Ligand-dependent corepressor LCoR

A modulator of estrogen and progesterone target gene expression

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August 2009

A thesis submitted to McGill University, Faculty of Graduate Studies and Research, in partial fulfillment of the requirements of the degree of Doctor of Philosophy (Ph.D.)

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To my parents, Antonija and Ivan, for always taking care of me and making sure that I can follow my dreams. Hvala.
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Abstract

Ligand-dependent corepressor LCoR was discovered almost ten years ago in the laboratory of Dr. John White through a yeast two-hybrid screen in which the ligand binding domain (LBD) of the estrogen receptor alpha (ER alpha) was used as bait. LCoR represses gene transcription by recruitment of the corepressor C-terminal binding proteins (CtBPs) and histone deacetylases (HDACs) through distinct domains. The action of HDACs strengthens the interactions of histones with negatively charged DNA resulting in stable nucleosomal and chromatin structures and repression of transcription. CtBPs interact with many transcriptional repressors through a common motif P/VLDLS/TXK/R, of which there are two in LCoR. We hypothesize that LCoR is a regulator of endogenous nuclear receptor target gene expression. Initial data characterized the function of LCoR as a nuclear receptor (NR) corepressor by using a series of in vitro and over expression assays. LCoR was also shown to have many protein-interacting domains that might contribute in its corepressor function. The goal of this thesis was to determine the role of LCoR in the control of endogenous estrogen and progesterone target gene expression. Through reporter gene assays with overexpression of LCoR and truncated forms of LCoR lacking the interacting domains, we have shown that both HDAC6 and CtBP1 contribute to transcriptional corepression observed with LCoR. To find out whether these cofactors are recruited to regulatory regions of endogenous ER alpha and PR target genes, ChiP and reChiP assays techniques were applied. These have shown a ligand-dependent and orderly corecruitment of LCoR, HDAC6, CtBP1 and corresponding NR on estrogen and progesterone
target genes. SiRNA-mediated gene silencing of LCoR, HDAC6 and CtBP1 in combination with reporter gene assay showed a transcriptional derepression in ERα- and PR-mediated gene transcription, confirming the transcriptional corepressor function of these coregulators. However, the investigation of siRNA-mediated knockdowns effect on endogenous gene expression also showed the LCoR transcriptional corepressor function to be gene-specific. Even though these results were somewhat unexpected, they are in concordance with recent findings by other groups studying corepressors, adding to the complexity of NR-mediated transcriptional control and the need of further studies in the field of coregulators.
Résumé

Il y a dix ans, le laboratoire de Dr. John White a découvert un corépresseur nucléaire surnommé LCoR. La fonction du corépresseur est dépendante de la présence d’agoniste. LCoR a été découvert grâce au système du double hybride de levure pour lequel le domaine de liaison du ligand (LBD) du récepteur estrogen alpha (ERalpha) a été utilisé comme appât. LCoR inhibe la transcription d’un gene en s’associant avec le corépresseur C-terminal binding proteins (CtBPs) et les histones déacétylases (HDACs). L’association de LCoR avec ces deux familles protéiques ce fait à travers deux domaines distincts. L’action enzymatique des HDACs stabilisent les structures du nucléosome et des histones en renforçant les liens entre ces derniers et l’ADN. Cette stabilité structurale a pour but d’inhiber la transcription génique. Les CtBPs interagissent avec un grand nombre de répresseurs transcriptionnel en s’associant au motif d’intéraction P/VLDLS/TXK/R, dont LCoR en possède deux. Nous émettons l’hypothèse que LCoR est un régulateur transcriptionnel des gènes cibles de récepteurs nucléaires in vivo. Les données initiales (basées sur des techniques in vitro) ont caractérisé LCoR en tant que répresseur de transcription médiée par les récepteurs nucléaires. Ces données ont également démontrée la contribution des cofacteurs CtBP1 and HDAC6 à l’activité transcriptionnelle répressive de LCoR. Le but de cette thèse est de déterminer le rôle de LCoR dans le contrôle transcriptionnel des gènes cibles des récepteurs nucléaires ERAlpha et progestérone (PR) in vivo. Les immunoprécipitations chromatiques des cofacteurs LCoR, CtBP1, HDAC6, ERAlpha et PR ont démontrée une association de ceux-ci aux éléments de réponses hormonales de manière ordonnées et séquentielles. L’ablation
d’expression de LCoR, HDAC6 and CtBP1 par rapproche RNA inhibiteur a démontrée une perte de répression transcriptionnelle médiée par les expressions transitoires d’ERalpha et PR. Cependant, les effets de ces inactivations géniques sur l’expression des gènes cibles in vivo sont spécifiques aux contextes géniques. Ces résultats surprenants appuient les données récentes dans le domaine de la régulation transcriptionnelle et démontre le niveau de complexité de celle-ci.
My doctoral rollercoaster ride started at five in the morning on September 6th 2004. These past five years have been both challenging and fulfilling and gave me an unique life experience that I would not change for the world. Eighteen hour days, pulling “all-nighters”, working seven days a week were no strangers. Even with its many lows (i.e. ChIP experiments not working, unable to grow Flag-LCoR MCF7 Tet-On clones, losing 600 Flag-LCoR T47D Tet-On clones due to no space in the liquid nitrogen, not able to get LCoR knockdowns, and the list goes on and on) I was lucky enough to be surrounded by wonderful people that offered me a great support system. And because of this, amidst all the pains and frustrations that PhD students are faced with, these past five years were a blast and a true gift.

