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Involvement of the SMRT/NCoR-HDAC3 complex in transcriptional repression by the CNOT2 subunit of the human Ccr4-Not complex

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Running title: SMRT/NCoR complexes are cofactors for the Ccr4-Not complex

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Synopsis

In eukaryotic cells, the Ccr4-Not complex can regulate mRNA metabolism at various levels. Previously, we showed that promoter targeting of the CNOT2 subunit resulted in strong repression of RNA polymerase II transcription, which was sensitive to the histone deacetylase (HDAC) inhibitor, trichostatin A. In this study, the cofactor requirement for CNOT2-mediated repression was investigated. We found that coexpression of SMRT or NCoR in combination with HDAC3 (or HDAC5 and HDAC6) augmented repression by CNOT2. This repressive effect is mediated by the conserved Not-Box, which resides at the C-terminus of CNOT2 proteins. We observed physical interactions of CNOT2 with several subunits of the SMRT/NCoR-HDAC3 complex. Our results show that the SMRT/NCoR-HDAC3 complex is a cofactor of CNOT2-mediated repression and suggest that transcriptional regulation by the Ccr4-Not complex involves regulation of chromatin modification.

Keywords: histone deacetylase, SMRT/NCoR, Ccr4-Not, transcription regulation, chromatin

Abbreviations used: pol II, RNA polymerase II; HDAC, histone deacetylase; SMRT, Silencing Mediator of Retinoid and Thyroid hormone receptors; NCoR, Nuclear hormone receptor CoRepressor; TBL1, Transducin β-like protein 1; TBLR1, TBL-related 1; GPS2, G protein pathway suppressor 2; trichostatin A, TSA.

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Introduction

Transcription of eukaryotic genes by RNA polymerase II (pol II) involves the action of an extensive set of transcription factors and chromatin regulatory proteins. Basal transcription only requires general transcription factors including the TFIID and Mediator complexes. In contrast, regulated transcription involves DNA-binding transcription factors, which can recruit corepressors or coactivators to the promoter. These cofactors can affect transcription by interacting with the basal transcription machinery or with chromatin. Chromatin regulatory proteins can remodel nucleosomal structures in an ATP-dependent manner or they can alter posttranslational modifications of the histones tails, which includes methylation, phosphorylation and acetylation/deacetylation [1, 2].

The evolutionary-conserved Ccr4-Not complex controls gene expression both at the level of transcription initiation and of mRNA degradation [3, 4]. The Not subunits of the complex were first genetically isolated in the yeast *Saccharomyces cerevisiae* as negative regulators inhibiting transcription from the TATA-less promoter of the *HIS3* gene. Several genetic and biochemical interactions have been observed between yeast Not proteins and transcription initiation complexes like TFIID and Mediator [3, 4]. The mammalian Ccr4-Not complex contains the orthologs of yeast Not1-4p proteins (CNOT1 to CNOT4) and also of the CNOT6(hCcr4), CNOT7(hCaf1), CNOT8(hPop2/Calif), CNOT9/RQCD1(hRcd1/hCaf40) and CNOT10(hCaf130) proteins. Besides the role in transcription regulation, the Ccr4-Not complex is involved in mRNA decay. Human CNOT6(hCcr4) and yeast Ccr4 possess a 3'- 5' exonuclease activity which shortens the poly(A) tails of mRNAs [3-5]. Together, this indicates that the Ccr4-Not complex exerts its functions both in the cytoplasm and in the nucleus. Interestingly, a substantial portion of Ccr4-Not proteins is localized in the cytoplasm [3-6] and cell-cycle progression regulates distribution of human CNOT7(hCaf1) between the nucleus and the cytoplasm [7]. In addition, human CNOT4 displays ubiquitin-protein ligase activity *in vitro*, but the importance for gene transcription and/or mRNA decay is not exactly known [8].

Genetic and biochemical studies provided evidence that the Ccr4-Not complex can regulate pol II transcription. Various subunits of the yeast Ccr4-Not complex are required for the expression of *RNR* genes after hydroxyurea treatment or DNA damage [9]. In mammalian cells, Ccr4-Not core subunits are involved in transcriptional activation by nuclear receptors [7, 10, 11]. In contrast, it was found that CNOT9/RQCD1(hRCD1/hCaf40) can interact with the c-Myb transcription factor and repress its transactivation function in human cells [12].

