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# Funktionelle Charakterisierung der C-terminalen-Domänen des Korepressors N-CoR

Dissertation

Zur Erlangung des Doktorgrades der Naturwissenschaften

Vorgelegt beim

Fachbereich Biochemie, Chemie und Pharmazie (14)

der Johann Wolfgang Goethe Universität Frankfurt am Main

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**Frankfurt am Main August 2006**

Vom Fachbereich 14 (Biochemie, Chemie und Pharmazie) der Johann Wolfgang Goethe Universität als Dissertation angenommen

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À Nicolas,

un Homme généreux et responsable,

Pilier de notre Famille

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# Zusammenfassung

In vielzelligen Organismen bewirkt die selektive Expression bestimmter Gene die Entstehung verschiedener Zelltypen, die in vielen verschiedenen Organen unterschiedliche Funktionen wahrnehmen. Die temporäre und zellspezifische Kontrolle der Genexpression ist einer der fundamentalsten Prozesse der Biologie. Die Frage der Regulierung, der Zusammensetzung aber auch der Kommunikation der verschiedenen Komplexe, die für die Genexpression erforderlich sind, ist elementar für das Verständnis der molekularen Mechanismen, die der Entstehung vieler Erkrankungen, wie zum Beispiel Krebs zugrunde liegen. Die DNA im Kern eukaryotischer Zellen ist mit Histonen und Nicht-Histon-Proteinen assoziiert. Dies ermöglicht die Verpackung und die Kondensierung der DNA aber auch die Regulierung der Genexpression. Die Chromatinstruktur stellt neben der Rekrutierung von DNA-bindenden Faktoren einen zusätzlichen komplexen Prozess der nukleären Regulierung in Bezug auf die Gentranskription, DNA Replikation, die Rekombination und die DNA Reparatur dar. Für die Transkription ist diese Struktur eine Barriere, da sie die Bindung von Transkriptionsfaktoren verhindert.

Nukleäre Hormonrezeptoren gehören zu einer Gruppe von Transkriptionsfaktoren, die die Fähigkeit besitzen, ihre Bindungsstellen in dieser kompakten Chromatinstruktur zu finden und zu binden. Die Aktivität solcher sequenzspezifischer Transkriptionsfaktoren, welche entweder reprimierend oder aktivierend auf die Expression wirken, wird von Kofaktoren beeinflusst, mit denen sie im Komplex vorliegen. Diese Kofaktoren verändern die Chromatinstruktur lokal und steuern so die Genexpression. Zusätzlich zu den erforderlichen Änderungen der Chromatinstruktur durch Histon-ATP-abhängige und unabhängige Modifizierungen sind die Zusammensetzung der Kofaktorkomplexe und die Regulierung ihrer Aktivitäten von Bedeutung für die Transkriptionsregulation. Die Transkriptionsaktivität nukleärer Rezeptoren ist nicht nur durch hormonale Signale reguliert. Sie wird sowohl von der Vielfalt der anwesenden Koregulatoren als auch von deren Aktivitätszustand beeinflusst. Die Integration der Vielfalt der anwesenden Koregulatoren und deren Aktivierungszustand an einem bestimmten Promoter führt zu einer zellkontext- und zelltypspezifischen Genexpression. Eine unausgewogene Rekrutierung von Koregulatoren (Koaktivatoren bzw. Korepressoren) an spezifischen Promotorregionen führt zu der Dysregulation der Expression bestimmter Gene und ist für die Entstehung vieler Krankheiten verantwortlich. Die fehlerhafte Rekrutierung von Korepressoren wie N-CoR ist die Ursache der genetischen RTH (resistance to thyroid hormone) Krankheit und verschiedener Leukämien. Darüber hinaus ist die N-CoR vermittelte Repression für die Entwicklung von Säugern entscheidend. N-CoR Knockoutmäuse sterben in der mittleren Phase der Embryonalenentwicklung und zeigen Defekte in der Reifung von Erythrozyten, Thymozyten und in der Entwicklung verschiedener neuronaler Strukturen. Dies ist zum Teil auf die Störung der Repression zurückzuführen, die

von nuklearen Hormonrezeptoren wie Retinsäure- und Thyroidhormonrezeptoren vermitteltet wird. Diese nukleären Rezeptoren können in Abwesenheit ihrer Liganden spezifische DNA Sequenzen binden und durch die Rekrutierung des N-CoR Korepressorkomplexes Repression vermitteln.

N-CoR ist in der Zelle mit Histondeacetylasen (HDACs) komplexiert. Diese Enzyme bewirken im Zusammenspiel mit den Histonacetyltransferasen durch Deacetylierung beziehungsweise Acetylierung von Histonen eine dynamische Modifikation des Chromatins und beeinflussen so die Transkription von Genen. Obwohl N-CoR ein seit längerer Zeit bekanntes Protein ist, enthält es noch uncharakterisierte Domänen wie die äußerste carboxyterminale Region, welche in dem homologen Protein SMRT konserviert ist. Diese Domäne spielt vermutlich in der Transkriptionsregulation eine Rolle und wurde als Köder in einem Hefe-Zwei-Hybrid Screen zur Identifizierung von Interaktionspartnern des N-CoR C-Terminus eingesetzt. Verschiedene potentiell interagierende Proteine wurden isoliert. Die Proteine, für die eine direkte Interaktion *in vitro* nachgewiesen worden ist, wurden in der vorliegenden Arbeit weiter untersucht. Da nur das Protein NonO/p54nrb (non POU domain containing octamer binding protein) in Koimmunopräzipitationen (Fig. 11) und Kolokalisationsstudien (Fig. 13) auch *in vivo* Interaktion zeigte, wurde die biologische Bedeutung der Interaktion dieses Proteins mit N-CoR erforscht. NonO ist ursprünglich als Splicing-Faktor charakterisiert worden, da es RNA Erkennungsmotive besitzt und in Splicing-Komplexen nachgewiesen wurde. Seitdem wurde beschrieben, dass NonO in verschiedenen Komplexen eine Rolle spielt und es wird jetzt als multifunktionales Protein bezeichnet. Die Wechselwirkungen zwischen NonO und Splicing-Faktoren, RNA Polymerase II und nukleären Rezeptoren, aber nicht Korepressoren, waren bei Beginn des Projektes schon beschrieben, allerdings blieb die genaue Funktion dieser Interaktion ungeklärt. Für das NonO homologe Protein PSF (polypyrimidine tract-binding protein-associated splicing factor) aber, welches mit NonO interagieren kann, wurde gezeigt, dass es rezeptorabhängig und ligandunabhängig Transkription reprimieren kann. NonO und PSF zeigen besonders in den RNA Erkennungsmotiven (RRM) hohe Homologie in der Aminosäureabfolge, PSF unterscheidet sich jedoch von NonO in den umliegenden Domänen und vornehmlich in seinem erweiterten Amino-Terminus. Folglich ist es nicht auszuschließen, dass beide Proteine unabhängige Funktionen übernehmen können.

Unter dem Einfluss des überexprimierten NonO Proteins oder von Deletionsmutanten wurde die Repressionskapazität des Korepressors N-CoR in 293T Zellen untersucht. Als alternative Strategien wurden mit Hilfe der RNA-Interferenz Technik funktionelle Untersuchungen in der Abwesenheit von in 293T endogen exprimiertem NonO oder N-CoR durchgeführt. Dies führte jedoch nicht zum Erfolg, da das Wachstum der Zellen bei der spezifischen Herunterregulierung der Proteine N-CoR oder NonO stark beeinträchtigt war. Aufgrund seiner hohen Stabilität und der niedrigen Umsatzrate (Fig. 22) sind selbst nach signifikanter Degradierung der entspechenden mRNA (Fig. 20), noch detektierbare Mengen des NonO

Proteins vorhanden, was aber keinen Einfluss auf die Regulierung der Transkription der Reportergene hat. Die Interaktion zwischen NonO und der N-CoR carboxyterminalen Domäne wurde zunächst in GST-Pulldown Experimenten auf die amino-terminale Hälfte des Proteins NonO eingegrenzt, die die RRM Motive enthält. Diese Motive können vermutlich sowohl bei der RNA-Interaktion als auch bei Protein-Protein Wechselwirkungen eine Rolle spielen. Es wurden Deletionsmutanten hergestellt, denen jeweils eines der zwei RRM Motive (RRM1 bzw. RRM2) fehlt. Im Pulldown-Experiment konnten Interaktionen gezeigt werden zwischen dem *in vitro* translatierten und radioaktiv markierten N-CoR C-Terminus und dem Glutathion-S-Transferase (GST)-fusionierten NonO Wildtyp oder NonOΔRRM2 aber nicht mit der NonOΔRRM1 Mutante (Fig. 26 A). Unter der Annahme, dass das RRM1 Motiv für die Interaktion mit N-CoR entscheidend ist, wurde dieses mit GST fusioniert und untersucht. Die beobachtete Assoziation des N-CoR C-Terminus mit dem rekombinanten GST-NonO RRM1 Protein bestätigte diese Hypothese (Fig. 26 B). Die Deletion der Domänen RRM1 und RRM2 zeigte keine Auswirkung auf die nukleäre Lokalisation der Mutanten. Allerdings wurde die Zellzyklus-abhängige posttranslationale Modifikation bei der ΔRRM2 Mutante beeinträchtigt. Beide Mutanten wurden in funktionellen Experimenten eingesetzt und deren Effekte wurden mit NonO Wildtyp verglichen. Zwei verschiedene transient transfizierte Reportergene wurden zur Untersuchung der Repressionskapazität von N-CoR eingesetzt. Der UAS-TK-Luziferasereporter, welcher in der Thymidin Kinase (TK) Promoterregion Erkennung-sequenzen des Gal4 Transkriptionsfaktors (UAS) enthält, ermöglicht die Bewertung der Transkriptionskapazität von Proteinen, wenn sie mit der Gal4 DNA-bindenden Domäne fusioniert sind. Der RARE-Luziferasereporter, dessen Expression von Retinsäure-Rezeptoren (RAR) reguliert wird, ermöglicht die Auswertung der Rekrutierung von N-CoR und Koregulatoren.

Im Gal4-reporterAssay wurde zunächst die Repressionskapazität des Fusionsproteins Gal4-DBD-N-CoR Wildtyp untersucht. Die Transkriptionsrepression hängt bei diesem Reporter von der Korepressorkomplexbildung und der Rekrutierung von Histondeacetylasen zum Promoter ab. Diese wurde nicht beeinträchtigt in Gegenwart von exogenem NonO und NonOΔRRM2; NonOΔRRM1 hingegen erhöhte die Gal4-DBD-N-CoR vermittelte Repression konzentrationsabhängig (Fig. 31). Da NonOΔRRM1 das RRM1 Motiv fehlt, das entscheidend für die Interaktion mit N-CoR ist, deutet dieser unerwartete Effekt darauf hin, dass es sich möglicherweise um einen dominant-negativen Effekt handelt. Aus diesem Experiment ist zu schließen, dass NonO nicht in die Bildung des Korepressorkomplexes involviert ist sondern eher in die Modulation der Kapazität von N-CoR die Transkription zu reprimieren.

Es ist denkbar, dass NonO und andere Kofaktoren an der Regulation der Aktivität des Korepressorkomplexes beteilig sind. NonOΔRRM1 könnte in einer deregulierten Weise mit diesen interagieren und ferner deren regulatorische Funktion beeinträchtigen. Im RARE-Reporterassay erhöht exogenes NonO die Transkriptionsaktivität des Reporters in

Abwesenheit des Liganden und in Anwesenheit des Agonisten. Dagegen bleibt die Antagonist-induzierte Repression unverändert (Fig. 33). Aus diesen Beobachtungen ist zu schließen, dass NonO eine regulatorische Rolle in der Hormonrezeptor-abhängigen Transkription spielt. Dass NonOΔRRM1 einen ähnlichen Effekt wie Wildtyp NonO zeigte, deutet darauf hin, dass NonO die Assoziation von N-CoR mit den nukleären Rezeptoren und die N-CoR Repressionskapazität beeinflussen kann. Im RARE-Reporter Assay, konnte ligandunabhängig ein stärkerer aktivierender Effekt mit exogenem NonOΔRRM2 gezeigt werden. Vermutlich konnte diese Mutante die Rekrutierung von Korepressoren zum Promoter verhindern. Allerdings zeigte die Proteinanalyse von Zelllysaten aus synchronisierten Zellen, dass die N-CoR Proteinmenge in Gegenwart von NonOΔRRM2 reduziert war, was das Ergebnis im RARE-Reporterassay erklären könnte.

NonO interagiert mit dem N-CoR C-Terminus. Gal4-DBD-N-CoR-Ct weist im UAS_TK-Reporterassay keine Repressionsaktivität auf. Allerdings reprimiert Gal4-DBD N-CoR weniger als Gal4-DBD fusioniert mit der amino-terminalen Hälfte des Proteins N-CoR, welche die Repressionsdomäne I enthält (Fig. 41). Diese Beobachtung ist überraschend, da von dem intakten Korepressorprotein durch die Bildung eines stabileren vollständigen Komplexes eine stärkere Repression zu erwarten wäre, es sei denn, dass der C-Terminus die Repressionskapazität des N-Terminus moduliert. Die Hypothese, dass beide Termini an einer intramolekularen Wechselwirkung beteiligt sind und sich gegenseitig regulieren könnten, wurde durch die Interaktionsstudien dieser Domänen *in vitro* getestet. In GST-Pulldown-Experimenten konnte eine direkte Interaktion von N-CoR C-Terminus und N-Teminus gezeigt werden (Fig. 40). Ferner weist die Überexpression der C-terminalen Hälfte von N-CoR, welche die Rezeptor Interaktionsdomäne (RID) enthält, einen Einfluss auf die Transkriptionsakitvität des RARE-Reporters auf. Die exogene Expression des N-CoR C-Terminus beeinträchtigte die basale und die aktivierte Transkription. Die agonistinduzierte Transkription war vermindert, während die Repression der Transkription in Abwesenheit von Ligand verstärkt war (Fig. 42). Dies deutet darauf hin, dass der N-CoR C-Terminus ohne den Amino-Teil nicht dissoziieren kann und die Regulierung der Repressionsaktivität des endogenen Proteins beeinflusst. Wenn diese Proteindomäne tatsächlich eine regulatorische Rolle spielt, könnte die Interaktion von modulierenden Faktoren mit dem überexprimierten Carboxy-Terminus der erhöhten Repressionsaktivität des endogenen Proteins zugrunde liegen. NonO könnte somit zu den N-CoR regulierenden Proteinen gehören.

Auf Grund der Vermutung, dass die Termini des N-CoR Proteins in der Tertiärkonformation nah beieinander liegen und der Beobachtung, dass bestimmte Kofaktoren sowohl mit dem Amino-Terminus als auch mit dem Caboxy-Terminus interagieren, wurde die Interaktion zwischen NonO und der amino-terminalen Hälfte von N-CoR untersucht. Diese Interaktion konnte in GST-Pulldown-Experimenten gezeigt werden und wurde auf die SANT Domänen eingegrenzt. Diese Domänen sind umgeben von den Repressionsdomänen I und II des N-

CoR Amino-Terminus. Das SANT1 Motiv ist sowohl für die Aktivierung von HDAC3 als auch für die Bindung von acetylierten Histonen von Bedeutung. Dagegen ist das SANT2 Motiv für die Bindung von SUMO-modifizierenden Enzymen verantwortlich. Die Transkriptionsaktivität von N-CoR und verschiedenen Koregulatoren kann durch post-translationale SUMO Modifizierung beeinflusst werden. Interessanterweise wurde eine Interaktion zwischen dieser regulatorischen Region und NonO nachgewiesen (Fig. 44) und eine mögliche SUMOylierungsstelle in NonO gefunden (Fig. 46). Ob die Bindung von NonO mit der N-CoR SANT Domäne und die SUMO Konjugation für die Modulierung der NonO abhängigen N-CoR Aktivität relevant sind, konnte im Rahmen dieser Arbeit nicht mit Sicherheit festgestellt werden. Es wurde jedoch gezeigt, dass dieses RNA-Erkennungsmotive enthaltende Protein eine regulatorische Rolle in der Hormonrezeptor-abhängigen Transkriptionsregulation spielt, und dass das RRM1 Motiv für die Interaktion mit N-CoR entscheidend ist. Ferner wurde festgestellt, dass NonO sowohl die Interaktion zwischen nukleären Rezeptoren und N-CoR als auch die N-CoR Repressionsaktivität beeinflussen kann. Diese Ergebnisse, unterstützt von publizierten Daten, weisen darauf hin, dass der extreme N-CoR C-Terminus möglicherweise eine regulierende Domäne ist, welche die N-CoR Repressionsaktivität des Amino-Terminus modulieren kann. Diese Region kann mit einem Sensor verglichen werden, der das Verhältnis von Koaktivator und Korepressor registriert. Die Akkumulation von Kofaktoren wie z.B. NonO am Promoter könnte die Sensitivität von nukleären Rezeptoren gegenüber transkriptionsaktivierenden Stimuli erhöhen und die N-CoR Repressionsaktivität beschränken. Dagegen würde eine lokale Verringerung der NonO Konzentration die Interaktion von Korepressoren mit nukleären Rezeptoren begünstigen und diese in eine repressive Form zurückzubringen. Die Konzentration von Koregulatoren wie NonO, die an nachfolgenden Ereignissen wie der RNA Polymerase II Komplexbildung und -aktivierung, Spliceosom Komplexbildung und der Bildung des RNA Polyadenylierungskomplexes beteiligt sind, könnte die Menge und Art der Splicing-Varianten eines RNA Transkriptes beeinflussen, abhängig von der Konzentration des Stimulus und dem Zellkontext.

Bevor die Koordination und Gleichzeitigkeit von Transkriptions- und Splicingprozessen bekannt wurde, waren Interaktionen von Splicing-Faktoren und Splicing-assoziierten Proteinen mit dem RNA Polymerase II Komplex beschrieben worden. In vergleichbarer Weise konnte anhand meiner Ergebnisse gezeigt werden, dass NonO die physische Verbindung zwischen transkriptionsakivierenden und -reprimierenden Prozessen sein kann, da es auch mit dem Korepressor N-CoR interagiert. Ferner wird ein Modell vorgestellt, welches die biologische Relevanz dieser Interaktion aufzeigt (Fig. 49). Die Modulation der N-CoR Repressionsaktivität durch NonO stellt folglich ein bislang nicht bekanntes Regulationselement in der Kontrolle der Genexpression dar.

# Summary

Although in general cells are genetically identical in multicellular organisms, the differential expression of genomic information enables cell type definition and specific organ function. In eukaryotic cells the DNA is associated with histones and non-histone proteins into a restrictive structure called chromatin. It is now clear that this structure does not only protect and package the linear double-stranded DNA in the nucleus but is fundamental for the execution of diverse genetic programs. Local chromatin modifications at the gene promoter region play a determinant role in the regulation of transcription. Nuclear receptors are transcription factors that bind specific target sequences on the DNA and recruit transcriptional coregulators at the promoter. These are able to modify the chromatin structure in an activating or repressing manner. Recruitment of coactivators mediates decondensation of the chromatin structure, which enhances the binding of general transcription factors and the formation of the RNA polymerase complex whereas corepressor recruitment enhances structural changes that mediate transcriptional repression. The contribution of corepressors to the biological actions of nuclear receptors has turned out to be essential. Impaired corepressor function can be the cause of endocrine malfunctions, neoplastic diseases or severe developmental abnormalities. To better understand the role of the nuclear receptor corepressor N-CoR, the unknown function of the extreme C-terminus was investigated.

During my thesis, I could confirm the *in vivo* interaction of the non-POU-domain containing octamer-binding protein NonO/p54nrb with N-CoR in co-immunoprecipitation and confocal microscopy experiments. NonO was previously found to interact with N-CoR C-terminus in a yeast-two-hybrid screen (Ducasse 2002). This protein contains two RNA recognition motifs (RRM) and is described as a multifunctional protein since it is involved in transcription initiation as well as in pre-mRNA processing. In GST-pulldown experiments, the RRM1 motif was determined to be essential and sufficient for the interaction with N-CoR. Therefore, deletion mutants were generated lacking RRM1 and RRM2, respectively. The biological significance of the NonO/N-CoR interaction was then investigated in different functional reporter assays expressing exogenous NonO wild type versus NonO deletion mutants. In the Gal4-reporter assay the repression activity of N-CoR full-length fused to the Gal4-DBD was enhanced in the presence of the ∆RRM1 mutant in a concentration-dependent manner whereas exogenous expression of the ∆RRM2 mutant or NonO wild type did not affect N-CoR repressive activity. As this effect is concentration-dependent and NonO RRM1 lacks the interaction domain, it may result from a RRM1 mutant-related dominant-negative effect. These observations support the possibility that NonO is involved in the modulation of the repression activity of N-CoR. In the retinoic acid receptor-dependent reporter assay (RARE-

reporter), the recruitment of N-CoR by the nuclear receptor is induced in the absence of ligands or in the presence of antagonists. In comparison to the Gal4-reporter assay, which was not influenced by NonO wild type overexpression, the result obtained in the RARE-reporter assay suggests that NonO can affect the nuclear receptor-dependent recruitment of N-CoR. Taken together these results suggest that NonO modulates the capacity of N-CoR to repress and its recruitment to targeted promoters by nuclear receptors.

N-CoR repressive activity is mainly contained in the N-terminal half. As it was observed that NonO, which interacts with the extreme C-terminus, affected N-CoR repressive activity, questions remained if NonO binds to the N-terminus and if both N-CoR termini are involved in intramolecular interactions. This was assessed in *in vitro* binding assays. Indeed, NonO interacts with the N-CoR N-terminus within the SANT domain and both N-CoR termini interact in GST-pulldown assays. Further results obtained in reporter assays suggest that the N- and C-terminus of N-CoR regulate each other. In the last few years the regulation of nuclear receptor-dependent transcription has turned out to be more complicated than expected. It is now clear that nuclear receptors integrate at the target promoter a multitude of parameters including their abundance, affinity and their transcriptional activity as well as those of recruited coregulators. This increases the number of possible levels of regulation and the cell-specific gene expression patterns. Together, my results supplemented by published data support the functional model proposed here. The capacity of the C-terminus of N-CoR to modulate the repressive activity of the N-CoR N-terminus would depend on the concentration of coregulators such as NonO in the nuclear receptor environments. Accumulation of NonO at the promoter would enhance nuclear receptor sensitivity to stimulation and reduce N-CoR repressive activity; whereas a decrease of NonO local concentration would favor the interaction of nuclear receptors with corepressors (Fig. 49). The variation of the local concentration of coregulators such as NonO that are involved in events downstream of the activation of nuclear receptors associating with the Pol II complex, spliceosome and polyadenylation complexes, and involved in the regulation of repression would enable the coordination of these different processes. This would be physiologically relevant to regulate the amount and the quality of transcribed RNAs in response to stimulation intensity and cellular context.

# 1 Introduction

In multicellular organisms, the differential expression of the genomic information is essential for cell type definition and specific organ functions. Even though most cells are genetically identical, specificity is ensured by the expression of a defined set of proteins. The synthesis of functional proteins is a multi-step process taking place in different cell compartments. In eukaryotic cells, DNA which contains the genetic information is located in the nucleus. Transcription and RNA processing enable synthesis of mature RNA messenger which translocates out of the nucleus. mRNA is then translated into amino acids and folding of the polypeptide chain completes protein synthesis. Thus, transcription and RNA processing are the determinant initial nuclear events that ensure protein synthesis. Each step of this process is tightly regulated; however the regulation of gene transcription remains the most important step. To achieve initiation of transcription a series of ordered events is required. The transcriptional machinery has to be recruited to target promoter regions and has to overcome the restrictive chromatin structure into which DNA is packaged. Transcription factors that bind to target DNA sequences are essential for both recruitment of protein complexes which mediate modifications of the chromatin and for the formation of the transcription initiation complex. Their presence in the nucleus and their binding to DNA is essential for target gene expression. A subfamily of transcription factors, the nuclear receptors are able to activate as well as to repress transcription actively. Bound to their response element they can recruit transcriptional coregulators including coactivators and corepressors. In this context it is expected that a dysregulation of coregulators is likely to influence the expression of target genes. Indeed, impaired nuclear receptor-dependent transcription is involved in the pathogenesis of a number of diseases, particularly cancer. Therefore, transcription control exerted by nuclear receptors continues to be an intensively investigated area in the perspective to find novel therapy targets. The key protein of this project is N-CoR, the nuclear receptor corepressor that mediates transcription repression in a nuclear receptor-dependent manner. To better understand the importance of N-CoR function in gene expression some details about the chromatin structure and its regulation will be described as well as the role of nuclear receptors in transcriptional regulation

## 1.1 Chromatin: more than DNA packaging

The organization of DNA into chromatin is an advantageous structure to protect and package the linear double stranded DNA (in the order of meters) into the nucleus (a few micrometers) with the lowest level of compaction. The hypothesis of R. Kornberg (1974) that chromatin structure is fundamental for the execution of diverse biological programs has turned out to be true. It is now clear that DNA packaging into chromatin plays a determining role in the regulation of nuclear processes such as transcription, replication, repair and recombination and this dynamic structure even contains heritable epigenetic information that is not contained in the DNA sequence.

### 1.1.1 Chromatin structure

The basic organization of chromatin is a succession of nucleosomes. A nucleosome consists of 146 bp of DNA wrapped 1.7 times around an octamer of core histones. Each nucleosome core particle is separated by a linker region of approximately 50 bp. The main histone variants that are involved in the assembly of a nucleosome are histone H2A, H2B, H3 and H4. These histones heterodimerize in H3-H4 and H2A-H2B dimers and each are represented twice in the nucleosome core particle. A H3-H4 tetramer assembles first onto the double stranded DNA (dsDNA) and two separate H2A-H2B dimers associate later in a cooperative manner. Each histone dimer contains three regions of interaction with the dsDNA and two additional interactions occur at the entry and exit points of the nucleosome with the histone tails from each H3 histone (Luger et al. 1997). Interaction is formed mainly by tight hydrogen bonds between the histone main chain amide and the phosphate oxygen of the DNA, assisted by electrostatic interactions between basic side chains and negatively charged phosphate groups and additional nonpolar interactions. This allows nucleosomes to form on any DNA sequences. The lowest compacted chromatin structure is the 10 nm fiber known descriptively as beads on a string (Kornberg 1974). This chain of nucleosomes can arrange to a more compact structure, the 30 nm fiber, which is stabilized by linker histones (H1) (Fig. 1 A and B) (Oudet et al. 1975; Schalch et al. 2005). For the organization of chromatin into a 30 nm fiber two models were suggested the solenoid and the zigzag model. The latter model is the nucleosome arrangement that is adopted in the cell (Bednar et al. 1998). The relative positioning of nucleosomes is based on the entry and exit paths of the DNA in the presence of histone H1 (Box. 1 C and D). Compared to the solenoid architecture based on protein-protein interactions, zigzag pattern results from the alternate positioning of the nucleosomes. Thus, nucleosomes are physically closer and present minimal internucleosomal attraction energy (Cui and Bustamante 2000). This structure allows dramatic changes in compaction level to occur without a concomitant change in topology. Further compaction levels are known and require additional interaction with non-histone proteins.

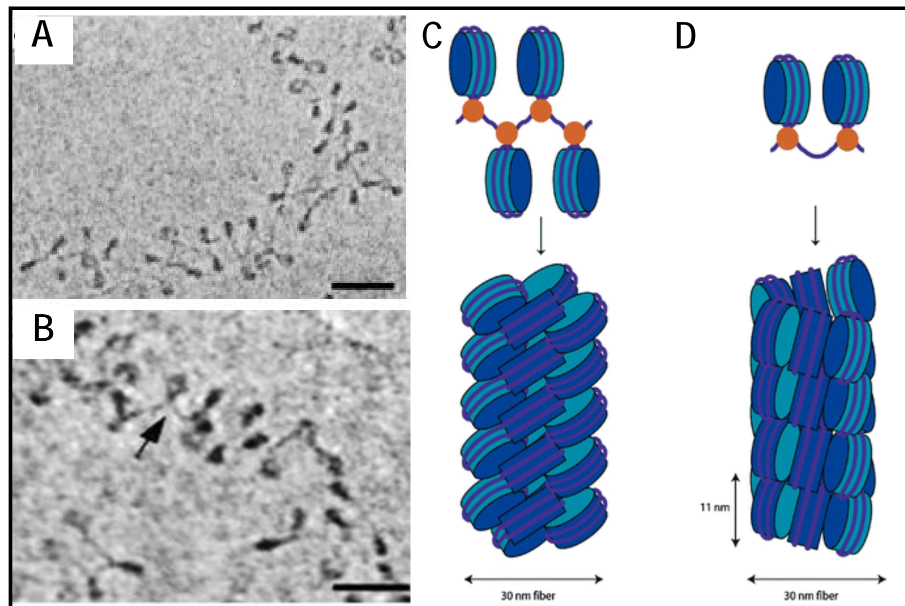



**Figure 1: Nucleosomes, linker DNA and linker histone form a unique structural motif that directs the higher-order folding and compaction of chromatin**

Left panel, linker histones induces a "stem" conformation of linker DNA segments **(A)** and **(B)** from (Bednar et al. 1998), electron cryomicroscopy images of segments of unfixed, unstained chromatin fibers released from chicken erythrocyte nuclei into low-salt buffer and observed in frozen hydrate state. Right panel from (Khorasanizadeh 2004), schematic representation of the 30nm fiber, the histone octamer is shown in two shades of blue an the DNA is shown in magenta. The linker histone H1 is shown in orange. **(C)** The alternating aspect of adjacent nucleosomes creates a zigzag pattern of packing. **(D)** The consecutive arrangement of six nucleosomes in a turn of helix can form a solenoid.

Chromatin is a structural support for a defined genomic organization in eukaryotic cells in which heterochromatin and euchromatin distinguish transcriptionally inactive from active chromatin, respectively. It provides also a great structural flexibility in dividing cells in which DNA has to be replicated (DNA is accessible to the replication machinery) and sequentially compacted into the highest ordered chromatin organization level which is the chromosome. The required modulations of the chromatin structure during cell growth, differentiation and division are ensured by modifications that occur on the DNA and mainly at nucleosomes, the structural unit of chromatin.

### 1.1.2 Chromatin regulation

In the past three decades a number of chromatin related events including DNA methylation, incorporation of histone variants, histone postranslational modifications and ATP-dependent chromatin remodeling have been discovered. These have been correlated to the regulation of chromatin structure and are involved in major reactions with chromatin substrates such as transcription, replication, recombination and repair. Distinct multiprotein complexes participate in the regulation of chromatin accessibility and higher order structure formation

ensuring proper regulation of downstream events. This regulation is mediated by modifications at the DNA and the nucleosome level.

**DNA modification**

Methylation is a covalent modification that can occur at cytosines within CpG rich regions in the DNA and is important for gene repression in mammals and plants although is does not occur in a number of eukaryotes including yeast. DNA-methylation status contributes to epigenetic inheritance, allele-specific expression, inactivation of the X chromosome, genomic stability and embryonic development. The loss of methylation control can contribute in initiation and progression of tumorigenesis (reviewed in (Plass and Soloway 2002)). The majority of methylated CpG islands are located within repetitive elements including centromeric repeats, satellite sequences and gene repeats encoding ribosomal RNAs. In the euchromatin CpG regions are found at the 5´end of genes and are typically unmethylated. DNA methylation enhances transcriptional repression recruiting methyl-CpG binding domain (MBD) proteins that in turn associate with corepressors. It is now clear that there is a connection between DNA methylation and other silencing mechanism including histone modifications and chromatin remodeling (reviewed in (Holmes and Soloway 2006).

**Nucleosome remodeling**

In contrast to histone posttranslational modifications (see below) nucleosome remodeling is an ATP-dependent process that modifies chromatin structure in a noncovalent manner. Nucleosome ATP-dependent remodeling complexes increase access of transcription factors to DNA sequences that originally interact with the histones by disrupting DNA-histone interactions. Loss-of-function mutations in several chromatin remodeling factors were reported to have serious consequences. This indicates that the role of ATP-dependent chromatin remodeling in activation and repression of transcription can affect the control of cell cycle, cell differentiation and hence the development of multicellular organisms. A number of complexes have been determined that can change the position of the nucleosomes on DNA. These complexes were divided into three classes based on the identity of their catalytic ATPase subunit. These ATPase subunits display homology only within the ATPase domain and contain different additional domains. For example, the SWI/SNF family contains an additional bromo domain whereas the ISWI family contains a SANT domain and the Mi-2/NURD family a chromo domain. Each ATPase associates with different subunits to form distinct multiprotein complexes. These other subunits may be involved in the regulation or targeting of the remodeling activity. Recent discoveries provide evidence for mechanistic differences between each of the ATP-dependent remodelers and this may be correlated to their specific biological tasks. Two models are considered for the mechanism of ATP-dependent nucleosome remodeling (Fig. 2).This first described

mechanism was the nucleosome sliding which result in changes in the position of the nucleosome on the DNA and a second mechanism which allows exposure of DNA within the region bound by a histone octamer (reviewed in (Narlikar et al. 2002)). Nagaich and colleagues studied *in vitro* the interaction between the glucocorticoid receptor and an array of highly positioned nucleosomes assembled on the mouse mammary tumor virus long terminal repeat. They observed that receptor binding to nucleosomal DNA is enhanced by SWI/SNF and is accompanied by sequential reorganization of histone proteins within the nucleosomes. The action of SWI/SNF is proposed to lead to changes in the position of histone H2B within the nucleosome that accompanies the recruitment of GR to a new binding site within the nucleosomal DNA. (Nagaich et al. 2004). In addition to promote DNA accessibility, the action of the SWI/SNF ATPase appears to prepare nucleosomes for further posttranslational modifications although it does not drive these reactions per se. A number of studies support the coupling of ATP-dependent remodeling with histone posttranslational modifications. Genetic studies in yeast suggest that remodelers and covalent modifiers function interdependently since combination of mutation in subunits of the ySWI/SNF complex and SAGA complex containing histone acetyltransferase (HAT) activity is lethal in contrast to single mutants that display no severe growth defects. Together nucleosome remodeling and histone covalent modification are the major processes involved in gene activation and repression or other reactions with chromatin substrate (reviewed in (Becker and Hörz 2002)).

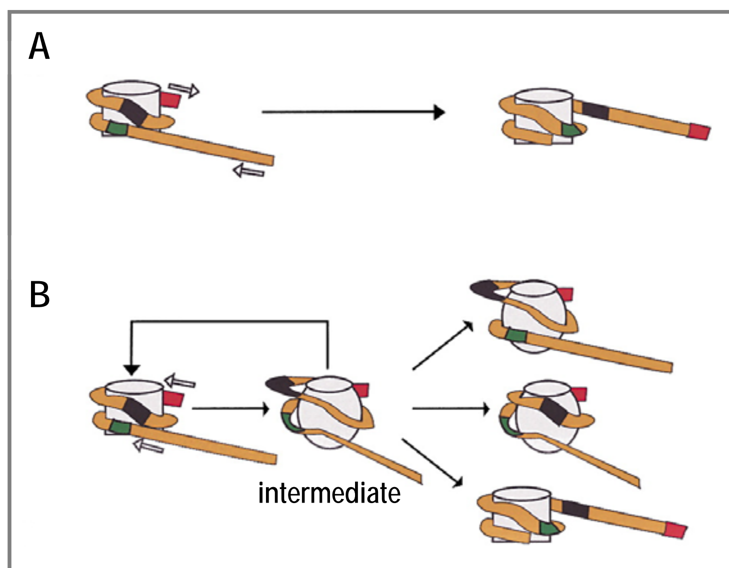



**Figure 2: Two models for the mechanism of ATP-dependent nucleosome remodeling**

(**A**) Nucleosome sliding.The histone octamer is repositioned on the DNA by sliding. DNA sequences which were originally interacting with the histones, becomes nonnucleosomal. (**B**) Nucleosome conformational change. Hypothetical structures for intermediates and products of a non sliding mechanism that could involve changes in the conformation of DNA, histone, or both. Models from (Narlikar et al. 2002)

### Histone posttranslational modifications

Histones are subjected to posttranslational modifications such as methylation, citrullination, acetylation, phosphorylation, SUMOylation and ADP-ribosylation (Fig. 3 B). The combination of different modifications is thought to constitute the histone code (Strahl and Allis 2000; Jenuwein and Allis 2001) and to correlate with specific events required for transcription, DNA repair or the regulation of chromosome condensation. These modifications do not exert their biological function by significantly modulating the nucleosome structure, but rather act as marks for the specific recruitment of proteins that bring additional regulatory functions to these units. The histone structure is highly conserved through evolution and relatively similar in the different variants. Two main domains are distinguished within the histone, the folded core and the unstructured tail. The histone core is a globular domain folding in a helix turn helix turn helix motif allowing easy dimerization. Histone tails or terminal extensions protruding from the nucleosome core particle do not adopt defined conformations in crystal structures except bound to their recognition proteins. The tail domains contain a number of conserved amino acid residues (lysine, arginine and serine) that can undergo postranslational modifications (Fig. 3 A). Covalent modifications at the N-terminus tail of all four histone proteins and at C-terminus tails of the histone H2A and the linker histone H1 are relevant in the regulation of chromatin structure and transcription.

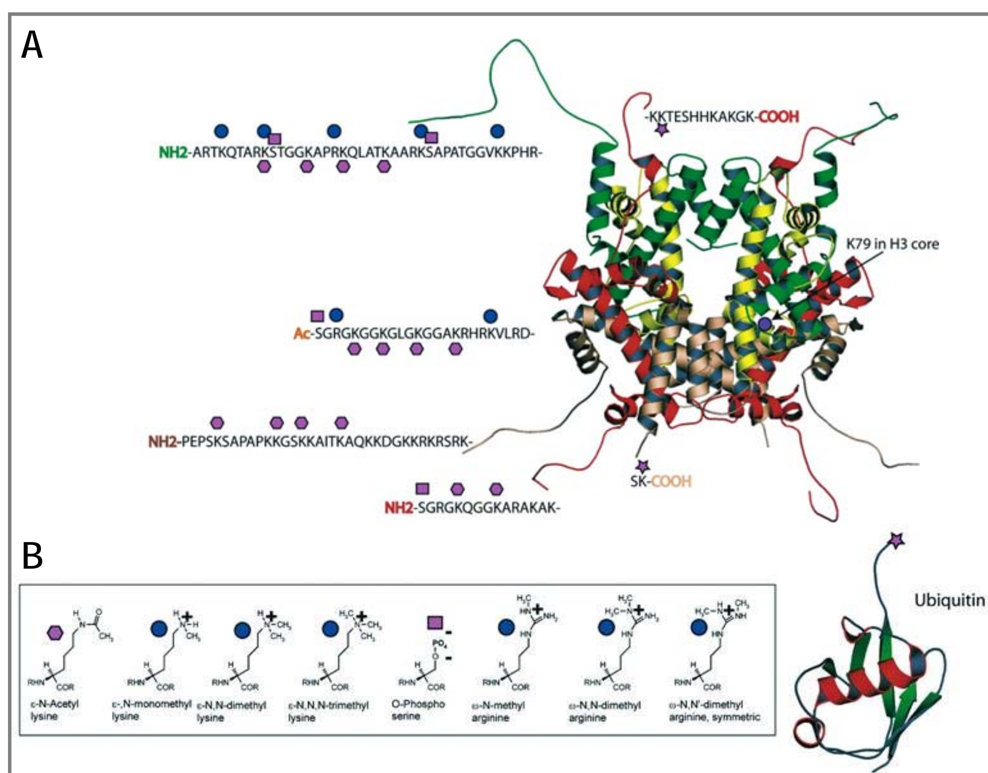



**Figure 3: Histone posttranslational modifications**

**(A)** The histone octamer portion of the nucleosome core particle is shown. The sites of modifications are marked. For clarity, the modifications are shown on one copy of each protein. **(B)** The covalent modifications of the amino acids are shown. Figure from S. Khorasanizadeh review (Khorasanizadeh 2004).

Due to their basicity histone tails can electrostatically interact with the polyanionic backbone of the core DNA; however they marginally contribute to the nucleosome stability (Hayes et al. 1991). Histone tails are thought to play a role in the regulation of nucleosome mobility. They mediate interactions outside the nucleosome core and in the condensation state of chromatin fiber, mediate interchromosomal interactions (Wolffe and Kurumizaka 1998). Furthermore the histone tails can either alter the accessibility of nuclear factors to DNA (Vitolo et al. 2000) or enable the recruitment of factors involved in transcription or chromatin assembly pathways. To better understand the central role of the nucleosome in the regulation of chromatin structure and transcription the most important reversible covalent histone modifications will be presented briefly.

Histone methylation on conserved residues of the histone amino tails was first thought to be mainly implicated in epigenetic inheritance. Indeed methylation is fairly stable in comparison to other modifications such as ubiquitination, phosphorylation, SUMOylation or acetylation and until recently no enzyme was identified which could actively remove methylation. In addition H3 lysine 9 methylation was found to be a mark of heterochromatin initiation and maintenance mediating interaction with the heterochromatin protein 1 (HP1) required for the formation of higher order chromatin structure (Nakayama et al. 2001) (Richards 2002). Similarly, polycomb proteins that are involved in gene silencing during development interact with H3 methylated at lysine 27 (Fischle et al. 2003). However, further investigations indicated that methylation is not a permanent mark on histones and can be removed by active processes necessary in the regulation of gene expression. Histone methylation at lysine and arginine residues has been reported to enhance transcriptional activation. Histone methylation at H3 lysine 4 (Wang et al. 2001) or H3 lysine 36 (Kizer et al. 2005) was correlated with transcriptionally active genes as were H3 methylated arginines (2,17 and 26) (Schurter et al. 2001) and H4 arginine 3 (Strahl et al. 2001). Although the precise mechanism by which histone methylation contributes to physiological processes is mostly unresolved, several chromatin associated factors and methyltransferases contain a well defined chromo domain which is a binding motif for methylated lysine. The state of methylation (mono- di- and trimethylation) was also determined to be of functional significance which was emphasized by the discovery of histone methyltransferases which catalyze distinct methylation states (Rice et al. 2003). In transcriptionally active chromatin, mostly mono- and di-methylation of lysine or arginine residues is found in contrast to histone trimethylation, which is thought to be a biochemically irreversible covalent modification. This observation supported that active turnover mechanisms for methyl groups on histone tails should exist. Indeed, enzymes have been recently discovered which can prevent arginine monomethylation (Cuthbert et al. 2004) or catalyze demethylation of mono- and dimethylated lysine (Shi et al. 2004). These findings indicate that methylation can be antagonized and

reversed by distinct enzymatic activities in addition to histone replacement that was proposed to be the most likely mechanism for the removal of methylated histones from chromatin. Histone deposition occurs mainly during DNA replication; however different histone variants are incorporated at different regions of chromatin by replication-independent histone assembly mechanisms (Ahmad and Henikoff 2002). More recently, response-mediated replacement of histones has been reported to require the process of active transcription (Schwartz and Ahmad 2005). Although further investigations are necessary to understand the function of histone methylation in details in various biological processes it is now clear that this modification is part of the histone code and offers a multitude of distinct combinations of marks.