I would like to thank my doctoral supervisor, Dr. John H. White. He is the Big Pearl of supervisors. It is very rare that a professor will make his priority to be available for his students, and even more to lend a friendly ear for both professional and private matters. I have learned and grown a lot in his lab. More importantly, he has taught me the most valuable life lesson, i.e. the theory of “four types of people”. Thank you John for giving me the opportunity of being part of your lab, for mentoring me and for giving me carte-blanche when I had new ideas. A special thank you to my thesis committee members, both past and present: Dr. Pejmun Haghighi, Dr. John Orlowski, Dr. Xiang-Jiao Yang, Dr. Mark Featherstone and Dr. Julie Desbarats. Thank you for your encouragements and constructive comments. I would also like to thank the Department of Physiology as a whole. What differentiates this
department from others is the feeling of being part of a big family. I want to thank the
department studies coordinators, Christine Pamplin and Sonia Viselli, for our excellent
morning conversations and your guidance.

A special thank you to my lab mates, both past and present: Dr. Tian Tian Wang, Dr.
Beum-Soo An, Dr. Hui-Jun Wang, Ari Bitton, Vassil Dimitrov, Mario R. Calderon, Mark
Verway, Basel Dabbas and Dr. Luz Elisa Tavera-Mendoza. From working together so closely,
strong friendships are made. Thanks to Tian Tian’s (a.k.a. T²) wisdom and super protocols, I
was able to get the data necessary to complete my thesis. I have a big thank you for Luz, my
Woman, for being my mentor, my motivator, my entertainer, for listening to my B-Team
stories, for encouraging me when my experiments did not work, and for being an awesome
friend. I am so proud of you and all your accomplishments. You are a true genius, but with
awesome hair and style. Also, a big thank you to my boy Basel. You are a great friend and
TH has never seen a duo like ours. I am really happy that you are following your dreams. A
student life cannot be without other students. Thank you all for making the time to come
together and having fun. I want to thank Eric Libby for your awesomeness, the many good
times, the good food and giving me good abs by sharing your funny stories. A special thank
you goes to my “monkey people”: Soroush, Marion, Mathieu, Jessica, Mohsen and Jerome.
Thank you for making me the unofficial Cullen Lab member.

I would not be here today writing the acknowledgment of my doctoral thesis if it
was not for my parents. They came from nothing and worked very hard all their lives to
ensure security for my sister and myself. Their sole goal was for their children to be happy,
healthy and able to achieve whatever they set their minds onto. And for this I will be eternally grateful to them. I have to thank my family as a whole for their support and understanding of my schedule and my priorities. I would like to especially thank my sister Nicole, my brother-in-law Vlado and my beautiful nephew and niece, Dominik and Natalie, for always being there for me, for helping me out and for giving me a second home. I also want to thank Jose, the dad of my boyfriend. Thank you for taking me under your wing, your encouragements and our wonderful conversations.