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Previously we reported that promoter targeting of CNOT2 or CNOT9/RQCD1(hRcd1/hCaf40) results in strong repression of pol II transcription [13]. The domain of ~100 residues at the C-terminus of CNOT2, called the Not-Box, is responsible for repression of promoter activity. This Not-Box is present in all orthologs of CNOT2 and CNOT3. CNOT2-mediated repression is sensitive to the histone deacetylase (HDAC) inhibitor, trichostatin A (TSA). In general, recruitment of HDACs to the promoter is associated with repression of transcription. HDACs can catalyze removal of acetyl-groups from lysines at the N-terminal tails of histone proteins. This reduces chromatin accessibility and recruitment of bromodomain-containing coactivators. To date, 18 different HDACs have been described in human cells [14]. They are divided in four classes: class I regroups the yeast Rpd3 orthologs and consists of HDAC1, -2, -3 and -8, class II consists of HDAC4, -5, -6, -9 and -10, and are the orthologs of yeast HDA1, class III is SIRT2 NAD^+-dependent HDAC family and class IV is represented by HDAC11. Biochemical studies demonstrated the existence of different HDAC-containing complexes in mammalian cells. HDAC1 and HDAC2 exist in similar complexes including mSin3A, Mi2/NuRD or CoREST. The Sin3A/HDAC complex was shown to mediate repression by a variety of DNA-binding transcription factors [15], like Mad1, MeCP2 and p53. HDAC3 is part of a complex containing the corepressor proteins SMRT/NCoR (Silencing Mediator of Retinoid and Thyroid hormone receptors/Nuclear hormone receptor CoRepressor), TBL1 (Transducin β-like protein 1), TBLR1 (TBL-related 1) and GPS2 (G protein pathway suppressor 2) [16]. Class II HDACs, like HDAC4, -5 and -7, can be associated to this complex by interacting with the SMRT/NCoR proteins [17]. Unliganded nuclear hormone receptors recruit SMRT/NCoR complexes to repress transcription, but transcription repressors such as Bcl-6, MyoD or ETO also use this complex as corepressors.

In this study, we decided to further investigate the involvement of HDACs in the CNOT2-mediated repression. In transient transfections, we found that coexpression of SMRT (or NCoR) in combination with specific HDACs increased transcriptional repression by CNOT2. The functional link between CNOT2 and the SMRT/NCoR-HDAC3 complex was confirmed in knockdown experiments using small interference RNA (siRNA). In addition, we provide evidence for physical interactions between CNOT2 and several proteins of the SMRT/NCoR-HDAC3 complex. Our results show that the SMRT/NCoR-HDAC3 complex is a cofactor for CNOT2-mediated repression.

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Experimental

Cell lines

The U2OS (human osteosarcoma) and HEK-293T (human embryonic kidney) cell lines were cultured in Dulbecco's minimal Eagle's medium (DMEM, BioWhittaker) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Plasmids

The mammalian expression vectors pCMVDBD, pCGDCNOT2, pCGDCNOT2 1-255, pCGDCNOT2 256-540, pCGDCNOT2 1-334, pCGDCNOT2 334-540, pCGDCNOT2 437-540 and pCGDyNot2, which expressed Gal4DBD fusion proteins under control of the CMV promoter have been described previously [13].

The expression vectors pCMX-NCoR and pCMX-SMRT were kindly provided by Dr. J. Wong (Houston). They encode C-terminal flag-tagged mouse NCoR and SMRT, respectively, under the control of CMV promoter. The expression vectors pCMV-flag-HDAC1-7 were obtained from Dr. E. Verdin (University of California) and have been described previously [18, 19]. The expression vector pEBB-Flag-mSin3A (obtained from Dr. H.G. Stunnenberg) encodes for the murine Sin3A and is under the control of the human EF-1α promoter.

The LexA CNOT2 437-540 plasmid was constructed by insertion of a *Bgl*II/*Xho*I PCR fragment encoding residues 437-540 from human CNOT2 into the *BamH*I/*Xho*I digested pEG202NLS. B42-NCoR RD1, B42-NCoR RD2, B42-NCoR RD3, B42-NCoR RD4 and B42-NCoR RID were constructed by insertion of restriction digested fragments obtained from pGAD10-NCoR 1/2 (5-373), pGAD10-NCoR 9/10 (743-1064), pGAD10-NCoR 11/12 (1056-1487), pGAD10-NCoR 13/14 (1495-1965) and pGAD10-NCoR 15/16 (2002-2440) [20] respectively into pJG4-5. B42-NCoR SANT (374-757), B42-SMRT RD1 (1-365), B42-SMRT SANT (366-750), B42-SMRT RD2 (751-1089), B42-SMRT RD3 (1090-1523), B42-SMRT RD4 (1528-2005) and B42-SMRT RID (2038-2473) were constructed by insertion into pJG4-5 of the respective PCR fragments obtained using the pCMX-NCoR or pCMX-SMRT as templates. B42-HDAC3 was obtained by insertion of the PCR fragment corresponding to human HDAC3 into pJG4-5. A GPS2 clone was obtained from the IMAGE Consortium (clone DKFZp434A0312Q2) and was used as template for PCR amplification using appropriate primers. The obtained PCR fragment was then inserted into pJG4-5. All

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plasmid constructs were verified by DNA sequencing across the cloning junctions. Further details on primer sequences and cloning strategy are available upon request.

Luciferase assay

U2OS or HEK-293T cells were plated on 3 cm dishes and transfected in duplicate by the calcium phosphate method. To correct for transfection efficiency, 50 ng (U2OS) or 5 ng (HEK-293T) pCMV-*Renilla*-luciferase was cotransfected. Forty hours after transfection, cell lysates were prepared, and luciferase activity was determined using the Dual-Luciferase Reporter Assay system (Promega) and a Lumat LB9507 luminometer (Berthold) according to the manufacturer's instructions. Luciferase values were corrected for transfection efficiency by determining the firefly luciferase:*Renilla* luciferase ratio. The fold repression by Gal4-DBD fusion proteins was calculated by normalization of the firefly luciferase:*Renilla* luciferase ratios to the ratio obtained by transfection containing only reporter plasmids and the empty pcDNA3.1 vector.