In 1964 Allfrey and colleagues found a correlation between increased histone acetylation and increased transcription (Allfrey et al. 1964). Since then several mechanisms by which histone acetylation and deacetylation regulate gene activity have been elucidated. Recently, new roles for histone acetylation have been uncovered, not only in transcription but also in DNA replication, repair and heterochromatin formation in yeast as well as in humans (reviewed in (Kimura et al. 2005)). Hyperacetylation of histone tails at lysine residues is associated with transcriptional activity. It is thought to weaken DNA-histone contacts by neutralizing the positive charge of the histone tails and decreasing their affinity for negatively charged DNA, thereby allowing access for transcription factors to promoters in the chromatin. Conversely, histone deacetylation is believed to prevent access by restoring the positive charge and strengthening the interaction between DNA and histones. Although this charge-neutralization model has become popular, more recent studies support that histone acetylation/deacetylation rather regulate transcription by altering higher-order folding properties of the chromatin fiber and by providing specific binding surfaces for the recruitment of repressors and activators. Observations by Turner and colleagues support this latter model. These suggest that histone modification mediates not only promoter-specific gene expression but also longer-range gene expression. They observed distinct lysine acetylation patterns in specific chromosomal regions (euchromatin, heterochromatin and hyperactive male X chromosome) in *Drosophila megalogaster* polytene nuclei (Turner 1991). Many of the enzymes that catalyze histone acetylation and deacetylation were previously identified as transcriptional cofactors. This observation underscores the importance of these dynamic modifications in transcriptional regulation.

Acetylated lysine residues of histones are recognized by bromodomains that are contained in a number of histone acetyltransferases and chromatin associated factors. In euchromatin at promoter sites, acetylation of histone amino-termini provides binding surfaces for transcription factors of the TFIID transcription initiation complex as well as for proteins in

chromatin-remodeling complexes. Agalioti and colleagues have shown progressive acetylation of the human interferon (IFN)-$\beta$ gene upon transcriptional activation. Each acetylation pattern correlated with the recruitment of a specific protein. The general transcription factors GCN5 and $TAF_{II}250$, the largest subunit of the TFIID complex, are recruited to target promoter regions and sequentially acetylate H4 lysine 8 and H3 lysine 9 and 14, respectively. In turn, H4 lysine 8 acetylation provides a binding site for BRG1 that is part of the SWI/SNF complex that promotes ATP-dependent nucleosome remodeling (Agalioti et al. 2002). The first cloned histone acetyltransferase (HAT) was from *Tetrahymena thermophilia* (Brownell et al. 1996) and sequence similarity with previously identified transcription factors, such as CBP/p300, $TAF_{II}250$ and SRC-1 revealed that these transcriptional coactivators all contained HAT activity (reviewed in (Kimura et al. 2005)). These findings strengthened the idea that local acetylation of histones by transcription factors contributes to the activation of promoter-specific gene expression. Histone acetylases act as part of large complexes *in vivo* and additional subunits can modulate HAT activity and substrate specificity. Homozygous deletions of the different histone acetylases in mice exhibit distinct developmental defects, suggesting differences in function of these highly regulated HAT subunits. Histone acetylase activity can directly be affected by sequence-specific transcription factors and adjacent histone modification. However, for the proper regulation of gene expression antagonistic activity is required. Histone deacetylases (HDACs) were identified almost in parallel to HAT enzymes and promote gene repression and silencing by catalyzing removal of acetyl moiety from acetyl-lysine within histones (reviewed in (Gregoretti et al. 2004)).

In 1996 the first mammalian HDAC enzyme was identified related to the yeast transcriptional regulator, Rpd3 (Taunton et al. 1996). Since then, a number of HDACs have been discovered and subdivided into subclasses. This classification was based on sequence homology with the yeast homologues. The human class I histone deacetylases include HDAC 1, 2, 3 and 8 that are similar to Rpd3, whereas class II including HDAC 4, 5, 6, 7, 9 and 10 are similar to the yeast Hda1. The third class also named the sirtuins shows significant sequence and functional divergence to class I and II subgroups. Class III HDACs present NAD-dependent deacetylase activity like the yeast Sir 2 protein and play an essential role in epigenetic silencing (Blander and Guarente 2004). Class II deacetylases have an additional level of regulation in comparison to class I and III since they shuttle between the nucleus and the cytoplasm and their subcellular distribution appears to be under control of the classic cellular signaling pathways. Substrate specificity of each HDAC remains unclear but they all can deacetylate acetylated lysine residues of histone *in vitro*. Phylogenetic analysis (Gregoretti et al. 2004) revealed that HDACs evolved in the absence of histone proteins suggesting that key HDAC substrates might not be histones, nevertheless they are

by far the most abundant substrate. Meanwhile it is clear that a variety of biological processes in addition to transcription such as DNA repair, replication or recombination are regulated by acetylation and deacetylation of nonhistone proteins (reviewed in (Minucci and Pelicci 2006)). HDACs and HATs that are involved in the chromatin changes that regulate transcription associate with corepressor and coactivator complexes, respectively. They are recruited to gene regulatory regions directly or indirectly by DNA binding factors. A number of complexes containing acetylase or deacetylase activity have been described and variations remain in the exact complex composition. The differences might reflect heterogeneity essentially due to cell type and stages of differentiation in which these studies were performed. In a number of cancers, correlation between pathogenesis and imbalance in acetylation/deacetylation has been characterized. The interest in these enzymes is growing because HDAC inhibitors appear to be promising therapeutic agents against cancer and a variety of other diseases (Krämer et al. 2001; Minucci and Pelicci 2006).

Strahl and Allis proposed that distinct histone modifications, on one or more tails, act sequentially or in combination to form a 'histone code' that is read by other proteins to initiate distinct downstream events. Although a number of combinations remain unelucidated, distinct patterns have been correlated to different events. For example, H3 lysine 9 and 14 acetylation, in combination with H3 lysine 4 methylation and H4 lysine 8 acetylation are marks for transcriptional activation, whereas H3 lysine 9 trimethylation is a heterochromatin mark and H2B serine 14 phosphorylation is a mark for apoptotic chromatin condensation (Fernandez-Capetillo et al. 2004). In addition to this level of regulation, other histone modifications such as ubiquitination, SUMOylation and phosphorylation have been described to be involved in the histone crosstalk which characterizes modification of histones that influence other histone modifications (Fischle et al. 2003). Indeed, methylation of H3 lysine 9 and 27 have been shown to be dependent of H2B and H2A lysine ubiquitination, respectively (Dover et al. 2002) (Wang et al. 2004), H3 serine 10 phosphorylation enhances H3 lysine 14 acetylation (Clements et al. 2003) and H4 SUMOylation induces HDAC1 recruitment (Shiio and Eisenman 2003). Together these few examples illustrate the dynamic and the complexity of the regulation of the chromatin structure by histone posttranslational modifications. The discovery of histone variants incorporation that has been shown to have several effects on chromatin expands this complexity. Locally it affects nucleosome structure as well as the propensity of variant containing chromatin to be remodeled through specific sequence changes in the histone domain. Hence, histone variant incorporation can alter nucleosome stability, mobility and histone modifications patterns, with possible effects on higher order structure or downstream events (Ahmad and Henikoff 2002) (Meneghini et al. 2003) (Chakravarthy et al. 2005). Regulation of the chromatin structure by nucleosome remodeling, histone posttranslational modification and the incorporation of histone variants emphasize

the central role played by the nucleosome and the histone code in the regulation of gene expression (reviewed in (Mellor 2005)).

While the regulation of the chromatin structure is necessary for regulating gene expression, it is not sufficient: functions of sequence-specific activators and repressors, mediator complexes and general transcription factors are also required to ensure proper regulation. When transcription of a gene is altered, a specific event, frequently the binding of gene-specific factors to specific DNA binding sequences triggers a cascade of spatially and temporally coordinated reactions. These reactions result in a chromatin template, appropriately remodeled, which is bound by regulatory factors and the general transcription machinery. The precise order in which ATP-dependent remodeling, covalent modification of histones, or binding by regulatory factors or the transcription machinery occur seems to depend upon the nature of the promoter, chromatin structure in which the promoter resides and the complement of present transcription factors. The sole requirement is that the end point, which is the structure of the template and association of appropriate components of the general transcription machinery, is reached in a timely manner (Narlikar et al. 2002). Thus, each cell type may establish its unique repertoire of expressed genes.

## 1.2 Nuclear receptor-dependent regulation of transcription

Transcription factors are able to bind to specific sets of short conserved sequences contained in each promoter and are divided into three groups. The general transcription factors are subunits of the Polymerase II complex which transcribes template DNA into messenger RNA. The second group, the upstream transcription factors are ubiquitous and recognize specific short consensus elements located in promoter regions and increase the efficiency of transcription initiation. General transcription factors and upstream transcription factors require accessible chromatin structure for DNA binding which is mediated by the third group of transcription factors. This consists of the inducible factors which have a regulatory role since they are synthesized or activated at specific times and in specific tissues. The nuclear receptors constitute a large family of ligand-inducible transcription factors. They regulate many biologically important processes in development and homeostasis by their bimodal function as repressors and activators of gene transcription.

### 1.2.1 Nuclear receptors are ligand-inducible transcription factors

Nuclear receptors are intracellular receptors that perform hormone-dependent signal transduction. The superfamilly of nuclear receptors is subdivided in three groups and the steroid and the non-steroid hormone receptors correspond to type I and II, respectively. Sex hormone receptors including androgen, estrogen, progesterone, glucocorticoid and mineralocorticoid receptors bind steroid hormones. The type II nuclear receptors include non-

steroid ligand binding receptors such as the vitamin A, D, retinoid and thyroid hormone receptors as well as receptors that bind diverse products of the lipid metabolism such as fatty acids and prostaglandins (peroxisome proliferators activated receptor (PPARs) and liver X receptors (LXRs)). A number of nuclear receptors that do not have any known ligands are so-called orphan receptors (reviewed in (Gronemeyer et al. 2004). All nuclear receptors can exert their effects via sequence-specific binding to target genes and via ligand-dependent regulation of transcription. Steroid and non steroid hormones are small lipophilic molecules that easily penetrate biological membranes. Type I receptors interact with their cognate ligand in the cytoplasm and then translocate to the nucleus whereas type II receptors reside in the nucleus in the presence or absence of their ligand. An essential step is the interaction of the receptors with specific DNA sequences, called hormone-response elements (HREs). These response elements position the receptors, and the complexes recruited by them, close to the genes that are targeted. HREs are bipartite elements that are composed of two hexameric core half-site motifs. The identity of the response element resides in three features: the nucleotide sequence of the two core motif half-sites, the number of base pairs separating them and the relative orientation of the motifs. Class I receptors, preferentially bind as homodimers to identical or very closely related response elements with a palindromic consensus sequence GGTACAnnnTGTTCT (Beato et al. 1989). In contrast, non-steroid receptors have the highest binding affinity for direct repeats that are asymmetric and can be variably spaced as heterodimers with the retinoic X receptors (Mangelsdorf et al. 1995). However, nuclear receptor structure plasticity enables either some nuclear receptor to heterodimerize with other transcription factors on composite response elements (Flick et al. 2002) or to regulate transcription in a DNA-independent manner being recruited by other DNA-bound transcription factors to target promoter regions (Almawi and Melemedjian 2002).

### 1.2.2 Nuclear receptor structure

Nuclear receptors share a common modular structure composed of independent but functionally related domains. The well conserved DNA-binding domain (DBD) centrally located comprises two zinc-finger motifs. Residues in the first module determine the specificity of the target DNA sequence, while residues in the second module are involved in dimerization. As mentioned previously homo-and heterodimerization are correlated with the response element type. Furthermore, the DNA-binding domain is involved in several other functions including nuclear localization, and interaction with transcription factors and co-activators. The DNA-binding domain therefore plays a central role, not only in the correct binding of the receptors to target genes, but also in the control of other activities of nuclear receptors. The ligand-binding domain (LBD) located in the carboxyl terminal half folds into a canonical $\alpha$-helical sandwich generally consisting of 12 helices (H1-H12). Helix 12 adopts

different positions depending on the presence or absence of ligand and the type of the ligand, agonist or antagonist. Its position is crucial in forming interaction surfaces for coactivators or corepressors. This LBD region also contains ligand-dependent nuclear translocation signals, binding surfaces for chaperone proteins, dimerization interfaces, and a potent ligand-dependent activation domain AF-2. A ligand-independent activation domain, AF-1 which synergistically acts with AF-2, is encoded within the amino-terminal region of the receptors which is the most variable region in terms of length and amino acid sequence (Fig. 4) (reviewed in (Smirnov 2002)). Receptor domains are functionally interlinked. After ligand and DNA binding, the interaction of distant receptor domains with other molecules may be changed due to conformational receptor plasticity. For example, unliganded steroid receptors are retained in the cytoplasm in a complexe with heat shock proteins, which dissociate upon ligand binding, receptors can subsequently translocate to the nucleus. For all nuclear receptors, the binding of ligand changes the affinity of the DNA bound receptors for other proteins that do not exclusively interact within the ligand binding domain.

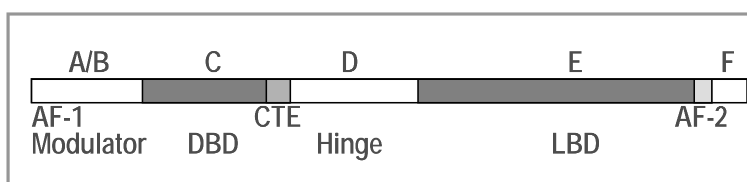



**Figure 4: Domain organization of nuclear receptors**

The A/B domain is the most variable region and contains the ligand-independent activation domain (AF-1) which mediates interactions with tissue-specific cofactors. The C region contains three modules, the DNA binding domain which recognizes enhancer-likes DNA sites called the hormone-response elements (HRE), a surface involved in receptor dimerization and a C-terminal extension (CTE) which is involved in the binding specificity of HRE and protein-protein interaction with other factors. The hinge region D provides great flexibility between the DBD and the ligand binding domain as a surface for receptor interaction with some coregulators. The E region contains the ligand binding motif and the ligand-dependent activation domain AF-2. The extreme carboxyl region F is not present in all receptors. Scheme adapted from Smirnov review (Smirnov 2002).

### 1.2.3 Nuclear receptor cofactors

In addition to their capacity to bind to condensed chromatin templates (Hebbar and Archer 2003) the response of a given receptor to a particular ligand depends on the set of proteins with which the nuclear receptor is able to interact. Recruited coregulators are factors that covalently modify histones and ATP-dependent chromatin remodeling factors. In turn, these determine the specificity of recruited factors and modulate the promoter accessibility to transcription factors as well as to the basal transcriptional machinery, respectively. Coregulators provide the appropriate mechanistic and enzymatic requirements for effective transcriptional regulation. In general, agonist binding is believed to increase the affinity for coactivators and inversely antagonist binding enhances interaction with corepressors. However, for some receptors variations are observed concerning their state of activation and

recruited coregulators. Receptors such as retinoid and thyroid hormone receptors, residing in the nucleus are capable to bind DNA in the unliganded state and are associated with repressor complexes that actively repress transcription. Moreover, distinct corepressor complexes have been identified to interact with agonist bound nuclear receptors (Traish et al. 1997). Although ligand-binding mainly controls association of the coregulators with nuclear receptors, composition of the DNA response element as well as posttranslational modifications of amino acid residues in different receptor domains can also affect transcriptional activation and/or nuclear receptor stability. The modulation of the nuclear receptor transcriptional activity through allosteric changes that occur upon specific DNA binding was reported for several nuclear receptors. Glucocorticoid, thyroid or retinoic acid receptors bound to their cognate agonist were found to repress transcription when bound to negative hormone-response DNA elements (reviewed in (Dostert and Heinzel 2004)). This suggests that binding to these DNA elements, differing from canonic HREs by nucleotide sequence in half-sites and in the size of the separating spacers, can affect the recruitment of coregulators. Posttranslational modifications such as phosphorylation, acetylation, ubiquitination and SUMOylation can also affect nuclear receptor transcriptional activity enhancing or inhibiting directly interaction or inducing conformational changes. For example serine phosphorylation in the DBD of thyroid receptor β1 (TRβ1) leads to allosteric changes in the proximal region of the LBD resulting in dissociation of corepressor (SMRT) from TRβ1 (Davis et al. 2000). On the other hand phosphorylation of a serine residue in the AF-1 domain of estrogen receptor α (ERα) enhances interaction with steroid receptor RNA activator (SRA) in a ligand-independent manner (Deblois and Giguere 2003). In addition acetylation of lysine residues in the LBD of androgen receptor regulates ligand sensitivity and specificity (Fu et al. 2003). Recently discovered as a posttranscriptional modification SUMOylation of lysine residues within the hinge region of ERα which occurs strictly in the presence of hormone has been described to modulate ERα-dependent cellular response (Sentis et al. 2005). The interplay between the functional domains and the allosteric changes that are induced by modification in the different regions suggest that nuclear receptor transcriptional activity is not simply due to ligand binding. Thus, nuclear receptors enable at the target promoter the integration of a variety of information derived from extracellular signals, cell context and chromatin environment.

Nuclear receptor-mediated transcription requires several different protein complexes that can act sequentially, combinatorially or in parallel. In recent years a multitude of proteins and protein complexes have been shown to be nuclear receptor cofactors. Upon ligand binding conformational changes in the ligand binding domain occur. The position of helix 12, which contains the transactivation domain AF2 was determined by crystal structure analysis to be

critical for the binding of coregulators. Agonist binding induces position variation of the helix 12 forming a hydrophobic coactivator-binding surface which allows recruitment of transcription factors containing the leucine-rich motif (L-X-X-L-L) (Fig. 5).

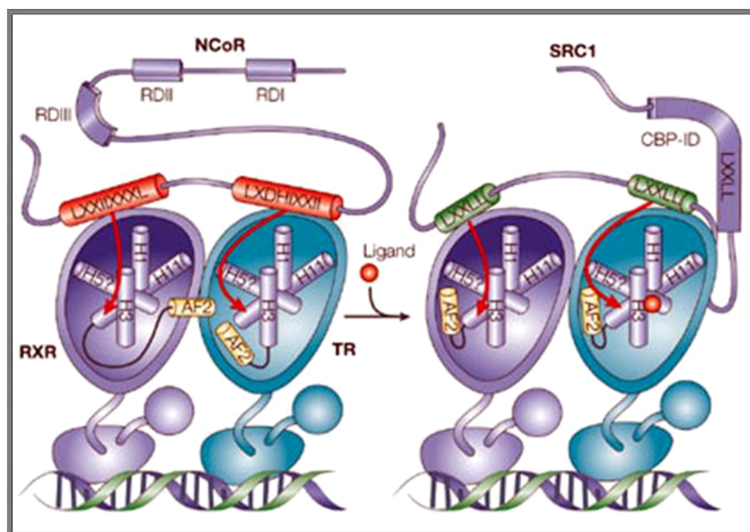



**Figure 5: Nuclear receptor coregulator interaction motifs**

The schematic representation illustrates conformational changes induced by ligand binding in the ligand binding domain of nuclear receptors which are critical for the interaction with coactivators and corepressors. Figure from (Perissi and Rosenfeld 2005) review.

ATP-dependent remodeling complexes such as SWI/SNF, complexes with histone acetylase activities (such as SRC/p160 family, CBP/p300 proteins or ADA complexes), and proteins with histone methylase activities (including CARM1 and PRMT1) are recruited to modify the chromatin structure. Subsequently, mediator complex (TRAP/DRIP/ARC) which coactivates NR-regulated gene expression are recruited. These facilitate the recruitment and the activation of the RNA polymerase II-associated basal transcription apparatus. In addition to these coactivators involved in the regulation of the chromatin structure and the assembly of the transcriptional initiation complex, factors containing E3 ubiquitin-protein ligase activities are required for efficient transcriptional activity. These are involved in the regulation of the nuclear receptor and coregulator turnover. Ubiquitination of nuclear receptors has been described for receptors from class I and II, and was linked to ubiquitin proteasome-mediated degradation (Reid et al. 2003). The nuclear receptor degradation is ligand-dependent and influenced by several activating events such as phosphorylation and acetylation, coactivator binding and crosstalk between different receptors. Whether all of these events contributing to the regulation of the nuclear receptor turnover are independent or are part of an interconnected mechanism is yet not clear (reviewed in (Nawaz and O'Malley 2004)). Preventing degradation has been shown to be deleterious to the regulation of transcription by certain receptors (Lonard et al. 2000). This suggests that nuclear receptor degradation and transactivation are mutually interdependent. It seems that this mechanism prevents cells

from overstimulation (Dennis et al. 2001); however coactivators also undergo proteasome-mediated degradation that may be required to exchange coactivator complexes for transcription initiation, elongation and RNA processing. Nuclear receptors in a repressive conformation (receptors bound to antagonist or unliganded thyroid and retinoid receptors) recruit repressive complexes to target promoters; these include ATP-dependent remodeling complexes and corepressors such as SMRT (silencing mediating of retinoid and thyroid hormones receptors) and N-CoR (nuclear hormone receptor corepressor) which associate directly or indirectly with histone deacetylases. These complexes generate a local chromatin environment that actively restricts transcription (Privalsky 2004).

### 1.2.4 Regulation of nuclear receptor-dependent transcription

Biochemical and structural studies have determined that initiation of transcription progresses through a series of ordered events. However, integrating time into the analysis of transcription with chromatin immunoprecipitation (CHIP) assays and fluorescence recovery after photobleaching (FRAP) revealed the dynamic, cooperative, functionally redundant and cyclical nature of gene expression. These techniques permit the evaluation of transcriptional processes kinetically with time resolutions from sub-seconds (FRAP) to minutes (CHIP). Real-time, single live cell imaging of transcription factors tagged with fluorescent proteins has shown that nuclear receptors are highly mobile in the nucleus and suggests that transcriptional activation is achieved through stochastic mechanisms. Whereas FRAP experiments mainly detect the bulk, rapid and potentially transient binding of factors, CHIP assays only detect the productive association of promoter sequences with specific transcription factors. With this latter technique, a number of groups analyzed the estrogen receptor-$\alpha$-mediated transcription of the pS2 promoter in MCF-7 cells in the presence of estrogen. They observed a cyclical recruitment of different complexes which correlated with defined histone modification patterns. In the presence of estrogen histone methyltransferase, histone acetyltransferase, transcription factors, RNA polymerase II, remodeling complex and histone deacetylases associate sequentially and periodically (Fig. 6). Interestingly coactivators and corepressors are alternately recruited; suggesting that promoter clearance provoking the promoter to return to a basal state is required for productive cycling. It was also observed that a given enzymatic function could be provided by functionally redundant enzymes which supports that coactivator or corepressor complexes may vary in their composition and that different routes may lead to transcription initiation (reviewed in (Metivier et al. 2006)).

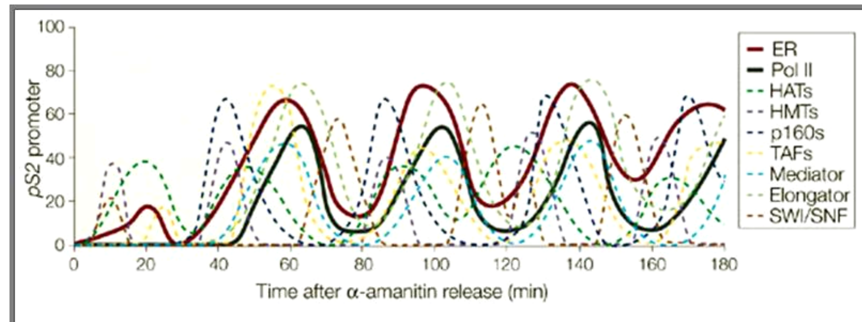



**Figure 6: Cyclical and combinatorial recruitment of cofactors on a natural target promoter**

Kinetics of cofactor recruitment to the pS2 promoter determined by CHIP analysis (Metivier et al. 2003) simplified and reviewed in (Perissi and Rosenfeld 2005). Different factors that have the same enzymatic activities have been grouped together. Cyclical and combinatorial recruitment of cofactors is required for transcription activation.

Metivier and colleagues proposed a model that integrates the high mobility of nuclear receptors as observed by FRAP with the longer cycle times determined by CHIP. They postulate that transcriptionally productive complexes have a slower mobility than transcription factors not engaged on the promoter. Progression through the cycle would depend on productive events that are less frequent than unproductive associations of factors resulting from rapid, stochastic and transient interactions. Consequently, progress is made when a specific and required factor becomes recruited at an appropriate time. The direction of the progression to transcription initiation would be determined by posttranslational modifications as well as by allosteric changes that occur on histones, chromatin associated factors, and interacting proteins at the promoter. This transcriptional cycling model is supported by discovery that nuclear receptors and coreguators can be ubiquitinated and degraded in the course of their activation. This proteasome-dependent degradation is required for the cycling of nuclear receptors on hormone-responsive promoters (Reid et al. 2003). Nuclear receptor turnover may also play a role in resetting the transcriptional apparatus in preparation for a subsequent response. Physiologically, a cycling mechanism and transcription-dependent degradation would enable continuous sampling of hormone exposure to ensure an appropriate response to stimulation. This mechanism suggests the strong interconnection between distinct processes including chromatin structure regulation and general transcription machinery which may be extended in the near future to downstream processes which are also regulated by nuclear receptors such as splicing and RNA maturation.

The regulation of nuclear receptor-dependent transcription results actually from the integration at the target promoter of a multitude of parameters including abundance, affinity and activity of nuclear receptors as well as coregulators ensuring gene-specific expression pattern cell-context and cell-type-dependent. In addition to previously described possible mechanisms of nuclear receptor transactivation modulation another level of regulation is

ensured by the differential expression of nuclear receptor types and isoforms. Distinct nuclear receptor isoform may be expressed depending on the cell type and/or the developmental stage. Each isoform can act as a functionally distinct transcription factor exerting synergistic or antagonistic effects. For example, the RARα isoform represses target gene expression in the absence of hormone, whereas RARβ and γ do not repress (Hauksdottir et al. 2003). The differences in their transcriptional regulation reflect the corepressor binding properties of each isoform. The tissue-specific variation of the cellular level of coregulators is believed to create cell- and temporal-specific transcriptional conditions. Indeed some coregulators are limiting factors for the transcriptional regulation of a specific receptor and are differently regulated. Coregulator expression can be modulated in a time and/or cell type-specific manner, resulting in the presence of different protein-protein complexes involved in transcriptional regulation. Variation of the nuclear localization and concentration of coregulators may also be controlled by regulation of the shuttling of coactivators and corepressors between the nucleus and the cytoplasm. This is still little understood; however recent discoveries support this supplementary level of regulation. Proteolysis of some cofactors has been shown to be a mechanism for modulating their cellular level. Transcriptional regulation by modulation of the enzymatic activity of nuclear receptor coregulators has also been investigated. Indeed, posttranslational modifications including phosphorylation, acetylation, methylation or SUMOylation under the influence of upstream signaling cascades are able to modulate the function of coregulators (Hermanson et al. 2002). In addition to regulation mechanisms altering protein-DNA interaction, complex formation and enzymatic activity, some preliminary data suggest that non-protein-coding RNAs are involved in the transcriptional regulation of steroid and non-steroid hormone receptors, besides their currently known regulatory functions in the cell (reviewed in (Mattick 2003)). The steroid receptor RNA activator (SRA) is an RNA transcript that functions as a eukaryotic transcriptional coactivator for steroid hormone receptors (Lanz et al. 2002). It was shown recently that SRA is also a thyroid hormone receptor coactivator directly interacting with a RNA binding motif lying between the DNA binding domain and the ligand binding domain of this receptor (Xu and Koenig 2004). It is now clear, that the transcriptional activity of nuclear receptors can be modulated in vatious manner. However, subsequent transcriptional regulation remains completely dependent on the structure of the promoter. Thus the combinatorial pattern of gene-specific factors and common coregulators on a promoter, in combination with expression levels and post-translational modifications, will establish a context-specific recruitment of required molecules and enzymatic activities.

Transcriptional regulation by nuclear receptors and coregulators is thus an extremely complex but very specific and delicately regulated event, involving many sophisticated modes of modulation to achieve and maintain homeostasis in the cell and the organism. In

this context, it is likely that any perturbation of the balance of coregulators influences the expression of target genes and thus participates in the development of disorders in an important manner. Indeed deregulated expression of corepressors is observed in cancers of the female reproductive system, leukemia, lymphomas and other cancers (reviewed in (Kumar et al. 2005)). Impaired localization of corepressors or altered interaction with corepressors can also affect transcriptional regulation and has been described to be involved in the pathogenesis of Huntington's nervous system disorder (Boutell et al. 1999). Furthermore, aberrant growth factor signaling which influences the functions of corepressors and their associated proteins can also dysregulate their functions. Altered kinase signaling and posttranslational modifications is a common feature of tumor cells. Pathogenesis can also be the consequence of mutations of receptors or chromosomal rearrangements. Nuclear receptor transcriptional activity can be impaired when receptor mutations result in a decreased ligand binding and/or defective ligand induced release of corepressor. Such mutations in the thyroxine-receptor (TRβ1) and the peroxisome proliferator-activated receptor-gamma (PPARγ) have been associated to resistance-to-thyroid-hormone syndrome (Clifton-Bligh et al. 1998; Safer et al. 1998) and to insulin resistance (Barroso et al. 1999), respectively. Acute promyelocytic leukemia and acute myeloid leukemia are caused by chromosomal translocations leading to the expression of transcription factors fused to the nuclear receptor RAR or to the zinc finger nuclear protein ETO, respectively, which contain corepressor interaction domains (Hiebert et al. 1996; Lin et al. 1999). The progression of these leukemias is linked to the aberrant recruitment of the N-CoR/SMRT corepressor complex containing histone deacetylase activity blocking differentiation and allowing uncontrolled growth of hematopoietic cells.

Although nuclear receptors are traditionally thought of solely as ligand transducers, unliganded thyroid hormone receptors and retinoid receptors are emerging as major players in many cellular processes and in development through their role as gene repressors. For instance, the dynamic and often abundant expression of the retinoid receptors does not necessarily correspond to the spatial and temporal availability of retinoic acid during the head formation in Xenopus or during skeletal development in the mouse (reviewed in (Weston et al. 2003)). Thus, these receptors have important function beyond transducing a retinoid signal. Receptor-mediated repression seems as important as activation throughout various embryonic processes. Nuclear receptor corepressor (N-CoR)-deficient embryos exhibit abnormalities in erythrocyte, thymocyte, and neural development and generally die around embryonic age e16 (Jepsen et al. 2000). Moreover, N-CoR has been categorized as a principal regulator of neural stem cell fate, in that its activity promotes self-renewal of these cells, repressing their differentiation into astrocytes (Hermanson et al. 2002). Knocking out other components of repressor complexes in vertebrates results mostly in embryonic lethality

attributed to major proliferation defects and developmental retardation caused at least in part by the derepression of otherwise silenced genes. It is now clear that corepressors are essential contributors to the biological actions of nuclear receptors and that aberration in corepressor function leads to endocrine malfunctions, neoplastic diseases, or severe developmental abnormalities.

## 1.3 Nuclear receptor corepressor N-CoR

### 1.3.1 N-CoR and SMRT

The best characterized nuclear receptor corepressor N-CoR (Hörlein et al. 1995) and the closely related protein SMRT (silencing mediator of retinoid and thyroid hormone receptors (Chen and Evans 1995) are involved in the active repression of basal transcription (Glass and Rosenfeld 2000). Both are recruited to promoter regions by receptors known to repress in the unliganded state, such as thyroid hormone receptor (TR), retinoic acid receptors (RAR) and the COUP-TF I/II orphan receptor. Additional nuclear receptors including peroxisome proliferator-activated receptors (PPARs) and vitamin D receptors (VDRs) among others have been identified to interact as well with N-CoR and/or SMRT in the absence of hormone. In addition, a number of nuclear receptors in the presence of their cognate antagonist also require N-CoR or SMRT correpressor complexes to mediate repression. Furthermore, N-CoR/SMRT are recruited by nonreceptor transcription factors that bind specific DNA sequences. For example, MyoD, NF-κb and AP-1 that play a role in the stimulation of cellular proliferation processes (mLee et al. 2000) or BTB/POZ domain containing protein such as PLZF or BCL6 (Melnick et al. 2002). HOX-PBX containing proteins regulating the expression of specific gene involved in cell fate and segment identity also recuit N-CoR/SMRT to mediate repression (Asahara et al. 1999). N-CoR was also found to be involved in active long-term repression of the growth hormone gene, recruited by the pituitary-specific POU domain factor Pit-1, in combination with other DNA binding factors (Scully et al. 2000). Together these observations indicate that N-CoR/SMRT repressive activity is required in the transcriptional regulation of a multitude of genes (reviewed in (Privalsky 2004). Both make direct contact with the ligand binding domain of many nuclear receptors and nucleate the assembly of a larger array of additional corepressor proteins including histone deacetylases (HDACs). N-CoR and SMRT are the principal point of contact between this larger complex and the nuclear receptors. Molecular events that regulate the interaction of N-CoR or SMRT with the nuclear receptor generally control the recruitment or release of the entire corepressor complex (Glass and Rosenfeld 2000; Privalsky 2001).

### 1.3.2 Corepressor function and regulation

In contrast to mediator or coactivator complexes, corepressor complexes interfere with the transcriptional initiation (Muscat et al. 1998; Wong and Privalsky 1998). These recruit histone deacetylases to the targeted promoter which mediate local chromatin structural changes and inhibit transcription (Nagai et al. 1990; Heinzel et al. 1997; Nagy et al. 1997). Guenther and coworkers reported that SMRT and N-CoR do not serve merely as platforms for HDAC recruitment. Both correpressors are able through their SANT domain to activate HDAC3 (Guenther et al. 2001) and enhance repression by increasing the affinity of HDAC3 enzyme for histone substrate (Yu et al. 2003). Recent studies emphasize the importance of the activation state of transcriptional coregulators which is altered by posttranscriptional modifications. DNA binding affinity, transcriptional activity and stability of transcription factors have been reported to be regulated by modifications such as acetylation (reviewed in (Minucci and Pelicci 2006)) or SUMOylation (reviewed in (Girdwood et al. 2004)) among other modifications. Transcription factor II B was shown to associate physically with N-CoR and the interaction resulted in the inhibition of transcription initiation (Muscat et al. 1998). Later, TFIIB was described to required autoacetyltransferase activity for full transcriptional activation (Choi et al. 2003). Although the direct link between N-CoR dependent deacetylase activity and the inhibition of TFIIB remains to be determined these data suggest that N-CoR may be involved in the regulation of the basal Pol II transcription machinery. These observations suggest that corepressors repressive activity may no be limited to mediate transcription repression through deacetylation of histone amino tails. It is likely that some of the corepressor complex-associated deacetylases are able to catalyze deacetylation of nonhistone proteins thereby contributing to the regulation of either corepressor complex-associated proteins and/or other factors that bind to the promoter region.

Recent publications report that a number of transcription regulators are SUMOylated and that this modification correlates in most cases with inhibition of the transcription. Histones, nuclear receptors, basal transcription factors and histone deacetylases have been reported to be substrates of SUMOylation. However, the exact mechanism by which the reversible covalent SUMO modification represses transcription remains unclear. Multiple mechanisms have been suggested in accordance with recent data (reviewed in (Gill 2005)). First, SUMO might compete with other posttranslational modifications (ubiquitination, acetylation) for substrate lysines. Consequently, SUMOylation might inhibit the interaction of the SUMOylated substrate with other binding partners, proteins or DNA, that are dependent on either an unmodified or an alternatively modified lysine. Second, modification by SUMO might induce critical conformational changes in important regulators of transcription. Third, covalent attachment of the SUMO polypeptide might confer new interactions, even independent of a conformational change in the substrate. In addition, increasing evidence

suggests that a complex crosstalk between acetylation and SUMOylation might be important for the regulated expression of many genes (reviewed in (Gill 2005)). SUMOylation enzymes have been newly described to interact and modify N-CoR (Tiefenbach et al. 2006) suggesting that these recently discovered enzymes (Okuma et al. 1999) may be an additional subunit of the corepressor complex. However, the direct link between SUMOylation enzymes recruited by N-CoR and SUMOylated substrates has not yet been determined. Although the repressive capacity of N-CoR/SMRT is mainly due to the recruitment of HDACs and the deacetylation of histones, other posttranslational modifying enzymes such as SUMO ligases and nonhistone deacetylases which can associate with the corepressor complexes and modulate the activity of transcription factors may also be involved.

Corepressors N-CoR and SMRT are paralogous products of a gene duplication event that occurred prior to the vertebrate evolutionary radiation. They are encoded by two distinct loci but share a common molecular architecture and approximately 45% amino acid identity. Since they share high structural homology and the complexes they form display analogous composition with only modest exceptions both utilize related modes of receptor interaction and transcriptional repression. Despite these similarities, a number of functional distinctions have been defined between N-CoR and SMRT. These two corepressors display different affinities for the different nuclear receptors. Additional differences have been detected in studies of their posttranslational modifications. Phosphorylation of SMRT in response to MAP kinase signaling leads to the dissociation of SMRT from its nuclear receptor partners and to its nuclear export; this is not true for N-CoR (Hong and Privalsky 2000). Conversely, Akt signaling can phosphorylate N-CoR directly resulting in nuclear export whereas the same domain of SMRT is not subject to this regulation (Hermanson et al. 2002). Important differences also exist between N-CoR and SMRT at the end of their lifespan. N-CoR interacts with RING protein Siah2 and is targeted for proteasomal degradation, whereas SMRT appears to be refractory to this form of regulation (Zhang et al. 1998). N-CoR and SMRT are differentially expressed in different tissues and at different times in development. This indicates that they are functionally distinct and the splicing variants they display further diversify the functional properties of these corepressors (reviewed in (Goodson et al. 2005)).

### 1.3.3 Structure and function of corepressor domains

N-CoR and SMRT are both ~270kDa in size, these large molecules can be divided into a C-terminal half that interacts with nuclear receptors and a N-terminal half that transmits the repression signal to the basal machinery and to chromatin. The corepressor complex consists of 10-12 associated proteins considered as integral subunits. Docking surfaces are principally located in the N-terminal and central regions. The corepressor core complex

includes histone deacetylases and other components that may serve as scaffolds, assist in substrat recognition, or regulate corepressor function. The WD-40 protein TBL1 (transducin-like protein and TBL1 related protein (TBL1-R) complex with N-CoR and SMRT and by making additional contacts with HDAC3, stabilize the quaternary structure of the corepressor assembly (Yoon et al. 2003). These factors also bind to histones H2B and H4 which may help in the chromatin substrate recognition. The G protein pathway suppressor 2 (GPS2) is another member of the corepressor complex which interacts with TBL1 and N-CoR (Zhang et al. 2002). As TBL1, GPS2 is a component of the G protein-coupled signal transduction pathways and may play additional roles as a regulator that couples signal transduction to transcriptional repression. The class I deacetylase HDAC3 and class II HDACs (HDAC4, 5 and 7) interact directly with N-CoR and SMRT (Li et al. 2000; Fischle et al. 2002) whereas HDAC1 and HDAC2, integral subunits of the mSin3 complex (Hassig et al. 1997), may associate indirectly. Although N-CoR/SMRT does not appear to be stably associated with the mSin3 complex, mSin3 and the SAP30 subunit interact with SMRT and N-CoR directly (Heinzel et al. 1997; Nagy et al. 1997; Laherty et al. 1998). Since the Sin3/HDAC complex lacks any DNA-binding activity, it must be targeted to gene promoters by interacting with DNA-binding proteins and is an important corepressor for many nonreceptor transcription factors (Laherty et al. 1997). It is thought that N-CoR/SMRT may serve as an adapter molecule between the core mSin3 complex and sequence-specific transcriptional repressors such as unliganded nuclear receptors. All previously discussed cofactors mainly interact in the regions determined as repression domains (RDs). The overall architectures of the N-CoR and SMRT proteins are similar. Depited in (Fig. 7), the detailed domain organization specific to N-CoR.

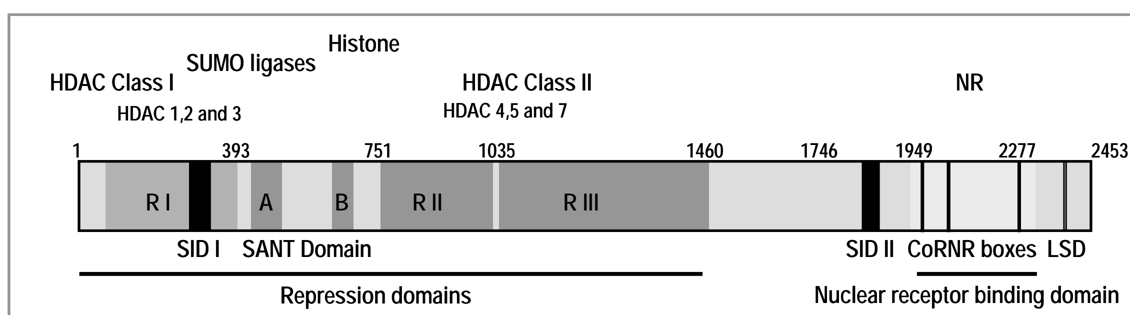



**Figure 7: Domain organization in the corepressor N-CoR**

Three repression domains (RD I, II and III) are contained in the amino-terminal half of N-CoR as is the SANT domain (**S**WI3, **A**DA2, **N**-CoR and **T**FIIIB). Two interacting domains with mSin3 (SID I and II) have been identified at the amino-terminus as well as at the carboxy-terminus. The nuclear receptors interaction domain (NID) is located at the carboxy-terminus and contains 3 CoRNR motifs in N-CoR. At the extreme C-terminus the lysine-serine-aspartic acid (LSD) motif which was determined to be essential for the interaction between SMRT and Sharp is also found in N-CoR.

Between the first and second repression domain a conserved SANT (SWI3, ADA2, N-CoR, and TFIIIB) domain is found (Aasland et al. 1996). This motif is found in a number of chromatin remodeling factors. It consists of two 50-amino acid motifs named SANT1 and SANT2. The N-CoR and SMRT SANT1 domain are part of the HDAC3 activating domain (DAD) (Guenther et al. 2001) and it is also involved in the interaction with the SUMO conjugating enzyme UBc9 and the SUMO ligase Pias1 (Tiefenbach 2003 August). The SANT2 domain functions as a histone interaction domain (Yu et al. 2003). SMRT and N-CoR bind to nuclear receptors, through the set of C-terminal “CoRNR box” motifs (or L-X-X-I/H-I-X-X-X-L/I) (Hu and Lazar 1999; Perissi et al. 1999). The CoRNR motif forms an extended α-helical domain compared to the coactivator nuclear receptor binding motif (L-X-X-L-L) (McKenna et al. 1999). Corepressor binding motifs dock into a complementary groove formed by helices 4/5/6 in the ligand binding domain of nuclear receptors (Xu and Lambert 2003) (Fig. 5). Dissociation of corepressor from the nuclear receptor is induced upon agonist binding resulting in conformational changes of the ligand binding domain and the occlusion of the corepressor docking surface by the reorientation of the NR helix 12. For the corepressor SMRT, Sharp (**S**MRT/**H**DAC1 **A**ssociated **r**e**p**ressor) was determined as a new interacting protein and was determined as a potent transcriptional repressor whose repression domain (RD) interacts directly with SMRT and at least five members of the NuRD (**nu**cleosome **r**emodeling and histone **d**eacetylation) complex including HDAC1 and HDAC2 (Zhang et al. 1998; Wade et al. 1999). A conserved LSD motif upstream of the last amino acid residue in SMRT was demonstrated to be necessary for the interaction between Sharp and the corepressor (Shi et al. 2001). According to the sequence analysis of N-CoR and SMRT, the high degree of homology observed at the extreme carboxy-terminal end of the proteins indicates that this domain is conserved because of its biological function. Although it is likely that this domain has a specific function it was not elucidated for N-CoR. Hörlein and coworkers could show that this domain is not required for the repression activity of N-CoR and contains no inherent repression activity. This finding and other observations promoted me to further investigate the function of the N-CoR extreme C-terminus.