I also want to thank my second family: my friends. First and foremost, I have to thank the person that was with me through thick and thin, both highs and lows, the person that beams with joy when I have good news, cries and holds my hand when I have bad news, my best friend Vicki Karaglanis. From Deng to White, we have seen it all. Thank you Babe for always being here for me and for making me part of your wonderful and loving family. I also have a special thank you to my girl, my little Greek goddess with the perfect hair, Maria Kourelis. You were the best student ever, but more importantly, you are a wonderful person, pure at heart and full of goodness. I cannot thank you enough for all your help throughout the years and I am so proud of you and all your accomplishments. Utopia would be reality if everyone was like you.

These past two years I am blessed to have a wonderful person in my life, my honey Frederic. I would have never thought that our paths would meet again after so many years. On the other hand, the best things in life are the ones we did not plan for. Thank you for your unconditional love, support, encouragement and belief in me. You gave me the last
push I needed to finish this chapter of my life by showing me all the possibilities in the chapters to come.
Preface

In accordance with *McGill Graduate and Postdoctoral Studies* thesis preparation guidelines and approval from the thesis committee and supervisor Dr. John H. White, the results of my doctoral research are presented in manuscript-based format. The texts of two original papers are included as chapters for this thesis (Chapters 3 and 4). These two papers were submitted to the *Journal of Biological Chemistry* (manuscript ID numbers JBC/2009/045526 and JBC/2009/051201, respectively) during the writing of this thesis. The manuscripts presented in this thesis are the following:


Contribution of authors

For Chapter 3, entitled *Function of histone deacetylase 6 as a cofactor of nuclear receptor coregulator LCoR*, the authors are: Palijan A, Fernandes, I, Bastien Y, Verway M, Tang L, Kourelis M, Tavera-Mendoza LE, Li Z, Bourdeau V, Mader S, Yang XJ, White JH. Co-author contributions: Fernandes I performed immunocytochemistry for the co-localisation studies of LCoR with HDAC3 and HDAC6. Bastien Y, Tang L and Li Z made truncated forms of LCoR and did GST pulldowns to determine the HDAC6 interacting domain in LCoR. Kourelis M did qRT-PCR analysis of LCoR and HDAC6 siRNA-mediated knockdowns for the pS2 and GREB1 genes. Verway M did the statistical analysis of qRT-PCR data. Tavera-Mendoza LE provided ERα binding promoter regions in estrogen target genes and designed primers for the BMP7 ChIP analysis. Bourdeau V provided a list of estrogen target genes in MCF7 cells and primers used for ChIP and qRT-PCR analysis. Bourdeau V works under the supervision of Dr. S Mader who did the genomic studies identifying estrogen target genes. Dr. XJ Yang provided expression vectors for HDAC3 and HDAC6 and antibodies for the latter. The mutant form of LCoR lacking the HDAC6 interacting-domain, Flag-LCoRΔHDAC6, was made by me. The transient transfections and Western blots with Flag-LCoRΔHDAC6 presented in Figure 3.3 were performed by me. All the ChIP and re-ChIP assays presented in Figures 3.4 and 3.5 were done by me. The siRNA knockdowns of LCoR and HDAC6, as well as the control scrambled siRNA, presented in Figures 3.6 and 3.7 were done by me. In addition, all the tissue culture work, including the treatments, cell harvesting and luciferase reporter assays, were performed by me.
For Chapter 4, entitled *Ligand-dependent corepressor LCoR is an attenuator of progesterone-regulated gene expression*, the authors are: Palijan A, Fernandes I, Verway M, Kourelis M, Bastien Y, Tavera-Mendoza LE, Sacheli A, Bourdeau V, Mader S, White JH. Co-author contributions: Fernandes I performed immunocytochemistry for the co-localisation and co-immunoprecipitations studies of LCoR with CtBP1 and CtIP, as well as the co-immunoprecipitation analysis of LCoR mutants (m1, m2 and m1m2). Bastien Y made truncated forms of LCoR to determine the CtBP interacting domains in LCoR. Kourelis M did qRT-PCR analysis of LCoR and CtBP1 siRNA-mediated knockdowns for the pS2, GREB1 and IGFBP1 genes. Verway M did the statistical analysis of qRT-PCR data. Tavera-Mendoza LE provided chip-ChIP data to identify estrogen and progesterone target genes. Sacheli A performed preliminary transient transfections LCoRΔHTH mutant in MCF7 cells treated with estrogen and progesterone. Bourdeau V provided a list of estrogen target genes in MCF7 cells and primers used for ChIP and qRT-PCR analysis. Bourdeau V works under the supervision of Dr. S Mader. The experiments performed by me are the following: co-immunoprecipitation of CtIP with LCoR (Figure 4.1C), expression analysis of full-length and truncated form (m1m2) of LCoR by Western blot (Figure 4.2D), GST pulldown investigating direct interaction of LCoR with CtBP1 and CtIP (Figure 4.2E), transient transfections of LCoR with CtIP, CtBP and m1m2 in MCF7 cells treated with estrogen and progesterone (Figure 4.3), ChIP and reChIP assays in MCF7 and T47D cells for the pS2 and IGFBP1 promoters (Figure 4.4) and BMP7 promoter (Figure 4.8G), deletion of HTH domain in LCoR (Figure 4.5A) and subsequent characterization of the deletion mutant by Western blot (Figure 4.5B), ChIP assay (Figure 4.5C), and transient transfections in T47D and MCF7 cells (Figure 4.5, panels D
and E, respectively), siRNA-mediated knockdowns of LCoR, CtBP1, CyPB and scrambled siRNA (Figure 4.6) and their respective regulation of endogenous progesterone target genes (Figure 4.7) and endogenous estrogen target genes (Figure panels 4.8 A to F). In addition, all the tissue culture work, including the treatments, cell harvesting and luciferase reporter assays, were performed by me.