Immunoprecipitation assays

HEK-293T cells were plated on 9 cm dishes and transfected with 10 μg of DNA using the PEI method [22]. Forty hours after transfection, cells were lysed in 1 ml lysis buffer (50 mM Hepes KOH pH 7.9, 100 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.1% NP-40, 5% glycerol, 1 mM PMSF and proteases inhibitors). Lysates were cleared by centrifugation at 14 krpm at 4ºC. Flag-tagged proteins and the associated proteins were immunoprecipitated overnight at 4ºC using 10 μl anti-flag M2 agarose (Sigma). Beads were washed three times with lysis buffer. Proteins associated with the beads were released by adding SDS sample buffer without β-mercaptoethanol and by elution at 65ºC for 10 minutes. Before loading on SDS-PAGE, β-mercaptoethanol was added and the samples boiled. Proteins were detected by immunoblotting using anti-Gal4DBD-RK5C1 (sc-510, Santa Cruz Biotechnology) and by using enhanced chemiluminescence (ECL, Amersham Biosciences).

Yeast two-hybrid interaction assay

Growth and manipulation of yeast strain EGY48 was carried out as described [23]. For quantitative determination of β-galactosidase activities, yeast transformants were disrupted with zirconia/silica beads using a mini beadbeater (Biospec Products, 1 min maximum speed at room temperature) in buffer containing 100 mM Tris-HCl pH 8.0, 20% (v/v) glycerol, 1

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mM β-mercaptoethanol, 0.5% SDS, and protease inhibitors. After removal of insoluble material by centrifugation, β-galactosidase activities were determined using the Galacto-Light Plus chemiluminescent reporter assay (Tropix) essentially according to the manufacturer's instructions and normalized to total protein content as determined by a Bradford protein assay (Biorad).

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Results

Effects of SMRT, NCoR and HDAC overexpression on CNOT2-mediated repression

In a previous study, we investigated the transcriptional effects of Ccr4-Not subunits when targeted to a promoter in transient transfection/reporter assays [13]. We found that CNOT2 is able to repress promoter activity and that this repression is sensitive to trichostatin A (TSA) suggesting a mechanism involving histone deacetylases (HDACs) [13]. To study this in more detail, we tested the effect of coexpression of different HDACs, alone or in combination with their respective partner on CNOT2-mediated repression. For this we employed plasmids expressing CNOT2 fused to the DNA-binding domain (DBD) of yeast transcription factor Gal4 and expression plasmids for flag-tagged versions of different HDACs (HDAC1 to -6), SMRT, NCoR or Sin3A. The plasmids were transiently transfected into human embryonic kidney cells (HEK-293T) together with a firefly luciferase reporter plasmid (7xTKLuc), carrying seven binding sites for Gal4 upstream of the minimal HSV thymidine kinase promoter, and a CMV promoter-controlled *Renilla* luciferase reporter for normalization of transfection efficiencies. A plasmid expressing only the DBD of Gal4 was included as a negative control in the experiments.

As expected the Gal4-CNOT2 fusion plasmid was able to repress promoter activity (~6-fold, Figure 1A). Cotransfection of Sin3A, HDAC1 or HDAC2 did not increase repression but rather resulted in relief of repression (Figure 1A). Whereas coexpression of NCoR or HDAC3 had little effect on CNOT2 repression, we observed an increase of repression upon SMRT expression, (from 6- to 14-fold). Repression of promoter activity was further augmented when CNOT2 was coexpressed with SMRT in combination with HDAC3. This resulted in a 25-fold repression, suggesting a synergetic effect of the two proteins. Coexpression of NCoR and HDAC3 gave a similar effect. In contrast, coexpression of Sin3A and its cognate catalytic subunits (HDAC1 and HDAC2) did not augment CNOT2-mediated repression.

Our next step was to study the effect of class II HDACs. Overexpression of HDAC4, HDAC5 or HDAC6 did not augment CNOT2-mediated repression (Figure 1B). Overexpression of HDAC7 resulted in a strong non-specific repression and was excluded from further analysis (data not shown). HDAC4 and HDAC5 have been shown to interact with the SMRT/NCoR-HDAC3 complex [17]. Co-expression of HDAC5 but not of HDAC4 with SMRT or NCoR increased CNOT2-mediated repression (Figure 1B). Surprisingly, coexpression of HDAC6 and SMRT or NCoR also resulted in a strong specific effect. HDAC6 was shown to localize

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in the cytoplasm and to deacetylate tubulin [24-26]. However, HDAC6 has been shown to interact with p300 and to repress transcription via its CRD1 motif [27, 28] suggesting that HDAC6 could have a nuclear function. Immunoblot of transfected cell lysates confirmed that the HDAC, SMRT and NCoR proteins were expressed and of expected sizes (data not shown).

In conclusion, overexpression of the SMRT and NCoR corepressor proteins with HDAC3, HDAC5 or HDAC6 augments CNOT2-mediated repression in HEK-293T cells. Similar observations were made when the human osteosarcoma U2OS cell line was used for transient transfection (data not shown).