## 1.4 Aims of the Ph.D Thesis

The C-terminal region of N-CoR and SMRT is located adjacent to the nuclear receptor interacting domain. Since this domain, according to its function, is at the surface of the protein it was assumed that the adjacent carboxy-terminal region would rather be exposed to the environment than buried in the molecule. Moreover, protease degradation analysis of N-CoR (Fletterick, R personal communication) suggested that the C-terminal region constitutes a functional domain. Furthermore, by homology to platform molecules that contain a succession of either functional and/or interacting domains it was postulated that this region is

involved in protein-protein interactions. Indeed, several proteins were identified in a yeast-two-hybrid screen which could interact with the extreme C-terminal domain of N-CoR (aa: 2290-2453) (Ducasse 2002). The set of putative interaction partners that interact directly with N-CoR in *in vitro* binding assays were further investigated during my thesis.

The aim of my Ph.D thesis was to characterize the function of the C-terminus of N-CoR. At first, interaction of potential candidates with endogenous N-CoR had to be confirmed in co-immunoprecipitation experiments and to be verified by confocal microscopy. Subsequently, the molecular mechanisms had to be characterized and functional assays had to conducted. Furthermore, the effect of the down-regulation of endogenous interaction partners had to be analyzed. Therefore, generation of specific siRNAs and evaluation of their efficacy in mRNA and protein downregulation after transfection had to be performed. In parallel, the N-CoR binding domains in interacting proteins had to be characterized, generating GST-fusion proteins and performing pulldown assays. Thereafter, deletions mutants could be generated and tested in reporter assays. Effects of the mutants on the repression capacity of N-CoR had then to be compared to those obtained with the wild type protein. Finally, a model of the potential molecular mechanisms had to be established supporting the biological relevance of the interaction of the N-CoR C-terminus with the novel identified partner.

# 2 Materials and Methods

If not mentioned otherwise, molecular biological methods were used according to Sambrook et al. or Ausubel et al. and modified when necessary.

## 2.1 General molecular biology techniques

### 2.1.1 Analytic and preparative isolation of nucleic acids (DNA/ RNA)

#### DNA isolation on anion exchange resin

DNA Plasmids are generally amplified in bacteria. Since these vectors contain a antibiotic resistance gene, they are amplified in transformed bacteria grown under selection pressure in media containing corresponding antibiotics. In the first step bacterial chromosomal DNA and proteins are separated from the plasmid DNA. Plasmid DNA is isolated from E. coli DH10ß cells using different commercially available kits, depending on the DNA amount needed. For small amounts up to 20 µg the NucleoleoSpin Plasmid Kit from Macherey-Nagel is used (mini preparation). For preparative isolation up to 500 µg the Maxi Kit from Genomed is used (maxi preparation). The purification of plasmid DNA from the bacteria is generally performed according to the manufacturer's instructions. Briefly, this purification procedure is based on alkaline lysis (NaOH-SDS buffer) of the resuspended bacterial cells in the presence of RNase A. Neutralization with potassium acetate causes precipitation of SDS, thereby forming large complexes containing denatured proteins, chromosomal DNA, and cellular debris, while plasmid DNA remains in solution. After clearing the lysate by centrifugation, the plasmid DNA is immobilized on a resin matrix (anion exchange resin), washed and eluted (high salt concentration). For large scale isolations the plasmid DNA is finally precipitated by adding 0.7 volumes of isopropanol to the eluate and centrifuging 20 min at 12000 g and 4°C. The DNA pellet is washed with 5 ml 70% ethanol and centrifugated for 5 more minutes. Plasmid DNA is air-dried and dissolved in sterile, deionized water (200-500 µl/maxi).(Shapiro 1981)

#### DNA Ethanol precipitation

Ethanol precipitation enables isolation of plasmid DNA from enzymatic reactions (digestion, dephosphorylation or PCR reaction). 2.5 times of the volume of the DNA solution of ice-cold 100% ethanol and 1/10 3 M sodium acetate are added to the DNA sample. Mixture is vortexed for 1 min and centrifuged in a cold microfuge (4°C). Afterwards, the pellet is washed with 70% ethanol centrifuged 5 min at 13,000 rpm and air-dried. It is then dissolved in an appropriate volume of $H_2O$ (10-20 µl).

#### DNA gel extraction

After separation of the DNA on a 1% agarose gel, the fragment of interest is cut out with a scalpel (minimize surrounding agarose) and the extraction is performed with gel extraction kit (Pec-Lab Erlangen) according to the manufacturer's protocol. Briefly, this procedure involves

the melting of the agarose gel slice followed by separation of the DNA from contaminants using spin columns containing a silica-gel membrane. Whereas the DNA fragments adsorb to this silica-gel membrane at high salt concentration, contaminants such as agarose, dyes, and ethidium bromide stay in the supernatant. DNA fragments are finally eluted with water and either directly used in ligation reactions or stored at -20°C.

**RNA Isolation**

To analyze endogenous gene expression, mRNA or total RNA is isolated from cells. Expression of target gene is analyzed by quantifying target mRNA by semi quantitative PCR or real time PCR. For RNA purification, cells are first lysed and then homogenized through QIAshredder spin columns (Qiagen). Ethanol is added to the lysate to provide ideal binding conditions. Afterwards, the lysate is loaded onto the RNeasy silica-gel membrane. After the adsorption of the RNA all contaminants are efficiently washed away. The pure and concentrated RNA is eluted with water.

Protocol:

| | |
|---|---|
| 1. | Harvest cells ($2x10^6$ cells /10 cm dish are splitted 48 h before), trypsinize and centrifuge 3000 g/ 5min |
| 2. | Lyse cells with 600 µl lyse buffer RTL (add fresh ß-mercaptoethanol, 10 µl/ml), vortex |
| 3. | Homogenize sample through QIAshredder spin column |
| 4. | Add same volume of 70% ethanol to lysate |
| 5. | Load mixture onto the RNeasy column centrifuge quickly |
| 6. | Proceed to DNase I digestion on the column |
| 7. | Wash twice with 500 µl RPE buffer centrifuge 15 s at 8000 g |
| 8. | Dry silica-gel membrane by centrifuging 2 min at 8000 g |
| 9. | Elute RNA with 50 µl with RNase free-water |

10 µl of sample are kept for RNA concentration determination and FA gel electrophoresis. The rest is stored at -80°C.

| | | |
|---|---|---|
| RNeasy Mini Kit | Isolation of RNA from cells or tissues lysates | Qiagen |
| RNAsin Ribonuclease Inhibitor | Inhibition of RNAses | Promega |
| QIAshredder spin columns | Homogenization of cell and tissue lysates | Qiagen |
| DNase RNase free DNase Set | Efficient on-column digestion of DNA | Qiagen |

### 2.1.2 Determination of DNA and RNA concentration

**Determination of DNA concentration**

To measure DNA concentrations, purified plasmid preparations are diluted 100-fold in $dH_2O$ and transferred into a quartz cuvette. The absorption measured at 260 nm in a spectrophotometer (WPA S2000) enables determination of the DNA concentration. The absorption of 1.0 at λ = 260 nm corresponds to a concentration of 50 µg/ml. The ratio of the absorptions measured at 260 nm and 280 nm indicates the quality of the DNA isolation. A value between 1.7 and 2.0 indicates a good quality.

Abs. (260 nm) x DNA extinction coefficient (50 ds DNA) x dilution factor = DNA concentration in µg/ml

### Determination of RNA concentration

The concentration is determined at two different dilutions (1:100 and 1:66) by spectrophotometry. The absorption measured at 260 nm in a spectrophotometer (*WPA S2000*) enables the determination of RNA concentration considering that an optical density of 1.0 at $\lambda$ 260nm corresponds to a concentration of 40 µg/ml (Sambrook, 1989). The quality of the RNA isolation is related by the ratio 260 nm/280 nm. A value between 1.7 and 2.0 indicates a good quality. The integrity of the total RNA isolation can also be assessed by electrophoresis followed by staining with ethidium bromide (see RNA formaldehyde agarose gel electrophoresis).

Abs. (260 nm) x RNA extinction coefficient (40 for RNA) x dilution factor = RNA concentration in µg/ml

## 2.1.3 DNA and RNA electrophoretic analysis

Nucleotide acids can be separated and quantified by agarose gel electrophoresis. This method is used during the procedure of cloning to isolate a fragment amplified by PCR, to isolate an insert or a vector after digestion, to check the isolated plasmid for its correct size, to control DNA quality and to compare DNA concentrations. In addition this method is also used to visualize semi quantitative PCR products or to control the quality of total RNA isolations.

### DNA agarose gel electrophoresis

DNA fragments are separated by gel electrophoresis in agarose gels of 1-1.5% (w/v). Electrophoresis is performed at 8 V/cm gel length with TAE running buffer. To visualize DNA under UV light, ethidium bromide is added to the melted gel (3 µl/100 ml Gel) before pouring it in a suitable chamber (*Mini Sub$^R$ cellGT Biorad*). The addition of ethidium bromide permits quantitative and qualitative detection of DNA at a wavelength $\lambda$ = 360nm, because ethidium bromide is fluorescent at this wavelength only when it is intercalated into the DNA backbone (Helling et al. 1974). 5 µl DNA ladder is used to verify the length of the and to estimate DNA concentration.

| | |
|---|---|
| TAE buffer (1x) | 40 mM Tris-HCl; 1 mM EDTA; adjusted to pH 8.0 with acetic acid |
| DNA loading buffer (6x) | 0.25% bromophenolblue (w/v); 0.25% xylenecyanol (w/v); 15% Ficoll |
| DNA molecular weight marker | Smart DNA ladder 10 kbp-200 bp(*Eurogentec*) |

### RNA formaldehyde agarose gel electrophoresis

Ribosomal RNA (rRNA) comprises over 80% of total RNA sample in contrary to mRNA comprising only 1 to 3%. The major species, in mammalian system, are the 28S and 18S ribosomal subunits which quality and quantity is assumed to reflect that of the underlying mRNA population. However, these rRNAs are more abundant and stable than the mRNA, which turns over much more rapidly.

The integrity of the total RNA isolation is controlled by formaldehyde agarose gel electrophoresis followed by staining with ethidium bromide. Two bands appear on the denaturing gel corresponding to the 28S rRNA and 18S rRNA that are both contained in the

isolate. The ratio 2:1 and sharpness of the bands are considered and compared to concentration and ratio. If notable degradation appears, the complete RNA isolation is renewed.

5 µl of sample are used to perform this control. 1 volume 5x RNA loading buffer is added to 4 volumes of RNA sample (5 µl loading buffer + 5 µl RNA + 15 µl RNase free-water) and is incubated for 3-5 min at 65°C, subsequently chilled on ice and loaded onto an equilibrated FA gel.

**Formaldehyde agarose gel preparation**

| | |
|---|---|
| **1.2% FA gel** | 1.2 g agarose (RNase free) |
| | 10 ml 10x FA gel buffer |
| | Add RNase-free water to 100ml |

The mixture is heated in order to melt the agarose and cooled in water bad before adding 1.8 ml of 37% (12.3 M) formaldehyde and 1 µl of a 10 mg/ml ethidiumbromide stock solution. Prior to running, the gel is equilibrated in 1xFA gel running buffer for 30 min. Gel is run at 5 V/cm.

**Buffers and solutions**

| | |
|---|---|
| **10 X FA gel buffer** | 200 mM 3-[N-morpholino]propanesulfonic acid (MOPS) |
| | 50 mM sodium acetate |
| | 10 mM EDTA |
| | pH to 7.0 with NaOH |

| | |
|---|---|
| **running buffer 1X FA gel** | 100 ml 10x FA gel buffer |
| | 20 ml 37% (12,3 M) formaldehyde |
| | 880 ml RNase -free water |

| | |
|---|---|
| **RNase free water** | 0,1 % DEPC (diethyl pyrocarbonate) in water, incubated 12 h at 37°C and autoclaved before use |

| | |
|---|---|
| **loading buffer 5x RNA** | 16 µl saturated aqueous bromophenol blue |
| | 80 µl 0,5 M EDTA, pH 8,0 |
| | 720 µl 37% (12,3 M) formaldehyde |
| | 2 ml 100% glycerol |
| | 3084 µl formamide |
| | 4 ml 10x FA gel buffer |
| | RNase -free water added to 10 ml |

## 2.1.4 DNA enzymatic analysis and modifications

### DNA Control digestion

Generally, 5 units /µg DNA of restriction enzyme are used for the cleavage of plasmid DNA. Restriction enzyme digestion is carried out in the supplied restriction buffer and, if recommended by the manufacturer, 10% BSA are added depending on the enzyme used (*New England Biolabs, Schwalbach or Fermentas*). To avoid unspecific enzymatic cleavage, it has to be ensured that the reaction mixtures contained less than 5% glycerin. For control digestion, enzymatic reaction is usually performed for 2-3 h at 37°C. Reactions are stopped by adding DNA loading buffer and loading the whole sample on an agarose gel. (Roberts. 1996)

### DNA preparation for cloning

PCR products are either precipitated with ethanol or extracted from an agarose gel before digestion. When the restriction enzymes are compatible, the insert is directly double digested overnight, if not the digestions are preformed sequentially. 5-10 µg of vector for cloning are prepared in parallel with the appropriate amount of enzyme.

| Enzymes | New England BioLabs or Fermentas |
| --- | --- |

### DNA dephosphorylation

To minimized self ligation events, the digested vector is dephosphorylated. The calf alkaline phosphatase (*CIP, New England BioLabs*) is used to catalyze the removal of 5`dephosphate groups from the linearized vector. After 1 h incubation at 37°C, enzyme deactivation is achieved by heating the sample for 20 min at 85°C. The dephosphorylated vector is then precipitated with ethanol and resuspended in $H_2O$. The concentration of the vector that is now ready for ligation is estimated by loading some µl of the sample onto an agarose gel. A DNA ladder that allows quantification by optical comparison of the bands is loaded together with the sample onto the gel.

### Ligation of DNA fragments

Ligation of a DNA fragment into the backbone of a vector of choice is catalyzed by the T4 DNA-ligase. Insert and vector have to contain compatible restriction sites. The T4 DNA ligase catalyzes phosphodiester bounds formation between the nucleotide 5'phosphates of the insert and the nucleotide 3'OH groups of the vector. The best ratio of vector and insert for ligation was calculated with the following formula:

Insert (ng) = Vector (ng) x (Insert length (bp) / Vector length (bp))

The insert and 20-200 ng vector are mixed in a 20 µl volume reaction containing the ligation buffer supplied with the ligase enzyme and 2 units T4 DNA ligase (*Fermentas*). The ligation reaction is incubated for 2 h at 22°C or alternatively overnight at 16°C. Deactivation of the T4 DNA ligase is obtained by heating the sample at 65°C for 10 min. 1-8 µl of the ligation reaction are used for transformation of 50-100 µl of an electrocompetent DH10ß E.*coli* strain. For blunt end ligation, 50% PEG 4000 and 4U T4 DNA ligase are required additionally.

### DNA recombination

The Gateway® Cloning Technology (*Invitrogen)* is based on site-specific recombinations of the phage lambda. The reaction is specific and directional, enabling DNA transfer between different cloning vectors, while maintaining orientation and reading frame. This method effectively replaces the use of restriction endonucleases and ligase, since excision and integration take place in the same reaction. The advantage of this method is that it is possible to transfer a sequence from one vector backbone to another time efficiently. The sequence of interest has first to be inserted in an Entry vector by classical cloning or by recombination of a PCR product flanked by two recombination sites. Transfer of the gene in a destination vector is then achieved by recombination. Entry vectors are transcriptionally silent, contain a kanamycin resistance gene, and are flanked by two recombination sites

(*att*L1 and *att*L2), whereas Destination vectors contain all the sequence information required for expression, an ampicillin resistance gene and two recombination sites (*att*R1 and *att*R2). All plasmid recombination sites flank a gene for negative selection, *ccd*B. Att1 and att2 sites confer directionality and specificity for recombination, so that only attL1 will react with attR1, and attL2 with attR2. LR Clonase™ reaction enables excision and insertion of the two sites and thereby exchanging DNA sequences flanked by the restriction sites. Selecting for ampicillin resistance eliminates the starting vector and the by-product. The presence of the negative selection marker ensures elimination of vectors that have not been recombined during the reaction.

Protocol

| | |
|---|---|
| LR clonase$^{TM}$ Reaction Buffer | 5 µl |
| Destination vector (linearized)* | 300 ng |
| Entry clone | 100 ng |
| LR Clonase$^{TM}$ Enzyme Mix | 4 µl |
| Add water to final volume | 20 µl |

* linearize the *Destination vector* within the *att*R cassette avoiding the *ccd*B gene.

| | |
|---|---|
| 1. | Mix and incubate for one hour at 25°C |
| 2. | Add proteinase K solution and incubate 10 min. at 37°C |
| 3. | Transform competent DH10ß; 1 µl per 30 µl cells |
| 4. | Grow for one hour and select on appropriate LB AMP plate |

To verify successful cloning control digestion is performed with *Bsp*1407I (*Bst*BI) which have restriction sites in the recombination boxes (*att* Boxes).

### 2.1.5 DNA and cDNA amplification by poly-chain reaction

#### Polymerase chain reaction (PCR)

The amplification of DNA fragments is performed in a GeneAmp 9600 thermocycler (*Perkin-Elmer*). Amplification reactions are set in a final volume of 50 µl in a 200 µl reaction tube, containing 2.5 units Pfx platinum DNA polymerase (*Invitrogen),* 20-100 ng of template DNA, 200 µM of each deoxyribonucleotides (dATP, dCTP, dTTP and dGTP), 50 µM $MgSO_4$) the corresponding polymerase reaction buffer (1x), and 0.5 µM of both oligonucleotide primers. The reaction mixtures are prepared on ice. Samples are set in a PCR machine that is programmed as shown below:

| Cycle | Temperature | Time |
|---|---|---|
| 1X | 94°C | 3 min |
| 35x | 94°C | 30 sec |
| | (Tm-2)°C | 30 sec |
| | 68°C | 1 min/kbp |
| 1X | 68°C | 10 min |
| | 4°C | unlimited |

The lid is heated to 110°C in order to avoid evaporation of the samples. Before proceeding to cycling, the DNA template is completely denaturated for 3 min at 94°C. Depending on the primers that are used in the PCR, annealing temperature has to be adapted usually to a

temperature 2°C below the primer melting temperature (Tm). Elongation step is executed at 68°C, which is the optimal catalytic temperature for the Pfx platinum DNA polymerase. This polymerase processes 1 kb per minute and the elongation time has to be adapted according to the length of the PCR product. Amplified DNA fragments are checked (4 µl from PCR reaction) by agarose gel electrophoresis. Before digestion, amplification products are isolated from PCR reaction by ethanol precipitation and resuspended in 20 µl of water. DNA can be stored at -20°C.

**Semi-quantitative PCR**

As a template for semi-quantitative PCR 1 µl of 1:40 diluted cDNA obtained by reverse transcription (RT PCR, see below) is used. The total volume of reaction is 25 µl (200 µl reaction tubes, Sarstedt). The conditions for the reaction are the same as for a PCR reaction (see above). Elongation time is 30 seconds because all the fragment lengths are smaller then 500 bp. The number of cycles has to be adapted to signal intensity and cDNA dilution. E.g., for the house keeping gene 18S 20-25 cycles are sufficient whereas GAPDH requires 33 PCR cycles.

**Reverse transcription (RT-PCR)**

To generate cDNA from an RNA template First-Strand cDNA Synthesis Kit (*Amersham Bioscience*) is used. This enables the reverse transcription of RNA into cDNA with random primer pd(N)$_6$. 0.5µg total RNA isolate are used in 15 µl final volume of first strand reaction. The resulting double-strand RNA-cDNA heteroduplex is directly used for amplification by PCR. cDNA samples are stored at -80°C.

Protocol

| | |
|---|---|
| 1. | RNA sample is brought to the final volume of 8 µl<br>Incubated 10 min at 65°C |
| 2. | 5 µl Bulk first-strand reaction mix |
| 3. | 1 µl DTT(from 200 mM solution) |
| 4. | 1 µl pd(N6 (1:10 dilute 0,02 µg) |
| 5. | Incubate 37°C 1 h |
| 6. | Store at -80°C |

1 µl of a 1:40 dilution of cDNA reaction is added to 25 µl PCR reaction.

**Site directed mutagenesis**

Using site-directed mutagenesis, the DNA sequence in genetic material can be changed. Two synthetic DNA oligonucleotides containing the desired mutation and annealing to the same DNA sequence on opposite strands of the plasmid are required for the reaction. Amplification of the plasmid with mutated primers permits deletion, insertion or point mutation in the plasmid DNA sequence. This reprogrammed DNA molecule can direct the synthesis of a protein with an exchanged, deleted or inserted amino acid or can present an additional restriction site at a determined position in its sequence. This method is composed of two essential steps. In the first step the template with an integrated point mutation is amplified, during the second step the non-mutated parental DNA template is destroyed.

Complementary oligonucleotides of 25-45 bp length containing the desired mutation flanked by unmodified nucleotides sequence (10-15 nucleatides on both sides) are designed and synthesized. This primer pair is used for the mutant strand synthesis by PCR with the PfuTurbo DNA polymerase (Stratagene). Cycling reaction is followed by DpnI digestion, which cleaves methylated and hemimethylated DNA strands only, thereby digesting the parental DNA template. Bacteria are transformed and afterwards screened for positive clones.

**Mutant strand synthesis**

| 5-50 ng plasmid template DNA |
|---|
| 1x mutagenesis buffer in a final volume of 50 µl in $H_2O$ (20 mM Tris HCl, pH 8.8, 10 mM KCl, 10 mM (NH4)2SO4, 2 mM $MgSO_4$, 0.1% triton X-100, 0.1 mg/ml BSA) |
| 125 ng of each primer |
| 250 µM each dNTP, |
| 2.5 U PfuTurbo DNA polymerase, (Stratagene) |

For the PCR conditions primer melting temperature and vector length are used to determine the temperature of hybridization and the duration of the elongation step while cycle number is used as indicated in the manufacturer's protocol for a single site mutation. *Dpn*I digestion is performed by adding 10 U *Dpn*I to the amplification reaction and incubating at 37°C for 1 h. One 1 µl of *Dpn*I- treated DNA is used afterwards for transformation.

| | |
|---|---|
| QuikChange® Multi Site-Directed Mutagenesis Kit | Stratagene |

## 2.1.6 pSuper RNAi system

In several organisms, the introduction of double-stranded RNA has proven to be a powerful tool to suppress gene expression through a process known as RNA interference (Sharp. 1999). However, high production costs limit the utility of this technology. Additionally, RNA interference provokes in most mammalian cells a strong cytotoxic response. Recently, several DNA-based plasmid vectors have been developed that direct transcription of small hairpin RNAs, which are processed into functional siRNAs (small interfering RNAs) by cellular enzymes. Therefore the pSuper RNAi System (Invitrogen) can be used to cause efficient and specific down-regulation of gene expression (4, Brummelkamp, R. Bernards, and R Agami, Science 296, 550 (2002)), resulting in functional inactivation of the targeted genes. The pSuper DNA plasmid provides a mammalian expression vector that directs intracellular synthesis of siRNA-like transcripts. The vector uses the polymerase-III H1-RNA gene promoter, as it produces a small RNA transcript lacking a polyadenosine tail and has a well-defined start of transcription and a termination signal consisting of five thymidines in a row (T5). Most importantly, the cleavage of the transcript at the termination site is after the second uridine, yielding a transcript resembling the ends of synthetic siRNAs, which also contain two 3' overhanging T or U nucleotides (nt).

### Design of oligonucleotides

To ensure the silencing of a specific gene, the pSuper vector is used in combination with a pair of custom oligonucleotides that contain, among other features, a unique 19-nt sequence derived from the mRNA transcript of the gene targeted for suppression (the “N-19 target sequence”). The N-19 target sequence corresponds to the sense strand of the pSuper-generated siRNA, which in turn corresponds to a 19-nt sequence within the mRNA. In the mechanism of RNAi, the antisense strand of the siRNA duplex hybridizes to this region of the mRNA to mediate cleavage of the molecule. These forward and reverse oligonucleotides are annealed and have to be cloned into the vector, between the unique *Bgl*II and *Hind*III enzyme sites positioned downstream from the H1 promoter’s TATA box to generate the desired siRNA duplex. The forward and reverse oligonucleotides are synthesized with *Bgl*II and *Hind*III overhangs at their 5’ ends respectively, therefore no digestion is required prior to cloning. To enable more efficiently the screening of positive clones the 5’ *Bgl*II overhang of the forward oligonucleotide contains a point mutation that destroys the *Bgl*II palindrome upon ligation. The sequence of the oligonucleotide includes the unique N-19 target in both sense and antisense orientation, separated by a 9-nt spacer sequence. The resulting transcript of the recombinant vector is predicted to fold back on itself to form a 19-base pair stem-loop structure. Analysis indicates that the stem-loop precursor transcript is quickly cleaved in the cell to produce a functional siRNA. General steps for cloning oligonucleotides in the double digested (*Bgl*II and *Hind*III) pSuper vector:

| | |
|---|---|
| 1. | Anneal the forward and reverse strands of the oligos that contain the siRNA-expressing sequence targeting your gene of interest |
| 2. | Linearize the pSuper vector with *BglII* and *HindIII* and dephosphorylate with CIP |
| 3. | Clone the annealed oligos into the vector |
| 4. | Transform the vector in bacteria |
| 5. | Transfect pSuper vector into mammalian cells |

### Oligonucleotides protocol

64 mers oligonucleotides are annealed and phosphorylated before ligation into pSuper. Complementary oligos are dissolved in water to a final concentration 1 mM. For annealing 1 µl of each oligos is used in 48 µl annealing buffer, incubated 4 min in 95°C and slowly cooled down. This is achieved by incubating the annealing reaction in a recipient field with boiling water and allowing to cool down to room temperature. Samples can be frozen at -20°C. Synthesized oligos do not contain any phosphate at their 5’ ends; therefore, they have to be phosphorylated. 2 µl of annealing reaction are mixed in a 10 µl volume reaction containing the PNK buffer, 1 mM ATP supplied with the T4 polynucleotide kinase (PNK) (NEB). This enzyme catalyzes the transfer and exchange of Pi from the γ position of ATP to the 5´-hydroxyl terminus of polynucleotides (double-and single-stranded DNA and RNA). After 30 min at 37°C T4 PNK is inactivated by heating 10 min at 70°C. 2 µl of annealed and phosphorylated oligos are added to 200 ng pSuper for ligation.

| | |
|---|---|
| Annealing buffer | 100 mM potassium acetate, 30mM HEPES-KOH pH 7.4, 2 mM Mg-acetate |

T4 polynucleotide kinase — New England Biolabs

### 2.1.7 DNA sequence analysis

The DNA sample was sequenced in an ABI377 sequencing machine. The used sequencing primers are marked with fluorescent dyes (‘dye primer chemistry’). The sequencing was done by the in-house institute service.

Gene banks existing sequences were used for primers design and to compare clone sequences. Sequence information is investigated via internet with BLASTN (comparison of the nucleotide sequence with the nucleotide sequence database) and BLASTX (comparison with the nucleotide sequence in all the reading frames with the protein sequence database, National Center for Biotechnology Information /NCBI http://www.ncbi.nlm.nih.gov/blast/blast.cgi). Information concerning amino acid sequences, protein domains and their secondary / tertiary structure was also obtained from protein databases. (Brookhaven Protein Data Bank/ PDB, http://www.rcsb.org/pdb/; SwissProt. http://www.expasy.ch/sprot/)

## 2.2 Cloning strategies, primers and plasmids

### 2.2.1 Primers used for cloning

**Cloning of the different N-CoR interaction partners out of the YTH screen**

Except for the ESET protein for which the mammalian expression plasmid was sent by Liu Yang, specific primers were designed and synthesised (Thermohybaid) for MBD3, CtBP and NonO protein. Full-length coding sequences were amplified by PCR for MBD3 and NonO from NIH 3T3 (mouse fibroblasts) total cDNA and CtBP from a plasmid sent by Criqui Filipe. Primers used for CtBP and MBD3 are listed in the following table:

<table>
<tr><td>CGGGAATTCAATGGGCAGCTCCAAC</td><td>CtBP Fw ATG (EcoRI)</td><td rowspan="2">CtBP, C-terminal binding protein</td></tr>
<tr><td>AAAGATATCCAACTGGTCACTCGTATGGTC</td><td>CtBP Rev no STOP (EcoRV)</td></tr>
<tr><td>GGGGACAAGTTTGTACAAAAAAGCAGGCTG-GAGGGCCACAATGGAGCGGAAGAGGTGGG</td><td>MBD3 FW recombination box for GATEWAY</td><td rowspan="2">MBD3</td></tr>
<tr><td>ACCACTTTGTACAAGAAAGCTGGGTC-CACTCGCTCTGGCTCCGGCT</td><td>MBD3 Rev recombination box for GATEWAY</td></tr>
</table>

PCR amplified sequences are digested and isolated before ligation in the appropriate Entry vector if primers contained restriction sites. If primers contained recombination box sequences the amplificat was directly added to BP reaction containing pDONOR vector.

**NonO constructs FL, RRM 1 and RRM2 deletion mutants**

Gateway cloning system was partially used for the cloning of NonO full-length and deletion mutants lacking RRM 1 (deletion of RNA recognition motif 1) or lacking RRM2 (deletion of RNA recognition motif 2) in different expression vectors. Full-length coding sequence was amplified by PCR out of NIH 3T3 cDNA (mouse fibroblasts) and was inserted into pENTR 2B entry vector between recombination boxes by conventional cloning (restriction/ligation). Deletion mutants were obtained by digestion of NonO full-length and relegation (blunt end restriction endonucleases were chosen that did not shift the coding sequence reading frame). RRM1 deletion mutant lacking amino acids 87-160 was obtained by double digestion with *Eco*RV (nucl:258) and *BSa*AI (nucl:480). RRM2 deletion mutant lacking amino acids 160-227

was obtained by double digestion with *BSa*AI (nucl:480) and *Nru*I after inserting *Nru*I restriction site at the position 681 by site directed mutagenesis.

The cloning in pENTR 2B enabled transfer of the different constructs in pEF6-DEST 51 and pDEST14 expression vectors through recombination. Primers used for NonO full-length cloning and *Nru*I mutation:

| | | |
|---|---|---|
| CGCGGATCCAAAAATGCAGAGCAATAAAGC | NonO Fw ATG (*BamH*I) | NonO Full-length without stop codon |
| GAATTCCATATCGGCGGCGTTTATTTG | NonO Rev (1419) (*Eco*RI) | |
| CTGTGACTGTGTCGCGAATGGACCAGTTAG | Fw mut *Nru*I (681) | NonO full-length NurI mutated (681) |
| GACACTGACACAGCGCTTACCTGGTCAATC | Rew mut *Nru*I (681) | |

**Gal DBD fused NonO constucts**

GAL4-DBD fused NonO fusion proteins were obtained by cloning PCR fragments amplified with the following primers from pENTR 2B NonO FI and deletion mutants into pCMX GAL4-DBD mammalian expression vector. Primers were designed considering reading frame and compatible restriction sites.

| | | |
|---|---|---|
| GCCTCTGTCGTCGACATGCAGAGCAATAAAGCC | Fw NonO Start (*Sal*I) | Gal DBD-NonO and Deletion mutants |
| CGAATTCCTAATATCGGCGGCGTTTATTTG | Rev NonO (1419) (*EcoR*I) | |

**GST fused NonO constructs**

GST fused NonO proteins or NonO domains were obtained by cloning PCR fragments amplified with the following primers from pENTR 2B NonO FI and deletion mutants into pGEX AHK bacterial expression vector. Primers were design considering reading frame and compatible restriction sites.

| | | |
|---|---|---|
| CGAATTCATGCAGAGCAATAAAGCCTTTAAC | Fw (start) NonO (*Eco*RI) | GST-NonO full-length and deletion mutants |
| GCTCTAGACTAATATCGGCGGCGTTTATTTG | Rev (1422) NonO (*Xba*I) | |
| CGCGGATCCATCACTGAGGAGGAAATGAG | Fw (259) RRM1 (*Bam*HI) | GST-NonO RRM1 (aa:87-160) |
| GCGAATTCGTACTGAGGAAGGTTGCGG | Rev (480) RRM1 (*Eco*RI) | |
| CGAATTCATGCAGAGCAATAAAGCCTTTAAC | Fw (start) (*Eco*RI) | GST-Nt NonO and Deletion mutants |
| CCGAATTCTCAGGGTGGCTGTTCTCTCTCC | Rev (771) (*Eco*RI) | |
| CGGAATTCCCACCCAGATTTGCACAACC | Fw (766) RRM2 (*EcoR*I) | GST-NonO-Ct (aa:255-472) |
| GCTCTAGACTAATATCGGCGGCGTTTATTTG | Rev (1422) NonO (*Xba*I) | |

| | |
|---|---|
| Synthetized oligonucleotides | Thermo electron corporation |
| Amplify | Software, allowing the check of designed primers |

## 2.2.2 Primers used for semi-quantitative PCR analysis

### Human RAR-β2

| | | |
|---|---|---|
| RAR-β2 Fw | TGGATGTTCTGTCAGTGAGTCCT | E. Pfitzner |
| RAR-β2 Rev | CCCACTTCAAAGCACTTCTG | E. Pfitzner |

### Mouse/Human GAPDH house keeping gene

| | | |
|---|---|---|
| GAPDH Fw | GATGACATCAAGAAGGTGGTG | T.Heinzel |
| GAPDH Rev | GCTGTAGCCAAATTCGTTGTC | T.Heinzel |

## 2.2.3 Primers used for pGEX and pCMX sequencing

### To verify GST and Gal4-DBD fusion in N-terminus

| | |
|---|---|
| Nt-GST fusion Fw | CTGGTTCCGCGTGGATCTCGT |
| Nt-Gal fusion Fw | CAAAGACAGTTGACTGTATCG |

## 2.2.4 Oligonucleotides used for siRNA expression

### NonO directed siRNA

| **Oligonulceotides for the expression of short hairpin containing Si I (nucl: 489-507)** |
|---|
| **5'GATCCCC**CGAACTGCTGGAAGAAGCC**TTCAAGAGA**GGCTTCTTCCAGCAGTTCG**TTTTTGGAAA** |
| **3'AGCTTTTCCAAAAA**CGAACTGCTGGAAGAAGCC**TCTCTTGAA**GGCTTCTTCCAGCAGTTC**GGGG** |
| **Oligonulceotides for the expression of short hairpin containing Si II (nucl: 1248-1266)** |
| **5'GATCCCC**AGGACCTGCCACTATGATG**TTCAAGAGA**CATCATAGTGGCAGGTCCT**TTTTTGGAAA** |
| **3'AGCTTTTCCAAAAA**AGGACCTGCCACTATGATG**TCTCTTGAA**CATCATAGTGGCAGGTCCT**GGGG** |

## 2.2.5 Plasmids used in this work

### Plasmids for mammalian expression

| **Name** | **Encoded protein** | **references** |
|---|---|---|
| pDEST 51 NonO Fl | V5tagged NonO full-length(aa:1-472) | this work |
| pDEST 51 ΔRRM1 | V5 tagged NonO Δ RRM1 (deletion aa:87-160) | this work |
| pDEST 51 ΔRRM2 | V5 tagged NonO ΔRRM2 (deletion aa:161-227) | this work |
| pDEST 51 MBD3 | V5 tagged MBD3 | this work |
| pSG5 FL ESET | flag tagged ESET full-length (*Eco*RI/*Sma*I) | L. Yang |
| pDEST 51 CtBP | V5 tagged CtBP | this work |
| pCMX N-CoR FL flag | flag tagged full-length N-CoR (aa: 1-2453) | Heinzel |
| pCR3.1 PSF HA | HA- tagged PSF full-length | P. Tucker |
| pcDNA3 N-CoR Ct NID | N-CoR Ct (1587-2453) | M. Grez |
| **pCMX Gal4** | **Protein or domain fused to Gal4-DBD** | **references** |
| pCMX Gal4-DBD | Gal4 transcription factor DBD | T. Heinzel |
| pCMX Gal-N-CoR$_{\text{Flag-Epitop}}$ | Gal4-DBD N-CoR (aa 1-2453) | T. Heinzel |
| pCMX Gal-N-CoR 1 | Gal4-DBD N-CoR (aa 1-312) | T. Heinzel |
| pCMX Gal-N-CoR 9+10 | Gal4-DBD N-CoR (aa 2174-2453) | T. Heinzel |
| pCMX Gal-NonO full-length | Gal4-DBD NonO (aa 1-472) | This work |
| pCMX Gal-ΔRRM1 NonO | Gal4-DBD NonO lacking (aa 86-160) | This work |
| pCMX Gal-ΔRRM2 NonO | Gal4-DBD NonO lacking (aa 160-227) | This work |

### Mammelian reporter plasmids

| | | |
|---|---|---|
| 2xUAS TK luciferase reporter | promoter contains 2x UAS (Gal4 RE) | T. Heinzel |
| RARE luciferase reporter | promoter contains retinoic acide RE | T. Heinzel |
| ERE luciferase reporter | promoter contains estrogen RE | E.Pfitzner |
| SV 40 beta galactosidase reporter | normalization vector | T. Heinzel |

### Supplementary vectors

| | | |
|---|---|---|
| GFP | Green fluorescent protein expressing vector | T. Heinzel |
| psp vektor | Fill vector | T. Heinzel |

### Plasmids for bacterial expression

| **Name** | **Protein or domain fused to GST** | **References** |
|---|---|---|
| pGEX 2TDK | GST | T. Heinzel |
| pGEX JDK N-CoR 1-393 | GST-N-CoR RD I | T. Heinzel |
| pGEX AHK N-CoR SANT1/2 | GST-N-CoR SANT (aa:435-683) | T.Heinzel |
| pGEX AHK N-CoR 1679-2453 | GST-N-CoR C-t NC-Nco2 | T. Heinzel |
| pGEX N-CoR Ct-2290 | GST-extrem C-terminus (aa 2290-2453) | T. Heinzel |
| pGEX NonO FL | GST-NonO full-length (aa:1-472) | this work |
| pGEX NonO Nt | GST-NonO (aa:1-237) | this work |
| pGEX NonO Ct | GST-NonO (aa:255-472) | this work |
| pGEX NonO Δ RRM1 | GST-NonO lacking (aa:87-160) | this work |
| pGEX NonO Δ RRM2 | GST-NonO lacking aa:161-227) | this work |
| pGEX RRM1 | GST-NonO RRM1 (aa: 87-160) | this work |
| pGEX RRM2 Ct | GST-NonO (aa: 161-472) | this work |

## Plasmids for *in vitro* translation (TNT)

| | | |
|---|---|---|
| N-CoR Nt (1-549) | pCMX Gal N-CoR (1-549) TNT | T. Heinzel |
| N-CoR Ct (1629-2453) | pCMX N-CoR 102 TNT | T. Heinzel |
| pDEST14 NonO FL | NonO (aa 1-472) | this work |
| pDEST14 NonO Δ RRM1 | NonO lacking (aa 86-160) | this work |
| pDEST14 NonO Δ RRM2 | NonO lacking (aa 160-227) | this work |

## Plasmids for Si RNA expression

| | | |
|---|---|---|
| pSuper Si I NonO | NonO target sequence-CGAACTGCTGGAAGAAGCC- | this work |
| pSuper Si II NonO | NonO target sequence-AGGACCTGCCACTATGATG- | this work |
| pSi N-CoR | N-CoR target sequence-AAGAAGGATCCAGCATTCGGA- | MA. Lazar |
| pSi SMRTB | SMRT target sequence-AAGGGTATCATCACCGCTGTG- | MA. Lazar |

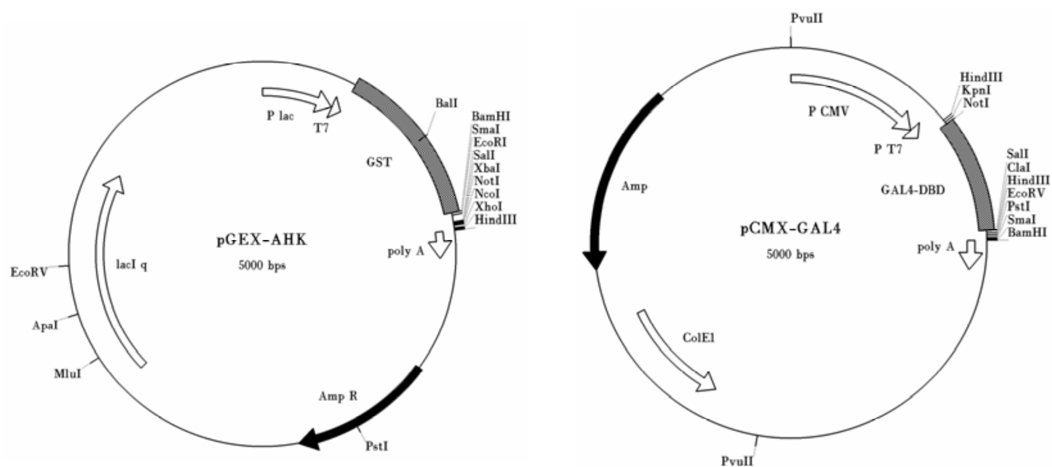
## Plasmids for antisense expression

| | | |
|---|---|---|
| pCR3.1AS NonO | NonO full-length antisense | P. Tucker |
| pCR3.1 AS PSF | PSF full-length antisense | P. Tucker |

## Vectors used for cloning

| | | |
|---|---|---|
| pENTR 2B | for recombination in various destination vectors | GATEWAY™ Cloning Technology Invitrogen |
| pEF6-DEST 51 | for expression of protein tagged in C-t withV5/His | Technology Invitrogen |
| pDEST 14 | for TNT translation | Technology Invitrogen |
| pDONOR | for generating attL-flanked entry clones | Technology Invitrogen |
| pGEX AHK | for expression of GST-fused proteins in bacteria | T.Heinzel |
| pCMX Gal DBD | for expression of protein fused in Nt to Gal4-DBD | T.Heinzel |
| pSuper | for expression of short interfering RNA | Oligoengine |
| pSuper+ | modified pSuper for efficient double digestion | this work |

## Vector description




**pGEX-AHK** **pCMX GAL4**

| | |
|---|---|
| **pENTR™ 2B /2718 nucleotides**<br>• Entry vector expression is silenced<br>• attL1/attL2 recombination sites<br>• ccdB gene<br>• Kanamycin resistance gene | **pEF6-DEST 51 /7464 nucleiotides**<br>• EF-1alfa promoter<br>• attR1/attR2 recombination sites<br>• ccdB gene<br>• V5 epitope / 6xHis tag<br>• Ampicillin resistance gene |
| **pDEST™14 /6422 nucleotides**<br>• T7 transcription termination region<br>• attR1/attR2 recombination sites<br>• ccdB gene<br>• Ampicillin resistance gene | **pDONOR™ 201 /4470 nucleotides**<br>• for Entry vector production by BP reaction<br>• attP1/attP2 recombination sites<br>• ccdB gene<br>• Kanamycin resistance gene |
| **pSuper basic /3176 bp nucleotides**<br>• BglII: 928 /HindIII: 934<br>• H1 promoter: 708 - 934<br>• Ampicillin resistance | |

Gateway empty vectors all contain the *ccd*B gene. These plasmids can only be propagated in modified *E.coli* strain such as DB3.1 cells containing the *gyr*A62 allele which renders the strain resistant to the toxic effects of the ccdB gene.