For Chapter 5, entitled *Domain analysis of LCoR*, the investigation of various domains in LCoR was performed through a series of deletion mutants. Maria Kourelis and Jonathan Grinstein helped with the clone selection, sequencing, transient transfections and luciferase reporter assays for the LCoR acetylation mutants (Figure 5.2). Preliminary transient transfections of LCoRΔHTH mutants with luciferase reporter assays were performed by Aaron Sacheli. Data presented in Figure 5.1 and 5.3 are results of experiments performed by me. All the primers for the various LCoR deletion mutants were designed by me. The results presented in this chapter are preliminary data for future investigations.
**Abbreviations**

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<tr>
<td>a.a.</td>
<td>Amino acid</td>
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<tr>
<td>AD1</td>
<td>Activation domain 1</td>
</tr>
<tr>
<td>AD2</td>
<td>Activation domain 2</td>
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<tr>
<td>ADORA1</td>
<td>Adenosine A1 receptor</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>bHLH</td>
<td>Basic helix-loop-helix</td>
</tr>
<tr>
<td>BMP7</td>
<td>Bone morphogenetic protein 7</td>
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<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation assay</td>
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<tr>
<td>CoIP</td>
<td>Co-immunoprecipitation</td>
</tr>
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<td>CoREST</td>
<td>REST corepressor 1</td>
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<td>CoRNR</td>
<td>Corepressor NR box</td>
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<td>COS7</td>
<td>Immortalized monkey kidney cell line</td>
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<tr>
<td>CtBP</td>
<td>C-terminal binding protein</td>
</tr>
<tr>
<td>CtIP</td>
<td>CtBP-interacting protein</td>
</tr>
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<td>CYP26B1</td>
<td>Cytochrome P450, family 26, subfamily b, polypeptide 1</td>
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<td>CyPB</td>
<td>Cyclophilin B</td>
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<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
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<td>E2</td>
<td>Estradiol</td>
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<td>Eip93F</td>
<td>CG18389 gene product from transcript CG18389-RB</td>
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<td>Abbreviation</td>
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<td>ER</td>
<td>Estrogen receptor</td>
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<td>ERα</td>
<td>Estrogen receptor alpha</td>
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<tr>
<td>G9a</td>
<td>Euchromatic histone-lysine N-methyltransferase 2</td>
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<td>GR</td>
<td>Glucocorticoid receptor</td>
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<td>GREB1</td>
<td>Gene regulated by estrogen in breast cancer protein</td>
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<td>GST-pulldown</td>
<td>Glutathione S-transferase-pulldown</td>
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<td>Histone acetyltransferase</td>
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<td>HRE</td>
<td>Hormone response element</td>
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<td>Heat shock protein 90</td>
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<td>HTH</td>
<td>Helix-turn-helix motif</td>
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<td>IGFBP4</td>
<td>Insulin-like growth factor binding protein 4</td>
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<td>LBD</td>
<td>Ligand-binding domain</td>
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<td>LCoR</td>
<td>Ligand-dependent corepressor</td>
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<td>LSD1</td>
<td>Lysine (K)-specific demethylase 1</td>
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<td>m1m2</td>
<td>LCoR mutant lacking both CtBP interacting domains</td>
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<td>MBLK-1</td>
<td>Honeybee MBlk-1 related factor</td>