Repression enhancement by SMRT or NCoR overexpression requires the Not-Box of CNOT2

Previous analyses indicated that the Not-Box of CNOT2 constitutes the minimal repression domain [13]. To test whether the observed effects of SMRT or NCoR on CNOT2 repression require this Not-Box, we cotransfected U2OS cells with plasmids for different Gal4-CNOT2 fusion proteins and the firefly luciferase reporter plasmid 7xTKLuc. We used in this experiments three different constructs of Gal4-CNOT2: an N-terminal construct, CNOT2(1-334), which was not able to repress promoter activity; the complementary construct containing the Not-Box, CNOT2(334-540) and the isolated Not-Box, CNOT2(437-540) [13]. These fusion proteins are expressed to similar levels [13]. We also included in this experiment Gal4 fusions with yeast Not2. As expected, transfection of all constructs except the CNOT2(1-334) construct resulted in repression of promoter activity (Figure 2). Coexpression of SMRT clearly increased repression for all constructs, except for CNOT2(1-334). The effect of SMRT overexpression is particularly strong for the C-terminal constructs of CNOT2. Again, coexpression of NCoR had little effect on the repression activity of full length CNOT2, but repression by isolated Not-box of CNOT2 was significantly enhanced by NCoR. Interestingly, the coexpression of SMRT or NCoR also augmented repression mediated by yeast Not2.

In conclusion, the enhanced repression observed upon SMRT or NCoR overexpression is dependent on the presence of the Not-Box. It is important to note that this correlates well with previous results, which showed a stronger relief of repression by TSA with the Not-Box of CNOT2 than with the full length CNOT2 [13].

CNOT2 physically interacts with HDAC3 in mammalian cells

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As a final step, potential physical interactions between CNOT2 and the SMRT/NCoR-HDAC3 complex subunits were tested in two different experimental setups. To test the interaction in human cells, we transfected HEK-293T cells with plasmids overexpressing flag-tagged HDAC3 and different Gal4-CNOT2 fusion proteins. Flag-HDAC3 was immunoprecipitated from transfected lysates and the precipitates were analyzed by immunoblotting using Gal4 antibodies. We observed strong coprecipitation of Gal4 fused to full length CNOT2 (Figure 3, lane 2) and not of the Gal4 only control. In addition, CNOT2 constructs containing the Not-Box also interacted with HDAC3. Analysis of the CNOT2(1-334) mutant was precluded by its co-migration of with the IgH-chains of the Flag antibody. Taken together, the co-immunoprecipitation analysis indicated that CNOT2 can interact, directly or indirectly with HDAC3.

CNOT2 interacts with multiple subunits of the SMRT/NCoR-HDAC3 complex in the yeast two-hybrid assay

Next, we employed the yeast two-hybrid assay to investigate interaction of CNOT2 with subunits of the SMRT/NCoR-HDAC3 complex. In yeast, full length CNOT2 acts as an activator when fused to the DBD of LexA [23]. Therefore, we used a LexA fusion of the isolated Not-Box of CNOT2 as bait. Fragments of NCoR [20], and the corresponding fragments of SMRT (Figure 4A) were fused to the B42 activation domain. All B42-fusion proteins were expressed to similar levels with the exception of the NCoR SANT fragment, which was excluded from further analysis (data not shown). We observed interactions of the Not-Box with the two C-terminal fragments of NCoR and SMRT corresponding to the nuclear receptor interaction domain (RID) and repression domain 4 (RD4) (Figure 4B). Comparison of beta-galactosidase activities indicated a stronger interaction with the SMRT fragments (Figure 4C). The interaction with other subunits of the SMRT/NCoR-HDAC3 complex was also tested. This showed that the Not-Box of CNOT2 interacts strongly with GPS2 but surprisingly, not with HDAC3 (Figure 4D). B42 fusions with TBL1 or TBLR1 were expressed to very low levels precluding the analysis of these subunits (data not shown).
Taken together, the coprecipitation and the yeast two-hybrid analysis indicate that the Not-Box of CNOT2 can interact with several subunits of the SMRT/NCoR-HDAC3 complex.

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Discussion

CNOT2-mediated repression has been shown to be sensitive to the histone deacetylase inhibitor trichostatin A (TSA), suggesting involvement of histone deacetylases [13]. Here, we present data indicating that the SMRT/NCoR-HDAC3 complex acts as a cofactor for repression by CNOT2. We observed that overexpression of SMRT or NCoR in combination with HDAC3 cooperatively enhanced CNOT2-repression (Figure 1). CNOT2 interacts with HDAC3 in mammalian cells (Figure 3), and by using the yeast two-hybrid assay we found interactions of the Not-Box of CNOT2 with GPS2 and with two regions of the NCoR and SMRT corepressors including the nuclear receptor interacting domain (RID) (Figure 4).

Together, our results indicate that the SMRT/NCoR-HDAC3 complex can act as a cofactor for transcriptional repression by the CNOT2 subunit of the human Ccr4-Not complex and that direct protein-protein interactions are responsible for this.