## 2.3 Biochemical analyses

### 2.3.1 Protein extract preparation

**Protein whole cell extract**

Cells are harvested from 10 cm dishes in PBS, transferred into falcon tubes and spun down by 1000 rpm for 10 min at 4°C. (Cell pellets can be frozen at -80°C directly after centrifugation and removal of PBS supernatant.) Cells are lysed adding 0.8-1 ml NETN, RIPA or Coimmunoprecipitation (Co-Ip) buffer with fresh added inhibitors and DTT (frozen pellets were thawed slightly on ice). Lysates are kept on ice for 15min before sonication and centrifugation. Sonication enables DNA sheering and improves protein solubilization. 10 pulses with Branson SONIFIER 250 from *G.HEINEMANN* (70% duty cycle, output control 4) are sufficient. Lysates were centrifuged at 13,000 rpm, 4°C for 20min and supernatants were transferred into fresh eppendorf tubes. Whole cell extracts can be stored at -80°C.

| | |
|---|---|
| NETN | 0.5% NP-40, 20 mM Tris pH8, 100 mM NaCl, 1 mM EDTA, 10% Glycerin add fresh 1 mM DTT,, 1x PIC, 0.5 mM PMSF |
| RIPA | 0.1%NP40, 20 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, SDS 0.1% deoxycholat 0.25% (w/v), add fresh 1 mM DTT, protease (PIC, PMSF) and phosphatase (NaF, Na-orthovanadate) inhibitors |
| Co-Ip buffer | TritonX-100 0.2%, 20 mM Tris pH 8, 25 mM NaCl, 1 mM EDTA, $MgCl_2$ 1.5mM glycerol 10%, add fresh 1 mM DTT, protease (PIC, PMSF) and phosphatase (NaF, Na-orthovanadate) inhibitors |
| PIC x1000 | 1 mg/ml Leupeptin, 2 mg/ml Antipain, 100 mg/ml Benzamidine, 10 000 U/ml Aprotinin (stored at -20°C) use diluted 1:1000 |
| PMSF | 0.5 M in ethanol (add fresh to final concentration 0.5 mM) stored at RT |
| NaF | Phosphatase inhibitor used at 1 mM final concentration |
| Na-orthovanadate | Phosphatase inhibitor used at 0.4 mM final concentration |
| DTT | 1 M dithiotreitol (stored at -20°C) |

**Nuclear extract short protocol**

Nuclear extracts and cytoplasmic extracts are obtained from cells with the following fractionated cell disruption method. The cytoplasmic fraction is obtained by adding hypotonic buffer to cell pellet and incubating on ice for 5 min. Nuclei stay intact whereas the cytoplasmic membrane of the cell was disrupted. Nuclei pellet is obtained by centrifugation. Supernatant was transferred in new tubes since it was the cytoplasmic fraction. To avoid any contamination with cytoplasmic proteins, nuclear pellets are washed once with the same hypotonic buffer. Lysis of the nuclear membrane is obtained by thoroughly vortexing and sonicating nuclei in the same buffer. Cell debris is spun down and the supernatant which constitutes the nuclear fraction is transferred in new tubes. In this buffer protein content could be determined with BCA assay before utilization or storage at -80°C.

**Procedure**

| | |
|---|---|
| 1. | Wash cells on the 100mm dishes with PBS, spin scraped cells in PBS, 10 min 1500rpm |
| 2. | Add 600 µl hypotonic buffer to cell pellet and resuspend with shorted tips. Incubate 7 min on ice |
| 3. | Centrifuge 2 min 14,000 rpm to obtain cytoplasmic fraction (supernatant) separated from nuclei pellet. |
| 4. | Wash nuclei pellet with hypotonic buffer before lyse, spin 2min 14,000rpm and remove supernatant |
| 5. | Add 600 µl hypotonic buffer to nuclei vortex and sonicate briefly (2x 10 pulses) to disrupt nuclear membrane |
| 6. | Spin at 14,000 for 20 min and collect supernatant |

| | |
|---|---|
| Hypotonic Buffer | 20 mM HEPES pH7.9, 10% glycerine, 0.2% NP40,10 mM KCl, 1 mM EDTA add fresh DTT, PIC and PMSF |

### 2.3.2 Protein analysis

#### SDS-polyacrylamide-gel electrophoresis (PAGE)

SDS polyacrylamid-gel electrophoresis is a technique used to separate macromolecules on the basis of electric charge and size. During electrophoresis, macromolecules are forced to move through the pores (gel-pore size depends on acrylamid, bisacrylamid containt) when the electrical current is applied. Their rate of migration through the electric field depends on the strength of the field, size and shape of the molecules.

Polyacrylamide gels (100 x 80 x 1.5 mm) used in this work consisted of 6%-12% separating gel, depending on the size of molecule to separate, and a 3% stacking gel:

| **Separating gel** | **6%** | | **8%** | | **10%** | | **12%** | |
|---|---|---|---|---|---|---|---|---|
| Mini gel number | **2x** | **4x** | **2x** | **4x** | **2x** | **4x** | **2x** | **4x** |
| 30%Acrylamide/ Bis | 3 ml | 5 ml | 4 ml | 6.7 ml | 5 ml | 8.3 ml | 6 ml | 10 ml |
| Separating buffer | 5.6 ml | 9.4 ml | 5.6 ml | 9.4 ml | 5.6 ml | 9.4 ml | 5.6 ml | 9.4 ml |
| 20%SDS | 75 µl | 125 µl | 75 µl | 125 µl | 75 µl | 125 µl | 75 µl | 125 µl |
| $H_2O$ | 6.3 ml | 10.3 ml | 5.3 ml | 8.6 ml | 4.2 ml | 7.0 ml | 3.2 ml | 5.3 ml |
| 10%APS | 75 µl | 125 µl | 75 µl | 125 µl | 75 µl | 125 µl | 75 µl | 125 µl |
| TEMED | 10 µl | 16.5 µl | 10 µl | 16.5 µl | 10 µl | 16.5 µl | 10 µl | 16.5 µl |

| **Stacking gel** | **3%** | |
|---|---|---|
| Mini gel number | **2x** | **4x** |
| 30%Acrylamide/ Bis | 830 µl | 1.33 ml |
| Stacking buffer | 625 µl | 1 ml |
| 20%SDS | 25 µl | 40 µl |
| $H_2O$ | 3.5 ml | 5.6 ml |
| 10%APS | 25 µl | 40 µl |
| TEMED | 5 µl | 8 µl |

Protein samples were denatured for 3-5 min at 95°C in sample buffer before separation by SDS-PAGE (Sambrook, 1989), using the Mini-Protean II system (*BioRad, Munich*). Electrophoresis was performed at 90V for the stacking gel and 120 V for the separating gel. Subsequently, proteins were either stained with Coomassie Brilliant Blue R-250 (*Serva, Heidelberg*) or transferred on nitrocellulose membrane (*Schleicher& Schuell*) for detection by immunoblotting.

#### Buffers and solutions

| | |
|---|---|
| Acrylamide/ bisacrylamide | 30% (w/v) : 0.8% (w/v) |
| TEMED | Tetramethyethyldiamine |

| Running gel buffer | 1 M Tris-HCl, pH 8.8 |
|---|---|
| Stacking gel buffer | 1 M Tris-HCl, pH 6.8 |
| 20% SDS | 20% (w/v) in $H_2O$ |
| 10%APS | 10% (w/v) in $H_2O$ |
| 5 x sample buffer | 250 mM Tris-HCl, pH 6.8; 50% glycerine; 10% SDS (w/v); 0.5% bromophenolblue (w/v); 3% 2-β-mercapto-ethanol |
| SDS-PAGE running buffer | 25 mM Tris-HCl; 250 mM glycine; 0.1% SDS (w/v); pH 8.3 |

| Protein molecular weight markers | Precision plus proteinTM standards | Biorad |
|---|---|---|
| | Prestained protein marker, broad range | NEB |

### Staining of polyacrylamide gels with Coomassie Brilliant Blue

After separation of protein mixtures by SDS-PAGE, the gels are incubated in staining solution for 15 min. To remove unbound dye the gel is washed 4 times in destaining solution for 10 to 15 min (Sambrook, 1989). The stained gels are kept in $dH_2O$ until they are dried.

| Staining solution | 30% ethanol; 10% acetic acid; 0.025% Coomassie brilliant |
|---|---|
| Brilliant blue | Blue R-250 (*Serva, Heidelberg*) |
| Destaining solution | 30% ethanol; 10% acetic acid |

### Western Blotting

Western blotting or immunoblotting is a technique permitting the simultaneous detection of a specific protein by means of its antigenicity, and its molecular mass. Therefore proteins are first separated using SDS-PAGE and transferred to a membrane suitable for detection. Protein or antigen will be recognized by specific primary antibodies and the secondary species specific conjugated antibodies allow detection.

After separation by SDS-PAGE, proteins are transferred onto nitrocellulose membrane suitable for immuno detection. Wet transfer with a Biorad transfer chamber is run 3 h at RT or overnight at 4°C. Gel, nitrocellulose membrane, Whatman paper and fiber pads all well soaked in transfer buffer are stacked as followed in a gel holder cassette:

| **Anode side (+)** |
|---|
| Fiber pads |
| Whatman paper (3mm) |
| Nitrocellulose membrane |
| Acrylamide gel |
| Whatman paper (3mm) |
| Fiber pads |
| **Cathode side (-)** |

All subsequent steps are performed with constant shaking. The complete protein transfer is checked by incubating the membrane 1 min in Ponceau red staining solution followed by incubation in 10% acetic acid before washing the membrane with distilled water until a clear staining appears. This staining is also used to compare amount of loaded protein or to render lane visible for membrane snipping or to visualized immunoprecipitation antibodies light and heavy chains. Ponceau staining is a reversible protein dye disappears by further rinsing with water or PBS/0.05% Tween buffer. Membrane is blocked in PBS 0.05% Tween with 5% low fat milk for at least 30 min to prevent non-specific binding of the antibodies. The primary antibody (antigen specific) is incubated with the membrane overnight at 4°C or 2-3 hours at room temperature diluted in a suitable manner (usually 1/500 to 1/10,000) in 2% milk / PBS

0.05% Tween. In 50 ml falcons 3 ml solution is sufficient for homogenous covering of the membrane during incubation on a roller incubator. The membrane is then washed 4 times in 15 ml PBS/0.05% Tween with constant shacking; changing buffer every 10 min. Secondary antibody diluted in 5ml PBS/0.05% Tween (usually 1/2,000 to 1/10,000 following instructions) is incubated with the membrane for 30-45 min at room temperature. The membrane is washed several times before detection of proteins with the ECL luminicence assay (*Pierce Amersham Biosciences*). Proteins which are recognized by the specific primary antibody can afterwards be detected by specific secondary antibodies that are coupled to a HRP (horseradish peroxidase) enzyme. In the presence of its substrate this enzyme catalyses a chemical reaction that leads to activation of luminol whose luminescence is detected by an X-ray film. The ECL reaction was performed as shown in the manufacterer's protocol. The exposition time is varied depending on the level of luminescence.

### Buffers and solutions

| | |
|---|---|
| PBS 10x buffer | 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM $KH_2PO_4$, pH 7.4 (autoclave solution for longer storage) |
| SDS 10x buffer | 1% SDS, Tris, Glycine. |
| Transfer buffer | 1X SDS Buffer, 20% ethanol |
| Wasching solution | PBS/0.05% Tween |
| Blocking solution | 5% low fat milk / PBS 0.05% Tween |
| Ponceau S staining solution | 20 mg/ml Ponceau S, 0,3 mg/ml trichloroacetic acid, 0,3 mg/ml sulfosalicylic acid |
| Ponceau Fixing solution | 10% acetic acid |

### Antibodies

| **Primary antibodies** | **Species** | **Dilutions** | **Reference** |
|---|---|---|---|
| Gal4-DNA-binding-domain (RK5C1) | mouse, monoclonal | 1:500 | Santa Cruz |
| V5-HRP | mouse, monoclonal | 1:5000 | Invitrogen |
| Flag- tag | mouse, monoclonal | 1:2000 | Sigma |
| N-CoR C-terminal affinity purified | rabbit, polyclonal | 1:1500 | Heinzel |
| NonO NMT-4/403° (N-terminal) | rabbit, polyclonal | 1:2000 | Traish (Boston) |
| PSF | rabbit, polyclonal | 1:1000 | Tucker (Austin) |
| PLC $\gamma$1 | mouse, IgG1 | 1:1000 | BD Biosciences |
| **Secondary antibodies (IgG-HRP)** | | | |
| Anti rabbit | goat, polyclonal | 1:15000 | Sigma |
| Anti mouse | sheep, polyclonal | 1:10000 | Amersham Biosciences |

### Membrane stripping

Membranes can be probed with different specific antibodies several times. Stripping is performed before incubating with another first antibody to eliminate preliminary signals. 7 µl β-mercaptoethanol per 1 ml stripping buffer is added freshly. Membrane is incubated in 15 ml stripping buffer at 50°C for 20 min. The membrane is afterwards rinsed tree times with $dH_2O$, 10 min with shaking, and finally once in PBS/0.05% Tween, before blocking it in 5% low fat milk / PBS 0.05% Tween.

| | |
|---|---|
| Stripping buffer | 2% SDS, 65 mM Tris-HCl pH 6.8, 100 mM ß-mercaptoethanol |

### Determination of protein concentrations

BCA$^{TM}$ protein assay (*Pierce*) is used to measure protein content of extracts. BCA reduces divalent copper ion to a monovalent ion under alkaline conditions. A molybdenum/tungsten

blue product is produced whose absorbance can be quantified at 562nm with a visible light spectrophotometer (*Biorad*). Sample and working reagent are incubated 30 min at 37°C and absorbance is measured in glass or polystyrene cuvettes.

### 2.3.3 Co-immunoprecipitation experiment

This method enables precipitation of specific proteins and their *in vivo* interaction partners. In a first step, proteins of interest are recognized by specific antibodies. Secondly, through interaction of conserved region (Fc) of antibodies (IgG) heavy chains with protein A and or protein G, which are coupled to sepharose beads, targeted proteins and interacting molecules can be co-precipited.

Cells are splitted 24 h to 72 h before harvesting depending on if transfection and or treatments which are required. Cells grown on 10 cm dishes are lysed in 0.5-1 ml NETN or co-immunoprecipiting buffer with 0.5 mM PMSF and a cocktail of protease inhibitors (PIC), sonicated twice 10 pulses with a Branson SONIFIER 250 from *G.HEINEMANN* (70% duty cycle, output control 4). Lysates are centrifuged at 13,000 rpm, 4°C for 20 min and supernatants are transferred into fresh eppendorf tubes (50 µl are kept in separate tubes and directly boiled with 2x protein loading buffer for the 10% inputs). 1 µg antibodies suitable for immunoprecipitation are added to 250 µl lysate (200 µg-500 µg protein) and incubated for 30 min on ice. As negative control the same amount of pre-immune sera, from corresponding species, is added to the lysate. 30-50 µl of protein A/G sepharose beads (Amesham biosciences) slurry in lysis buffer are afterwards added and incubated several hours up to over night on a spinning wheel at 4°C. Reaction volume is supplemented to 500 µl. Before adding the beads, they are equilibrated in the lysis buffer and blocked with 0.1% BSA for 30 min on a spinning wheel at 4°C. 1 ml lysis buffer is added and spun 30 s at 5.000 rpm at 4°C. Supernatant is discarded. Washing step is repeated five times and final centrifugation is done at 13,000 rpm for 30 s at 4°C. 30 µl 2x protein loading buffer are added and samples are subsequentially boiled at 95°C for 3 min. After short centrifugation, supernatants are loaded on a polyacrylamide SDS gel.

| Immunoprecipitation antibodies | Species | reaction | Reference |
|---|---|---|---|
| V5-tag | mouse, monoclonal | 0.5µl | Invitrogen |
| Flag- tag | mouse, monoclonal | 0.5µl | Sigma |
| N-CoR C-terminal (SA92) | guinea pig, polyclonal | 2µl | Heinzel |
| N-CoR N-terminal (SA 90) | guinea pig, polyclonal | 3µl | Heinzel |
| NonO 78-1 (C-terminal) | mouse, monoclonal | 0.5µl | Traish (Boston) |
| RNA Pol II | | 0.5µl | |

| | |
|---|---|
| Co-immunoprecipiting Buffer | 20 mM Tris-HCl pH 8, 25 mM NaCl, 1,5 mM $MgCl_2$,1 mM EDTA, 1% Triton X-100,10% Glycerine, add fresh 1 mM DTT, PIC and PMSF |
| Protein A sepharose | Lyophilized powder was suspended in $dH_20$, washed several times, final slurry 50% settle medium to 50% Co-Ip buffer |
| Protein G sepharose | Preswollen in ethanol, beads were washed several times in Co-Ip buffer, final slurry 50% settle medium to 50% Co-Ip buffer |

| | |
|---|---|
| Protein A sepharose CL-4B | Amersham biosciences |
| Protein G sepharose 4 fast flow | Amersham biosciences |

### 2.3.4 GST-pulldown assay

GST-pulldown assays enable determination of protein interactions *in vitro*. This method was used for mapping the interaction domain between NonO and N-CoR. One protein is radioactively labeled with $^{35}$S-methionine and the interaction partners or interaction domains are fused to GST, expressed in bacteria and isolated on glutathion agarose beads.

**Expression and purification of recombinant proteins**

BL21 codon+ bacteria strain is engineered to contain extra copies of genes that encode certain tRNAs necessary for efficient high level expression of heterologous proteins in E.coli. Single colonies (or 10 µl of glycerine stocks) containing plasmids encoding the different recombinants are grown overnight in 3 ml selective LB medium at 37°C shacking at 220 rpm. Overnight cultures are diluted 1:100 into 300 ml LB medium with antibiotics and grown to an $OD_{600}$ of 0.7 to 1 ($OD_{600}$ doubles about every 20 min). To induce bacterial expression of fusion-proteins, IPTG is added to the culture to a final concentration of 0,5 mM. Expression is performed at 30°C for 3 h under continuous shaking. Bacteria are collected by spinning at 5000 rpm for 10 min and supernatant is removed before freezing pellets for 30 min (or longer) at -80°C. Cells are thawed on ice before resuspension in 20 ml LysS buffer (containing freshly added ß-mercaptoethanol, PMSF and PIC protease inhibitor cocktail). Lysozym is added to a 0.2 mg/ml final concentration to destroy bacterial cell walls efficiently. Suspensions are directly transferred to a centrifugal tube and incubated 5 min at room temperature. To obtain an efficient lysis (samples become very viscous) it is mixed several times with a pipette. Samples are set on ice for 15 min while continuing mixing every 5 min untills it becomes very viscous. To clear lysates the samples are spun in a precooled (2°C) ultracentrifuge at 45000 g for 30 min at 2°C. Ultracentrifugation supernatants containing soluble recombinants are aliquoted and stored at -80°C or used immediately. 0.5-1 ml glutathione agarose beads are added to 5 ml supernatant in 14 ml falcon tube and incubated for 1 h on a spinning wheel at 4°C. Agarose beads are spun at 400 rpm for 2 min at 4°C. Supernatant is removed and beads are washed 5 times with 5 ml of 0.5 LysS buffer without protease inhibitors. Between washing steps samples are mixed by inverting tubes. Finally the beads are transferred in 2 ml Eppendorf tubes in 0.5 LysS buffer containing 1 mg/ml BSA. Proteins stored at 4°C are usually stable for up to 6 months.

Comparable protein amounts are necessary for the GST-pulldown assay; therefore beads are equilibrated in 0.5 LysS slurry 50%. 50 µl are spun and boiled with 2x protein loading buffer before loading on a SDS acrylamide gel and staining the gel with coomassie. Band intensity is estimated and glutathione beads are added for normalization of protein amounts. SDS PAGE and coomassie staining are repeated to check amounts of recombinants contained in 50 µl normalized glutathione-agarose beads.

| **Lys-S buffer 1x** | 50 mM Tris pH 7.8, 0.4 M NaCl, 0.5 M EDTA, 10% Glycerol, 0.1% NP40. |
|---|---|
| Add fresh before use | 5 mM ß-mercaptoethanol,0.5 mM PMSF and PIC (1:1000) |
| Lysozyme | 100 mg/ml (store at -20°C) |
| IPTG | 1 M isopropylthiogalactoside (store at -20°C) |
| 10% BSA solution | 100 mg/ml (store at -4°C) |
| Glutathion agarose beads (*Sigma*) | swollen overnight in $dH_2O$, washed 4x times thoroughly with $dH_2O$ before resuspended in LysS buffer slurry 50% |

### *In vitro* transcription and translation (TNT)

For cell free *in vitro* translation 'TNT Coupled Rabbit Reticulocyte Lysate System' from Promega (*Heidelberg*) is used according to the manufacturer's protocol. (Pelham and Jackson 1976; Jackson et al. 1983). It contains all necessary cellular components required for protein translation. This kit enables *in vitro* transcription and translation. Vectors suitable for *in vitro* expression require a T7 promoter. Coding sequences of protein of interest were cloned in pDEST 14 (Invitrogen) for this purpose. Some other proteins relevant for this project Gal-fused are also used in GST-pulldown assays and TNT translated since pCMX-Gal vector backbone contains the requisite promoter. Reaction is incubated for 90 min at 30°C and stored at -80°C.

**Protocol for a 25 µl TNT reaction**

1 µg template DNA
12.5 µl rabbit reticulocyte lysate
1 µl TNT reaction buffer
0.5 µl T7 RNA polymerase (10 U/µl)
0.5 µl RNasin inhibitor (40 U/µl)
1 µl amino acid mixture lacking methionine (1 mM)
2 µl [$^{35}$S]-methionine (10 µCi/µl specific activity >1000 Ci/mmol. Amersham)
Rnase free $H_2O$ is added to 25 µl

1 µl of the protein sample is boiled for 3 min at 95°C with 2x protein loading buffer and separated by SDS PAGE. The gel is shaken in gel dry solution (Bio-rad) for 30 min and dried on 3 mm Whatman paper in a gel vacuum dryer for 45 min to 1 h at 80°C. The dried gel was exposed to an X-ray film overnight.

| | | |
|---|---|---|
| TNT® T7 Coupled Reticulocyte Lysate System | *in vitro* Transcription-Translation Reaction; Protein expression | Promega |
| [$^{35}$S]Methionin | >1.000 Ci/mmol; 10 mCi/ml | Amersham |

### GST-pulldown assay

The whole procedure was performed with isolated and normalized recombinant proteins and unfused GST on glutathion agarose beads. 10% of the radiolabeled protein that was used for reaction was loaded as inputs on SDS-polyacrylamide-gel to compare with the intensity of the interaction signal.

**protocol**

| | |
|---|---|
| 1. | Prepare GST agarose beads fusion protein in PPi (0.02% NP40), BSA 1 mg/ml mix 20 min and then resuspend as 50% slurry |
| 2. | To 50 µl of GST agarose beads fusion protein 50% slurry add 100,000-500,000 cpm TNT product (usually 1-3 µl TNT reaction) |
| 3. | Make up to 100 µl with PPI |
| 4. | Incubate at 37°C for 20 min while shaking at 8000 rpm (longer incubation at 4°C) |
| 5. | Add 1 ml PPI and spin down (30 sec at 5000 rpm) and aspirate supernatant |
| 6. | Repeat washing step 5 five times with PPI (0.05% NP40) |
| 7. | Remove supernatant and add 25 µl 2x SDS protein sample buffer |
| 8. | Boil 3 min at 95°C and spin down |
| 9. | Run 20 µl on a minigel, 70-90 min, 120 V |
| 10. | Fix protein on gel by incubating in fixing solution for 15 min on a shaker or proceed to coomassie staining |
| 11. | Destaining is followed by several washes with water to remove all trace of acetic acid |
| 12. | Incubate the gel for 20 min in amplify solution |
| 13. | Dry gel on whatman paper (3 mm) in vacuum dryer for 45 min at 80°C |
| 14. | Expose autoradiography X-ray film overnight |

| | |
|---|---|
| PPI incubation buffer | 20 mM Hepes pH 7.9, 100 mM NaCl, 1 mM EDTA, 4 mM $MgCl_2$, 1 mM DTT, 10%glycerol, 0.02% NP40 |
| PPI wash buffer | 20 mM Hepes pH 7.9, 100 mM NaCl, 1 mM EDTA, 4 m M $MgCl_2$, 1 mM DTT, 10%glycerol, 0.05% NP40 |
| PMSF | 0.5 M in ethanol (add fresh to final concentration 0.5 mM) |
| Fixing solution | 10% acetic acid, 40% methanol (not necessary if Comassie stained) |

| | | |
|---|---|---|
| Gel dry solution | Prevents excessive gel swelling and cracking during drying | Biorad |
| Amplify | Increases detection efficiency, enhances conversion of ß-emissions to light efficiently recorded on film | Amersham biosciences |

## 2.3.5 Luciferase assay

In order to determine luciferase gene expression in transfected cells, the activity of the luciferase enzyme in cell lysates was measured. Prior to lysis, the growth medium of transfected cells (and treated) was removed. In each well of 12 well plates cells were lysed in 150 µl of lysis buffer and shaken 15 min at room temperature. Lysates were transferred into 1.5 ml tubes (*Eppendorf, Hamburg*) and cell debris was pelleted by brief centrifugation. 10µl extract were transferred on a 96-well microlyte$^{TM}$2 plate (*ThermoLabsystems, Franklin, MA, USA*). Luciferase activity was then measured at room temperature for 20 s in a luminometer (Microlumat LB 96P, *Berthold, Munich*) with automatic injection of 50 µl of luciferase solution. To consider the transfection efficiency that may vary depending on the plasmid DNA mix which was transfected, beta galactosidase encoding vector (SV40-ßgal) was cotransfected. This vector is under the control of SV40 promoter. Normalizing luciferase activity through beta galactosidase activity resulted in a relative luciferase activity taking in consideration the transfection efficiency.

| | |
|---|---|
| Harvest buffer | 50 mM Mes-Tris pH 7.8, 10% (v/v final) glycerol, 1 mM DTT, 0.1% Triton X-100 in $H_2O$ |
| Luciferin solution | 0.3 mg/ml Luciferin in 5 mM $KHPO_4$ pH 7.8 |
| Luciferase buffer | 130 mM Mes-Tris pH7.8, 30 mM MgCl2, 2.5 mg/ml ATP |
| Luciferase solution | 1:1 luciferin solution+luciferase buffer |

| | | |
|---|---|---|
| Luciferin | Beetle luciferin, potassium salt | Promega |

### 2.3.6 β-Galactosidase assay

β-Galactosidase assay is performed to normalize transfection efficiency. (SV40ß-Gal has to be co-transfected). Cell lysates in harvest buffer for luciferase assay are used directly. 10 µl extract are transferred on a 96-well flat bottom microtest plate (*Sarstedt*) and 90 µl ß-galactosidase reaction mix is added. Reaction is stopped with 200 µl of 1 M $Na_2CO_3$ after 30 min incubation at 37°C (protein alkaline denaturation). The reaction mix contains an artificial ß-galactosidase substrat ONPG (o-Nitrophenyl-β-D-Galactopyranosid). In the presence of ß-galactosidase ONPG is converted to galactose and Ortho-nitrophenyl (ONP). ONP is bright yellow in alkaline solution and its absorbance can be measured at 420 nm in a spectrophotometer.

**ß-Galactosidase reaction mix:**

**Mix for 10µl extract / 96 well**
1 µl 100x Mg solution
22 µl ONPG (Substrat)
72 µl 100 mM NaPi pH 7,5

**Buffers**

| 100x Mg | 100 mM $MgCl_2$, 4,5 M β-mercaptoethanol |
|---|---|
| ONPG (o-Nitrophenyl-β-D-Galactopyranosid) | 4 mg/ml ONPG in 100 mM NaPi pH 7,5 |
| NaPi pH7,5 Natriumphosphate buffer pH 7,5 | 41 ml 200 mM $Na_2PO_4$ + 9 ml 200 mM $NaHPO_4$ |
| $Na_2PO_4$ | 200 mM $Na_2PO_4$ |
| $NaHPO_4$ | 200 mM $NaHPO_4$ |
| $Na_2Co_3$ stop solution | 1 M $Na_2CO_3$ |

## 2.4 Bacterial transformation

Bacterial transformation is the process by which bacterial cells take up naked foreign DNA molecules. Bacteria which are able to uptake DNA are called "competent" and are made so by different treatments in the early log phase of growth.

### 2.4.1 Bacteria transformation by electroporation

**Preparation of electrocompetent cells**

A single colony of DH10ß E.coli cells is inoculated into 5 ml LB medium. Since these bacteria do not contain any resistance gene all materials and reagents coming into contact with bacteria must be sterile. Bacteria are grown over night at 37°C shaking by 220 rpm. Bacteria are diluted 1:100 into 500 ml and grown to an $OD_{600}$ of 0.7 ($OD_{600}$ doubles about every 20 min). Before proceeding to centrifugation of cells, bacteria culture is chilled on ice for 10 to 15 min. Two sterile polypropylene 500 ml Beckman bottles are used for centrifugation at 5,000 rpm and 4 °C for 20 min in a J2-21 centrifuge (*Beckman*) equipped with a JA-10 rotor (*Beckman*). Pellets are resuspended first in 5 ml before adding 250 ml of ice cold sterile bidistilled water. This step is repeated before transferring cells into 50 ml polypropylene tubes. 40 ml ice cold 10% glycerol is added and cells are centrifuged for 10 min at 5,000 rpm (rotor JA -12). Pellet volume is then estimated and an equal volume of ice-cold 10% glycerol is added. Aliquots of 50-300 µl are frozen in liquid nitrogen before storage at -80°C.

**Protocol:**

The electrocompetent bacteria are thawed on ice. In pre-chilled Bio-Rad electroporation cuvettes (0.2 cm wide) 10 pg-0.5 µg plasmid DNA or 1-7 µl of the DNA ligation mix are added to 30-100 µl bacteria (for new ligation use more DNA, for already amplified vector use less than 10 pg). The Bio-Rad Gene Pulser$^{TM}$ setting is 2.5 kV, 25 µF and 400 ohm. To help bacteria recover after transformation, cells are immediately resuspended in 1 ml of SOC non-selective medium and incubated at 37 °C for 30 min at 1000 rpm in a Thermomixer (*Eppendorf*). Finally cells are spun in a microcentrifuge at 5000 rpm for 20 seconds, supernatant is discarded and cell pellet is resuspended in 100 µl LB and plated on appropriate LB-agarose selective medium (containing 100 µg/ml ampicillin or 50 µg/ml kanamycin) (Dower et al. 1988). Plates are afterwards incubated at 37°C overnight. Bacteria colonies appear the next day.

| **SOC medium:** | 0.5% yeast extract, 2% tryptone (*Peptone*), 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose (steril filtrated) |
|---|---|

### 2.4.2 Bacteria transformation by heat shock

**Preparation of chemocompetent cells**

A single colony of BL21 codon positive E.coli cells is inoculated into 5 ml LB medium. Bacteria are grown over night at 37°C shaking at 220 rpm. Bacteria are diluted 1:100 into 300 ml and grown to an $OD_{600}$ of 0.7 ($OD_{600}$ doubles about every 20 min). The following procedure is performed similar to the preparation of electrocompetent cells. Culture is chilled before harvesting cells by centrifugation. Total culture is divided in two fractions for more facility. After removal of the supernatant cell pellet is resuspended in 100 ml solution 1 and kept on ice for 2 h. Cells are then centrifuged and resuspended in 5 ml of solution 2. Aliquots at 200 µl each are frozen at -80°C.

| **Solution 1:** | 100 mM $RbCl_2$, 50 mM $MnCl_2$, 30 mM Kac, 10 mM Ca $Cl_2$, 13% glycerine |
|---|---|
| prepared before use and equilibrated at pH 5.8 with acetic acid before sterile filtration | |

| **Solution 2:** | 10 mM MOPS pH7, 10 mM $RbCl_2$, 75 mM $CaCl_2$, 13% glycerine |
|---|---|
| prepared before use and equilibrated at pH 7 with NaOH before sterile filtration | |

**Protocol**

Chemocompetent bacteria stock vials are thawed on ice. 10 ng Plasmid DNA is added to 100 µl (1 µl mini DNA preparation is added to 50 µl Bacteria) and the mixture is incubated for 5 min on ice. Transformation is achieved by incubating 5 min at 37°C followed by 2 min chilling on ice before adding 500 µl SOC medium and incubating for 30 min at 37°C with gentle shaking. 100 µl of the mixture is then spread on plates with adequate selective LB-agar medium. Plates are incubated over night at 37°C.

### Bacteria strains used in this work

| *E.coli* Strain | Genotype | References |
|---|---|---|
| BL-21 Codon Plus | F$^-$ *ompT* *hsd*S$_B$ ($r_B^-$ $m_B^-$) *dcm* Tet$^r$ *gal*λ (DE3) endA Hte [*arg*U *ile*Y *leu*W Cam$^r$] | Stratagene |
| DH10B | F$^-$ *mcrA*Δ(*mrr-hsd*RMS-*mcr*BC)ϕ80d *lac*ZΔM15 Δ*lac*X74 *deo*R *rec*A *end*A1 *ara*D139 Δ(*ara,leu*)7697 *gal*U *gal*K λ$^-$ *rps*L *nup*G | Life Technologies |
| DB3.1* | F- *gyr*A462 *end*A Δ(sr1-recA) *mcr*B *mrr* *hsd*S20 (rB-, mB-) *sup*E44 ara14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Smr ) *xyl*5 Δ*leu* mtl1 | Invitrogen |

*Gateway empty vectors all contain the *ccd*B gene. These plasmids can only be propagated in modified *E.coli* strains such as DB3.1 cells containing the *gyr*A62 allele which renders the strain resistant to the toxic effects of the *ccd*B gene.

Bacteria containing Gal4-UAS-TK-reporter plasmid are only grown efficiciently in Superbroth LB medium.

| **Superbroth LB medium** (1 litre) | 32 g tryptone, 20 g yeast extract, 5 g NaCl, 5 ml (1M) NaOH |
|---|---|

A single bacteria colony is grown overnight shacking at 220 rpm and at 37°C in LB medium with the appropriate antibiotics (3-5 ml medium for analytic DNA isolation or preculture, 300 ml for preparative DNA isolation). For bacteria culture stocks, cells are frozen at -80°C in 25% glycerin LB-medium.

## 2.5 Cell culture

Different cell lines are used for protein overexpression, cell extracts, transient reporter assays, RNA isolation and confocal microscopy:

| Cell line | Origin and properties | DSMZ-Nr./ Reference |
|---|---|---|
| HeLa | Human cell line, isolated from the aggressive glandular cervical cancer of a young woman (**He**nrietta **La**cks) | ACC 57 (Gey et al., 1952) |
| 293T | Human embryonic kidney cell line, transformed with large T antigen | ACC 305 (Graham et al. 1977) |
| MCF7 | Human breast cancer cell line, retained several characteristics of differentiated mammary epithelium, including the ability to process estradiol via cytoplasmic estrogen receptors | ACC 115 (Soule et al. 1973) |
| NIH 3T3 | Mouse fibroblaste cell line ,developed from NIH Swiss mouse embryo cultures | ACC 59 (Andersson et al 1969) |

### 2.5.1 Basic handling

#### Splitting cells

293T cells (derived from a human kidney carcinoma) are cultivated in DMEM medium (dulbeccos minimal essential medium) supplemented with 10% decomplemented FCS (foetal calf serum), 5% glutamine and 1% penicilline/streptomycine and grown in an incubator at 37°C with 5% $CO_2$. When the adherent cells reach confluency, they are splitted into new flasks (T75 250 ml). The medium is removed and the cells are washed with sterile 1x PBS (37°C) to eliminate trypsine inhibiting factors produced by the cells and serum adhering to their surface. Trypsine is added (1ml of trypsine/ flask) to the cells to release them into suspension from monolayers. When cells have lost contact, they are thoroughly resuspended in 10 ml of fresh DMEM medium to inactivate trypsine and to obtain a homogenous single

cell suspension. 1 ml of diluted cells is transferred into new flask supplemented with 14 ml new medium. When cells are cultured in a small flask, cells are passaged 1:10 in new flask.

**Counting cells**

For applications that require a precise cell number counting chambers were used to determine the number of cells per unit volume. Cell sample was diluted 1:1 (v/v) in Trypan bleu dye exclusion medium. Dead cells are blue and can be distinguished from living cells that are birefringent under the microscope. The hemacytometer was carefully and continuously filled. The charged chamber was placed on the microscope stage for counting. Calculations were performed according to the manufacturer's instructions.

| | | |
|---|---|---|
| DPBS | Dulbecco's PBS; identical to PBS, but contains 8.1 mM $Na_2HPO_4$, pH 7.4, sterile filtered | Cambrex |
| DMEM | Dulbeccos modified eagle medium | Boehringer |
| RPMI | Roswell Park Memorial Institute | Boehringer |
| L-glutamine | 200mM | Boehringer |
| Pen/Strep | 10 000 U Penicilline, 10 000 µg/ml Streptomycine | Bio*Whittaker |
| Trypsine/EDTA | 0.02% Trypsine; 0.05% EDTA | Seromed |
| FCS | Fetal calf serum | GibcoBRL |
| Counting chamber | Hemacytometer | Hycor Biomediacal Inc |

### 2.5.2 Long storage

Cell lines can be frozen and stored at -80°C for a short period (view months) or at -160 (liquide nitrogen tank) for longer storage. From a 10 cm dish confluent cells are trypsinized. Medium is removed after 5 min centrifugation with 1000 rpm. Cell pellet is afterwards resuspended in 1 ml freezing medium and transferred immediately in cryotubes on dry ice. Cells are stored overnight at -80°C before being transferred into a liquid nitrogen tank. By thawing, cells are quickly thawed, washed with 10 ml fresh complete medium in 15 ml test tubes and spun at 1000 rpm. Whole vial content is resuspended in 5 ml medium and transferred into a small flask. The next day all dead cells are removed by changing medium if confluency is reached, cells will be splitted immediately.

| **Freezing medium** | Minimal essential medium (DMEM or RPMI) containing 47% FCS and 13% DMSO (Dimethyl sulfoxide *Sigma*) |
|---|---|

| | | |
|---|---|---|
| DMSO | Dimethyl sulfoxide | Sigma |

### 2.5.3 Transfection

There are several methods to deliver exogenous DNA into cells. The methods that are used facilitate DNA binding to cell membranes and entry of the DNA into the cell via endocytosis. Calcium phosphate transfection (Chen and Okayama, 1987; Wigler et al., 1977) shows high transfection efficiency in different cell lines, probably protecting DNA against intracellular and serum nucleases. This method is also considered as being only minimally toxic to the cells. Since we observe also high transfection efficiency in 293T cells with the PEI (polyethyleneimine) transfection method, it can be used as an alternative method. The different cell lines that are used in this work grow differently. An adequate cell number is splitted, depending on the cell line and the dish size in which transfection is performed.

| Dish size | 22 mm | 100 mm |
|---|---|---|
| Cell line | Cell number/ well | |
| HeLa | $2.10^4$ | $3.10^5$ |
| 293T | $2.10^5$ | $3.10^6$ |
| MCF7 | $3.10^5$ | $3.10^6$ |

**Calcium phosphate-mediated transfection**

Cells are spitted 24 h before transfection. Culture medium has to be removed before adding transfection complex to the cells. Water, $CaCl_2$ and DNA are mixed before adding gently 2x HEPES-buffered saline while blowing bubbles for 30 s. Serum free medium containing (1:3000) chloroquine is added to the precipitate and the complete mixture is gently poured into the well (adherent cells can be loosened). 5 h later medium is changed into complete medium. Precipitates are visible in the microscope. For experiments including hormone treatments cells are grown and kept in complete medium containing charcoal-stripped FCS instead of complete FCS (see charcoal-stripped FCS).

**Protocol**

| Number of cells/ dish | $2\text{-}3.10^5$ | $3.10^6$ |
|---|---|---|
| Transfection reaction | 4x 22 mm dishes | 10 cm dish |
| DNA | 1-1.5 µg x 4 | 10-15 µg |
| H2O | 250 µl | 500 µl |
| 2M $CaCl_2$ | 30 µl | 60 µl |
| HBS 2x | 250 µl | 500 µl |
| DMEM(serum free) | 1500 µl | 3 ml |
| chloroquine | 1 µl/3 ml DMEM | 1 µl/3 ml DMEM |
| Pour into each well | 500 µl | 4 ml |

| 2x HEPES Buffered Saline | 280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO_4$, 12 mM glucose, 50 mM HEPES, pH 7.2 (sterile filtrated) |
|---|---|
| 2 M Calcium Chloride | dissolve 14.7 g $CaCl_2$ in 100 ml $H_2O$. (autoclave) |

**Polyethyleneimine (PEI)-mediated transfection**

Polyethyleneimine (branched) with an average molecular weight of 25 kDa was obtained from Aldrich (Taufkirchen). PEI-mediated gene transfer is considered most efficient at N/P ratios between 9 and 13.5 (N = positively charged amine nitrogens in PEI, P = negatively charged phosphates of the DNA backbone) (Boussif et al. 1995). The N/P ratios used in this work was 9.

Pipette separately DNA/PBS and PEI/PBS. Both solutions are then mixed and incubated for 15 min at RT. Growing medium is removed and cells are gently recovered with serum free medium. Transfection mix is then dropped equally on the cells and incubated for 4 h at 37°C, before changing medium. Cells are treated 24 h after transfection. Protein analysis or reporter assays are performed 48 h after transfection.

**Procedure**

| Protocol | 22 mm dish | 100 mm dish |
|---|---|---|
| Solution A (DNA) | 30 µl PBS + 1-2 µg DNA | 240 µl PBS + 5-15 µg DNA |
| Solution B (PEI) | 30 µl PBS + 5.4 µl 10 mM PEI | 240 µl PBS + 43.2 µl 10 mM PEI |
| Transfection mix | Mix A and B solution vortex and incubate 15 min RT | |
| Transfection | 1 ml serum free medium | 5 ml serum free medium |
| | Drop equally transfection mix on the cells | |

| **100mM stock solution** | 0.45 g PEI in 100 ml $dH_2O$, pH 7.0 (HCl) sterile filtrated with 0.22 µm filter |
|---|---|

Transfection reaction was done in triplicates. 1-2 µg of DNA was used to transfect cells on 22 mm dishes (12 well plates). In order to estimate transfection efficiency, Green fluorescent protein expressing vector was cotransfected. GFP expression was monitored by fluorescence microscopy 24 or 36 h after addition of the transfection complexes to the cells.