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<td>MCF7</td>
<td>Immortalized breast cancer cell line, positive for ERα</td>
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<td>MMP-2</td>
<td>Matrix metalloproteinase-2</td>
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<tr>
<td>MMTV</td>
<td>Mouse mammary tumor virus</td>
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<td>NCoA1</td>
<td>Nuclear receptor coactivator 1</td>
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<td>MR</td>
<td>Mineralocorticoid receptor</td>
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<tr>
<td>NCoR</td>
<td>Nuclear receptor corepressor</td>
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<td>ND</td>
<td>Nuclear receptor interacting domain</td>
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<td>PRA</td>
<td>Replication protein A</td>
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<td>PRE</td>
<td>Progesterone response element</td>
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<td>pS2</td>
<td>Trefoil factor 1</td>
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<td>PSA</td>
<td>Prostate-specific antigen</td>
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<td>PSQ</td>
<td>Pipsqueak motif</td>
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<td>QPCR</td>
<td>Quantitative PCR</td>
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<td>QRT-PCR</td>
<td>Quantitative real-time PCR</td>
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<td>RD</td>
<td>Repression domain</td>
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<td>reChIP</td>
<td>Re-chromatin immunoprecipitation</td>
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<td>RIP140</td>
<td>Receptor interacting protein 140</td>
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<tr>
<td>RLU</td>
<td>Relative luciferase units</td>
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<tr>
<td>SGK3</td>
<td>Serum/glucocorticoid regulated kinase family, member 3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>SMRT</td>
<td>Silencing mediator for retinoid and thyroid-hormone receptors</td>
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<td>SRC</td>
<td>Nuclear receptor coactivator</td>
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<td>T47D</td>
<td>Immortalized breast cancer cell line, positive for PR</td>
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<td>TR</td>
<td>Thyroid hormone receptor</td>
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<td>TRAP</td>
<td>Trapoxin</td>
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<td>Trichostatin A</td>
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<td>VDR</td>
<td>Vitamin D receptor</td>
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<tr>
<td>Wnt</td>
<td>Wnt oncogene</td>
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Chapter 1

Introduction
Ligand-dependent corepressor (LCoR) was discovered ten years ago in the laboratory of Dr. John White through a yeast two-hybrid screen where the ligand binding domain (LBD) of the estrogen receptor alpha (ERα) was used as bait. The initial paper, published in Molecular Cell, characterized the function of LCoR as a nuclear receptor (NR) corepressor by using a series of in vitro and over expression assays (1). LCoR was also shown to have many protein-interacting domains that might play a role in its corepressor function (a review of the initial studies on LCoR is given in section 2.4.3). The current doctoral work is a continuation of these initial studies.

The goal of this thesis was to determine the role of LCoR in the control of endogenous estrogen and progesterone target gene expression. To answer this question, an array of techniques was applied and examples of these are the following: chromatin immunoprecipitation (ChIP) assays to investigate the recruitment of LCoR and other coregulators on regulatory regions of endogenous estrogen and progesterone target genes, to examine the importance of protein interacting domains found in LCoR, a series of deletion mutants were made and tested using in vitro binding assays and transient transfections, siRNA mediated-knockdowns of LCoR and its partners were performed to investigate their mechanistic relevance and physiological effects.