The Ccr4-Not complex regulates gene expression at multiple levels

Genetic and biochemical studies demonstrated links between the Ccr4-Not complex and transcriptional repression in yeast. To circumvent the lack of cognate Ccr4-Not responsive promoters in mammalian cells, we used an artificial targeting approach to study the mechanism of CNOT2-mediated repression of transcription to identify the SMRT/NCoR-HDAC3 complex as a corepressor. Several observations support the validity of this approach. Mutants of the *Drosophila* ortholog of CNOT2, *Regena*, display a position-variegation effect phenotype, which is linked to chromatin regulation [29]. Secondly, the repression function of CNOT2 resides in the Not-Box representing a highly conserved part of the protein. While full length yeast Not2 inhibits transcription in mammalian cells, promoter targeting in yeast cells results in SAGA-dependent transcriptional activation [30]. The SAGA complex harbors the Gcn5 subunit, which can acetylate histone H3 tails [31]. Strikingly, yeast cells lack orthologous SMRT/NCoR-HDAC3 complexes, which can remove the acetyl groups from H3 tails [32].

Besides transcription initiation, the Ccr4-Not complex is also involved in mRNA degradation via the Ccr4p and Caf1 subunits, which were identified as the major cytoplasmic deadenylases in yeast [3, 4]. Not2p, Not4p and Not5p were recently involved in the stimulation of mRNA decapping resulting in enhanced decay of specific transcripts [33]. It is tempting to speculate that the transcription and mRNA degradation functions of the Ccr4-Not

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complex could be linked. Interestingly, CNOT4 harbors a highly conserved RNA-recognition motif [23], which could act to bridge these separate functions.

The SMRT/NCoR-HDAC3 complex acts as a cofactor for CNOT2-mediated transcription repression

The NCoR and SMRT corepressor proteins were first identified as cofactors for transcriptional repression by the unliganded receptors for thyroid hormone and retinoid acid hormones [16, 34]. Subsequent analysis indicated that the homologous NCoR and SMRT corepressors reside in similar protein complexes harboring histone deacetylases, in particular HDAC3. Within these large corepressor proteins different domains carrying distinct functions have been recognized [16, 34]. Several reports showed that the corepressor function of SMRT/NCoR proteins is not restricted to the nuclear receptors. The RID represents a domain responsible for interaction with unliganded nuclear receptors, but it can also interact with a variety of transcription factors like MyoD and Bcl-6 [35, 36]. In addition, CNOT2 interacts with RD4 region (residues 1495-1965 or 1528-2005 of NCoR or SMRT, respectively), which carry an autonomous repression function [21]. Interestingly, the RD4 region of NCoR also harbors one of the interaction sites for the TBL1 and TBLR1 subunits, which can interact directly with core nucleosomes [21]. The two-hybrid interaction of CNOT2 with SMRT was stronger than with NCoR fragments (Figure 5). This agrees with observations that SMRT overexpression enhances CNOT2-repression, whereas NCoR overexpression had a more limited effect (Figures 1 and 2). We also found an interaction with GPS2 in yeast two-hybrid assays. GPS2, first known as a protein involved in intracellular signaling pathway, was shown to be part of the SMRT/NCoR-HDAC3 complex [37]. Remarkably, HDAC3 did not interact in the two-hybrid assay. This could indicate that the CNOT2/HDAC3 interaction observed in mammalian cells is indirect.

In this study we focused on the HDAC3 and GPS2 proteins as subunits of SMRT/NCoR complexes. However, it has been reported that NCoR can also be part of different complexes including Sin3A, BRG1 and KAP1 complexes [34, 38-40]. We observed that CNOT2-mediated repression was slightly relieved by overexpression of Sin3A, HDAC1 or HDAC2 (Figure 1). Possibly, overexpression of Sin3A complex subunits sequesters the SMRT/NCoR proteins. This would limit the amount of endogenous SMRT/NCoR-HDAC3 complex available for CNOT2, thus relieving transcriptional repression.

It is important to note that neither NCoR, SMRT or HDAC3 proteins have been found in biochemical purifications of the Ccr4-Not complex as stable interaction partners [41](Albert

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and Timmers, unpublished results). Conversely, the Ccr4-Not proteins have not been detected in preparations of NCoR-HDAC3 or SMRT-HDAC3 complexes [20, 21, 34, 37, 40]. This suggests that the interaction between Ccr4-Not and SMRT/NCoR-containing complexes is transient. Together, this leads us to a model in which promoter association of the Ccr4-Not complex recruits SMRT/NCoR histone deacetylase complexes via its CNOT2 subunit to selected promoter regions resulting in local deacetylation and restriction of access of chromatin remodeling activities and transcription factors including pol II.

Involvement of other HDACs in CNOT2-mediated repression?

Overexpression of HDAC5 and HDAC6 also enhanced CNOT2-mediated repression (Figure 1). These proteins have been classified as class IIa and IIb HDACs, respectively [14]. In contrast to class I HDAC3, the HDAC4 and HDAC5 proteins reside in both nuclear and cytoplasmic compartments and are expressed in a tissue-specific manner. Both HDAC4 and HDAC5 are catalytically inactive by themselves, but they require association into SMRT/NCoR-HDAC3 complexes for activity [17]. Possibly, the HDAC5 protein behaves similarly in this assay explaining the enhancement of CNOT2-mediated repression by HDAC5, but it remains surprising that HDAC4 does not display this effect (Figure 1).