### 2.5.4 Transient reporter assays

**Gal4-reporter assay**

This conventional reporter assay is used to determine whether a protein has transcriptional repressing or activating activity. This experiment can also be extended to investigate protein interactions or substance effects. The protein or protein domain of interest is fused to the Gal4 transcription factor DNA binding domain (DBD). The reporter plasmid encodes luciferase under the control of a TK promoter and Gal4-specific response elements UAS. Reporter, Gal4-DBD fusion proteins and interaction factors are co-transfected to determine if the interaction influences Gal4-DBD fused protein activity. Luciferase activity is measured 48 h after transfection. Calcium phosphate transfection or PEI transfection is used to transfect the basic DNA mix for on 22 mm well ; experiment were performed in triplicats on 12 well plates:

| | |
|---|---|
| UAS TK Luc Reporter | 0.3 µg |
| Gal DBD N-CoR/ Gal DBD | 0.1-0.3 µg |
| pDest-V5 NonO / deletion mutants | 0.1-0.3 µg |
| SV40 ß galactosidase reporter | 0.1 µg |
| GFP expressing vector | 0.1 µg |
| Psp fill vector | Add to 1.4 µg |

**RARE-reporter assay**

A retinoic acid response element controls the expression of the luciferase on the RARE-reporter plasmid. Retinoic acid receptor co-transfection is omitted since 293T cells contain sufficient endogenous receptors (RARs, RXRs). Upon stimulation with retinoic acid receptor agonist or antagonist, transcriptional activation respectively repression occurs. 24 h after transfection cells are treated and luciferase activity is measured 24 h after treatment. For time course experiments cells are harvested at different times.

**RARE-reporter DNA basic mix for 1 well /12well plate:**

| | |
|---|---|
| RARE Luc Reporter | 0.3 µg |
| Protein expressing vector | 0.1-0.3 µg |
| Silencer plasmid | 0.5-0.8 µg |
| SV40 ß galactosidase reporter | 0.1 µg |
| GFP expressing vector | 0.1 µg |
| Psp fill vector | Add to 1.6 µg |

**TRE-reporter assay**

The TRE-reporter encodes for the luciferase under the control of thyroid hormone receptor response elements (TRE). 293T cells do not express thyroid hormone receptor (THR); therefore THR encoding plasmids have to be co-transfected to obtain responses of the

reporter upon stimulation. The transcription activation is obtained treating cells with Triac (triiododothyroacetic acid). Luciferase activity is measured after transfection and treatment.

**TRE-reporter DNA basic mix for 1 well /12well plate:**

| TRE Luc Reporter | 0.8-1 µg |
|---|---|
| Protein expressing vector | 0.1-0.3 µg |
| TR beta | 0.1 µg |
| SV40 ß galactosidase reporter | 0.1 µg |
| GFP expressing vector | 0.1 µg |
| Psp fill vector | Add to 1.8 µg |

### ERE-reporter

This estrogen response element (ERE) reporter encodes for luciferase under estrogen receptor control. ERE-reporter assays are performed in MCF7 cells, since they express estrogen receptor naturally. Cells are transfected with Fugene, since only low transfection efficiency is achieved with the others transfection methods. Luciferase activity is measured after transfection and treatment.

**ERE-reporter DNA basic mix for 1 well /12well plate:**

| ERE Luc Reporter | 0.3 µg |
|---|---|
| Protein expressing vector | 0.1-0.3 µg |
| SV40 ß galactosidase reporter | 0.1 µg |
| GFP expressing vector | 0.1 µg |
| Psp fill vector | Add to 1.4 µg |

## 2.5.5 Treatment of cells

All cells which are being hormonal treated have to be splitted in medium containing stripped serum in order to avoid the influence of naturally in FCS contained steroid hormones.

### Charcoal-stripped FCS

Serum is commonly used as a supplement to basal growth medium in cell culture. The serum used for cell growth was fetal calf serum (FCS). Serum provides a wide variety of macromolecular proteins, low molecular weight nutrients, carrier proteins for water–insoluble components, and other compounds necessary for in vitro growth of cells, such as hormones and attachment factors. Serum also adds buffering capacity to the medium and binds or neutralizes toxic components. Attempts to replace serum entirely with serum-free medium had only limited success. Hormones like retinoic acid or thyroid hormones are to be found in FCS, therefore charcoal-stripped FCS is added to the medium used in assays where cells were treated. Using stripped serum minimizes the effect of hormones contained endogenously in serum and increases the response to specific treatment.
To remove hormones from the serum, it is incubated with activated carbon (1 g/10 ml decomplemented serum, use 50 ml falcons) for 2 h at RT on a roller. Lipophilic molecules such as steroid hormones adhere to the carbon and can thereby be separated from the

serum. Charcoal is spun down by centrifugation (30 min/ 4000 rpm twice/ tree times). Serum is finally sterile filtered (0.22 µm) before storage at -20°C in aliquots.

**Hormonal treatment of cells**

Since adherent cells can be lost partially by changing medium and to avoid a variation of ligand concentration, 500 µl containing 3-fold final concentration of the specific hormone are applied to the cells in 1 ml stripped FCS medium in each well on the 12 well plate. Treatment is generally performed 24 h after transfection.

The luciferase expression encoded by the RARE-reporter can be activated by ATRA (all-trans retinoic acid). Different concentrations of agonist are applied to the cells by adding ATRA to medium containing stripped FCS. To enhance transcriptional repression, antagonist ligand mix109 is added to medium containing stripped FCS. In a similar manner TRE-reporter transcription can be activated by Triac and the ERE-reporter transcription is stimulated upon treatment tamoxifen with.

Final hormone concentration used in corresponding assays:

| | |
|---|---|
| ATRA | $5.10^{-7}$M |
| ATRA | $1.10^{-7}$M |
| RA antagonist mix 109 | $1.10^{-7}$M |
| Triac | $5.10^{-7}$M |
| Tamoxifen | $5.10^{-8}$M |

For endogenous gene analysis by semi quantitative PCR, RNA is isolated from cells treated in a similar manner.

**Protein turnover**

To observe how stable proteins are, cells were treated with different substance like cyclohexmide and MG which are known to inhibit mRNA translation and proteasome degradation respectively. Since cycloheximide was dissolved in ethanol, as control cells were treated with identical concentration of ethanol in medium. Cells were treated 24 h before being harvesting.

| | |
|---|---|
| cycloheximid | $5.10^{-7}$M |
| MG 132 | $1.10^{-7}$M |
| Ethanol | $1.10^{-7}$M |

| | | |
|---|---|---|
| Cycloheximide | Antibiotic derived from microbial sources inhibits eukaryotic, but not prokaryotic protein synthese | SIGMA |
| MG 132 | Proteasome inhibitor Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal Z-LLL-CHO | Calbiochem |

## 2.5.6 Cell synchronization

Several chemicals can be added to proliferating cells to arrest them in certain stages of the cell cycle. Double thymidine-nocodazole block was used to arrest cells in mitosis. These reversible inhibitors were added to culture medium before being applied to cells. 293T cells were grown on 10 cm dishes one day before incubation. Medium containing thymidne was applied overnight (16 h) and removed on the next day. 8 h release in normal medium preceded nocodazole treatment overnight (16 h).This procedure enables at first to arrest 293T cells in G1/S with thymidine which inhibits DNA replication fallowed by a synchronize

growth during the releazed phase and a final block in G2/M with nocodazole which promotes tubulin depolymerization. Mitotic 293T cell round up and detach from the bottom of the dish. Synchronization was monitored by microscopy and chromatin condensation was verified by Hoechst staining.

| chimicals | Stock solution | Final concentration | Cell cycle stage |
|---|---|---|---|
| Thymidine | 50 mg/ml $H_2O$ | 0,5 mg/ml in culture medium (dilution: 1:100) | Bock in G1/S phase |
| Nocodazole | 1 mg/ml DMSO | 50 ng/ml culture medium (dilution: 1:20 000) | Block in G2/M phase |

Thymidine and Nocodazole SIGMA-Aldrich

## 2.6 Optical and Confocal Microscopy

### 2.6.1 Fluorescence microscopy

#### GFP expression monitoring

Twelve-well plates or 100 mm culture dishes were positioned, lid closed, under a Nikon eclipse TE300 fluorescence microscope (Nikon, Düsseldorf) to investigate GFP expression in transfected cells. EGFP fluorescence was obtained with a FITC-fluorescence filter set (Nikon TE-FM Epi-fluorescence attachment, excitation 480/40 nm, mirror 505 nm, barrier 535/50 nm). Pictures were captured using a SONY DXC-9100P camera (Sony, Cologne). Processing of the images was performed using the LUCIA software and Adobe Photoshop.

#### Hoechst/DAPI staining

Hoechst 33342 (bisbenzimide H 33342) is a specific stain for AT-rich regions of double-stranded DNA like DAPI and also the fluorescence properties are similar to DAPI. It can be used preferentially with living, unfixed cells. The absorption maximum is at 340 nm, the emission maximum at 450 nm. Condensed chromatin in mitotic cells was visualized by this method. In an Eppendorf tube a drop of medium containing cells arrested in G2/M was sufficient for verification. Cells were fixed in 4% paraformalfehyde for 20 min after removing medium and proceeding to gentle wash with PBS and centrifugation. For permeabilization cells were incubated 5 min. in PBS containing 0.1% Triton. After gentle washing in PBS cells were incubated at least 30 min in 0.1 mg/ml Hoechst/PBS. Staining solution was discarded before resuspending cells in PBS in a 200 µl. Place a drop on a microscope slide cover with a coverslip. A Zeiss Axiovert 200M microscope was used to investigate Hoechst stained chromatin in 293T cells. Fluorescence was obtained with a DAPI/Hoechst filter set (01Ex 365/12) by an excitation wavelength of 365 ± 6 nm. Pictures were captured using a SONY DXC-9100P camera (Sony, Cologne). Processing of the images was performed using the LUCIA software and Adobe Photoshop.

### 2.6.2 Laser scanning microscopy

Confocal microscopy analysis was performed to investigate localization of endogenous or exogenous proteins and their colocalization in cells. Cells were grown and transfected on coverslips in 12 well plates. 24 h after transfection cells were used for analysis. Medium was removed and cells were washed shortly with PBS. Fixation was then performed incubating

cells in 3% Formaldehyde PBS (1 ml/well) for 10 min. at room temperature followed by 5 min washing in PBS. Cells permeabilization is obtained by incubating 5 min in 0.1% Triton X-100/PBS. Cells were again washed with PBS before immunostaining. To minimize the quantity of antibody necessary for efficient staining, coverslips were circled with a hydrophobic pen after removing all traces of buffer by aspiration. The hydrophobic barrier enables recovery of the sample with a minimal volume. For blocking and washing steps 1 ml solution was used per well, whereas for immunostaining 150 µl antibody-containing solution were sufficient. To reduce unspecific binding of the antibodies, cells were incubated in 3% BSA PBS block solution for 1 h before incubation with primary antibodies diluted 1:100 in 150 µl block solution for 1-3 h or overnight at 4°C. Prior and post incubation with secondary antibodies diluted 1:1000 in 150 µl block solution, cells were washed in 1 ml PBS at least tree times for 5 min. Secondary antibodies are coupled to fluorophores whith different excitation wavelengths. For colocalization experiment the whole procedure must be repeated and the first antibodies must differ in species and the fluorophore bound to secondary antibodies have to differ in their excitation wavelengths.

| **Antigen** | **Secondary antibodies** | **Fluorophore** | **references** |
|---|---|---|---|
| rabbit IgG | Goat, polyclonal | Alexa Fluor 546 | Molecular probes |
| rabbit IgG | Goat, polyclonal | Alexa Fluor 488 | Molecular probes |
| mouse IgG | Sheep, polyclonal | Alexa Fluor 546 | Molecular probes |
| mouse IgG | Sheep, polyclonal | Alexa Fluor 488 | Molecular probes |

After immunostaining, coverslips are then rinsed in $H_2O$ destillated and dried gently. The coverslips were soaked in mowiol solution (mounting medium for fluorescence) which protect samples from photobleaching and placed on the slide. TO-PRO-3 can be directly added in this solution for staining of the nuclei. The preparation was sealed with nail polish. Preparations can be stored in the darck at 4°C for up to 2 weeks.

| Moviol solution | 9 g glycerine, 3.6 g mowiol, 9 ml H2O, 18 ml 0.2 M Tris-HCL pH 8.5, 0.1% (v/v) DABCO |
|---|---|

| | |
|---|---|
| Moviol 4-88 | Aventis |
| DABCO | Fluka |
| TO-PRO-3 | Molecular probes |

A Leica DM IRBE confocal laser scanning microscope was used to analyze protein localization. Processing of images was performed using Leica confocal software (LCS).

## 2.7 Common drugs and equipment

Acrylamide/ bisacrylamide (Roth)
Agarose ultra pure (*Life Technologies*, *Gibco BRL*)
Ampicillin (*Serva Electrophoresis*)
Bromophenol blue (*Roth*)
Charcoal *(Roth)*
DEPC (*Sigma*)
Dimethylsulfoxid, DMSO, $C_2H_6SO$ (*Roth*)
Di sodium hydrogenphosphate dodecahydrate, $Na_2HPO_4.12\ H_2O$ *(Merck)*
DTT, dithiotreito (*Roth*)l
ECL western blotting detection reagent (*AmershamPharmacia*)
EDTA (*Sigma*)
Ethanol 100% (*Roth*)
Formaldehyde 37% (*Sigma*)

Formamide (*Roth*)
Glycerol (*Roth*)
HEPES (*Roth*)
Hydrochloric acid 1 M, HCl (*Roth*)
Kanamycine
Lithium Chloride, LiCl (*Roth*)
Lysozym (*Roth*)
Manganese (II) chloride dehydrate, $MnCl_2$. $H_2O$ (*Merck)*
Methanol (*Roth*)
MOPS, $C_7H_{15}NO_4S$ (*Roth*)
Nonfat dried milk powder (*AppliChem*)
Phenol and buffer pH 10.5 for saturated Phenol (*Sigma*)
Phosphatase alkalin, AP, (*Roche*)
PMSF (*Aldrich*)
Ponceau S, *(Sigma)*
Potassium acetate, $C_2H_3KO_2$ (*Roth*)
Potassium dihydrogen phosphate, $KH_2PO_4$ (*Roth*)
2-Propanol, Isopropanol (*Roth*)
Proteinase K (*Roche*)
RNase (*Roche*)
Rotiphorese Gel 30, polyacrylamid (*Roth*)
Rubidium chloride, RbCl *(Fluka)*
SDS ultra pure 20% (Roth)
Sodium acetate, $C_2H_3NaO_2{\cdot}3H_2O$ (*Roth*)
Sodium dihydrogenphosphate monohydrate, $NaH_2PO_4$. $H_2O$(Merck)
Sodium hydroxide, NaOH (*Roth*)
TEMED p.a. (*Roth*)
Tri reagent (*Sigma*)
Trichloromethan/Chlorophorm (*Roth*)
Tris, $C_4H_{11}NO_3$ (Roth)
Tris hydrochlorid, $C_4H_{11}NO_3HCl$ (*Roth*)
Tween$^R$ 20 (*Roth*)
Other more specific reagents and drugs are mentioned in the protocol text with companies of purchase.

**Plasticware and filter systems for laboratory and cell culture use**
Eppendorf (Hamburg); Costar (Bodenheim); Greiner (Frickenhausen); BD Biosciences (Heidelberg); Nalgene (Rochester, NY, USA); Schleicher & Schuell (Dassel)

**Tools and equipments**
Biofuge *pico* (*Heraeus*)
Vortex REAXcontrol (*Heidolph*)
Pipetus$^R$-akku (*Hirschmann Laborgeräte*)
Electrophoresis power supply- EPS 301 (*Amersham pharmaciabiotech*)
Hoefer HE33, mini horizontal submarine unit (*Pharmacia Biotech*)
MV120, mini vertical gel system (*LTF*)
Rocking table “Rocky”, to wash membranes (*Fröbel Labortechnik*)
Thermomixer compact, for 1.5 ml tubes (*Eppendorf*)
Dri-Block$^R$ DB-2D (*Techne*)
Biocentrifuge/J2-21 centrifuge with rotor JA-10 and JA-20 (*Beckman*)
Ultracentrifuge TL-100 (*Beckman*)
Centrifuge 2K15 (*SIGMA)*
Heraeus Typ. Nr.4400, minifuge GL Heraeus Christ (*Heraeus*)
iCycler PCR machine (*Bio-RAD*)
1500 TR-CARB$^R$, liquid scintillation analyser (*PACKARD*)

# 3 Results

The yeast-two-hybrid screen (Fields and Song 1989) is a suitable method to determine protein-protein interactions and was performed with N-CoR amino acids 2290-2453 as bait to identify novel potential interaction partners. The obtained yeast transfection efficiency was less then twice the diversity of the library implying that the library was only partially screened. Nevertheless, more than 100 clones remained after several rounds of selection. After determining the specificity of the interaction by blue/white assay and retransformation 80 clones were sequenced. The putative interacting proteins belong to various protein families such as extracellular matrix proteins, heat shock proteins, transmembrane proteins, splicing factors, response element binding proteins and transcription factors. It can not be excluded that in yeasts some factors enable an indirect interaction between bait and prey proteins. Therefore, direct interactions were verified by *in vitro* Glutathion-S-Transferase pull-down assays with a subset of proteins known to play a role in transcriptional regulation. After the initial analysis of the yeast-two-hybrid screen during my diploma thesis, the aim of my Ph. D. thesis was the functional characterization of the N-CoR extreme carboxy-terminus.

## 3.1 *in vivo* interaction of N-CoR with putative binding partners

Co-immunoprecipitation (Co-IP) is a commonly used immunochemical method to analyze protein-protein interactions *in vivo*. This technique is based on the capture of selective antigens with specific antibodies, and the subsequent isolation of the immune complex via protein A and/or protein G immobilized on a solid support (sepharose beads). Analysis of the co-immunoprecipitated proteins is performed by SDS PAGE followed by Western blotting. For this experiment, several different specific antibodies would have been necessary and not all of which were commercially available. In addition, the DNA sequences that were isolated from the library vector did not always encode the full-length protein. Therefore, complete coding sequences of proteins of interest were cloned into a V5-His tag mammalian expression vector (pEF6-DEST 51) and were detected by Western blot with an anti-V5 antibody. The proteins that were further investigated are listed in table1.

**Table 1:** Further investigated proteins, known to play a role in transcriptional regulation and to interact directly with the N-CoR extreme C-terminus in GST-pulldown assays.

| **Putative N-CoR interacting proteins** | |
|---|---|
| Methyl-CpG binding domain-containing protein 3 | (MBD3) |
| Similar to ERG-associated protein SET domain, bifurcated | (ESET) |
| C-terminal binding protein 1 | (CtBP1) |
| Non-POU-domain-containing octamer-binding protein | (NonO, p54nrb) |

### 3.1.1 Cloning full-length coding sequences into mammalian expression vector

Full-length sequences were obtained from plasmids or from NIH 3T3 (embryonic mouse fibroblasts) total cDNA. CtBP and ESET complete coding sequences were amplified from acquired plasmids used in published works (Criqui-Filipe et al. 1999) (Yang et al. 2002). The complete coding sequences from CtBP, MBD3 and NonO were successfully cloned into the mammalian expression vector pEF-DEST- 51 (Invitrogen GATEWAY) whereas the plasmid encoding ESET full-length protein (Yang et al. 2002) suitable for mammalian protein expression tagged with the Flag epitope was directly used for transfection. Different cloning strategies were used since the gateway technology provides two cloning options through site-directed recombination or restriction sites. Primers were designed depending on the cloning strategy. The coding sequences were first inserted into a transcriptionally silent entry vector by classical cloning or BP recombination before being transferred into the destination vector of choice by LR recombination. PB and LR reactions are based on site-specific recombination of the phage lambda. Each clonases BP and LR required specific recombination sites to mediate gene transfer. They enable excision of a sequence flanked by recombination sites, *att*B or *att*L, and insert DNA fragment between corresponding sites *att*P or *attR* respectively. These sites are modified to ensure DNA exchange in a specific manner maintaining orientation and reading frame.

### 3.1.2 Exogenous protein expression in 293T cells

Protein overexpression was analyzed in NETN whole-cell extracts from 293T cell transfected with the calcium phosphate transfection method. With this method 70% to 90% transfection efficiency was achieved in 293T cells (Fig. 8).

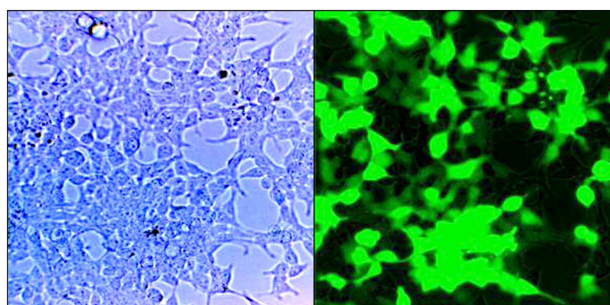


**Figure 8: Calcium phosphate transfection of 293T cells**

14 µg expression plasmid and 1 µg GFP expression vector were transfected into $5.10^6$ 293T cells. Over 70% of the cells express GFP indicating high transfection efficiency. GFP expression was visualized by fluorescent microscopy.

Expression of the different proteins and their molecular weight were controlled by Western blotting. CtBP, MBD3 and NonO were successfully overexpressed in 293T cells and detected with an antibody directed against the V5 epitope (Fig. 9) whereas ESET protein was detected with a Flag epitope-directed antibody.

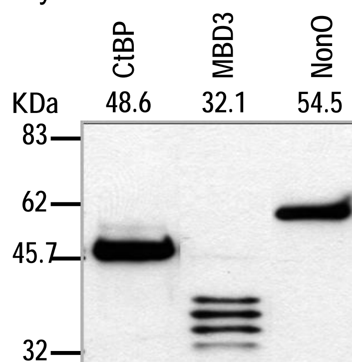



**Figure 9: Overexpression of CtBP, MBD3 and NonO in 293T cells**

Transfected cells on a 10 cm dish were lysed in 1 ml NETN buffer supplemented with protease inhibitors. 20 µl of each samples (1:1 in 2 x SDS loading buffer) were loaded on an 8% SDS polyacrylamid gel and separated by electrophoresis before transfer on nitrocellulose membrane for Western blotting. Invitrogen mouse V5 tag directed primary antibody was diluted 1:5000.

The molecular weight (MW) of proteins was verified by comparing bands to the prestained protein marker bands. For the different constructs specific single bands were obtained except for MBD3. Since extracts were made in parallel and the MBD3 ladder was always observed in different experiments, it is likely that the laddering was rather due to posttranslational modifications than degradation.

### 3.1.3 *In vivo* analysis of protein interaction

**Co-immunoprecipitation of interacting proteins**

N-CoR is a platform protein essential for the recruitment of histone deacetylases and for the formation of the transcription repressor complex, which is endogenously expressed in 293T cells. Therefore, endogenous N-CoR was immunoprecipitated from NETN whole cell extract (WCE) and co-immunoprecipitating factors were analysed by Western blotting. N-terminus directed specific polyclonal antibodies were used to immunoprecipitate endogenous N-CoR rather than available efficient carboxy-terminal directed antibodies which could have disrupted or impeded the interaction with potential partners. Although, the different V5 tagged proteins were successfully expressed and N-CoR was effectively immunoprecipitated; no co-immunoprecipitation was observed with MBD3, ESET (Fig. 10) or CtBP (data not shown).

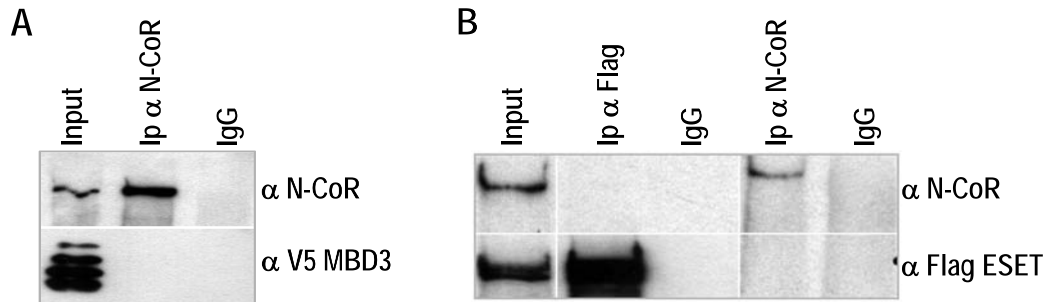



**Figure 10: N-CoR immunoprecipitation**

293T cells were lysed in NETN buffer 48 h after transfection with vectors encoding for the different interaction partners. Endogenous N-CoR was immunoprecipitated with guinea pig-specific antibody against N-terminal of N-CoR and detected by Western blot with rabbit-specific antibody against the C-terminal region of N-CoR. The interacting partners were detected with mouse anti-tag epitope antibodies. As negative controls, comparable amounts of preimmune sera are used (IgG). **(A)** Overexpressed MBD3 is nicely detected in the input lane but not with the immunoprecipitated N-CoR (Ip) in the middle lane. **(B)** Although expressed ESET protein and N-CoR are detected and specifically immunoprecipitated, no co-immunoprecipitation of both proteins is obtained.

Interestingly, *in vivo* interaction was determined with the RNA recognition motif containing protein NonO. Exogenous NonO-V5 was first observed to associate with endogenous N-CoR (Fig. 11 A). Since no antibody was commercially available, antibodies from previously published works were required and kindly provided by A. M. Traish (Pavao et al. 2001). With these specific antibodies NonO could be detected and immunoprecipitated, respectively. Endogenous NonO was co-immunoprecipated with both overexpressed Flag-N-CoR and endogenous N-CoR (Fig. 11 B).

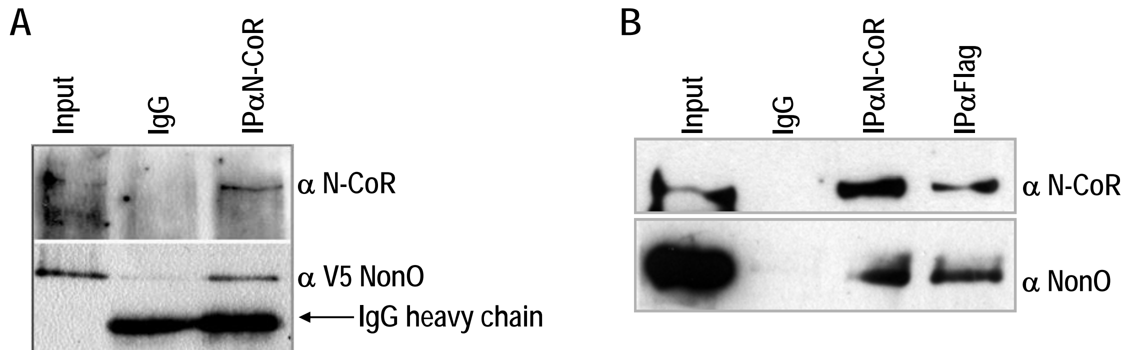



**Figure 11: NonO interacts with N-CoR *in vivo***

**(A)** N-CoR was immunoprecipitated from 293T cell lysat expressing exogenous NonO-V5. Co-immunoprecipitated NonO is detected with anti V5 antiboy. Immuno-cross reaction renders heavy chain of antibodies used for immunoprecipitation visible. Comparable amounts of antibody are observed in the control reaction (IgG). **(B)** Flag-N-CoR was overexpressed in 293T cells and was immunoprecipitated using guinea pig serum directed against N-CoR N-terminus or antibody against the Flag epitope. Endogenous NonO co-immunoprecipitates in both cases.

Receiving NonO-specific antibodies suitable for immunoprecipitation we could performed the reverse immunoprecipitation. Endogenous NonO was successfully precipitated from 293T NETN whole cell extract and endogenous N-CoR was specifically co-immunoprecipitating (Fig. 12).

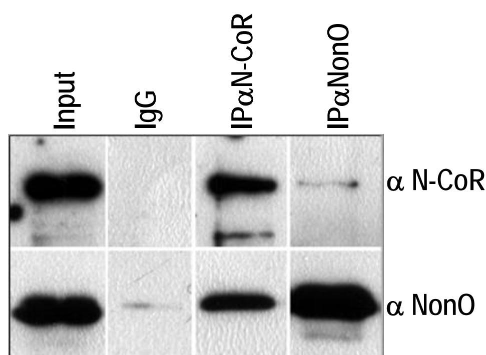



**Figure 12: endogenous NonO coimmunoprecipitates with endogenous N-CoR**

Immunoprecipitation of N-CoR and reverse immunoprecipitation both show association of endogenous NonO with N-CoR. N-CoR immunoprecipition was performed as previously whereas endogenous NonO was pecipited with 0.5 µl mouse-specific antibody (78-1-C6) and detected in the Western blot with rabbit-specific antibody (403A/NMT-5 aa 371-386).

### Co-localization of interacting proteins

To confirm co-immunoprecipitation experiments, colocalization of both interacting proteins was analyzed by confocal laser scanning microscopy in NIH 3T3 epithelial cells which contain high endogenous levels of N-CoR and have large nuclei. NonO-V5 was exogenously expressed and optimal immunostaining conditions were established to specifically detect endogenous N-CoR and tagged NonO. Colocalization of endogenous N-CoR with overexpressed NonO was observed (Fig. 13). Based on the results obtained in coimmunoprecipitation and colocalization experiments, NonO/p54nrb RNA/DNA binding protein was the focus of further investigations.

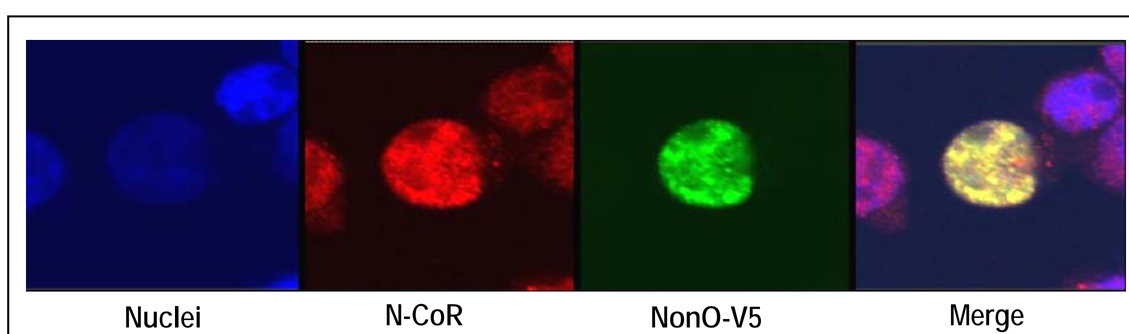



**Figure 13: Endogenous N-CoR and overexpressed NonO-V5 co-localize in NIH 3T3 cells**

Immunofluorescence images of endogenous N-CoR and exogenously expressed NonO-V5 shows co-localization of these proteins in NIH 3T3 mouse epithelial cells. Cells were grown and transiently transfected with mammalian NonO-V5 tag expressing vector in 12 well dishes on cover slips. 24 h after transfection, cells were fixed, permeabilized and immunostained. Nuclei (bleu) were visualized by TO-PRO-3 iodide (*Molecular Probes*) staining which excitation wavelength is 633 nm. Cross reaction and secondary antibodies specificity were tested and did not show any background signal. The subcellular localisation of N-CoR (red) and NonO (green) and colocalization (merg in yellow) were assayed by confocal microscopy sequential scan.

## 3.2 Role of NonO in the regulation of N-CoR repressive activity

### 3.2.1 Analysis in the presence of exogenous NonO

The co-immunoprecipitation and colocalization of NonO with N-CoR strongly support that these proteins interact *in vivo*. To determine the functional relevance of this interaction, it was first analyzed whether N-CoR repressive activity is affected by NonO in the well established Gal4-reporter assay.

**Functional analysis using the Gal4 UAS-TK reporter assay**

In this Gal4-reporter, the luciferase expression is under the control of the thymidine kinase (TK) promoter and a repeat of two Gal4 response elements (UAS). Proteins or domains of the protein of interest are fused to the Gal4-DNA binding Domain (DBD). This region of the transcriptional activator Gal4 is sufficient for binding of the fusion protein to the response element on reporter DNA. Transcriptional activity is determined by measuring luciferase activity. It is known that N-CoR repressive activity is mostly due to corepressor complex formation and the recruitment of HDACs. The well-defined repression domains that are essential for corepressor complex formation, are contained in the N-terminal half of N-CoR. In the Gal4 luciferase reporter assay N-CoR amino-terminal half fused to the Gal4-DBD represses whereas fusion protein containing the conserved extreme C-terminus does not (Fig. 14).

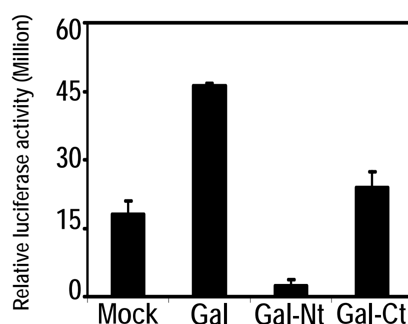



**Figure 14: N-CoR repressive activity can be measured with the Gal4-reporter**

To analyze the transcriptional activity of different N-CoR domains, Gal4-reporterwas cotransfected with the different constructs fused to the Gal4-DBD. Luciferase activity obtained while overexpressing Gal4 transcription activator DBD is considered as the basal reporter activity. Gal4-DBD-N-CoR Nt (Gal-Nt) has repressive activity in comparison to Gal4-DBD and Gal4-DBD-N-CoR Ct (Gal-Ct). The N-CoR C-terminal region has a similar activity as the reporter alone (mock transfected). Relative luciferase activity was determined by normalization to levels of $\beta$ galactosidase expression directed by the SV40 $\beta$-galactosidase internal control. The graph represents the mean of three independent experiments done in triplicates.

The effect of NonO on N-CoR repressive activity was assessed by expressing increasing amounts of exogenous NonO with the Gal4-reporterin the presence of Gal-N-CoR full-length (FL). NonO overexpression, even at the highest concentration (0.5 µg), did not significantly affect either the fold repression or transcriptional activity of the reporter (Fig. 15).

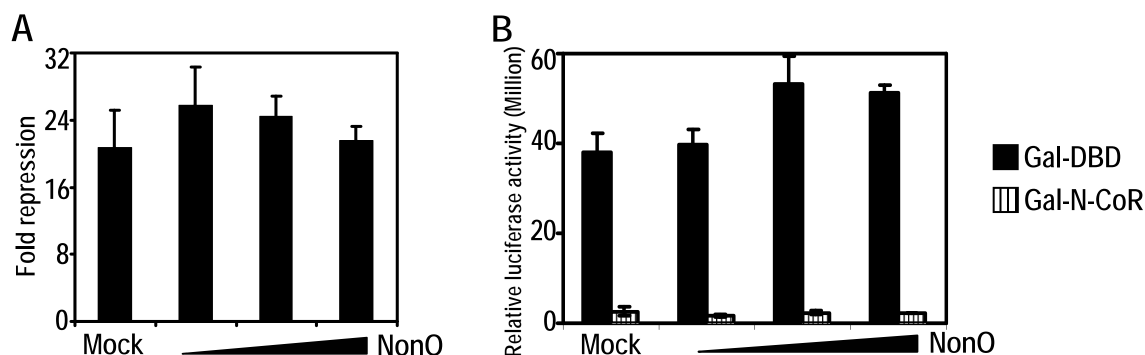



**Figure 15: N-CoR repressive activity is not affected by NonO overexpression**

0.3 µg Gal4-repoter and 0.3 µg Gal4-DBD or Gal-N-CoR FL expression plasmid with increasing amounts of NonO expression vector (0, 0.1, 0.3, and 0.5 µg) were transiently transfected into 293T cell. Empty expression vector was used as a mock control. **(A)** Gal4-N-CoR FL-dependent fold repression is similar in the absence or presence of overexpressed NonO. The indicated standard deviations represent two independent experiments done in triplicates. **(B)** Although luciferase activity shows some variations within an experiment, these are not NonO concentration-dependent.

With this assay it was also tested if NonO itself harbors a repressive activity. Therefore, the NonO full-length coding sequence was cloned into the pCMX-Gal vector. A Gal-N-CoR-Nt expressing vector was double digested with *Sal* I / *Eco* RI and the insert encoding N-CoR was replaced with NonO full-length sequence. The NonO insert was amplified with primers containing *Sal* I and *Eco* RI restriction sites. pCMX-Gal-NonO was cotransfected with the Gal4-reporter and luciferase activity was measured 48h after transfection. We observed strong repressive activity of the Gal-NonO fusion protein in this reporter assay (Fig. 16 A). However, the observed repression remained upon TSA treatment (Fig. 16 B), suggesting that the observed repression is histone deacetylase-independent.

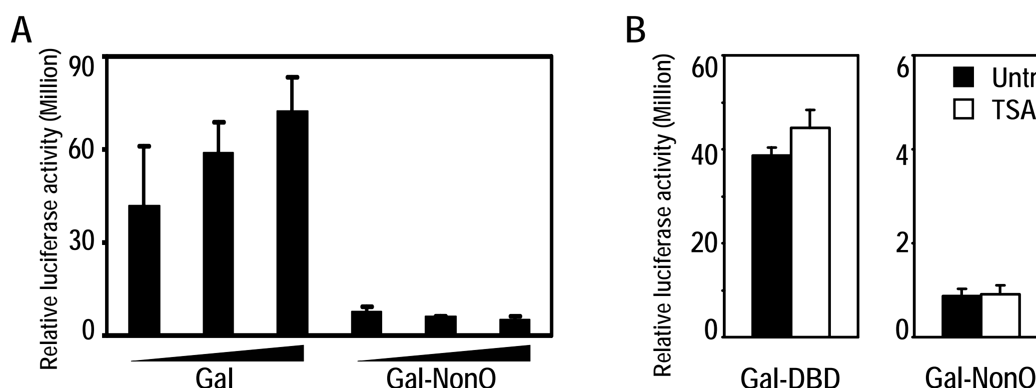



**Figure 16: NonO shows repressive activity in the Gal4-reporter**

**(A)** Increasing amounts (0.1, 0.2, 0.3 µg) of Gal4-DBD and Gal-NonO were co-transfected with Gal4-reporter (0.8 µg) in 293T cells. The basal reporter transcriptional activity is dependant on the Gal4-DBD concentration. In contrast Gal-NonO repression activity barely changes while increasing expression vector amount. Average induction values were determined from two experiments done in triplicates. **(B)** 24 h after transfection of Gal4-reporter with 0.1 µg Gal4-DBD versus Gal-NonO expressing vector, 293T cells were treated with TSA (100 nM) for 24 h. HDACs inhibitors as TSA can enhance slightly basal transcription, which is observed in panel B left. Right panel, TSA treatment does not relieve repression that is obtained in the presence of Gal-NonO. Average induction values were determined from one experiment done in triplicates.

It is conceivable that Gal-NonO recruits N-CoR to the promoter region and the presence of the nuclear receptor corepressor is sufficient for repression. Alternatively it is also possible that Gal-NonO enhances stable complex formation with other factors that are involved in other processes such as splicing. The resulting occupancy of this regulatory region may be sufficient to affect the accessibility of the promoter to the basal transcription machinery.

It is thought that transiently transfected plasmids assemble with histones into a nucleosome-like structure but that the chromatin context is thereby only partially achieved. Since N-CoR is involved in co-repressor complex formation and in the histone deacetylase mediated transcriptional repression, we have analyzed if NonO affects N-CoR repressive activity in a chromatin context dependent manner. A similar reporter assay was performed in HeLa cell lines containing the Gal4-reporter stably integrated into their genome. The calcium phosphate transfection method was used to introduce Gal4-DBD or Gal-N-CoR expression plasmids into HeLa cells. Unfortunately, due to the stability of the luciferase protein and transfection efficiency, the basal luciferase activity was not significantly affected upon exogenous Gal-NCoR expression (Fig. 17). Thus, this cell line could not be used to investigate transcriptional repression and the effect of deacetylase inhibitors such as trichostatin A (TSA) or valproic acid (VPA) in the presence of exogenous NonO in a chromatin context. A cell line containing stably integrated Gal4-reporter and Gal-N-CoR coding sequences may have been suitable to determine if NonO affects N-CoR histone deacetylase-dependent repression activity. A HeLa cell line was available but was resistant to transfection although a variety of efficient transfection methods were tested.

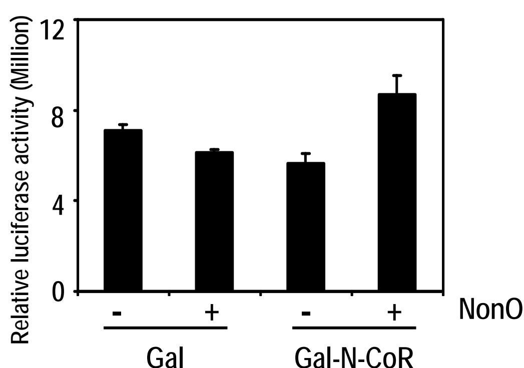



**Figure 17: Transcriptional regulation of the stably integrated Gal4-reporter in HeLa cells**

$4.7 \times 10^4$ HeLa cells containing a Gal4-reporter integrated into their genome were grown on 12 well plates 24 h before transfection. 1.4 µg total DNA per well were calcium phosphate transfected including 0.3 µg Gal4-DBD or Gal-N-CoR FL with or without 0.5 µg NonO expression vector, 0.1 µg SV40 β-galactosidase control vector, 0.1 µg GFP expression vector and psp fill vector to supplement to equal amount of DNA. No significant Gal-N-CoR FL-dependent repression was measured. Two independent experiments were performed in triplicates and one representative experiment is shown.

With the Gal4-reporter Gal-N-CoR FL repressive capacity could be evaluated. In this assay strong transcriptional repression is obtained in the presence of Gal-N-CoR-Nt containing HDAC interaction domains whereas no effect was observed in the presence of Gal-N-CoR Ct

which contains the nuclear receptor interaction domain. However, with the transiently transfected Gal4-reporter no significant variation of the Gal-N-CoR full-length repression was measured upon exogenous NonO expression. This may suggest that NonO does not affect corepressor complex formation or that endogenous NonO which is abundantly expressed in 293T cell is sufficient. To ensure that NonO is effectively not involved in the corepressor complex formation reporter experiment should be performed in the absence of NonO using RNA interference to specifically down regulate this protein.

### 3.2.2 Analysis in the absence of endogenous NonO

**Specific down-regulation of protein expression by small interfering RNA**

Interfering RNA is a novel tool to determine protein function by down-regulation of gene expression. Recently, several plasmid vectors have been developed that direct transcription of small hairpin RNAs, which are processed into functional small interfering RNAs (siRNAs) by cellular enzymes (Brummelkamp et al. 2002). Therefore, the pSuper RNAi System (oligoengine) was chosen to analyze NonO function by specifically down-regulating endogenous NonO expression. The pSuper vector had to be modified to achieve successful cloning (Fig. 18). This modified pSuper vector (pSuper +) was only used to obtain large amounts of double digested vector required for cloning.