The thesis is divided in four sections. The first section is a literary review of NRs and coregulators in which the history of the field and the discovery of new concepts of NR-mediated transcriptional control are explored (Chapter 2). The third chapter investigates the interaction of LCoR with one of its binding partners, histone deacetylase 6 (HDAC6), and
its relevance in ERα-mediated transcriptional control. The fourth chapter studies the roles of C-terminal binding protein (CtBP) domains and helix-turn-helix (HTH) motif in ERα- and progesterone receptor (PR)-mediated transcriptional control. The studies performed in Chapter 5 are a continuation of the previous chapter where a new series of CtBP and HTH deletion mutants of LCoR are investigated.

This thesis has brought substantial advancements in our understanding of LCoR. For the first time, LCoR was shown to be recruited to endogenous regulatory regions of estrogen and progesterone target genes. The recruitment was ligand-dependent and cyclical, making LCoR a bonafide NR coregulator. In order to investigate the mechanistic nature of LCoR corepression of NR-induced transcription, siRNA-mediated knockdowns of LCoR and its cofactors were performed. Interestingly, the effect of LCoR ablation on various estrogen and progesterone target gene expressions was shown to be gene-specific. Even though these results were somewhat unexpected, they are in concordance with recent findings by other groups, adding weight to the complexity of NR-mediated transcriptional control and thus the need of further studies in the field of coregulators.
Chapter 2

Literature Review
2.1 Nuclear receptors

Nuclear receptors (NRs) form the largest family of transcription regulators in eukaryotes (2, 3). They are DNA-bound transcription factors that regulate the expression of specific genes in response to ligands. These are usually small lipophilic molecules which include steroid hormones (estrogens, progestagens, glucocorticoids, mineralocorticoids, androgens and vitamin D), retinoids, thyroid hormone, oxysterols, eicosanoids and bile acid (2, 4, 5). Upon release of ligand (endocrine, paracrine, autocrine or intracrine), the ligand is transported to peripheral tissue where it binds to NRs through which gene regulation occurs (6, 7).

There are 48 NRs identified in humans (8, 9). The great majority of these transcription factors are ligand-modulated, making them an optimum system to study transcription control (8, 10). Upon binding of the ligand, and subsequent binding of coregulators, NRs can either activate or repress gene expression (see Figure 2.1) (11, 12). Gene expression depends greatly on the architecture of regulatory regions: presence of transcription factors and co-factors binding sites, the distance separating the binding sites, the accessibility of binding sites and the presence of cis-acting elements (13, 14). These promoter characteristics, in combination with the presence or absence of ligand and availability of NRs and coregulators, determine whether a gene will be activated or repressed (10, 15). Coregulators play an integral role part in NR signaling by ensuring proper NR function and gene regulation (16). The main functions of coregulators are to modify the three-dimensional structure of promoter architecture (or regulatory regions) and to interact with the transcriptional initiation complex (15, 17, 18).
Figure 2.1- Transcription regulation by nuclear receptors. Schematic view of NR mediated transcription control in the promoter region of a target gene. Upon binding of ligand (represented with a red circle), NRs bind specific sequences in promoter region of target genes, known as hormone response elements (HRE, as indicated in the figure). NRs bind DNA as monomers, homodimers or heterodimers. A selection of coregulators is recruited to NRs to ensure proper gene regulation and interaction with transcriptional initiation machinery. Through these interactions, gene transcription can be either activated or repressed. The purple arrow indicates the transcriptional starting site (TSS).
NRs play essential roles in many physiological and cellular processes: reproduction, embryonic development, growth, maintenance of homeostasis, cell differentiation, proliferation, and apoptosis (see Figure 2.2) (18, 19). Improper activation or disruption of receptor signaling have implicated NRs in many diseases, including inflammation, osteoporosis, hormone resistance syndromes, metabolic syndrome, and cancers (19-22). Environmental factors, such as pollution and diet, have been linked to deregulation of proper NR signaling, where chemicals can act as either NR agonists or antagonists (23, 24). Due to their ligand-dependent activity, NRs are good pharmaceutical targets. Many diseases are currently treated with synthetic antagonists such as asthma, atherosclerosis, diabetes and some cancers (3, 9, 19, 25, 26).