The HDAC6 protein was believed to be a dedicated cytoplasmic HDAC, involved in regulating microtubule-dependent cell motility by deacetylating tubulin [24-26], and in linking protein acetylation and ubiquitination signaling pathways [42, 43]. However, many studies show that HDAC6 can function also in the nucleus. For example, HDAC6 interacts with the transcription factor Runx2 to repress the $p21^{CIP1/WAF1}$ promoter [44]. Also, HDAC6 can be recruited by the repression domain of p300, named Cell Cycle Regulatory domain 1 (CRD1), when this domain is SUMO-modified [27]. HDAC6 could shuttle from the cytoplasm to the nucleus as was shown for other class II HDACs. An alternative explanation is that HDAC6 interacts with the cytoplasmic form of the Ccr4-Not complex and shuttles to the nucleus associated with this complex. However, further studies are needed to decipher the relationships of the HDAC5 and HDAC6 proteins with the SMRT/NCoR-HDAC3 and the Ccr4-Not complexes.

Targets for regulation of gene expression by the Ccr4-Not complex

Our observations support the view that the evolutionary-conserved Ccr4-Not complex can regulate gene expression at the promoter level [3, 4], but the identity of natural promoters

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targeted by the Ccr4-Not complex *in vivo*, has remained unknown. Several subunits of the human complex were shown to interact with DNA-sequence specific transcription factors. Murine CNOT9/RQCD1(Rcd-1/Caf40) interacts with c-Myb transcription factor and can repress c-Myb and AP1-dependent transcription activation [12]. CNOT9/RQCD1(Rcd-1/Caf40) can also interact with several retinoic acid receptors and the ATF-2 transcription factor, and is involved in activating transcription from c-Jun promoter [10]. The CNOT7(mCaf1) subunit can interact with retinoic X receptor β (RXRβ) and ligand-dependent RXRβ-mediated transcription is reduced in CNOT7-/- cells [11]. CNOT7(hCaf1) can also interact with estrogen receptor α (ERα). Depending on the promoter this results in activation or repression by ERα activity [45]. Taken together, this indicates that the Ccr4-Not complex could be recruited by several transcription factors. Detailed studies are required to determine whether CNOT2 and the Ccr4-Not complex are important cofactors for these transcription factors. Genomic approaches, like mRNA expression profiling combined with ChIP-on-Chip approaches, will help to identify target genes for the Ccr4-Not regulatory complex. Identification of *bona fide* promoter targets for the Ccr4-Not complex will allow rigorous testing of functional links between the CNOT2 protein and the SMRT/NCoR-HDAC3 complex, which has been uncovered by this study.

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Figures Legends

Figure 1: Overexpression of SMRT, NCoR and HDAC3 (or HDAC5 and HDAC6) augments transcriptional repression activities of CNOT2

(A) Effect of overexpression of class I HDACs, SMRT, NCoR and Sin3A on the repression activities of CNOT2. HEK-293T cells were transiently cotransfected with 2 µg p7xGalTKLuc reporter plasmid, 1 µg pCMV expression plasmid for Gal4DBD (white bars) or Gal4-CNOT2 fusion protein (black bars) and 3 µg pCMV expression plasmid for corepressor proteins. For a purpose of clarity, for each cofactor combination, the obtained value for Gal4-CNOT2 repression was divided by the value obtained with the Gal4DBD protein. As a consequence, on the graph, the fold repression of Gal4DBD is 1 for each cofactor. The effect of the repressors on the activities of Gal4DBD alone is less than two-fold, except for SMRT/HDAC3, HDAC4 combinations, Sin3A, HDAC1 and Sin3A/HDAC1. The experiments shown are representative of at least three independent assays, performed in duplicate. **(B)** Effect of overexpression of class II HDACs, SMRT and NCoR on CNOT2-mediated repression. The assays were performed and the results are displayed as in panel A.

Figure 2: Enhancement of repression by SMRT and NCoR requires the Not-Box

U2OS cells were transiently transfected as described in the legend of Figure 1. The effect of SMRT and NCoR on Gal4DBD alone was 0.97- and 1.2- fold respectively. The assays were done in duplicate. The experiment shown is representative of at least three independent assays.

Figure 3: The Not-Box of CNOT2 interacts with HDAC3 *in vivo*

HEK-293T cells were transiently transfected with plasmids expressing flag-tagged HDAC3 and different Gal4 fusion proteins: Gal4DBD (lane 1), Gal4-CNOT2 (lane 2), Gal4-CNOT2(334-540) (lane 3), Gal4-CNOT2(437-540) (lane 4), Gal4-CNOT2(256-540) (lane 5) or Gal4-CNOT2(1-255) (lane 6). Flag-tagged HDAC3 was precipitated using anti-flag beads in the presence of 100 mM KCl and the interacting proteins were analyzed by SDS-PAGE and immunoblotting using the RK5C1 antibody. Asterisks indicate positions of IgG proteins (heavy and light chains) and the arrow the position of Gal4DBD protein. Positions of co-migrating marker proteins are indicated by their molecular weight in kDa to the left of the figure.