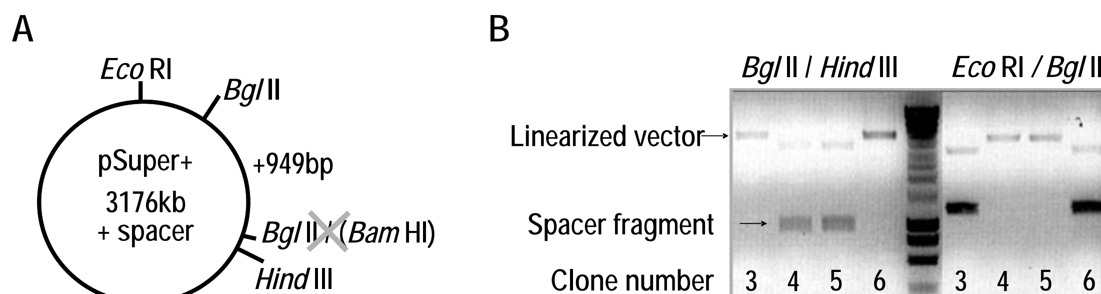



**Figure 18: pSuper modifications to optimize cloning**

In the original vector cloning restriction sites *Bgl* II and *Hind* III were spaced only by 6-nt which drastically reduced double digestion efficiency and successful cloning. **(A)** A DNA fragment out of N-CoR coding sequence was cloned in pSuper to separate (949-nt) *Bgl* II from *Hind* III restriction sites. **(B)** The inserted fragment was digested with *Bgl* II and *BamH* I generating *Bgl* II compatible cohesive ends and was cloned into *Bgl* II digested pSuper. *Bgl* II (A/GATCT) / *BamH* I (G/GATCC) ligation product is no longer a recognition site for these endonucleases. Insert orientation was checked by digesting recombined vector with *Bgl* II / *Hind* III and *Eco* RI / *Bgl* II. Clone 4 and 5 contain the spacer DNA fragment in the advantageous orientation (B left panel). *Eco* RI / *Bgl* II digestion of these both clones shows the vector backbone lacking a 281 bp fragment which is not visible (B right panel).

Two NonO mRNA specific target sites were determined (Fig. 19) for which corresponding oligonucleotides were synthesized and cloned into pSuper vector. NonO and PSF antisense (AS) containing vectors were also tested for comparison and were kindly provided by Phil Tucker (unpublished). These vectors contain full-length NonO and PSF antisense coding sequences. The antisense transcripts can interact with the complementary mRNA and thereby inhibit its translation and enhance double strand RNA degradation. This method is

now considered to be less efficient and more toxic for cells than inducing selective degradation with small interfering RNAs (Kurreck 2003).

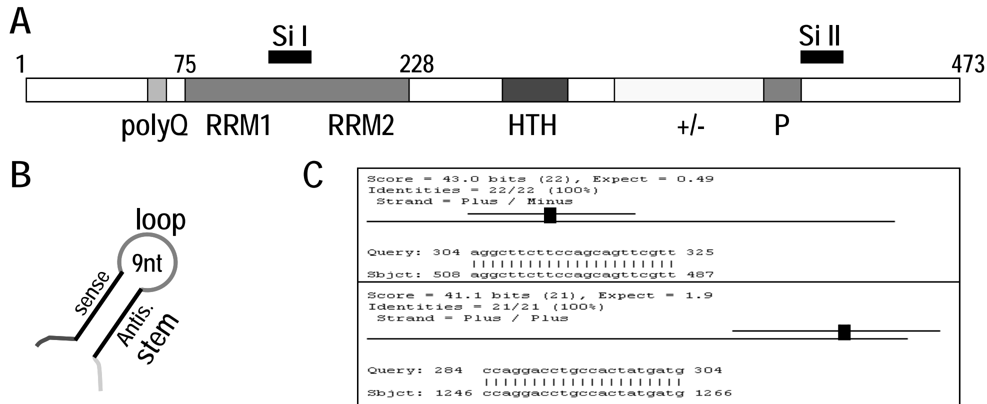



**Figure 19: Two different target sequences were chosen for siRNA design**

**(A)** Two different target sequences were chosen to design NonO-specific small interfering RNA. **(B)** Oligonucleotides are designed to fold once transcribed into a RNA hairpin structure which is processed to functional siRNA in the cell. Therefore they contain a unique target sequence of 19 to 23 nucleotides (19-23nt) in both sense and antisense orientation separated by 9 nucleotides (9 nt) spacer sequence. The resulting transcript of the recombinant vector folds back onto it self to form a 19-23 base pair stem-loop structure. **(C)** Oligonucleotide I and II contain a unique sequence (Si I and Si II) derived from the mRNA transcript of mouse NonO (sp|Q99K48|NONO_MOUSE). Corresponding sites for Si I and Si II are 487-508 (exon 5) and 1246-1266 (exon 9) nucleotides, respectively. These regions are identical in mouse or human NonO coding sequence and differ, partially for Si I and totally for Si II, from the sequence of the homologue PSF (polypyrimidine tract-binding protein-associated splicing factor) which shares high homology with NonO particularly in the RRM region.

The unmodified pSuper vector was transfected into cells as a negative control and not pSuper+ which contained a DNA fragment out of N-CoR coding sequence that may cause undesirable effects in the cells. 293T cells were harvested 48 h and 72 h after transfection with the silencing vectors, pSuper, pSuper NonO Si I and Si II, and the antisense expressing vectors. Efficacy of the siRNAs was first checked at the mRNA level and subsequently at the target protein level (Fig. 20 A).

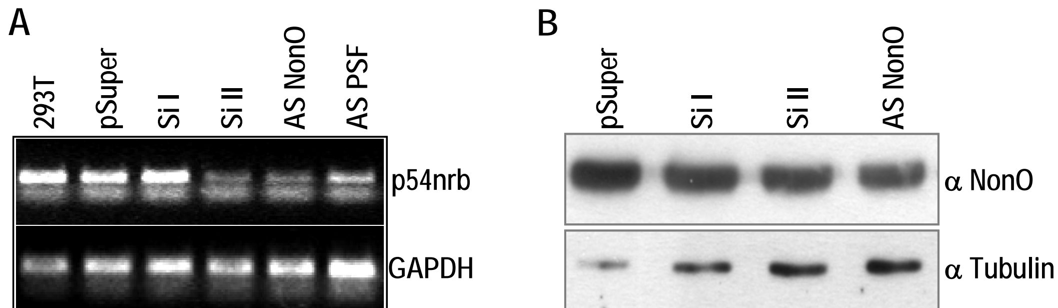



**Figure 20: Analysis of the potency of siRNAs directed against NonO**

**(A)** Total RNA was isolated from 293T cells grown on 10 cm dishes and harvested 48 h after calcium phosphate transfection with 10 µg DNA. 0.5 µg total RNA was reverse transcribed in cDNA which was analyzed by semi-quantitative PCR (25 µl reaction contained 1 µl cDNA diluted 1:40 and 0.1 U polymerase). For each primer pair cycling and Tm were optimized (p54nrb: 35 x at 62°C / GAPDH: 34 x at 55.8°C). Untransfected 293T cells show the same amount of NonO mRNA as pSuper or Si I transfected cells. Si II and AS-NonO affect effectively NonO mRNA content. AS-PSF reduces also NonO mRNA. **(B)** No extensive NonO protein down regulation was observed even 72 h after transfection. Tubulin was detected as a protein content loading control.

The mRNA analysis showed that Si II and NonO-AS were efficiently mediating NonO-specific mRNA degradation in 293T cells whereas degradation was observed neither in pSuper nor in Si I transfected cells. The NonO-mRNA down-regulation which was observed after transfecting PSF-AS vector is presumably due to the high homology of both protein coding sequences. Unfortunately, efficacy of Si II and NonO-AS was low at the protein level even 3 days after transfection (Fig. 20 B). Although no significant specific down-regulation of the protein NonO was archieved an unspecific toxic effect of the NonO-AS silencing vector could be observed by microscopy (Fig. 21). Cells were not growing properly after transfection of the vector containing NonO full-length antisens, limiting protein analysis over 3 days after transfection.

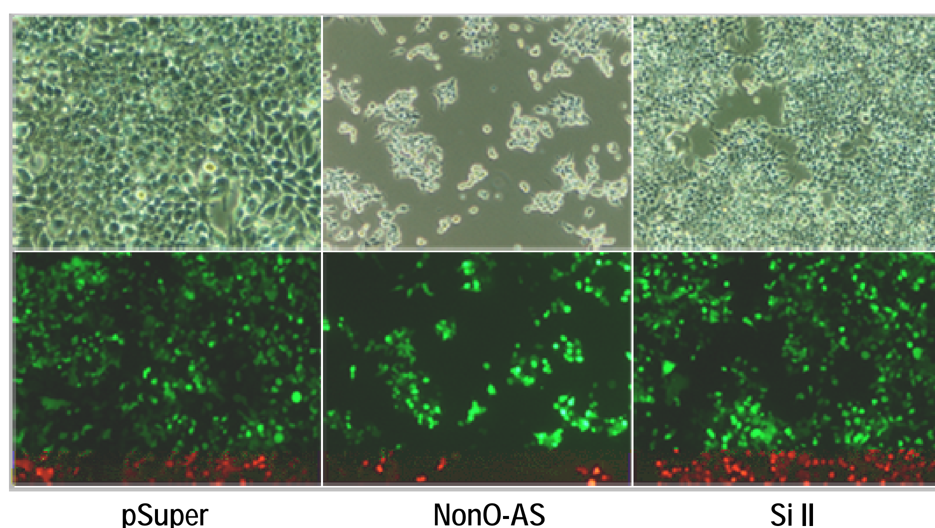



**Figure 21: Toxic effects are observed after transfection of NonO AS but not NonO Si II**

293T cells were PEI transfected with the different pSuper constructs and the NonO-AS expressing vector. GFP was cotransfected to verify transfection efficiency. 72 h after transfection noticeable cell growth differences were observed by microscopy. Cells expressing NonO-AS did not grow properly and the AS transcript seemed to be toxic for the cells whereas cells expressing Si II were growing in a comparable manner to cells transfected with pSuper.

Because of the variability in protein stability and turnover rates in biological systems, the time course and degree of protein reduction may differ significantly from that of the target mRNA. To verify that NonO protein level differ significantly from that of its mRNA due to its stability and low turnover rate, 293T cells were treated with translational (cycloheximide) and proteasome (MG132) inhibitors over 24 h and compared to HDAC2 stability (Krämer et al. 2003) (Fig. 22). In this experiment we observed that the protein NonO is rather stable and has a low turn-over rate.

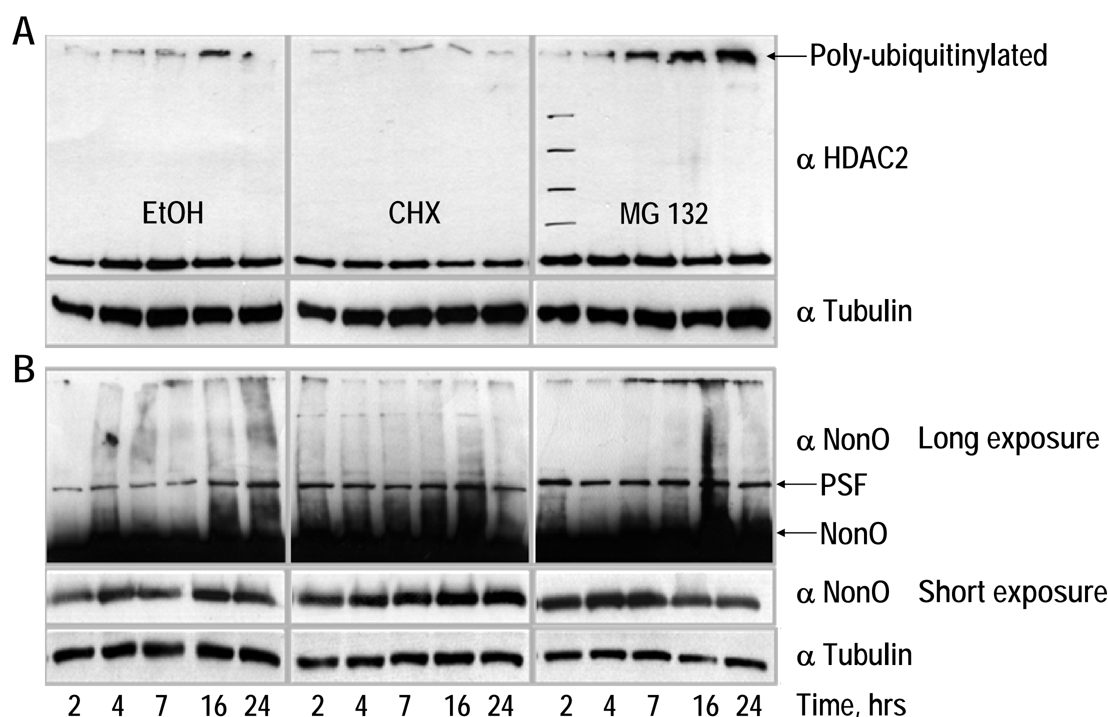



**Figure 22: NonO protein has a low turnover rate**

5 x $10^5$ 293T cells were seeded on 6 well plates and were exposed for the indicated period of time to the ethanol (0.05%), translation inhibitor cycloheximide (1 µg/ml) and proteasome inhibitor MG 132 (5 µM). Cells were harvested all simultaneously boiled in 250µl 2 x SDS loading buffer, twice sonicated (10 puls) and 5-8 µl were loaded on 8% SDS acrylamide gel. Protein levels of HDAC2, NonO and tubulin were determined by Western blot analysis. **(A)** The expression of HDAC2 relative to Tubulin expression was reduced after 24 h cycloheximide treatment and its degradation was proteasome-dependent. **(B)** NonO translation and degradation were not sensitive to protein translation inhibitor and to proteasome inhibitor treatment, respectively. A shorter exposure in the middle of panel B, shows rather constant amount of endogenous NonO at 55 kDa. A long exposure is shown in B above, to observe if higher migrating bands like potential polyubuquitinylated form of NonO were detectable. No significant time-dependent signal appeared. PSF is detected by NonO-specific antibody and appears in all samples at 100 kDa. Tubulin protein levels were determined to compare signal intensity and protein content.

This result indicates that no effective down-regulation of protein expression was achieved in an appropriate time span to analyze variations in functional assays performed 48 h after transfection although the Si II specifically induced NonO mRNA degradation. In addition it was observed that NonO down-regulation induced senescence ($G_0$ arrest) in transformed human cells (P. Tucker personal communication). Therefore, no functional assays were performed by sequential double transfection experiments with NonO directed siRNA, which would efficiently down-regulate NonO protein but led to cell growth arrest. Since neither overexpression nor silencing strategies were conclusive, mapping of N-CoR/NonO interaction regions was performed in *in vitro* assays in order to define potential interaction regions and create deletion mutants that were then tested in reporter assays looking for dominant-negative effects.

### 3.2.3 Construction of NonO deletion mutants

**Mapping of interaction regions by *in vitro* binding assay**

In the NonO protein (non POU domain containing octamer binding protein/ p54nrb) several domains and motifs have been defined (fig. 19). Considering the different defined modules constituting its structure, two Glutathion-S-transferase (GST) fusion proteins were first tested containing the N-terminal half (1-237 aa) with the two RNA recognition motifs and the NonO C-terminal half (238-472 aa) containing the helix turn helix motif. Recombinant proteins were expressed in *E.coli* BL21 Codon Plus and purified using glutathion-agarose beads. The N-CoR C-terminal region (1629-2453 aa) was translated *in vitro* using the TNT-coupled reticulocyte system and labeled with $^{35}$S methionine. Both TNT translated protein and fusion proteins were incubated together, washed several times and GST-pulldown reactions were analyzed by SDS PAGE followed by autoradiography. The NonO region interacting with N-CoR was determined to be in the NonO amino-terminal half (Fig. 23).

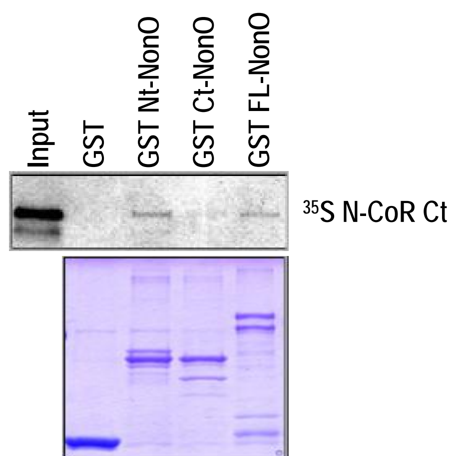



**Figure 23: The carboxy-terminus of N-CoR interacts in the N-terminal region of NonO**

$^{35}$S labeled *in vitro* translated N-CoR Ct (5 µl) was incubated 20min at 37°C with GST or GST-fusion proteins bound to glutathione agarose beads (50 µl) in PPi (0.02% NP40) buffer. After 5 washes proteins retained by the GST-fused proteins were analysed by SDS PAGE (8-10% gel) followed by autoradiography. Coomassie staining of the gel (lower panel) shows equal amounts of fusion protein used for this assay. (Upper panel) The input band intensity corresponds in this experiment to the total amount of TNT translate added to each reaction. No background signal was obtained with GST. GST-NonO FL (1-472) and GST-NonO Nt (1-237) show direct interaction with $^{35}$S N-CoR Ct (1679-2453) in contrast to GST-NonO Ct (238-472) which does not.

**Deletion of the N-CoR interaction region in NonO**

Since the N-CoR interacting region of NonO contains two RNA recognition motifs (RRM) and these domains could be involved either in RNA binding or in protein-protein interaction two deletion mutants lacking one of these RNA recognition motifs were constructed (Fig. 24). Required sequence and structure information for cloning were obtained from NCBI, Expasy and PROSITE data bases.

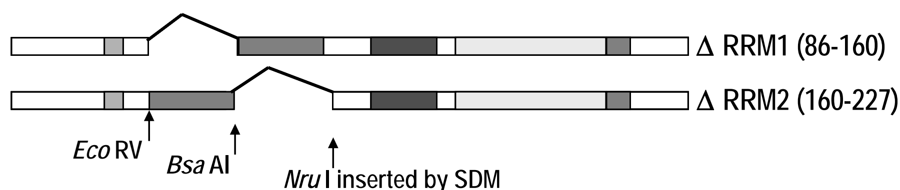



**Figure 24: NonO RRM deletion mutants**

NonO deletion mutants lacking RRM1 (86-160 aa) and RRM2 (160-227 aa) were obtained by deletion of the coding sequences (258-480 bp) and (480-681 bp) in the pENTR NonO full-length (1-1422 bp), respectively, using endogenous restriction sites (*Eco* RV and *Bsa* AI) or inserted restriction site (*Nru* I) by site directed mutagenesis (SMD). After digestion control and sequencing, inserts were transferred in Gateway expression vectors (pEF6-DEST 51 / pDEST 14) by recombination. For cloning in the pGEX AHK vector deletion mutant sequences were amplified by PCR with the adapted primer pair (Fw (start) NonO (*EcoR*I) / Rev (1422) NonO (*Xba*I)).

It was possible to modify the NonO full-length coding sequence within the pENTR vector with restriction endonucleases since the required enzymes presented a limited number of specific restriction sites in the insert and vector sequences. Restriction sites of interest were surrounding RRM target sequences, producing blunt-ends in reading frame. RRM1 and RRM2 sequences were deleted from the NonO full-length sequence in pENTR. After relegation, amplification in *E.coli* plasmids was verified by enzymatic digestion and sequencing (Fig. 25). From the pENTR plasmids, the coding sequences of NonO RRM1 and RRM2 deletion mutants were transferred by recombination in compatible Gateway vectors (pEF6-DEST 51 and pDEST14) or by classical cloning using adated primers for amplification and cloning in pGEX and pCMX vectors.

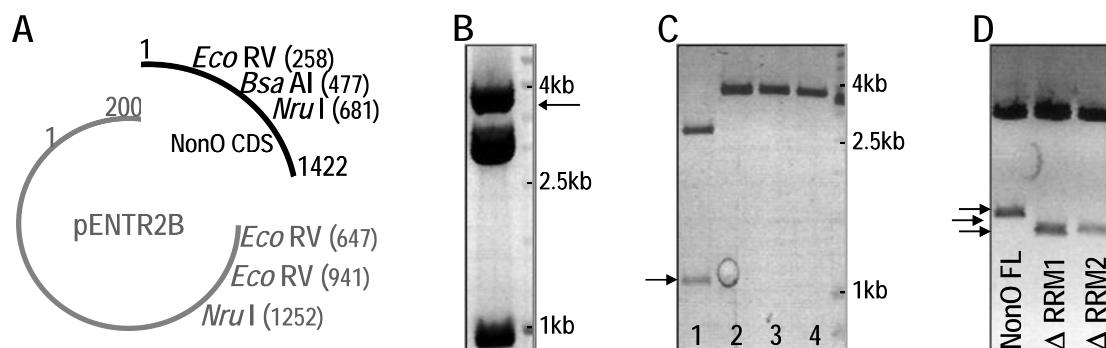



**Figure 25: Deletion mutant cloning strategy**

**(A)** Position of the different restriction sites of interest for deletion in the insert and vector. **(B)** Deletion of RRM1 (258-480 bp) coding region was achieved by digesting with *Bsa* AI (480) and *Eco* RV (258). Since *Bsa* AI was a unique restriction site, 10 µg pENTR NonO (3700 bp) was first linearized (overnight digestion) before 1 minute partial digestion with *Eco* RV that presents two additional sites in the vector backbone. From the different digestion products the linearized fragment lacking 219 bp was isolated, religated and amplified. For RRM2 an additional *Nru* I restriction site was incorporated by site-directed mutagenesis at position 681 of the NonO coding sequence. **(C)** By *Nru* I digestion control positive clones show an insert loss (1346 bp). *Nru* I mutated vector was linearized with *Bsa* AI and sequentially partial digested for 2 minutes with *Nru* I. Largest fragment lacking 204 bp was amplified after agarose gel extraction and ligation. All pENTR NonO constructs were control digested with *Bam* HI / *Not* I **(D)** and sequenced before cloning into expression vectors.

### 3.2.4 Analysis of NonO deletion mutants

#### *In vitro* binding assay

GST-pulldown assays were performed with the purified NonO deletion mutants fused to the GST catalytic domain. The i*n vitro* translated $^{35}$S methionine labeled N-CoR (1679-2453) was specifically retained by GST-NonO full-length (FL), GST-NonO N-terminus (1-237) and GST-Δ RRM2 immobilized on glutathion-agarose beads, whereas no interaction was detected with GST-Δ RRM1 (Fig. 26 A). Since interaction between N-CoR Ct and NonO was impaired with the mutant lacking the RRM1 motif, this region was fused to GST and tested for binding with $^{35}$S N-CoR (1679-2453). N-CoR Ct retention could be detected with GST-RRM1 suggesting that the RRM1 motif is essential and sufficient for the interaction (Fig. 26 B).

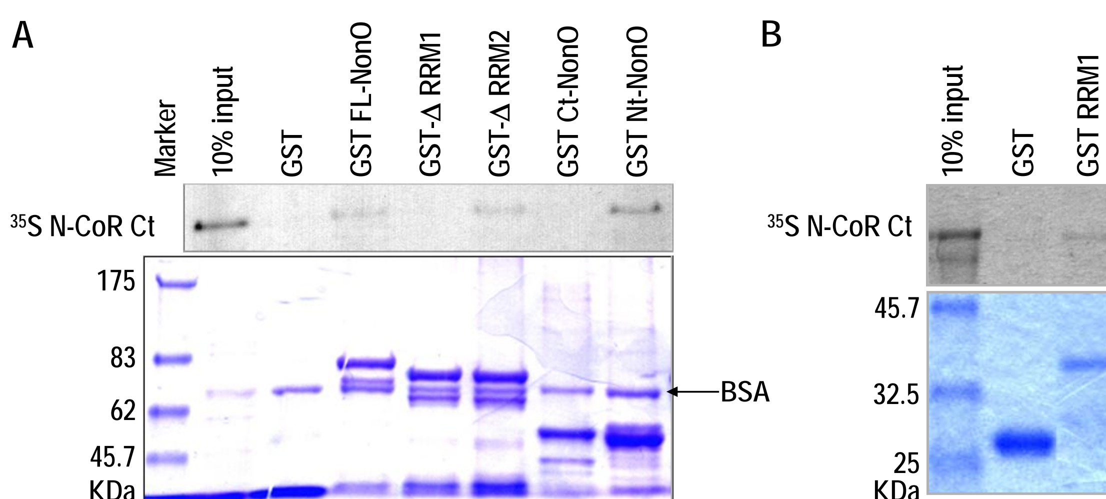



**Figure 26: N-CoR interaction is disrupted by deletion of the RRM1 motif of NonO**

GST-pulldowns were executed as previously described. The input lane represents 10% of total $^{35}$S-labeled protein used in each interaction. No unspecific association with GST alone was observed. **(A)** Coomassie staining of the gel (lower panel) shows equal amounts of fusion proteins that were used for this assay. The autoradiography (upper panel) of this experiment shows that GST-ΔRRM2 was able to retain $^{35}$S N-CoR Ct (1629-2543) to the same extent as GST-NonO FL. In contrast $^{35}$S N-CoR C-terminus is not able to interact with the deletion mutant lacking the RNA recognition motif 1 (GST-ΔRRM1). **(B)** The NonO RRM1 motif is sufficient for the interaction with N-CoR Ct.

#### *In vivo* analysis

Since the deletions were performed in the pENTR-NonO vector the modified coding sequences were easily transferred into the pEF6-DEST 51 mammalian expression vector by recombination. Overexpression of the different NonO mutants in 293T cells was analyzed by Western blot using an antibody directed against the V5-tag. Both RRM1 and RRM2 deletion mutants were detected in whole cell extracts and in the nuclear fractions, in which NonO full-length is found, (Fig. 27). As expected, nuclear localization was not impaired since the predicted nuclear localization signals were downstream of the deleted regions.

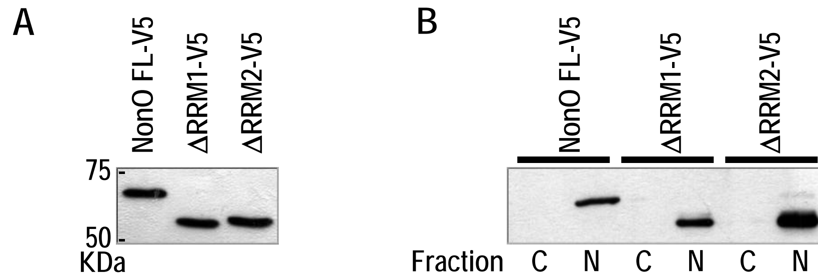



**Figure 27: Nuclear localization of NonO and RRM deletion mutants**

On 10 cm plates 293T cells were PEI transfected with 15 µg DNA and harvested 48 h after transfection. **(A)** In whole cell extract all the different NonO constructs (full-length, RRM1 and RRM2 deletion mutants) were efficiently detected with anti V5 antibody at the different expected molecular weights. **(B)** A fractionation short protocol was used to verify localization of the deletion mutants. As the exogenous NonO wild type deletion mutants are localizing in the nuclear fractions. For NonO detection in Western blot analysis a total amount of 5µg of protein is sufficient.

NonO was described to be modified in a cell cycle-dependent manner. It is hyperphosphorylated upon mitosis (Proteau et al. 2005). Protein analysis of synchronized 293T cells was performed to determine wether the deletion affects posttranslational modifications of the NonO mutants. 293T cell synchronization was monitored by microscopy and Hoechst chromatin staining (Fig. 28).

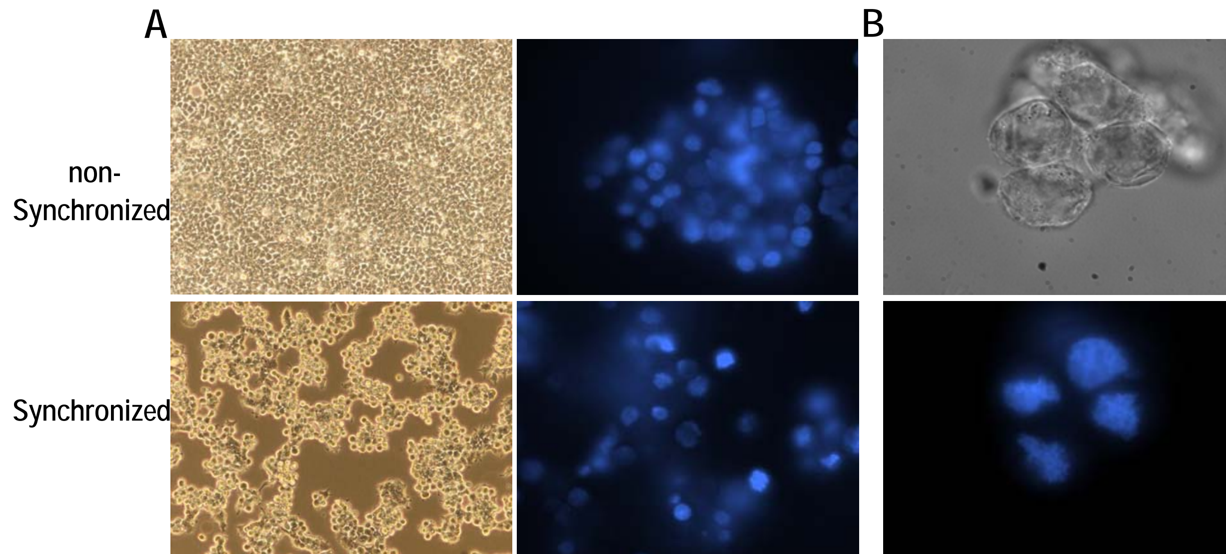



**Figure 28: 293T cell synchronization with thymidine / nocodazole treatment**

$3 \times 10^6$ 293T cells were grown on 10 cm dishes. Cells were synchronized by sequential treatment with thymidine (16 h) and nocodazole (16 h) after an intermediate release step of 8 h. **(A, left panel)** Untreated 293T cells grow asynchronously to confluency whereas thymidine and nocodazole treated cells arrested in the G2/M phase are no longer adherent and adopt a spherical shape. **(A, right panel)** Hoechst/DAPI staining enables chromatin visualization under UV illumination. Imaging was performed with a Zeiss Axiovert 200M microscope and a Zeiss Axiocam camera. Filter setting was 01Ex 365/12 for Hoechst/DAPI staining and excitation was optimal at 365 ± 6 nm wave length. Condensed chromatin events are very frequent in the synchronized cells blocked in the G2/M phase in comparison to asynchronously growing cells. **(B)** Higher magnification of synchronized cells after double block shows typical mitotic condensed chromatin in the nuclei.

For the protein modification analyses cells were harvested with RIPA buffer containing freshly added protease and phosphatase inhibitors. SDS PAGE and immunoblotting were performed with equal amounts of protein. Endogenous NonO protein showed a clear additional band after Nocodazole treatment (Fig. 29).

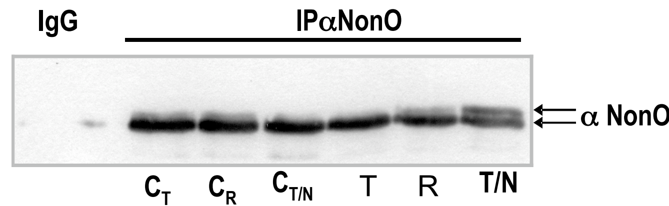



**Figure 29: Posttranslational modification of NonO during mitosis**

Cells were lysed at different treatment time points (T: 16 h thymidine; R: 8 h release; N: 16 h nocodazole) in RIPA buffer containing additional phosphatase inhibitors such as sodium fluorid and sodium orthovanadate. To ensure detection of posttranslationally modified endogenous NonO protein was immunoprecipitated (mouse α NonO) and detected with rabbit NonO-specific antibody (1:5000) in Western blot. The same protein amounts were used for the immunoprecipitation with the same amount of precipitating antibody, mouse IgG were used for the negative control. As a control, non-synchronized cells were lysed at the different time points in the same buffer ($C_T$: 16 h thymidine; $C_R$: 8 h release; $C_{N/T}$: 16 h nocodazole). A double band is noticeable in all control samples whereas this band is not visible in extracts from thymidine-arrested cells. Modified NonO levels (upper arrow) increase between G1/S phase (thymindine-arrested) and G2/M phase (nocodazol arrested).

Cell cycle-dependent modifications were also detected for the RRM1 deletion mutant in contrast to the RRM2 deletion mutant (Fig. 30).

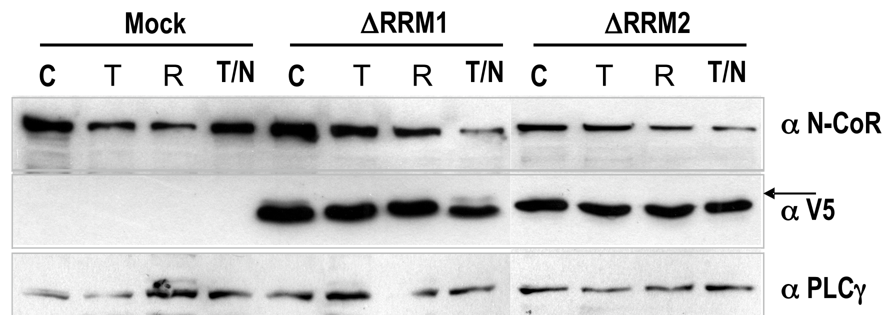



**Figure 30: Cell-dependent modification of NonO deletion mutants**

RIPA extracts were made from cells harvested at different treatment time points (T: 16 h thymidine; R: 8 h release; T/N: T+R+16 h nocodazole and C: untreated). 50µg of protein were loaded on an 8% SDS acrylamide gel. Electrophoresis was run until the 47.5 kDa marker bands reached the bottom of the gel. Separated proteins were then transferred onto a nitrocellulose membrane for Western blotting. Protein loading control was checked by detecting PLCγ (148 kDa). NonO overexpressed deletion mutants were detected with anti-V5 antibody (1:5000). Mock transfected cells do not express any V5 tagged proteins (left) but show variations in N-CoR level. ΔRRM1 is nicely expressed and shows cell cycle phase dependent modification whereas ΔRRM2 does not. In addition significant differences in N-CoR protein levels are observed compared to those in mock-transfected cells especially in the presence of ΔRRM2.

Interestingly, when N-CoR protein levels were analyzed at different time points, a clear reduction of signal intensity was observed in the presence of overexpressed RRM2 deletion mutant in comparison to mock-transfected cells or to cells transiently expressing the RRM1 deletion mutant. This suggests that overexpression of the RRM2 deletion mutant may impair

N-CoR protein regulation and/or stability. Whether this effect is due to the impaired function of the mutant lacking the RRM2 domain and altered cycle-dependent modification could not be elucidated. In protein analysis of synchronized cells, we observed that N-CoR protein level varies in G2/M arrested cells (T/N) in the presence of the NonO deletion mutants. To assess whether NonO mutants can affect N-CoR repressive activity reporter assays were performed with both mutants and compared to the effect obtained in the presence of NonO wild type.

### Analysis in Gal4-reporter assay

The deletion mutants were first tested in the Gal4-reporter assay in the presence of Gal-N-CoR full-length. The N-CoR repressive activity in this assay was affected by overexpression of NonO deletion mutants although exogenous NonO expression did not. Both mutants enhanced repression in a concentration-dependent manner although ΔRRM2 to a lesser extent then ΔRRM1 (Fig. 31). Since in the Gal4-reporter assay effects obtained in the presence of exogenous NonO and mutants differed, the effect of these mutants was further investigated in additional functional assays. Therefore, reporters under the control of nuclear receptor response elements such as thyroid receptor (TRE), retinoic acid (RARE) or estrogen nuclear receptor (ERE) were employed to investigate NonO and mutant effects.

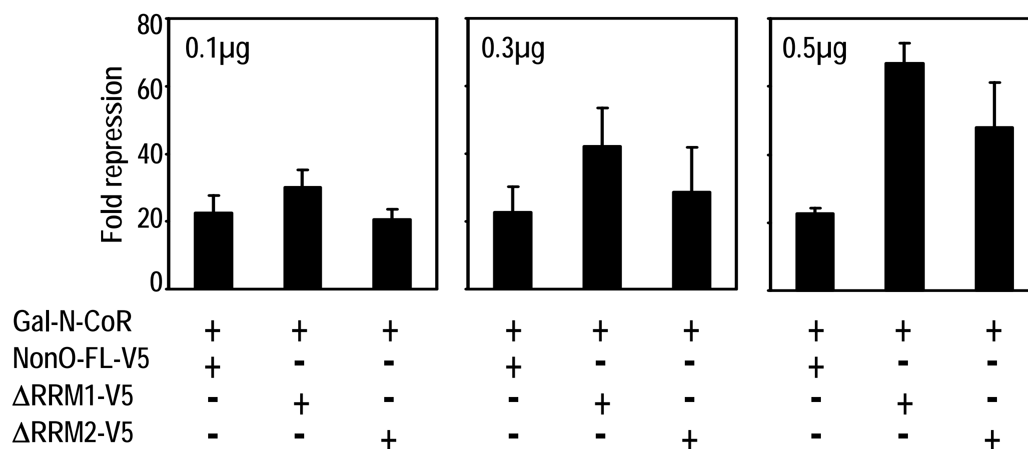



**Figure 31: Gal-N-CoR repression activity is affected by overexpression of RRM deletion mutants**

Reporter assays were performed on 12 well plates in triplicates. Fold repression was calculated comparing normalized luciferase activity obtained with Gal4-DBD and Gal-N-CoR full-length (1-2543). 0.5 µg Gal4-repoter and 0.3 µg Gal4-DBD or Gal-N-CoR FL expression plasmid were cotransfected in 293T cells with increasing amounts of NonO or RRM deletion mutant expression vectors (0, 0.1, 0.3, and 0.5 µg) transiently cotransfected into 293T cell. Exogenous wild type NonO does not affect Gal-N-CoR full-length dependent repression whereas ΔRRM1 expression enhances repression in a concentration-dependent manner. ΔRRM2 also impairs N-CoR repression but to a lesser extent. Fold repression is increased in the presence of NonO RRM deletion mutants lacking the first or second RNA recognition motifs. The graphs represent the mean of three independent experiments.

## 3.3 Role of NonO in nuclear receptor-dependent transcription

### 3.3.1 Nuclear receptor-dependent reporter assays

N-CoR is involved in a number of transcriptional repression processes and it is recruited by different types of nuclear receptors and transcription factors to target gene promoters. Recruitment of N-CoR mediates transcriptional repression which is associated with co-repressor complex formation, the recruitment of HDACs and the resulting histone modifications. Nonsteroid receptors like TR or RAR in the absence of ligand reside in the nucleus as heterodimers with RXR (Kliewer et al. 1992) and associate with N-CoR to actively repress target genes. However, upon agonist binding they undergo conformational changes and become potent transcriptional activators. In contrast, steroid hormone receptors such as glucocorticoid and estrogen receptors are retained in the cytoplasm complexed with heat shock proteins in the absence of ligands. Ligand binding is required to induce homodimerization and translocation of these receptors to the nucleus. N-CoR recruitment by this type of receptor is ligand- and DNA binding element-dependent. The association or dissociation of cofactors to the nuclear receptors can be investigated in NR-dependent transcription assays. To assess whether NonO affects N-CoR nuclear receptor-dependent repression, different reporter assays were performed comparing exogenous NonO and deletion mutant effects. Thyroid hormone and retinoic acid receptor-dependent reporters were used in 293T cells for these investigations as well as an estrogen receptor-dependent reporter in MCF7 breast cancer cells.

The thyroid hormone receptor-dependent reporter 2x DR4 TK Luc (Jow and Mukherjee 1995) was tested in 293T cells. Only in the presence of exogenous TR $\alpha$ or $\beta$ a specific response to agonist stimulation with TRIAC (Tri-iodo-thyro-acetic acid) was obtained. Namely, 293T cells do not express endogenously thyroid receptor isoforms although they express the heterodimerizing partner retinoic X receptor. Exogenous RXR expression with exogenous TRs did not improve resulting luciferase activity and the RXR expression vector was therefore no longer cotransfected. However, the basal transcriptional activity of this transiently transfected reporter was rather low and was not suitable for investigation of transcriptional repression modulation (data not shown).

The RARE-reporter assay (Mangelsdorf et al. 1991) was adapted to cell type and purposes. The amounts of cotransfected DNA plasmids (reporter and nuclear expression vectors) were modified. Because 293T cells are a highly transfectable cell line, reporter quantity was minimized in order to obtain valid luciferase activity measurements. In addition it was observed that an excessive amount of reporter renders it less sensitive to stimulation with RAR agonist (ATRA) or antagonist (synthetic RAR antagonist mix 193840) for which efficient concentrations were determined. Furthermore, in 293T cells endogenously expressing RAR and RXR receptors, no significant improvement of the reporter assay was observed upon expression of exogenous receptors. Therefore, nuclear receptor expression vector cotransfection was omitted. RARE-reporter transfection and stimulation of cells were carried out in medium supplemented with 10% charcoal stripped fetal bovine serum to increase response specificity. Since RAR/RXR can recruit N-CoR and its homologue SMRT which are endogenously expressed in 293T cells it was verified whether this established assay was

sensitive to N-CoR overexpression or down-regulation by siRNA. Exogenous N-CoR expression enhanced repression in the absence of ligand and upon antagonist whereas transfecting an N-CoR silencing vector (Ishizuka and Lazar 2003) induced opposite effects (Fig. 32). These results confirm that this assay is suitable to investigate the function of N-CoR/NonO interaction.

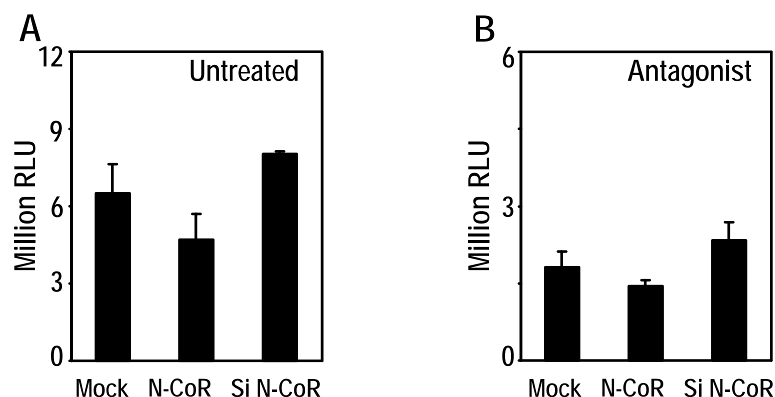



**Figure 32: RARE is a suitable reporter assay to investigate the modulation of N-CoR function**

0.3 µg RARE-reporter was cotransfected with 0.2 µg N-CoR expression vector or 0.8 µg N-CoR pSilencing vector, SV40 ß-galactosidase internal control and GFP expression vector 24 h before treatment. Transfection of 293T cells were carried out in the presence of charcoal stripped serum. Cells were harvested 24 h after treatment and luciferase activity was measured. Relative luciferase activity (RLU) was determined by normalization to levels of $\beta$-galactosidase expression directed by the internal control. **(A)** Exogenous N-CoR expression reduces luciferase transcription in the absence of ligand and **(B)** in the presence of RAR synthetic antagonist (0.1 µM). The opposite effect is observed reducing endogenous N-CoR level by transfecting N-CoR silencing vector; however, N-CoR homolog SMRT is also endogenously expressed in 293T cells and can be recruited by the retinoic acid receptor. Mean of induction values were determined from three independent experiments.

Interestingly, expression of exogenous NonO affected RAR-dependent reporter regulation (Fig. 33). General observed tendency is an enhanced transcription in the absence of ligand and in the presence of agonist suggesting that NonO overexpression enhances basal transcription as ATRA induced transcription at the different concentrations. However, in the presence of antagonist transcription repression remains unchanged upon exogenous NonO expression.