Figure 2.2- Diverse effects of nuclear receptors. Schematic view representing the various functions of NRs. They are shown to be essential for proper development and physiological functions. Aberrations in their functioning are the source of many diseases. Being ligand-dependent, NRs are excellent pharmaceutical targets where synthetic agonists and antagonist can be used as therapy. However, their signaling can easily be corrupted by chemicals found in the environment.
Estrogen and progesterone receptors, the two NRs focused on in this thesis, are implicated in many physiological processes. There are two estrogen receptors in humans: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) (27). They are products of two separate genes and share relatively similar tissue expression patterns (27). ERs are widely distributed in the organism (endometrium, breast tissue, ovaries, hypothalamus, kidney, brain, bone, heart, lung, intestinal mucosa, prostate and fat tissue, to name a few) and as a result are implicated in many various physiological processes and diseases (28-30). Conditions implicating changes in ERs expression or signaling include heart disease, osteoporosis and many cancers (breast, ovarian and prostate for example) (28, 31, 32). The progesterone receptor (PR) is also widely expressed in the organism and its expression is induced by estrogen. There are two PR isoforms (PR-A and PR-B), however contrary to the ERs, the two isoforms are products of a single gene where PR-A is the amino-terminal truncated form (33). PRs have many physiologic actions, including regulating carbohydrate, protein and lipid metabolism. Low expression of the PR has been linked to enhanced tumor aggressiveness (29).

2.1.1 Early endocrinology

In 1902, British physiologists Ernest Henry Starling (1866-1927) and William Bayliss (1860-1924) isolated the first hormone that they named secretin (34). At that time, salivary and alimentary glands, including pancreatic secretion, were believed to be controlled by the nervous system, a theory emitted by Russian physiologist Ivan Petrovich Pavlov (1849-1920) (34). The term hormone was used for the first time during a *Croonian Lectures of the Royal*
College of Physicians in 1905 by Starling (35, 36). Starling’s inspiration for the term hormone is believed to be the classical world, where “ormao” is the verb to excite or arouse in Greek (34, 36, 37). The term hormone defines chemical messengers secreted from endocrine glands that regulate functions in target tissues (38, 39).

While hormone secretion and effect on target tissues were complete mysteries until their discovery and characterization in the twentieth century, the physiological effects of hormones were described thousands of years ago, with fertility being the earliest example (40). Description and characterization of basic endocrinological processes were achieved with simple observation and association of cause and effect. This can be seen with the practice of castrati, sixteenth century male singers castrated before puberty in order to keep their soprano vocal range (36). An earlier example of association between hormone secretion and effect on target tissues is observed with the first attempts of treating cancer. Even though there are many examples of earlier cases of cancer and treatment (dating back to 3400 B.C.) (41, 42), the terminology and the disease were described for the first time in 400 B.C. in the Hippocratic writings as karkinos or karkinoma (42). Hippocrates was the first clinician to differentiate benign and malignant tumors and to observe that young woman with malignant cancer have better prognosis versus post-menopausal women (41, 43). Similarly, the physiological effects of vitamin A have been known for over 3000 years. For example, dietary supplements were used to treat night blindness, a common symptom of vitamin A deficiency (44, 45).
In the late nineteenth century, strong advances in endocrinology were made thanks to more stringent medical and scientific methodologies (36). In 1822, the Polish physician Sniadecki observed irregularities in the distribution of rickets among children, a disease that was then characterized by bone and skin deformities. Through careful observation of subjects living conditions, he concluded that sunbathing provided a cure for rickets (46). A few years later, Trousseau came to the same conclusion when he determined that poor sun exposure and diets were at the origin of rickets, a disease that was later characterized as deficiency in vitamin D (46). In the 1870s, the symptoms of hypo- and hyper-thyroidism were described. However, due to lack of biochemical studies, no link was made between the symptoms and thyroid secretions (35, 36). As an interesting side note, during the early years of hormone and nuclear receptor discoveries, there were many examples of misinterpretation of observations that resulted in incorrect hypotheses. An example of this is the “organo-therapy” phenomenon in the late 19th century (36, 47). Charles Brown-Séquard proposed that organs produced chemicals that can be used for therapeutic use. He came to this conclusion by using testicular extracts as rejuvenating agents, which he tested on himself by injecting subcutaneously testicular extracts (36, 37, 47). However, the concept of internal secretion and its maintenance of physiological effects dates back to 1500 where Paracelsus, with his dictum of *similia similibus*, believed that diseased tissue replaced with healthy tissue can restore proper physiological function (37). Even though Brown-Séquard’s “organo-therapy” theory was not well received by the scientific community, the concept of organ secretion and the physiological effect of the latter influenced the field. In 1891, Horsley and Murray demonstrated that thyroid extracts can
treat symptoms observed in hypothyroid patients (36). In 1895, through detailed and properly controlled experiments, Shäfer and Oliver demonstrated that thyroid extracts lowered blood pressure and extracts from the adrenal medulla raised blood pressure (35, 36).