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Figure 4: The Not-Box of CNOT2 interacts with different subunits of the SMRT/NCoR-HDAC3 complex in a yeast two-hybrid assay

(A) Schematic drawing showing the known functional domains of SMRT and NCoR proteins. **(B)** The Not-Box of CNOT2 interacts with the repression domain 4 (RD4) and with the nuclear receptor interacting domain (RID) of SMRT and NCoR. EGY48 cells were transformed with LexA and B42 expression plasmids together with a reporter plasmid. Galactosidase activity was detected by X-gal staining of yeast cells. Indicated to the left are the LexA fusion proteins, above the panel the B42 fusion proteins. Full-length CNOT2 could not be tested as the LexA fusion protein behaves as a strong transcriptional activator in this assay. **(C)** Quantification of β-galactosidase activity. Lysates of yeast cells were prepared as described in Materials and Methods. The indicated fold induction is relative to the activity of yeast cells expressing LexA and B42 control. (**D**) The Not-Box of CNOT2 interacts with GPS2 but not HDAC3 in the yeast two-hybrid assay. The assay was performed and the interactions were quantified as in panels B and C.

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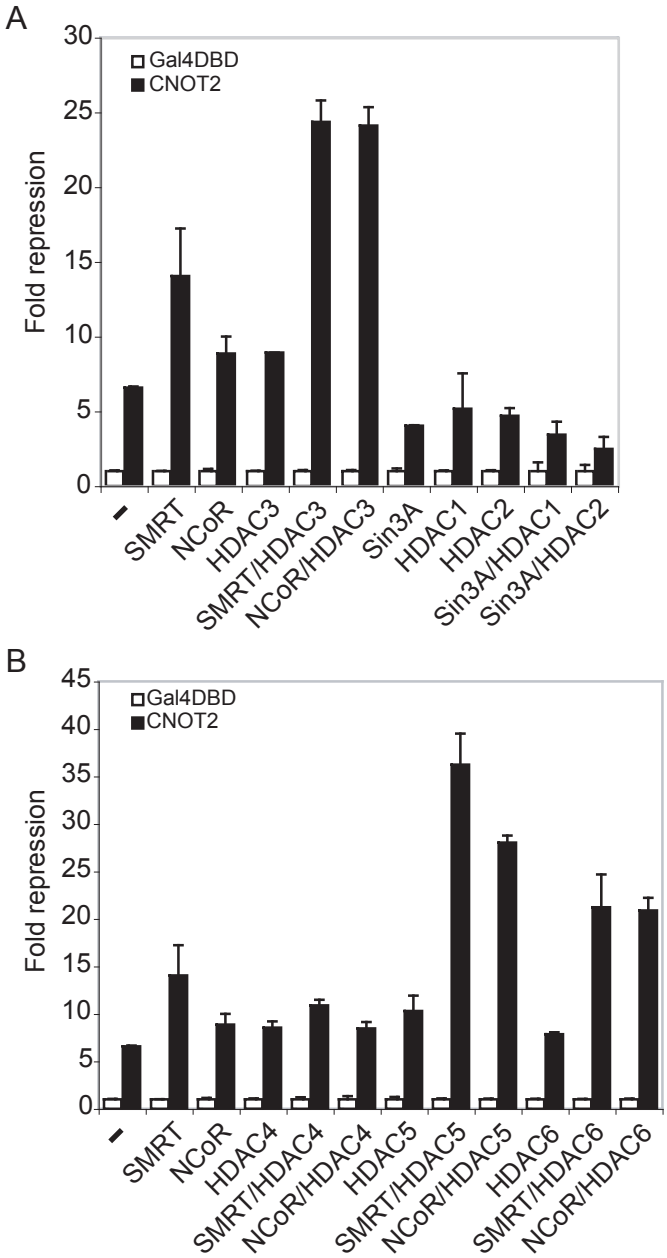

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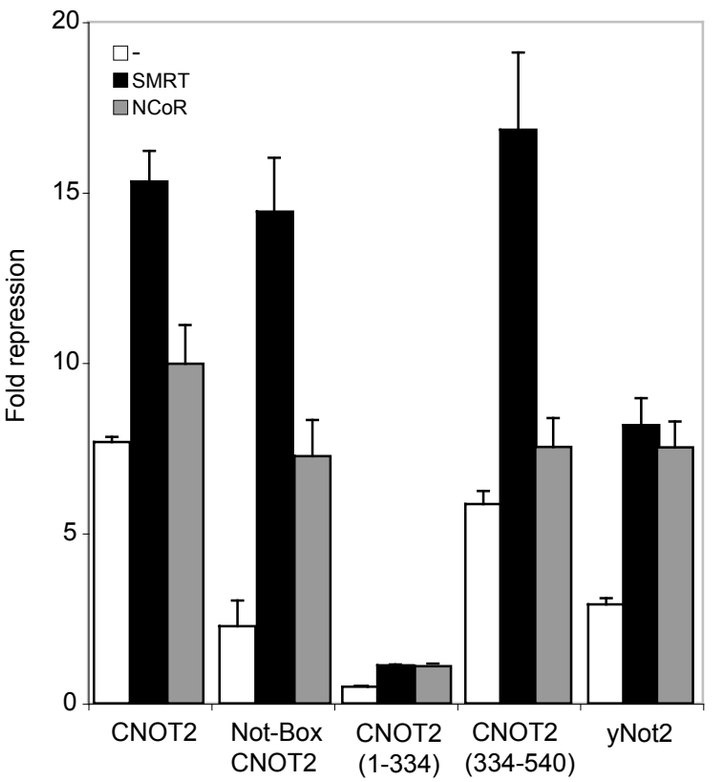