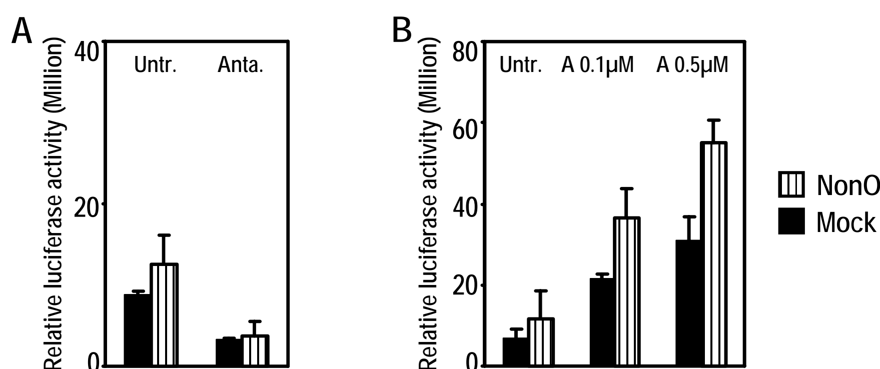



**Figure 33: RARE-reporter response to stimuli is not affected upon exogenous NonO expression**

The reporter assay was executed as preciously decribed in the presence and absence of exogenous NonO (0.2 µg). Cells were treated for 24 h with RAR antagonist mix 193840 (0.1 µM) (Anta.) and with ATRA at two different concentrations (0.1-0.5 µM) (A 0.1 µM; A 0.5 µM). Relative luciferase activity was determined by normalizing measured luciferase activity to levels of $\beta$-galactosidase expression directed by the internal control. **(A)** Exogenous NonO enhanced basal transcription in the absence of ligand but not in the presence of antagonist. **(B)** In the presence of overexpressed NonO ligand

concentration-dependent reporter transcriptional activation is enhanced. Mean of induction values were determined from two independent experiments.

Interestingly, in gel filtration assays endogenous NonO was detected in the same high molecular weight fractions containing N-CoR. However, upon treatment with retinoic acid receptor agonist a shift of NonO to higher molecular weight fractions was observed (Fig. 34) suggesting that it is sensitive to agonist simulation. This effect was also seen for RNA Polymerase II (Pol II) where complex formation and activity is likely to be enhanced upon agonist treatment. NonO was described to interact with Pol II (Emili et al. 2002). To assess whether NonO shift was due to its interaction with Pol II co-immunoprecipitation was performed with the fractions. Although NonO and Pol II were successfully immunoprecipited no co-immunoprecipitation was detected precluding any firm conclusion (data not shown).

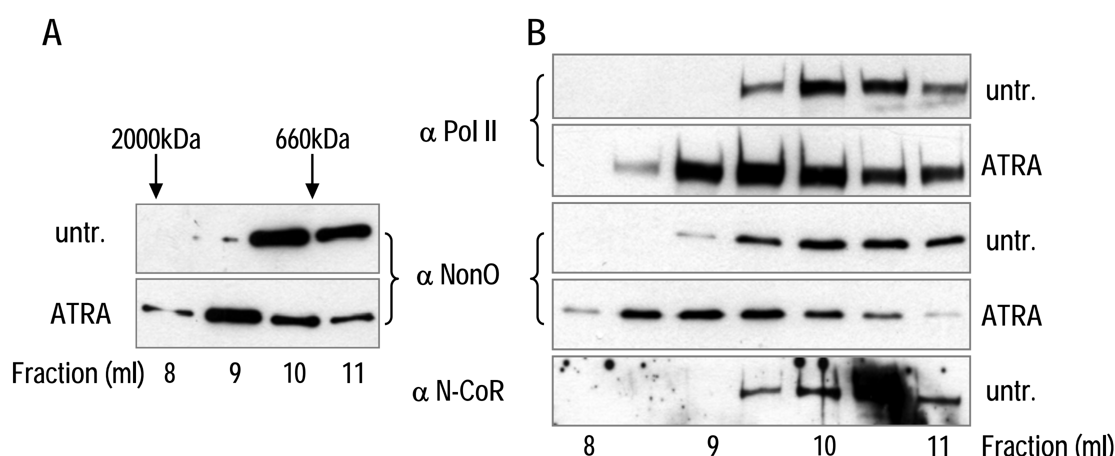



**Figure 34: Gel filtration analysis upon treatment with the retinoic acid receptor agonist ATRA**

293T cells were grown in medium containing stripped FCS on 10 cm dishes and were treated with ATRA (100 nM) for 24 h. Cells were harvest in 600 µl gel filtration lysis buffer. After two rounds of sonication (10 pulses) lysates were clarified by sedimentation (10 min at 14000 rpm) and 450 µl lysate was applied to a calibrated SUPEROSE 6HR gel filtration column and run by a FPLC apparatus (ÄKTA, Amesham Biosciences). One column volume (24 ml) was collected in 1 ml aliquots. Equal volumes of collected fractions were subjected to SDS-Page and immunoblotting. **(A)** Since NonO shifted to higher molecular weight fractions upon ATRA treatment, the same experiment was performed collecting 500 µl aliquots. **(B)** 0.5 ml aliquots were collected upon elution to confirm the shift obtained with NonO and RNA polymerase II (Pol II) upon ATRA treatment.

Since N-CoR was not always detectable in gel filtration fractions, especially in the presence of ATRA we verified that this was not due to treatment-dependent degradation. Therefore, cells were lysed in Laemmli loading buffer and analyzed by Western blot after SDS PAGE (Fig. 35). N-CoR protein levels in 293T cells were not affected by ATRA treatment suggesting that the lack of detection may be due to antibody detection limits or protein degradation occurring during experimental procedures. However, we continued the functional assays with the RRM deletion mutants and exogenous NonO wild type.

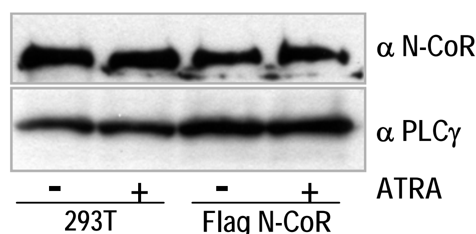



**Figure 35: N-CoR protein levels are unchanged upon treatment with ATRA**

Endogenously and exogenously expressed N-CoR level were anylized in 293T cells upon ATRA treatment. Cells were grown and treated with 100 nM ATRA in medium containing stripped serum. Cells were scraped from the dish 24 h after treatment and directly boiled in Laemmli buffer for SDS PAGE and Western blotting. Electrophoresis on a 6% acrylamid gel was run over 4 h. N-CoR (270 kDa) was detected with N-CoR-Ct directed affinity purified polyclonal antibody diluted 1:2000. As a loading control PLC γ (148 kDa) was detected.

Steroid hormone receptor transcriptional regulation differs from that of other nuclear receptor classes. Thus, comparable experiments were performed with the estrogen receptor dependent reporter (ERE-reporter). For this purpose the ERE-reporter assay was established in MCF7 cells which endogenously express estrogen receptors (ER). To ensure that the reporter response was ligand-specific and ER-dependent the reporter was transfected in MCF7 and 293T cells. In 293T cells which do not express ER ligand-independent luciferase activity could be measured whereas in MCF7 cells transcription activity was estradiol-dependent (Fig. 36).

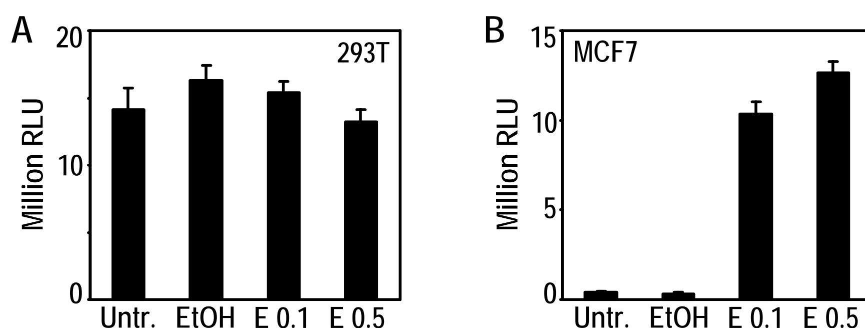



**Figure 36: ERE-reporter ligand dependant stimulation in MCF7**

MCF7 cells were plated in RPMI medium supplemented with 10% stripped serum one day before FuGENE (Roche) transfection (2 x $10^5$ cells per 12 well). 1.4 µg total DNA including 0.6 µg ERE-reporter, 0.1 µg SV40 β-galactosidase reporter, 0.1 µg GFP expression vector and 0.2 µg NonO FL or deletion mutant expression vectors were added to the cells with a reagent/DNA ratio of 3:2. Serum-free medium was used during transfection and replaced with medium containing stripped-serum 6 h later. On the next day cells were treated for 24 h. As estradiol is solubilized in ethanol containing 10% DMSO ($10^{-2}$M) and diluted to 1.5 mM in 100% ethanol and applied to a final concentration in medium of 0.5 µM, cells were treated as control with 100% ethanol diluted 1:3000 in medium. **(A)** 293T cells transfected with the same ERE-reporter reaction mix do not respond to estradiol stimulation whereas MCF7 cells do since they contain estrogen receptors **(B)**. The graphs represent the mean of two independent experiments done in triplicates.

NonO wild type and mutants were tested in the ERE-reporter assay and effects were compared with those obtained with the RARE-reporter. To facilitate comparison of the results obtained in the different reporter assays, normalized luciferase activity was converted to per-cent, considering NonO wild type values as 100% at the highest agonist concentration. The effects that were obtained expressing-NonO or RRM deletion mutants with the different reporters differ. Retinoic acid receptor-dependent transcription was affected (Fig. 37 A)

whereas estrogen-dependent transcription was not (Fig. 37 B). NonO ΔRRM1 exerted similar effects as in the presence of exogenous NonO wild type in the RARE-reporter and ERE-reporter. However, NonO ΔRRM2 enhanced RAR-dependent transcription to a larger extent than in the presence of exogenous wild type NonO and NonO ΔRRM1. This effect was not observed with the steroid receptor-dependent reporter.

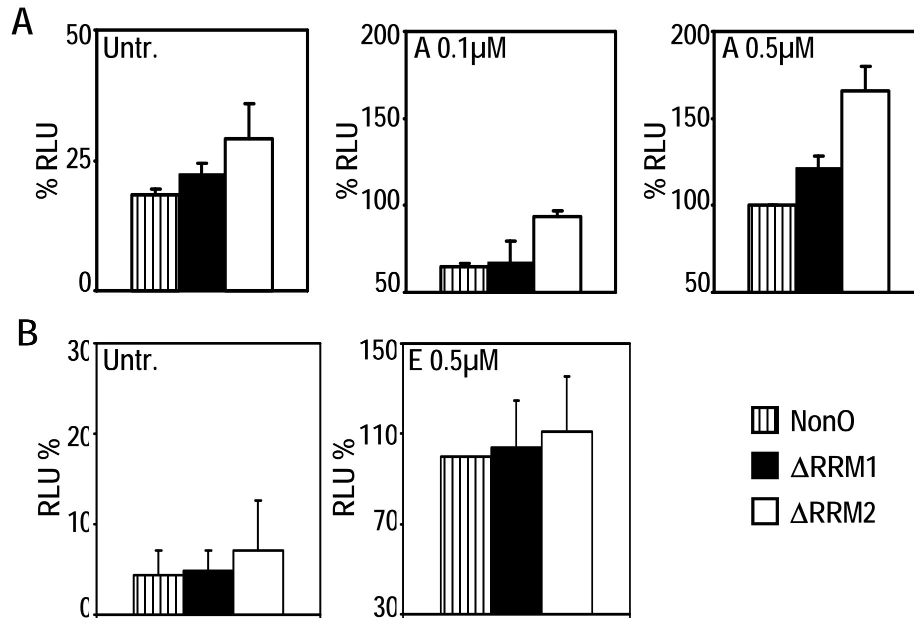



**Figure 37: ΔRRM2 mutant enhances transcription in the RARE-reporter**

Reporter assays were performed as described previously in 293T cells and MCF7 cells. For comparison of the results obtained in the different reporters in the cell lines normalized luciferase activity was converted to percent. **(A)** In the RARE-reporter (0.2 µg) the RRM2 deletion mutant enhances baseline activity and agonist-stimulated transcription. **(B)** In contrast comparable effects were not observed with the ERE-reporter. Mean of induction values were determined from two independent experiments for the RARE-reporter and from four independent experiments for the ERE-reporter.

The significant increase in luciferase activity in the presence of the RRM2 deletion mutant compared to NonO wild type and the RRM1 deletion mutant seems to be reporter-specific and ligand-independent since this effect is observed only with the RARE-reporter and upon any treatment, untreated, with agonist or with antagonist (Fig. 38).

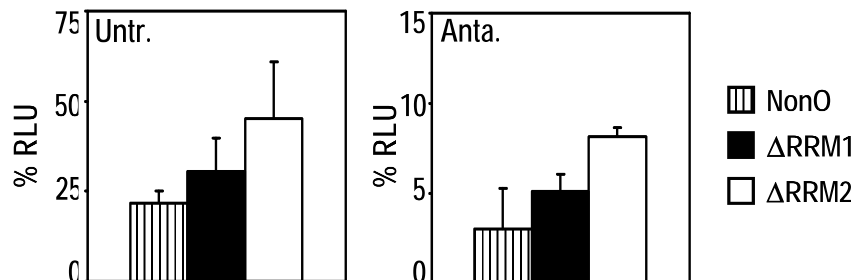



**Figure 38: The ΔRRM2 mutant enhances transcription also upon antagonist treatment**

In the RARE-reporter assay NonO RRM2 deletion mutant overexpression significantly enhances transcription in a ligand-independent manner. Either the basal transcription (left panel) or the antagonist-dependent repression (right panel) are affected by overexpression of the RRM deletion mutants and notably by ΔRRM2 in comparison to NonO wild type. Mean of induction values were determined from two independent experiments done in triplicates.

These observations suggest that NonO may be a modulator of N-CoR function. The stability of the nuclear receptor-dependent N-CoR complex seems to be affected rather than co-repressor complex formation itself since this effect was not observed in the Gal4-reporter assay. Supposing that NonO influences nuclear receptor dependent repression level, it is conceivable that ΔRRM2 overexpression, lacking the RNA recognition motif 2 which in turn impaires its function and affects endogenous NonO function. In order to confirm this hypothesis, regulation of endogenous genes was investigated.

### 3.3.2 Endogenous gene expression analysis

Since 293T cells respond to RAR ligand stimulation in RARE-reporter assays without exogenous expression of retinoic acid receptors, the regulation of known retinoic acid receptor-dependent genes was analyzed in this cell line. Therefore, semi-quantitative PCR analysis was performed and established in the human kidney embryonic 293T cell line to investigate the regulation of the RARβ2 gene, amongst others genes. Total RNA was isolated from 293T cells and randomly reverse-transcribed into DNA. Mature messenger RNAs are spliced and lack the intronic sequences that are contained in the corresponding genomic DNA sequence. To ensure the distinction of PCR fragments amplified complementary DNA (cDNA) or genomic DNA, primers were designed to span intron regions. The RARβ2 gene showed a specific and time-dependent response to stimulation with ATRA in 293T cells (Fig. 39 A), therefore the analysis of this gene was used to further investigate the effects of different NonO constructs in this assay. As internal control the GAPDH house keeping gene was chosen, since its RNA expression level was not altered in the determined experimental conditions. 293T cells were grown in medium supplemented with stripped serum, transfected with empty expression vector (pDEST) versus NonO wild type or NonO RRM deletion mutant expression vectors. 24 h after transfection cells were stimulated for 24 h or 16 h with ATRA. To obtain comparable cell numbers, cells were harvested at the same time point. Total RNA was isolated and its concentration measured by spectrophotometry at λ 260 nm. Quality of the isolated RNA products was also checked by formaldehyde agarose gel electrophoresis (Fig. 39 B). 0.5 µg RNA was reverse-transcribed into cDNA using random hexamers as primers to transcribe the total RNA population so that different PCR analyses could be performed on the same cDNA sample. 1 µl of the 1:40 diluted cDNA reaction was used for PCR analysis. PCR products were loaded onto Ethidium bromide-stained, 1.5% agarose gels. Gel images were captured with a CCD camera. A mean of several experiments is represented with the corresponding internal control (GAPDH) and control samples (pDEST transfected cells) (Fig. 39C). No significant variation of the stimulated gene was observed in the presence of overexpressed NonO or RRM1 deletion mutant comparing RARβ2-specific signal intensity to that of GAPDH internal control. However exogenous expression of RRM2 deletion mutant seems to increase transcription which confirms results found in the transient RARE-reporter assay.

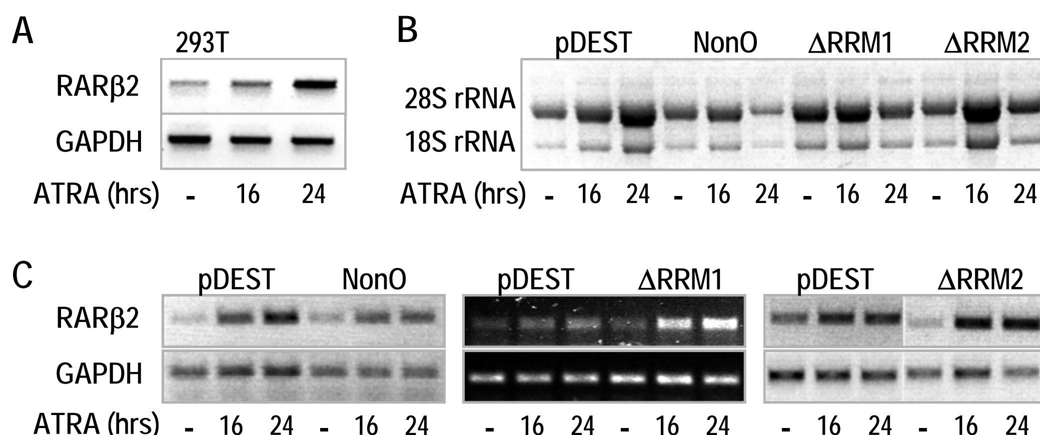



**Figure 39: Semi-quantitative PCR analysis of endogenous gene expression in 293T cells**

In 293T cells retinoic acid-dependent gene expression was analyzed upon ATRA treatment. mRNA analysis was performed by semi quantitative PCR. **(A)** RARβ2 gene is responsive to agonist treatment in 293T cells. Up-regulation of the gene is observed in a time dependent manner whereas house keeping gene expression (GAPDH) is unchanged. **(B)** Total RNA was isolated from transfected and treated cells and its quality was checked by formaldehyde agarose gel electrophoresis. Ribosomal RNA subunits 28 S and 18 S appear at a 2:1 ration in clean RNA products. **(C)** Semi-quantitative PCR analysis of the cDNA in the presence of exogenously expressed proteins. Empty expression vector pDEST was transfected in control samples. Considering house keeping gene band intensity and RARβ2-specific signal, no significant difference in gene regulation is observed comparing mock transfected cells to cells overexpressing NonO wild type or the RRM1 mutant and slight transcription enhancement is observed in the presence of RRM2 deletion mutant.

## 3.4 N-CoR Ct is involved in intramolecular regulation

Some N-CoR interaction partners have been shown to interact not only with the N-CoR amino-terminal region (Nt) which is sufficient for binding but can also interact with N-CoR carboxy-terminus (Ct) *in vitro* (e.g. Sin3A, HDAC3) (Wen et al. 2000). This suggests that these two domains may fold independently however are adjacent in the final protein tertiary structure. It is conceivable that the carboxy-terminal region is involved in N-CoR intramolecular regulation. In GST-pulldown assays, TNT-translated N-CoR Nt (1-549) region interacts with the GST fused C-terminal N-CoR region (1954-2453) (Fig. 40).

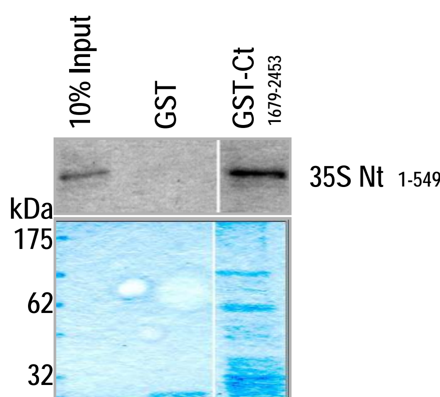



**Figure 40: N-CoR N-terminal and C-terminal regions interact *in vitro***

GST-pulldown assays were performed as previously described. The input lane represents 10% of total $^{35}$S-labeled protein used in each interaction. Coomassie staining of the gel (lower panel) shows some degradation products of the GST-Ct N-CoR (1679-2453) in the sample but also the intact full-length construct migrating at the correct molecular weight (77kDa) and in comparable amount to GST. Autoradiography (above panel) shows direct, specific interaction of the *in vitro* translated N-CoR Nt (1-549) with the GST-fused N-CoR Ct.

In addition in Gal4-reporter assays, the Gal-N-terminus region containing the HDAC interaction domains has greater repression potency then the full-length N-CoR protein which contains both Nt and Ct regions, suggesting that in the presence of the carboxy-terminus some modulation occurs (Figure 4c from (Tiefenbach et al. 2006)) (Fig. 41).

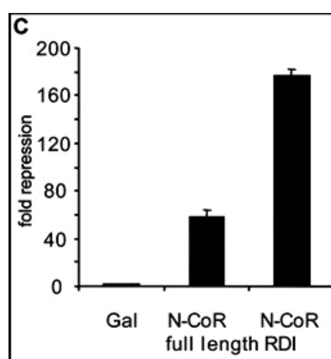



**Figure 41: Gal-N-CoR full-length represses less than the Gal-N-CoR amino-terminus**

To compare repression potency of different N-CoR regions fused to the Gal4-DBD, UAS-TK-Luc reporter (1 µg) was co-transfected with an internal control (SV40 reporter) and the different Gal4 construct expressing vectors (0.2 µg) into 293T cells on 12 well plates. Fold repression was determined relative to the basal reporter activity in the presence of the Gal4-DBD. Gal-N-CoR full-length and Gal-N-CoR (1-549) (Gal-RDI) show transcriptional repression. However, the repression potency or fold repression is greater in the presence of Gal-N-CoR Nt than Gal-N-CoR full-length. This was observed in at least three independent experiments. Mean of induction values were determined from one experiment done in triplicates. This experiment was performed by Jens Tiefenbach.

Furthermore, in the RAR-dependent reporter overexpression of Ct-N-CoR (1587-2543) impaired transcription activation in the presence of ATRA (Fig. 42). The N-CoR carboxy-terminus 1587-2543 containing the nuclear receptor binding domain is thought to interact with unlignaded receptors at the promoter region. Agonist activation with ATRA induces nuclear receptor conformational changes enhancing N-CoR dissociation and coactivator recruitment. The equilibrium of dissociation and association is affected in the presence of exogenous N-CoR carboxy-terminus expression.

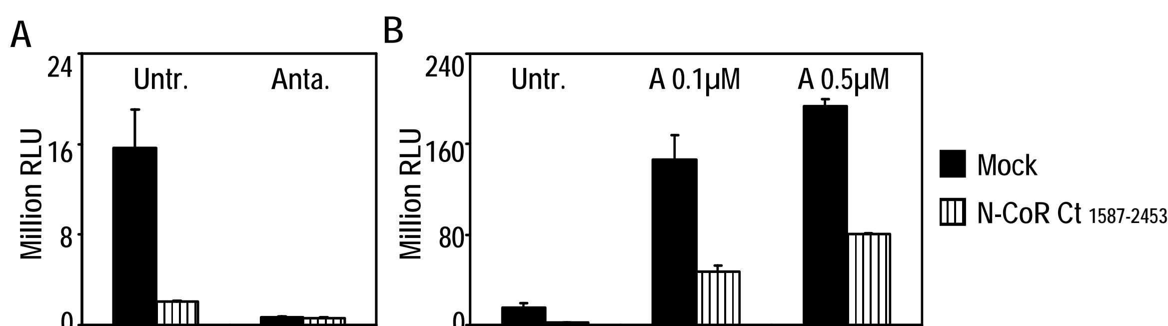



**Figure 42: N-CoR carboxy-terminus does not dissociate upon activation**

The RARE-reporter assay was performed as previously described. 0.3 µg N-CoR Ct (1587-2453) expressing vector was cotransfected with DR5 reporter and an internal control. Normalized luciferase activity (RLU) obtained upon stimulation is shown **(A)** Basal transcription is impaired in the presence of N-CoR Ct whereas active repression is not. **(B)** Transcription activation upon agonist treatment is affected upon N-CoR Ct overexpression. Mean of induction values were determined from one experiment done in triplicates.

Taken together, these results indicate that the N-CoR carboxyl terminal-half requires the amino-terminal domain to dissociate from activated receptors. The results support that Nt- and Ct-regions may be adjacent and regulate each other. Therefore, the NonO and N-CoR amino-terminal interaction was tested *in vitro*. Indeed, TNT translated N-CoR-Nt (1-549) was pulled down by the GST-NonO amino-half containing the RRM motifs which also interacts with the N-CoR carboxy-terminus (Fig. 43). In addition the RRM1 fused to GST seems to be sufficient for this interaction.

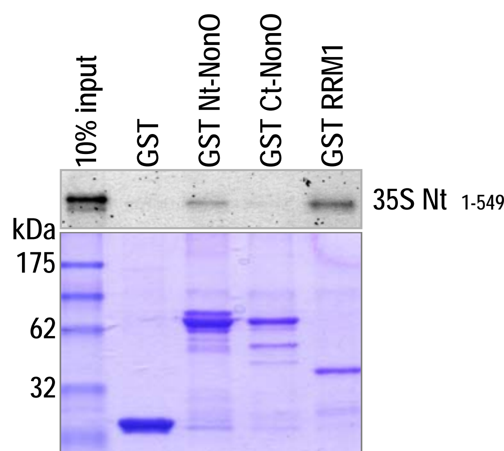



**Figure 43: The N-CoR amino-terminus interacts with the GST-fused NonO-RRM1 domain**

GST-pulldowns were performed as previously described. Coomassie staining of the gel (lower panel) shows the amounts of fusion protein used for this assay. The input lane represents 10% of total $^{35}$S-labeled protein used in each interaction. The autoradiography (upper panel) of this experiment shows that GST-NonO Nt (1-237) retains $^{35}$S N-CoR Nt (1-549) in contrast to GST-NonO Ct (255-472). Interestingly, GST-RRM1 (86-160) is sufficient to bind N-CoR Nt. No unspecific association with GST alone was observed.

The RRM1 domain consists of 80 amino acids which are known to fold into a $\alpha\beta$ sandwich (Maris et al. 2005). It is conceivable that the interaction with the N-CoR N-terminus or C-terminus occurs within the same region of NonO without necessarily involving identical interacting surfaces. It is likely that NonO plays a regulatory role in the nuclear receptor-dependent gene regulation, since it interacts with both N-CoR-Nt and -Ct regions that in turn may be engaged in intramolecular interaction and exert reciprocal regulation. Since the N-CoR-Nt region which interacts with NonO contains two distinct domains, corresponding GST constructs were used in binding assays to assess which of both interacts with $^{35}$S NonO full-length. The GST fused repression domain I (RDI: 1-393) and the SANT domain containing SANT1 and SANT2 motifs (SANT 1/2: 435-683) were tested (Fig. 44). TNT-translated NonO preferentially bound to the SANT domain and an additional, although weaker, specific signal was obtained with the repression domain I. Interestingly, the SANT domain of N-CoR is a regulatory region in the N-CoR amino-terminus. The SANT1 motif was described to be involved in HDAC3 activation (Guenther et al. 2001) and in recruitment of SUMO ligases (Tiefenbach et al. 2006) whereas the SANT2 motif is involved in histone interaction and enhances N-CoR repressive activity (Yu et al. 2003). Whether there is a direct correlation between the effects observed in the RARE-reporter by exogenously expressing NonO and

the recruitment of SUMO ligases at the N-CoR SANT1 domain remains to be elucidated; however I performed RARE-reporter assays expressing exogenously Gam-1 which inhibits the SUMOylation pathway (Boggio et al. 2004) (Fig. 44).

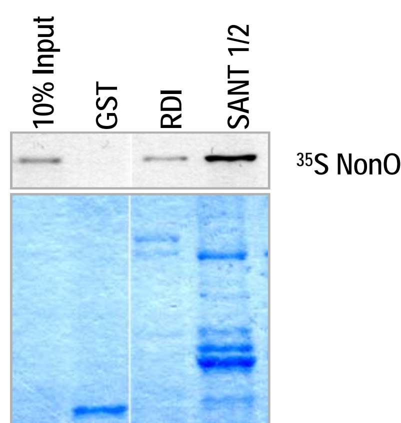



**Figure 44: NonO interacts preferentially with the SANT1/2 domain within the N-CoR-Nt**

GST-pulldowns were performed as previously described. Coomassie staining of the gel (lower panel) shows the amounts of fusion protein used for this assay. The input lane represents 10% of total $^{35}$S-labeled protein used in each interaction. The autoradiography (upper panel) of this experiment shows that GST-NCoR SANT1/2 (435-683) retains strongly $^{35}$S NonO full-length. Interaction is also observed with GST-N-CoR RDI (1-393) however to a lesser extend. No unspecific association with GST alone was observed.

Surprisingly, similar effects as those obtained upon exogenous NonO expression were observed in the presence of Gam-1 (Fig. 45)(published in (Tiefenbach et al. 2006)).

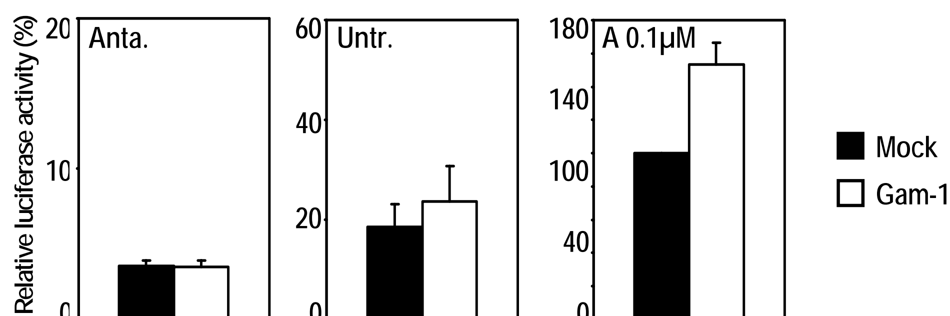



**Figure 45: Effect of SUMO modifications in the regulation of RAR-dependent transcription**

0.5 µg RARE-reporter was cotransfected with Gam-1 expressing vector and SV40 internal control. Normalized luciferase activity (RLU) obtained upon stimulation is represented in percent. Left panel, in the presence of antagonist mix 193840 (100 nM) Gam-1 expression does not affect transcriptional repression. Middle panel, luciferase expression is enhanced in the presence of Gam-1. Right panel, inhibition of SUMOylation increases RAR-dependent transcription. Mean of induction values were determined from three experiments done in triplicates.

Full repression induced by antagonist treatment was unchanged whereas in the absence of ligand and in the presence of agonist increased transcriptional activity was obtained in the presence of Gam-1. These observations suggest that post-translational modification such as SUMOylation can affect nuclear receptor-dependent transcription, particularly in the absence of ligand and in the presence of agonist. Whether NonO modulates N-CoR repressive

capacity by affecting SUMO-ligase recruitment, thereby reducing N-CoR nuclear receptor affinity or inhibiting SUMOylation of corepressors remains to be determined. Since lysine residues in the consensus ΨKXE sequence are subjected to SUMO conjugation NonO amino acid sequence was checked for potential sites (Fig. 46).

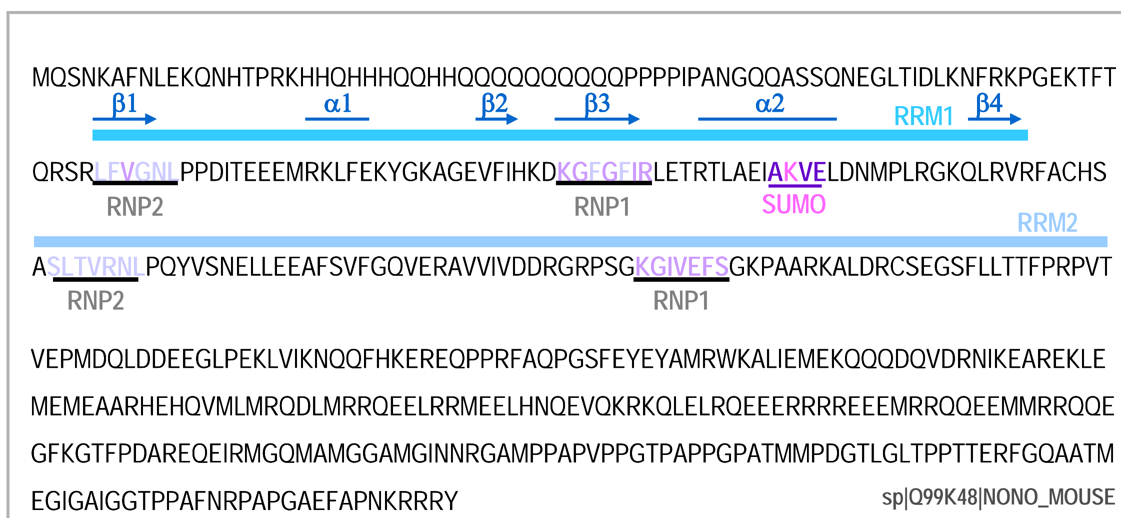



**Figure 46: NonO contains a SUMO-acceptor-site**

In the Sp|Q99K 48| NonO_Mouse coding sequence a potential SUMO-acceptor site was found that corresponds to the consensus SUMO-acceptor-site which consists of the sequence ΨKXE, where Ψ is a large hydrophobic residue, K is the lysine to which SUMO-1 is conjugated, X is any amino acid and E is glutamic acid. The potential SUMO acceptor site is in the RRM1 domain in the helix $\alpha_2$ folding region.

Interestingly, a SUMO-acceptor-site was found in the RRM1 domain which is sufficient for NonO/N-CoR interaction *in vitro*. RRM is composed of one four stranded antiparallel β sheet and two α-helices packed against the β sheet (Fig. 40 s. discussion). Once conjugated with SUMO target lysine (K127) in the α helix 2 possibly affects the interaction capacity of the RNA recognition motif and may alter interaction between N-CoR and NonO.

# 4 Discussion

The yeast-two-hybrid screening system is an efficient method to define new protein-protein interactions. When we screened the clontech mouse embryonic (e.17) library with the N-CoR extreme C-terminus (2290-2453) in AH109 *Saccharomyces Cerevisae* we found several interacting proteins that belong to various protein families (Ducasse 2002). From these potential interaction partners, only a subset was considered which could be directly linked to the transcription repressive activity of N-CoR. Other proteins which are involved in folding, degradation or protein localization and which play roles in the regulation of protein life span were not explored in detailed. Therefore, during my PhD. thesis, I further investigated selected proteins including C-terminal binding protein (CtBP), methyl-CpG binding domain-containing protein 3 (MBD3) or ESET domain containing protein (ESET/SETDB1) which were recently discovered. At that time no precise function was described but these proteins were thought to be involved in transcriptional regulation or chromatin remodeling. Since transcription regulation and RNA processing was considered to be mechanistically coupled (Maniatis and Reed 2002) the non-POU-domain-containing octamer binding protein (NonO/p54nrb) was also considered as an interesting candidate. NonO belongs to the heterogeneous nuclear ribonucleo (hnRNP) -like proteins which were identified in transcriptional and spliceosomal complexes.

For the CtBP, MBD3 and ESET proteins no *in vivo* interactions with N-CoR could be observed in co-immunprecipitation experiments. These results are concordant with new publications which support that these proteins do not directly interact with N-CoR. In 1995 CtBP was discovered as the C-terminal domain of the human E1A adenovirus binding protein (Boyd et al. 1993) and a consensus sequence (PXDLS) was determined to be an essential motif for interaction with CtBP (Schaeper et al. 1995). This sequence was not found in N-CoR. Meanwhile a variety of promoter-specific transcription factors have been shown to interact with CtBP which regulates their activity (reviewed in (Chinnadurai 2002)). CtBP is now considered to play a widespread role in transcriptional repression in a histone deacetylase (HDAC)-dependent and independent manner (Kumar et al. 2004). New data suggest that CtBP can also repress transcription inhibiting CBP (CREB binding protein) histone acetyltransferase activity with which it interacts directly (Meloni et al. 2005). For the methyl CpG binding domain protein 3 (MBD3) which does not possess appreciable methylated DNA binding activity as other family members do, its function remains unclear. MBD proteins are thought to serve as the bridge between histone modificating enzymes (histone deacetylases, histone methyltransferases) and hypermethylated DNA which is the most common epigenetic modification of vertebrate genomes that is associated with transcriptional repression. However, it is crucial to mammalian development as MBD3

knockout mice fail to develop to term (Hendrich et al. 2001). It is now clear that MBD3 assembles in a distinct MDB3 Mi-2 NURD like complex (nucleosome remodeling deacetylase complex). This complex is involved in ATP-dependent chromatin-remodeling either in a specifically targeted manner or upon constitutive association with chromatin (Li et al. 2002; Le Guezennec et al. 2006). Differences in MBD3 localization at the chromatin and cellular levels in lung cancer and normal cells were observed to lead to differential transcription and cancer-selective toxicity (Noh et al. 2005). When positive clones were analyzed out of the yeast-two-hybrid screen performed with the N-CoR C-terminus the ERG-associated protein containing a SET domain (ESET) was found as a potential N-CoR partner. A few months later, the full-length mouse cDNA was isolated by Yang L. et al. and was identified as the mouse homologue of the human SETDB1. This protein possesses evolutionarily conserved SET, preSET, and postSET domains implicated in histone methylation and was demonstrated to be a novel histone methyltransferase (Yang et al. 2002). ESET/SETDB1 has been described to be recruited by MBD1 and the catalyzed histone H3-K9 methylation is heritably maintained through DNA replication to support the formation of stable heterochromatin at methylated DNA (Sarraf and Stancheva 2004). MBD1 predominantly localizes to methylated DNA regions and recruits co-repressors and chromatin-associated factors. Recently, ESET/SETDB1 has been determined to belong to the histone di- tri-methyltransferases that can be recruited by heterochromatin protein1 (HP1), which binds to methylated histones, to induce heterochromatin formation (Verschure et al. 2005).

Interestingly, NonO was found to co-immunoprecipitate with overexpressed and endogenous N-CoR (Fig. 11; Fig. 12). Both, endogenous N-CoR and exogenous NonO, were visualized to co-localize in cell nuclei (Fig. 13) by confocal scanning microscopy. NonO is thought to be multifunctional, since it is found in several multiprotein complexes which are involved in different biological processes. The definite function of NonO however remains unclear. After confirming the interaction of N-CoR with NonO *in vivo* the aim of my work consisted in determining the biological relevance of this interaction.

## 4.1 N-CoR interaction partner NonO is a multi-functional protein

The deletion of an essential octamer motif contained in promoters and enhancers of immunoglobulin (Ig) genes results in a drastic reduction of Ig expression in B cells. This motif is recognized by a DNA binding motif, the POU domain (Herr et al. 1988). Looking for octamer binding factors that are involved in Ig expression regulation during early B-cell differentiation, Tucker et al. (Yang et al. 1993) isolated the non POU domain containing nuclear protein NonO (identical to the human p54nrb protein) that footprinted the octamer motif indistinguishably from Oct-2 a known Ig expression regulator in mature B cells. NonO was shown to bind polynucleotides, either RNA or single stranded DNA, via its ribonuclear

protein (RNP) binding motifs or double stranded DNA through a different region containing a helix turn helix domain followed by charged residues. Within NonO, there is a stretch of 10 glutamine residues near the amino-terminus and a proline-rich region near the carboxy-terminus. Both motifs are surrounding the POU domain in octamer binding factors. They are involved in protein-protein interactions within transcription initiation complexes and enhance transcriptional activation (Mitchell and Tjian 1989; Muller-Immergluck et al. 1990). Though, the role for NonO in Ig gene expression remains to be established it is meanwhile thought to be a multi-functional protein since it has been identified in complexes that operate in different cellular processes, including splicing and transcription.

NonO contains 2 copies of the RNA recognition motif (RRM) which is one of the most abundant protein domains in eukaryotes. Since this motif was thought to be a common feature of splicing factors NonO was first categorized as a protein involved in RNA processing. Indeed it was found in splicing protein complexes (Lindsey et al. 1995). Subsequently, it was described to bind not only to single stranded RNA but as its homologue PSF (PTB-associated splicing factor) (Patton et al. 1993) to bind a conserved RNA secondary structure as the stem b1 in U5 snRNA (Peng et al. 2002). This small nuclear RNA molecule associated to protein U5 is an essential component of the spliceosome core (Stanford et al. 1988). In addition to the high homology of NonO and PSF especially in their RRM domain, both proteins share functional similarity and can heterodimerize. However, PSF is a larger protein than NonO and presents an extended amino-terminus. Both proteins were described to be involved in the maturation of pre-mRNA as well as being implicated in other RNA-related processes. They have been determined to play a role in retention of hyperedited RNAs in the nucleus (Zhang and Carmichael 2001). NonO was found to be a key player in cleavage/polyadenylation during pre-mRNA processing (Liang and Lutz 2006) and PSF to be able to down-regulate human immunodeficiency virus type 1 (HIV) mRNA after transcription (Zolotukhin et al. 2003).

Besides its role in a number of steps in RNA metabolism, several reports suggest that NonO is involved in gene regulation through its binding to regulatory elements in promoter regions such as octamer motifs or enhancer elements (Basu et al. 1997). However, neither direct targeting of NonO to specific endogenous gene regulatory regions *in vivo*, nor the functional relevance of these interactions has been shown yet. Depending on the cell type that was observed NonO gene regulation varies (Lamb et al. 1992; Basu et al. 1997). Increase or decrease of NonO expression was observed in several cellular models and correlates with cell fate. For example, NonO gene transcription increases in response to prolactin stimulation in rat lymphoma cells (Too et al. 1998). Upon neuronal differentiation in P19 embryonal carcinoma cells NonO mRNA is decreased similar to some other general splicing factors

(Shinozaki et al. 1999). Additionally, in human breast cancer reduced expression of NonO/p54nrb or alteration of its protein structure in association with hER$\alpha$ expression was observed to contribute to tumor growth and progression (Pavao et al. 2001). In papillary renal cell carcinoma a defined X chromosome inversion results in the fusion of the almost complete NonO (p54nrb) to the TFE3 transcription factor DNA binding domain (Clark et al. 1997). However, whether NonO chimeric protein expression that sequesters TFE3 to new localizations within the nucleus, leading to a TFE3 null phenotype, is sufficient to mediate transformation is still under debate (Mathur et al. 2003).

Interestingly, NonO is thought to be involved in nucleic acid-protein complex formation and activation and was described to interact with a number of DNA or RNA-binding proteins (Yang et al. 1997). These nucleic acid binding proteins are involved in different but not completely unrelated cellular processes. For example Straub et al. suggested that the topoisomerase I DNA relaxation activity is enhanced by PSF and its smaller homologue p54nrb/NonO and that the recruitment of these factors was necessary to convert topoisomerase I to a true catalyst (Straub et al. 2000). Both, NonO and PSF were found to facilitate the association of RNA with the C-terminal domain (CTD) of RNA polymerase II (Pol II). This domain is tightly regulated by phosphorylation which is a determinant for the Pol II complex formation. NonO and PSF can interact at the CTD in both activated (hyperphosphorylated in the elongation phase) and inactivated state (hypophosphorylated in the transcription initiation phase) leading to the suggestion that these RRM binding proteins are the direct physical link between RNA polymerase and other pre-mRNA processing components during the initiation and elongation phases of transcription (Emili et al. 2002). More recently, it was shown that PSF in contrast to NonO does not only couple transcription to splicing but also stimulates pre-mRNA processing in a RNA pol II CTD-dependent manner (Rosonina et al. 2005).

