

In vitro transcription with chromatin template was performed according to the previous report (Kitagawa *et al*, 2003; An and Roeder, 2004) using histone octamers from HeLa cell (Fujiki *et al*, 2005). Template DNA pG₅ML was kindly provided by Dr Robert G Roeder. Recombinant GAL-PNR LBD was expressed by pET system (Novagen), and purified by Protino Ni column under denaturing condition. Then the protein was refolded in a native buffer (20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 10% glycerol, 0.5 mM DTT, 0.5 mM PMSF). Co-repressor activity of the

purified Ret-CoR complex for PNR was attenuated in the presence of 500 nM TSA.

HDAC assay

HDAC assay was performed using HDAC Fluorescent Activity Assay/Drug Discovery Kit (AK-500, BIOMOL) according to the manufacturer's instructions. Briefly, cell extracts prepared from Y79 cells in 24-well plate were immunoprecipitated with antibodies indicated and incubated with the substrate at 30°C for 30 min. After incubation, the reaction was stopped and the fluorescence was analyzed by microplate reading fluorimeter (Perkin Elmer).

Retroviral production and infection

shPNR, shRet-CoR and shControl expressing retroviruses were produced using pSIREN and 293gp-2 cells (Clontech). shPNR (corresponded to nucleotides 757–781), shRet-CoR (corresponding to nucleotides 2384–2408) and shControl (LacZ from *Escherichia Coli*: corresponding to nucleotides 291–311) was inserted in the pSIREN vector. Retina primary culture cells were infected by incubating them with the virus and 5 µg/ml hexadimethrine bromide (Sigma) following the manufacture's protocol (Clontech).

Retinal culture and cell proliferation assay

Neural retinas were dissected from postnatal day 0 mice or 3-month-old mice and cultured as pellets. The culture medium was a 1:1 mixture of Dulbecco's modified Eagle's medium (with Glutamax) and Ham's F12 (Gibco), supplemented with 10% FBS, insulin (10 µg/ml) and transferine (100 µg/ml). The cells were cultured 3 days by replacing half of the medium in the dish with fresh medium and used in cell proliferation assay.

The cell proliferation assay by incorporation of BrdU was performed using BrdU labelling and detection kit III (Roche) according to the instruction manual. All values are mean ± s.d. of at least three independent experiments.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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