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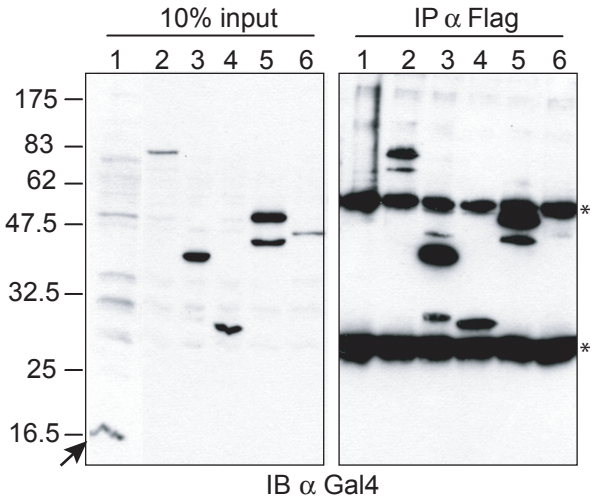

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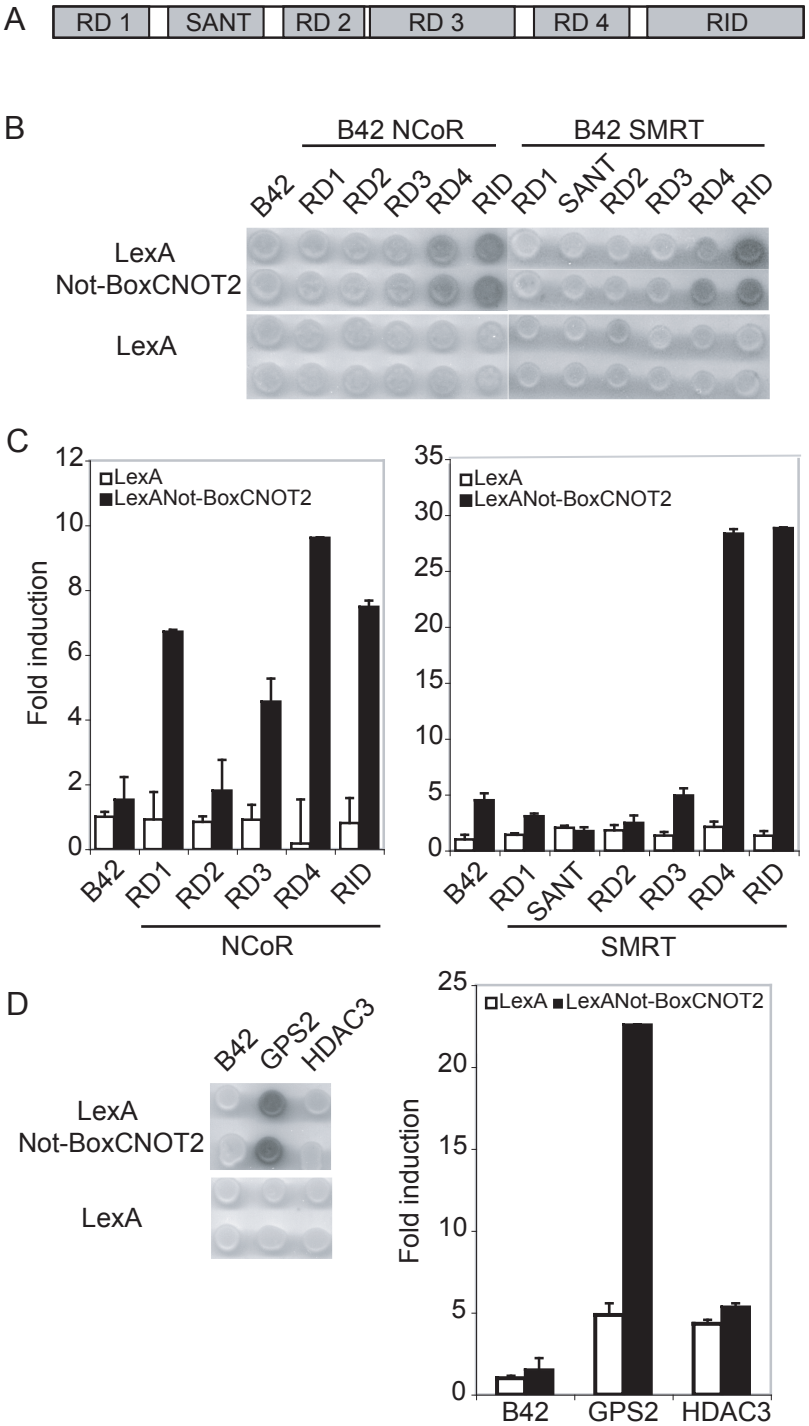

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