Publications about NonO and PSF interactions with transcription factors are increasing and some details about their function too. NonO can interact with DNA binding transcription factors such as SF1 (Sewer et al. 2002) or nuclear receptors such as retinoic X receptor (RXR), thyroid hormone receptor (TR) (Mathur et al. 2001), androgen receptor (AR) (Ishitani et al. 2003) and progesterone receptor (PR) (Dong et al. 2005). Class I and II nuclear receptors interact with NonO and PSF through their DNA binding domain (DBD) and interaction modulates their transcriptional activity. NonO potentiates AF-1 function of the AR (Ishitani et al. 2003) whereas PSF represses thyroid hormone receptor-dependent transcription interfering with receptor/DNA binding, and recruiting histone deacetylases through Sin3A interaction (Mathur et al. 2001). More recently, PSF was described to repress nuclear receptor dependent transcription inducing receptor degradation (Dong et al. 2005).

Since NonO and PSF show high homology mainly in their RRMs and less or no similarity in the C- and N-terminus, respectively it is likely that they differ in their functions. It is now believed that PSF and NonO in addition to their role in linking and coordinating transcription with pre-mRNA splicing are also involved in the regulation of gene expression. How multiple functions of NonO/p54nrb and PSF are regulated is not yet clear and several regulation mechanisms such as limiting protein level, post-translational modifications, nuclear sublocalization and RNA binding are under investigation. As other RNA-binding proteins such as RTA (Norris et al. 2002), SHARP (Shi et al. 2001) and GRIP120 (Eggert et al. 1997) have been shown to either coactivate or corepress nuclear receptor-dependent transcription it is likely that NonO is also involved in N-CoR-dependent transcriptional regulation.

## 4.2 N-CoR and NonO are essential factors for cell viability

At first Gal4-reporter assays were performed to examine if N-CoR transcriptional repression activity was affected by NonO overexpression. The transcriptional repression that is measured in this transiently transfected reporter is primarily due to the recruitment of N-CoR fused to the Gal4-DBD to the upstream activator sequence (UAS) in the promoter region together with the different associated proteins which are part of the corepressor complex. No unspecific effect was observed by expressing exogenous NonO at different concentrations with Gal4-DBD and the Gal4-reporter. In the presence of Gal-N-CoR full-length repression was about 20 fold relative to the basal reporter activity in the presence of Gal4-DBD alone and even at the highest NonO concentration this repressive activity remained unchanged (Fig. 15). Thus, we may conclude that NonO is not involved in N-CoR complex formation. However, to verify this hypothesis, specific NonO expression down-regulation experiments were required. Namely, endogenous NonO which is abundantly expressed in 293T cell could ensure N-CoR regulation. We therefore designed small interfering RNA targeting specifically NonO and its human homologue p54nrb mRNA. These corresponding oligonucleotides were synthesized and cloned successfully into the pSuper vector which was modified for this purpose.

Interestingly, the target sequence (Si I: 487-508) which is contained in the middle of exon 5 in a conserved region showing high similarity with a number of other protein coding sequences was inefficient. In contrast, the other sequence (Si II: 1246-1266) which targeted a part of the extreme end of exon 9 in a nonconserved region which does not code for any known motifs enhanced specific mRNA degradation. Si RNA that targeted sequences within a specific mRNA that shows low homology to other protein mRNA sequences may be more efficient. Since RNA transcripts are mainly associated with proteins during their lifespan, which are involved in RNA processing (splicing, nucleocytoplasmic transport, localization, stability, translation, and degradation), it is likely that designing small interfering RNA

considering not only the polynucleotide physical properties but also its position on the transcript may improve siRNA efficacy.

Although efficient degradation of the NonO mRNA in 293T cells was obtained with the pSuper Si II construct, no efficient specific down-regulation of the protein was achieved (Fig. 20). This is most likely due to the stability and the rather low turn-over rate of NonO which were examined in 293T cells. Levels of the NonO protein were in fact stable upon 24 h treatment with translation or proteasome inhibitor (Fig. 22). P. Tucker and collaborators observed similar phenomena in human cell lines stably transfected with a NonO antisense construct. In addition they observed that loss of colony growth after 6 to 10 days selection correlated with a very low level of NonO which induces senescence (Cells are blocked in the $G_0$ phase of the cell cycle) (personal communication). This latter observation suggests that NonO is an essential factor for cell growth and division. However, for comparable induced mRNA degradation we observed that NonO full-length antisense was more toxic to cells than the presence of pSuper Si II expressing small interfering RNAs (Fig. 21). The toxic effect was confirmed testing expression of the internal control reporter SV40 in the presence of the antisense coding vector which showed impaired transcriptional activity (data not shown). However, to achieve significant down-regulation of protein expression double transfection experiments could be performed. Cells are transfected twice with the silencing vector sequentially (cell are passaged before second transfection) and by the second transfection reporter plasmids are added. Due to the essential role of NonO for cell viability this was not performed. In addition several observations indicate that the NonO/N-CoR interaction may be transient and the effect of efficient NonO knock down may not be directly correlated to N-CoR.

An initial observation indicated that although NonO and N-CoR specifically interacted *in vivo*, they did not systematically co-immunopricipitate even in the presence of exogenous protein, after crosslinking or addition of phosphatase inhibitors or RNase inhibitors, suggesting that defined cellular conditions are necessary for interactions. Moreover, the reverse immunoprecipitation showed that a large amount of NonO could be precipitated whereas only a small amount of N-CoR co-immunoprecipitated. Furthermore, in confocal scanning microscopy experiments, only partial colocalization of both endogenous proteins was visualized (data not shown). In transiently transfected Gal4-reporters NonO fused to the Gal4-DBD showed strong transcriptional repression that was not relieved upon HDAC inhibitor treatment. This suggests that Gal-NonO can recruit cofactors which inhibit initiation of transcription by the basal transcription machinery in a histone deacetylase-independent manner. It is thought that transiently transfected plasmids are partially bound to nucleosomes and thereby present chromatin-like structures. However, this partial chromatinization of

reporter plasmids renders them less sensitive to HDAC inhibitors such as valproic acid (VPA) or trichostatin A (TSA) than reporters integrated into the genome. Thus, it is difficult to distinguish in transient transfected reporters whether repression is rather due to co-factor recruitment than to post-translational modifications of histones. In addition it is possible that interaction of Gal-NonO with N-CoR, PSF and/or other factors would be sufficient to repress transcription of transiently transfected reporter plasmids. PSF was described to repress transcription by direct interaction with Sin3A (Mathur et al. 2001). Since PSF and NonO can heterodimerize we can not prove that Gal-NonO repression is exclusively due to the recruitment of N-CoR.

To assess whether NonO influences post-translational histone modifications rather than co-repressor complex assembly, HeLa cells stably transfected with the Gal4-reporter were used. Unfortunately, no Gal-N-CoR-dependent repression could be measured in this stable cell line. Namely, the luciferase basal transcription is quite high in the absence of Gal-N-CoR and synthesized luciferase is very stable. No significant reduction of the luciferase activity could be measured even after transfecting Gal-N-CoR with high transfection efficiency. Consequently, a HeLa cell line that was stably transfected with both Gal-N-CoR and the reporter was used in order to avoid high luciferase background activity. Regrettably, no conclusion could be drawn regarding the function of NonO in the regulation of the histone tail modifications with this double stable transfected cell line since it is resistant to additional transient transfection. This was concluded after testing several different transfection methods.

As an alternative for investigating the function of NonO and N-CoR a stable cell line lacking N-CoR expression was required. A N-CoR knockout cell line was provided by Geoff Rosenfeld (University of California, San Diego). Its very low viability however limited its use for transfection experiments. The fact that N-CoR knockout mice are embryonic lethal and stable N-CoR down-regulation inhibits cell proliferation demonstrates that N-CoR is as NonO an essential factor for the cell. Therefore, no further experiments with the silencing vectors were performed and other strategies were required such as overexpression of NonO mutants to determine the biological significance of the NonO/N-CoR interaction.

## 4.3 NonO modulates the repression capacity of N-CoR

The NonO N-terminal half which contains two RNA recognition motifs (RRM) was determined to be sufficient for the interaction with the N-CoR carboxy-terminus in GST-pulldown assays (Fig. 23). Interestingly, these domains are also present in the SHARP protein (SMRT/HDAC1 Associated Repressor Protein) that was found to interact with SMRT C-terminus in a yeast-two-hybrid screen (Shi et al. 2001). The RNA recognition motif was first thought to be a simple rigid RNA binding domain. However, an increasing number of structures determined

by NMR and X-ray crystallography revealed unexpected structural variations correlated to primary and secondary structure changes. In addition, more recent work supports that this domain can also be involved in protein-protein interaction. The structure and the main consensus sequences (RNP1 and RNP2) of the approximately 90 amino acids domain were determined in the late 1980s (Adam et al. 1986) (Dreyfuss et al. 1988) and the first crystal structure of the RRM domain of the U1 small nuclear ribonucleoprotein A (snRNP) was solved in 1990 shedding light on a typical $\alpha\beta$ sandwich structure with a specific $\beta_1\alpha_1\beta_2\beta_3\alpha_2\beta_4$ topology (Nagai et al. 1990) (Fig. 47). The high plasticity of this domain ensures high RNA-binding affinity and specificity that is not necessarily linked to a specific RNA sequence since RNA secondary structure can be required for binding. In addition although the small compact domain is a central component of RNA recognition it is not the only determinant. N- and C-terminal extensions, multiplication of the RRM domains or protein cofactors can play an important role in the RNA binding specificity. These surrounding regions are often crucial in enhancing or inhibiting RNA binding (reviewed in (Maris et al. 2005)). However, additional biochemical and structural studies are required to elaborate a more comprehensive picture of this domain and its role in the cell.

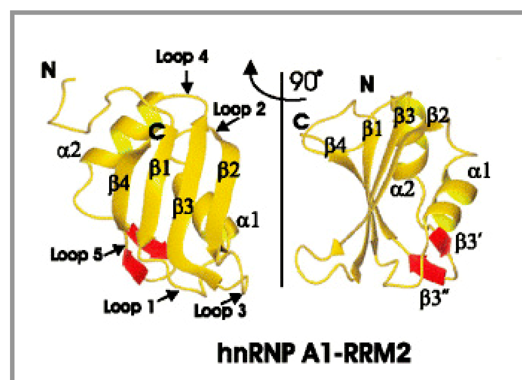



**Figure 47: hnRNP A1 RNA recognition motif 2**

A typical RRM fold in the hnRNP A1 (Xu et al. 1997). Figure out of (Maris et al. 2005) review. The RRM folds into an $\alpha\beta$ sandwich structure composed of one four-stranded antiparallel $\beta$-sheet spatially arranged in the $\beta_4\beta_1\beta_3\beta_2$ from left to right when facing the sheet and two $\alpha$-helices ($\alpha_1$ and $\alpha_2$) packed against the $\beta$-sheet.

Involvement of the RRMs in protein-protein interactions was also reported. Three distinguished cases have been described: between two RRM motifs within a protein, between RRM motifs of distinct proteins which are capable of RNA binding or not. Criteria which allow the distinction of RRM domains that are true RNA-binders from those that are not, remain to be defined. It is still not clear if RNA binding is required for specific protein recognition or whether these functions are mutually exclusive. Recent work supports that the RRM can be engaged concomitantly in RNA and protein binding. For the CBP20 subunit of the cap-binding protein complex, it was shown that the RRM domain becomes structured only when it is in a complex with both RNA and a non-RRM interaction partner (Calero et al.

2002). In addition new data about the La protein support that this protein does not use the typical binding surface of the RRM for RNA recognition, leaving this surface potentially available to bind other ligands, such as DNA and/or proteins (Maraia and Bayfield 2006). In this regard considering that RNA binding may play a role in the N-CoR/NonO interaction, co-immmunoprecipitation experiments were performed in the presence of RNAse inhibitors and conditions suitable for RNA stability. However, these conditions did not enhance association of NonO with N-CoR (data not shown) and since required RNA may be present in the *in vitro* transcription/translation reaction and in the GST-pulldown assays one can not exclude that RNA plays a role in NonO/N-CoR binding. Alternatively, it is also conceivable that NonO is usually associated in other complexes in a RNA-dependent manner and only a fraction of free NonO interacts with N-CoR. It would have been interesting to perform RNase digestion in cell lysate prior to immunoprecipitation experiments to verify if released NonO interacts more strongly with N-CoR. This experiment remains to be done since it was thought that RNA is rapidly degraded in lysates in the absence of RNase inhibitor and it was not considered that RNA digestion could be required for dissociation of RNA-dependent protein complexes.

The RRMs of the coactivator CoAA were described to play a major role in the regulation of reporter transcriptional activity (Auboeuf et al. 2004) and that these domains are not only involved in RNA recognition but also in protein-protein interaction. Therefore, two NonO deletion mutants were constructed that lack RRM1 (aa: 86-160) and RRM2 (aa: 160-227), respectively. *In vitro*, the RRM1 domain was determined to be essential and sufficient for NonO/N-CoR interaction in contrast to the RRM2 motif, the deletion of which did not impair the binding of $^{35}$S N-CoR Ct (1629-2543) to the GST fused NonO ΔRRM2 (Fig. 26). Nuclear localization signals of the NonO protein were predicted downstream of the deleted regions, however domain deletion can result in protein misfolding that in turn buries localization signals. Therefore, cellular localization of the mutated mutants was verified. Western blot analysis of cytoplasmic and nuclear fractions of 293T cells transfected with vectors expressing deletion mutants confirmed that NonO ΔRRM1 and NonO ΔRRM2 localized in the nucleus (Fig. 27). Cell cycle phase-dependent NonO posttranslational modifications (Proteau et al. 2005) were also verified. It seemed that this was impaired in NonO ΔRRM2 but not in the NonO ΔRRM1 mutant (Fig. 30). Both constructs were then cotransfected with the Gal4-reporter and Gal-N-CoR expressing vector in 293T cells. Interestingly, significant effects occurred in the presence of the RRM deleted mutants in contrast to NonO wild type. The NonO ΔRRM1 expression strongly enhanced the fold repression of Gal-N-CoR full-length in a dose-dependent manner. In contrast NonO ΔRRM2 enhanced repression only at the highest concentration, however to a lesser extent then NonO ΔRRM1 (Fig. 31). These results

were unexpected since the deletion of RRM1 correlated with a loss of interaction with N-CoR-Ct *in vitro*. Nevertheless, these first results showing that NonO deletion mutants are able to impair Gal-N-CoR-dependent repression support the hypothesis that NonO is involved in the regulation of N-CoR transcriptional repression activity. Thus, from this reporter assay it was concluded that NonO has a modulator role in the N-CoR transcriptional repression which is impaired upon overexpression of the deletion mutants. Consequently, these effects were verified in a nuclear receptor-dependent reporter assay.

NonO/p54nrb was shown to activate transcription in MMTV-luc reporter (containing the mouse mammary tumor virus promoter that can be activated by glucocorticoid and progesterone receptors) in the presence of progesterone receptors upon progesterone simulation. Auboeuf et al. could also show that NonO can activate transcription in a promoter-specific and nuclear receptor-dependent manner (Auboeuf et al. 2004). For our experiments a retinoic acid receptor (RAR)-dependent reporter was used. The luciferase expression was under the control of RAR response elements (RARE) that are contained in the promoter region. Retinoic acid receptor and heterodimer retinoic X receptor (RXR) are class II receptors which reside in the nucleus and can bind to specific cognate DNA sequences in the absence of ligand. Since RAR/RXR can recruit corepressors such as N-CoR or SMRT in their unliganded form and in the presence of antagonist, three different states of transcriptional regulation were considered. In the RARE-reporter assay transcription activation is enhanced in the presence of RAR agonist (all-trans retinoic acid ATRA), partial repression is obtained in the absence of ligand and full repression of transcription is enhanced in the presence of antagonist (Fig. 48).

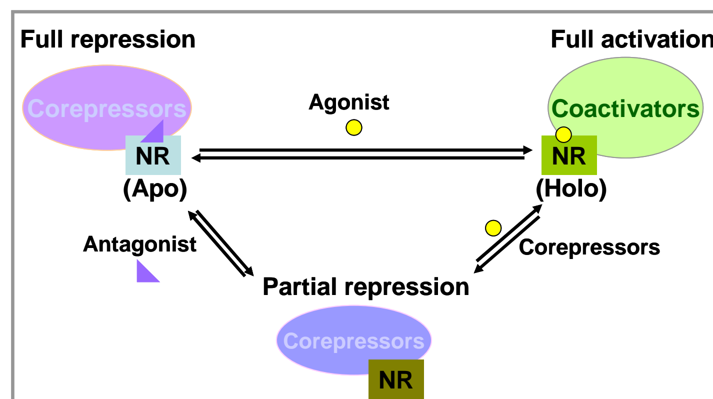



**Figure 48: Nuclear receptor-dependent transcription regulation**

Agonist binding induces conformational change to nuclear receptors that result in the exposition of the coactivator binding surface. Corepressor can directly facilitate the conversion of receptor from the holo (active) form to apo (repressive) form. Class II receptor and especially RAR/RXR receptor heterodimer reside in the nucleus, bind to response element and recruit corepressors in absence of ligand. This repression state is the partial repression distinguished from the full repression in the presence of antagonist.

Thus, in 293T cells endogenously expressing RAR, RXR and N-CoR, reporter and NonO versus deletion mutant expression plasmids remained to be transfected. In contrast to the Gal4-reporter, RARE-reporter activity was affected upon NonO wild type overexpression. Enhanced transcriptional activity was observed particularly in the absence of ligand or in the presence of agonist but not in the presence of antagonist. The main difference between the RARE and the Gal4-reporter is the recruitment of N-CoR to the promoter region. In the RARE-reporter N-CoR recruitment is RAR-dependent suggesting that NonO is involved in the regulation of transcriptional repression in a receptor-dependent manner.

The past model postulated that ligand-bound nuclear receptors are associated with coactivators and unliganded nuclear receptors with corepressors (Schulman et al. 1996). Indeed, dramatic conformational changes occur in the ligand binding domain in the presence of ligands and are determinants for cofactor association. However, it was recently shown that ligand binding stabilizes the activated liganded (holo) receptor structure, and that corepressor binding also stabilizes the unliganded (apo) receptor structure (Pissios et al. 2000). Furthermore, Sohn et al. propose that nuclear receptors are in dynamic equilibrium between apo- and holo-states and that both ligand and corepressor binding are the driving forces (Sohn et al. 2003). According to the latter model and the observation that NonO slightly enhanced basal and activated transcription but not full repression, it is conceivable that NonO reduces corepressor binding to nuclear receptors in the absence of ligand. This could explain why exogenous NonO expression did not affect Gal-N-CoR full-length repression activity in the Gal4-reporter assay where the recruitment of N-CoR to the promoter region is tethered by the Gal4-DBD fusion. Thus, we conclude that NonO rather plays a role in the equilibrium between nuclear receptors bound to coactivators or corepressors than in the full repression in the presence of antagonist. This hypothesis is supported by the results which were obtained in the presence of NonO RRM deletion mutants in both reporter assays.

Using the Gal4-reporter, NonO ∆RRM2 enhanced repression only at the highest concentration (0.5µg) though, to a lesser extent than NonO ∆RRM1 did (Fig.31). In contrast, in the RARE-reporter NonO ∆RRM2 ectopic expression enhanced transcription activity by about 30% in the absence of ligand and in the presence of agonist or antagonist whereas NonO ∆RRM1 expression did not (Fig. 37; Fig. 38). These results suggest that NonO ∆RRM2 which can still interact with N-CoR is able to impair nuclear receptor-dependent transcription in a ligand-independent manner. This suggests that this mutant may impede not only the recruitment of corepressors to the promoter region by nuclear receptors but also reduces the corepressors capability to compete with coactivators for binding to nuclear receptors. This hypothesis would be consistent with both reporter assay results for comparable concentration (0.2µg) and with the results obtained by endogenous RAR-

responsive gene analyses. In a semi-quantitative PCR analysis of the endogenous RARβ gene activated transcription is slightly enhanced in the presence of NonO ΔRRM2 (Fig. 39). Since the deletion of the RRM2 domain in PSF was correlated with impaired subnuclear localization (Dye and Patton 2001) further investigations would be necessary to elucidate the exact underlying molecular mechanism of the NonO ΔRRM2 induced effect. In cell synchronization experiments NonO ΔRRM2 posttranslational modification seemed to be impaired as the protein level of endogenous N-CoR. It is therefore conceivable that NonO ΔRRM2 induced incorrect N-CoR subnuclear localization or a decrease of the N-CoR protein level which would produce similar results.

For NonO ΔRRM1, according to the hypothesis that this mutant competes with endogenous NonO for cofactor binding but not for N-CoR interaction similar effects were obtained in the RAR-dependent reporter as with exogenous wild type NonO expression (Fig. 37; Fig. 38). Interestingly, NonO ΔRRM1 fused to the Gal4-DBD showed repression as strong as that obtained with Gal-DBD-NonO wild type in the Gal4-reporter (data not shown). This indicates that the RRM1 deletion mutant is able to recruit cofactors comparably to NonO wild type and reinforces the assumption that the observed repression is rather due to promoter occupancy than corepressor recruitment. Unexpectedly, NonO ΔRRM1 mutant enhanced Gal-N-CoR repression in a dose-dependent manner in the Gal4-reporter although deletion of the RRM1 motif abrogates interaction between NonO and N-CoR *in vitro*. This suggests that NonO and other factors may be involved in the regulation of N-CoR activity. It is likely that the enhanced Gal-N-CoR repression results from the squelching of NonO cofactors by the NonO ΔRRM1 mutant impairing endogenous NonO-dependent modulation of N-CoR activity. However, this effect was not observed in the RAR-dependent reporter. It is conceivable that NonO ΔRRM1-dependent squelching effect differs in the RARE-reporter assay in which N-CoR recruitment and repression capacity is nuclear receptor-dependent. Interaction-dependent conformational changes that may stabilize association of N-CoR with NonO and its cofactors may come into play in the presence of nuclear receptors and would differ from that with N-CoR fused to the Gal4-DBD. Considering the results obtained with NonO ΔRRM1 in both reporters, NonO not only seems to affect N-CoR association with nuclear receptors but also to regulate the capacity of N-CoR to repress transcription. Whether this regulation results from competition between NonO and other N-CoR cofactors to interact with N-CoR, interaction-induced conformational changes or altered N-CoR post-translational modifications is unclear.

## 4.4 N-CoR amino- and carboxy-termini regulate each other

Whereas the repression domains are contained in N-CoR amino-terminal half the nuclear receptor binding sites are carboxyl terminal. Interestingly, although the N-CoR C-terminus does not retain repressive activity it contains additional binding sites for proteins which are mainly interacting in the N-CoR amino-terminus, such as mSin3A and HDAC3 (Li et al. 2000; Wen et al. 2000) which are critical components of the N-CoR corepressor complex. This latter observation suggests that the N- and C-terminal ends may be in close proximity in the

tertiary protein conformation. Indeed, in GST-pulldown experiments the N-CoR N-terminus interacts with the C-terminal domain (Fig. 40). Since NonO was found to interact with the C-terminus of N-CoR, it was verified *in vitro* whether it can either interact with the N-terminus. Indeed, GST-fused NonO retains TNT translated N-CoR Nt (1-549) and the RRM1 domain is essential and sufficient for this interaction (Fig. 43). Since the N-CoR amino acid sequence 1-549 contains the repression domain I (RDI) and a part of the SANT domain, *in vitro* binding assays were performed to assess which region is involved in the interaction with NonO. In these experiments TNT translated full-length NonO strongly interacts with the GST fused SANT 1/2 domain, suggesting that it can interact with this regulatory region which recruits SUMOylation enzymes and HDAC3. However, interaction between the SANT domain and NonO was not found in the yeast-two-hybrid screen that was performed by Jens Tiefenbach with the N-CoR region containing SANT 1 and 2 (Tiefenbach 2003 August). These interesting findings led us to ask how NonO binding can affect N-CoR repressive activity. Our results and some published data suggest that N-CoR N- and C-termini are involved in intramolecular interactions and regulate each other. In a Gal-4 reporter assay (Tiefenbach et al. 2006) (Fig. 41) Gal-N-CoR N-terminus showed greater repression activity than Gal-N-CoR full-length although it contains only one out of three repression domains (RDI) and lacks the C-terminal half containing the NR interacting domain (NID). This observation is surprising since one would expect that the full-length platform protein N-CoR may repress more efficiently than the amino-terminal moiety, forming a larger and more stable corepressor complex at the promoter unless the C-terminal part is regulating repression capacity of the N-terminus. In addition, Li et al. showed, with an assay established by Wong J. (1998) to analyze transcription of microinjected DNA in *Xenopus* oocytes, that TR/RXR unliganded repression could be relieved by injection of αN-CoR purified antibodies that recognized the C-terminus in contrast to injections of N-CoR-N-terminal directed antibodies (Li et al. 2000). This indicates that this region may be either more accessible and/or plays a regulatory role. We show in a reciprocal manner that exogenous expression of the N-CoR Ct (1587-2453) affects transcriptional regulation of the RARE-reporter assay (Fig. 42).

Unexpectedly, the N-CoR C-terminal moiety which retains no repressive activity inhibited transcription activation of the RARE-reporter upon agonist treatment and reduced basal transcription in the absence of ligand. In contrast, in the presence of antagonist full repression was not affected. These results point out that there are differences between transcriptional repression mediated by unliganded nuclear receptors and antagonist bound receptors. Supposing that the N-CoR-Ct is able to associate in an unregulated manner with nuclear receptors, dissociation of N-CoR Ct enhanced by conformational changes in the ligand binding domain (LBD) upon agonist treatment may be impaired. This could explain the reduced transcriptional activation that is obtained in the presence of N-CoR Ct (Fig. 42 B). However; this would not explain the enhanced repression that is obtained in the absence of ligand (Fig. 42 A). The latter observation supports that regulation of endogenous N-CoR repressive activity and its dissociation from activated nuclear receptors is impaired in the presence of the overexpressed C-terminal moiety. It is conceivable that the observed effects

result from squelching of regulatory factors considering the conserved extreme carboxy-terminal region of N-CoR as a docking surface for modulators. Interactions within this region with factors such as NonO may play a determinant role in the regulation of N-CoR and its nuclear receptor-dependent recruitment. It is possible that NonO, which is found in several down stream events of nuclear receptor transcription activation such as RNA polymerase II initiation complex, elongation complex, splicesome and polyadenylation takes alsopart in earlier events such as nuclear receptor activation, corepressor dissociation and/or coactivator association.

## 4.5 Biological relevance of the interaction of NonO with N-CoR

N-CoR is a platform protein which is essential for the recruitment of the corepressor complex to promoter regions by unliganded or antagonist-bound class II nuclear receptors. Its repressive activity is mainly due to the recruitment of HDACs which modify histone tails and thereby promote a more condensed chromatin structure which is in turn less accessible to transcription factors and the transcription machinery. However, recent publications challenge whether the repressive activity of N-CoR is limited to the deacetylation of histone amino tails. It was shown that the SANT domain surrounded by the repression domains in the amino-terminal region of N-CoR is involved in the activation of HDAC3 which directly interacts with N-CoR (Guenther et al. 2001). This domain was more recently shown to recruit SUMO E2 and E3 ligases, and N-CoR SUMO modification contributes to repression by N-CoR (Tiefenbach et al. 2006). A number of recent publications report that SUMO modifications of transcription factors, corepressors, HDACs and histones mainly enhance their repressive activity whereas SUMOylation of coactivators reduces their activating capacity (reviewed in (Girdwood et al. 2004; Gill 2005; Minucci and Pelicci 2006)). It is conceivable that HDACs may deacetylate nonhistone substrates such as SUMOylation enzymes recruited to the corepressor complex and may thus be involved in the regulation of the transcriptional activity of transcription factors and coregulators. Together this suggests that N-CoR could be involved in the regulation of the activity of transcription coregulators in addition to the recruitment of histone deacetylases. According to my data, NonO seems to enhance RAR/RXR transcriptional activation capacity by affecting N-CoR binding to nuclear receptors. This may correlate to the inhibition of N-CoR conformational changes or post-translational modifications that favor N-CoR to interact with unliganded nuclear receptors and to compete with coactivators at agonist bound nuclear receptors. Postulating that N-CoR modulates coregulator activity, NonO interaction with N-CoR may affect either N-CoR association with modifying enzymes or the modification by deacetylation and SUMOylation of transcription factors.

Interestingly, NonO contains a potential SUMO site in the $\alpha$ helix 2 ($\alpha_2$) packed against the RRM1 $\beta$-sheet (Fig. 46). The potential SUMOylated lysine residue is conserved in its

homologue PSF (polypyrimidine tract-binding protein-associated splicing factor). PSF was identified as a putative SUMO substrate by proteomics studies (Rosas-Acosta et al. 2005) and more recently PSF corepressor activity was correlated to its SUMOylation-dependent recruitment of HDAC1 (Zhong et al. 2006). These reports strongly suggest that NonO can be conjugated to SUMO and that its function could be modulated by this posttranslational modification. Unfortunately, whether NonO is SUMOylated and if this modification alters NonO/N-CoR interaction was not determined during my thesis. However, results obtained in RARE-reporter assays upon NonO wild type expression were similar to those obtained in the presence of Gam-1. Gam-1 is an adenoviral protein, the expression of which leads to the general inhibition of SUMOylation in the cell (Boggio et al. 2004) and not exclusively SUMO E2 and E3-ligases that are recruited by N-CoR. Inhibiting SUMOylation pathways affected TR and RAR-dependent transcriptional regulation in transient reporter assays (Fig. 45) (published in (Tiefenbach et al. 2006)). In the presence of Gam-1 enhanced transcriptional activation of both TRE and RARE-reporters was observed as well as reduced repression in the absence of ligand. In contrast, inhibition of SUMOylation did not affect full repression upon antagonist treatment in the RARE-reporter. These results support that SUMOylation plays an important role in the nuclear receptor-dependent transcription regulation but seems not essential for full repression. Whether there is a direct correlation between NonO-dependent modulation of N-CoR and SUMOylation remains to be established.

Although further investigations are required to support the proposed mechanism one can speculate that NonO interacts with the extreme carboxy-terminus of N-CoR independently of the recruitment of corepressors or modifying enzymes that occurs at the amino-terminus. This interaction may in turn enhance stabilization of N-CoR in a less repressive conformation for example with a reduced affinity to nuclear receptors. It is also conceivable that NonO interaction reduces the activity of SUMOylation enzymes and/or recruitment by N-CoR to the promoter. Whether these are the mechanisms by which NonO regulates N-CoR has to be confirmed; nevertheless our findings suggest that the RRM containing protein NonO is involved in the fine-tuning of receptor-dependent transcription modulating N-CoR repressive capacity. The C-terminus of N-CoR is likely a regulatory region that regulates repressive activity at the N-CoR N-terminus. This region may be comparable to a sensor that evaluates the ratio of corepressors and coactivators in the nuclear receptor environment. For class II nuclear receptors (TR; RAR), their binding to DNA in the absence of ligand enables transcriptional repression and a rapid answer to ligand stimulation. Their presence at the promoter ensures an appropriate limitation to responsiveness which is not only influenced by the concentration of ligand but also availability and state of activation of transcription coregulators. The disposal of coregulators depends on the cell context, cell differentiation states or cell type. Auboeuf et al. have shown that the concentration of coregulators can be

critical for mRNA processing decisions in a nuclear receptor and promoter-dependent manner (Auboeuf et al. 2002). The recruitment of both, transcription and splicing factors by nuclear receptors is essential to ensure coordination between RNA synthesis and the nature of the final product. In a comparable manner, it is possible that N-CoR recruitment by nuclear receptors is not only determined by the ligand-induced conformational changes in the ligand binding domain but is also influenced by the local concentration of transcriptional coregulators. NonO could belong to such factors whose accumulation at the promoter would enhance nuclear receptor sensitivity to stimulation and reduce N-CoR repressive activity; whereas a decrease of NonO local concentration would favor corepressors to compete and draw back the nuclear receptor to its apo form (Fig. 49). The variation of the local concentration of coregulators such as NonO that are involved in events downstream of nuclear receptor activation associating with Pol II complex, spliceosome and polyadenylation complexes and affecting corepressor recruitment is a realistic mechanism to coordinate the amount and the quality of transcribed RNA in response to stimulation intensity and cell context.

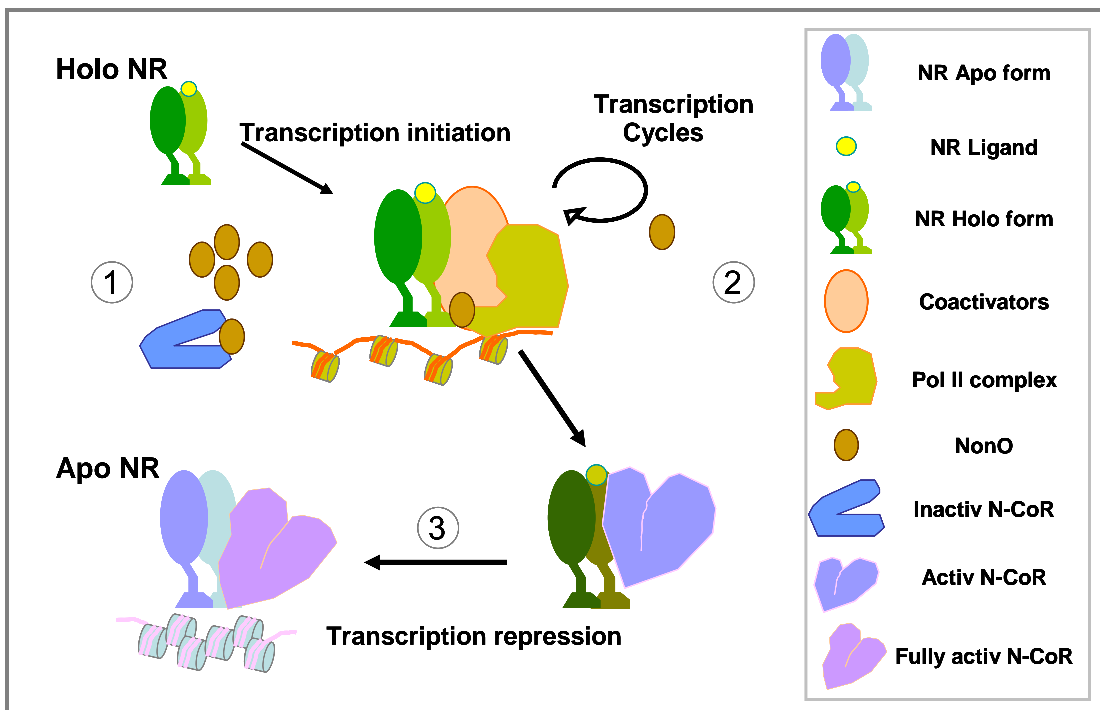



**Figure 49: N-CoR repressive activity is modulated by NonO**

Upon agonist binding, nuclear receptors recruit coactivators that decondense chromatin in the promoter region and render DNA accessible to the basal transcription machinery. 1) High local concentrations of modulating factors such as NonO may maintain N-CoR in a repressive conformation that cannot bind to NR in an active form (holo). 2) Decrease of NonO concentration that is involved in the transcription initiation complex formation and further downstream events enables N-CoR to adopt a conformation that favours N-CoR binding to NR and 3) enhances its capability to stabilize NR in an inactive form (Apo).

In the last decade the understanding of mechanisms which are involved in gene regulation tremendously increased. The sophistication and development of techniques have greatly contributed to this new picture. The initial proposed rigid chromatin structure has turned out to be a highly dynamic structure constituted of nucleosomes that have an inherent capacity to breathe (reviewed in (Mellor 2005)). It is now clear that a series of ordered events is required to activate or repress transcription and this is in part regulated by the chromatin structure. In euchromatin, discovery of reversible histone post-translational modifications and their combination that dictate the sequential recruitment of subsequent transcriptional factors, has increased the complexity of the information contained in chromatin. Moreover, unexpected layers of regulation in the molecular events were discovered. The capacity of nuclear receptors to switch from transcriptional repression to activation can be modulated suggesting that nuclear receptors are not only able to respond to hormonal stimulation, but integrate information from a large variety of external stimuli to achieve specific profiles of gene expression (reviewed in (Perissi and Rosenfeld 2005; Rosenfeld et al. 2006)). Furthermore, a number of studies provided evidence that distinct processes that were thought to operate sequentially and independently such as transcription and splicing are actually physically and functionally linked. RNA Polymerase II is now considered as a main player in the orchestration of transcription and RNA processing (reviewed in (Hirose and Manley 2000)). The carboxy-terminal domain (CTD) of RNA polymerase II (pol II) plays an important role in coupling transcription with precursor messenger RNA (pre-mRNA) processing. Efficient capping, splicing, and 3'-end cleavage of pre-mRNA depend on the CTD. Moreover, specific processing factors are known to associate with this structure (Rosonina and Blencowe 2004). However, it has been shown that promoter type and assembly of transcription coregulators in this region are also decisive in splicing events. Splicing factors or related proteins were first found to associate with the Pol II complex before the functional coupling of transcription and splicing was understood. In this work, I could show that NonO, a RRM containing protein, is able to interact with N-CoR and modulate its repressive capacity. Taken together, the results presented here combined with several recent studies indicate that the observed interactions are not the consequence of cotranscriptional splicing but point out a supplementary unexpected level of regulation in the control of gene expression.

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# 6 Abbreviations

| | |
|---|---|
| aa | amino acid(s) |
| Ab | antibody |
| AAV | adeno-associated virus |
| Ab | antibody |
| ADA | adenosine deaminase |
| APC | antigen presenting cell |
| APL | acute promyelocytic leukemia |
| APS | ammoniumperoxysulfate |
| ATCC | American type culture collection |
| ATP | adenosinetriphosphate |
| ATRA | all-trans retinoic acid |
| bp | base pair(s) |
| BSA | bovine serum albumin |
| cDNA | complementary DNA |
| CHIP | chromatin immunoprecipitation |
| CtBP | C-terminal binding protein |
| CTD | C-terminal domain |
| DBD | DNA-binding domain |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | dimethylsulfoxide |
| DNA | desoxyribonucleic acid |
| ds | double-stranded |
| DTT | dithiothreitol |
| ECL | enhanced chemiluminescence |
| *E. coli* | *Escherichia coli* |
| EDTA | ethylenediaminetetraacetic acid |
| EGF | epidermal growth factor |
| EGFR | epidermal growth factor receptor |
| EGFP | enhanced green fluorescent protein |
| ER | estrogen receptor |
| ERE | estrogen receptor response element |
| FCS | fetal calf serum |
| Fig | figure |
| FITC | fluoresceinisothiocyanate |
| FL | full-length |
| FPLC | Fast Performance Liquid Chromatography |
| FRAP | fluorescence recovery after photobleaching |
| Fw | forward primer |
| g | gravity |
| GST | glutathione-S-transferase |
| h | hour |
| HAT | histone acetyltransferase/ acetylase |
| HDAC | histone deacetylase |
| HRP | horseradish peroxidase |
| Ig | immunoglobulin |
| Ip | immunoprecipitation |
| IPTG | isopropyl-β-D-thiogalactopyranoside |
| kDa | kilodalton |
| M | Molar |
| MCS | multiple cloning site |
| MBD3 | methyl-CpG binding domain-containing protein3 |
| min | minute |
| mM | milimolar |
| MOPS | 4-morpholinepropanesulfonic acid |
| mRNA | messenger RNA |

| | |
|---|---|
| MW | molecular weight |
| N-CoR | nuclear receptor corepressor |
| NLS | nuclear localization sequence |
| NonO | non-POU-domain-containing octamer binding protein |
| NR | Nuclear receptor |
| Nucl | nucleotide |
| OD | optical density |
| ORF | open reading frame |
| PAGE | polyacrylamide gelelectrophoresis |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PEG | polyethyleneglycol |
| PEI | polyethyleneimine |
| PKA | protein kinase A |
| PKC | protein kinase C |
| PLC | Phospholipase C |
| PMSF | phenyl-methyl-sulfonylfluoride |
| Pol II | RNA polymerase II |
| PSF | PTB-associated splicing factor |
| PTB | polypyrimidine trac binding protein |
| R | release |
| RAR | retinoic acid receptor |
| RARE | retinoic acid receptor response element |
| RE | response element |
| Rev | riverse primer |
| RNA | ribonucleic acid |
| RNP | ribonucleoprotein |
| rpm | revolutions per minute |
| RRM | RNA recognition motif |
| rRNA | ribosomal RNA |
| RT | room temperature |
| RXR | retinoid X receptor |
| s | seconds |
| SANT-domain | SWI, ADA2, N-CoR and TFIIIB-domain |
| *S.cervisiae* | *Saccharomyces cerevisiae* |
| SDM | site directed mutagenesis |
| SDS | sodium dodecylsulfate |
| siRNA | small interference RNA |
| SMRT | silencing mediator of retinoic and thyroid receptor |
| snRNP | small nuclear ribonucleoprotein |
| SUMO | small ubiquitin-related modifier |
| SV40 | simian virus 40 |
| T | thymidine |
| TAE | Tris-buffered saline |
| TBL1 | transducin (beta)-like protein |
| TEMED | N,N’-tetramethyl-ethylene-diamine |
| TK | thymidine kinase |
| T/N | thymidine nocodazole |
| TR | Thyroid hormone receptor |
| Tris | tris(hydroxymethyl)-amino-methane |
| TSA | trichostatine A |
| UAS | upstream activating sequence |
| UV | ultra violet |
| VPA | valproic acid |
| v/v | volume/volume |
| WB | Western Blot |
| w/v | weight/volume |

***Acknowledgment***

*I would like to specially thank great Professors of the Goethe-University Frankfurt, Prof. Dr. Fasold, Prof. Dr. Rüterjans, Prof. Dr. Ludwig and Prof. Dr. Bereiter-Hahn who trusted my capacities to finish my study in Germany. Their initiative enabled me to become accepted at this university and to realize my self in this country. My gratitude goes to excellent students I met during my study, Dr. Jens Tiefenbach and Dr. Oliver Anderka, discovering next to them how exiting research can be. Kind thanks to Annemarie Schimpf for her devoted technical assistance and her attendance. Thanks also to Prof. Dr. Groner who set up this comfortable and competent institute in which I felt at home for a non-neglectable period of my life. Last but not least, I would like to thank my supervisor Prof. Dr. Thorsten Heinzel for giving me the opportunity to work in his lab from the very first beginning and enabled me to realize this very special adventure and education.*

*This work would not have been possible without all providential encounters and encouraging kindness of many persons from the university, administration, meetings and the Georg Speyer Haus which contributed to flavor my scientific quotidian. Sincere thanks.*

*Yours Miryam*

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**Dissertation:**

„Funktionelle Charakterisierung der C-terminale-Domäne des Korepressors N-CoR“

**Publication:**

**“SUMOylation of the Corepressor N-CoR Modulates Its Capacity to Repress Transcription“ Mol Biol Cell 17(4): 1643-51** Jens Tiefenbach, Natalia Novac, Miryam Ducasse, Maresa Eck, Frauke Melchior, and Thorsten Heinzel

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