2.1.2 History of nuclear receptors

The beginning of the twentieth century represented a peak of hormone discovery and isolation from which ultimately led to cDNA cloning of NRs several decades later. In 1913 vitamin A, originally known as “fat soluble factor A”, was isolated (48). McCollum and Davis from University of Wisconsin-Madison, and Osborne and Mendel from Yale, identified the fat-soluble nutrient in butterfat, cod liver oil and egg yolk (48). In 1914, Kendall isolated the thyroid hormone from more than 3 tons of porcine thyroid glands (49). In 1919, he identified the chemical structure of the hormone. Till this day, Kendall remains renowned for his discovery and characterization of cortisone for which he won the Nobel Prize in 1950 (38, 50, 51). In 1929, Butenandt and Doisy isolated estrogen and in 1933 Allen isolated progesterone (49, 52).

With the discovery of hormones, the next phase in the field of endocrinology was the study of their molecular action. The research now focused on how hormones exerted their physiological effects on target tissues. In other words, what were the mechanisms of the biological actions of hormones? In the middle of the twentieth century, the discovery of enzymes and their activity made them the central focus of molecular mechanisms. Therefore, it was believed that hormones had tissue specific effects by activating an
enzymatic signaling cascade where enzymes in the target tissue would activate the hormone by conformational or allosterical modifications (38, 53). It was then believed that enzymes present in target tissues would explain the tissue specific effects of hormone. Through a series of elegant experiments studying the mechanisms of estrogen action, Jensen and Jacobson demonstrated that target tissue contained a component that bound estrogen and that estrogen action was exerted without its modification (54). By this, they were able to disprove the metabolic action of estrogen and establish the two-step mechanism of steroid hormones action through nuclear receptors: upon administration of steroid hormones, first step of dissociation of the nuclear receptor with its associated proteins in the cytoplasm, and second step of nuclear receptor dimerization and localization to the nucleus where it binds DNA and acts as a transcription factor (38, 53). In 1977, Jensen made his second great contribution to the field of endocrinology by making the first mono- and polyclonal nuclear receptor antibodies (38, 53, 55). The antibodies permitted the cloning of NR cDNAs. The first NR cDNA cloned in 1985 by Ronal Evans group encoded the human glucocorticoid receptor (GR) (49). Shortly after, Chambon group cloned the human ER cDNA and later that same year, O’Malley cloned the chicken PR cDNA (38, 53, 55-62).

As seen in this short historical review, endocrinology is a critical element of physiology, and its study contributed to the fields of biochemistry and molecular biology.
2.1.3 Nuclear receptor structure

A/B domain

NRs have a general structure of five domains named A/B to F (see figure 2.3). The N-terminal A/B domain contains an autonomous activation (AF-1, also known as a ligand-independent transactivation domain). The latter contributes to the activation of the receptor in a constitutive ligand-independent fashion, and has the ability to bind coregulators and transcription factors (63). The length and sequence of A/B domain varies greatly between NRs (63, 64). Many NRs that have multiple isoforms resulting from alternative splicing or promoters vary in their A/B region, as is the case for the PR (65). The PR has two isoforms, named PR-A and PR-B, where the latter is the full length form of the protein and PR-A lacks the first 164 amino acids of the A/B region (66).

Figure 2.3- Nuclear receptor structure. Schematic view of the five domains in NRs. The two transactivation domains (AF1 and AF2) are shown as red circles.

C domain

The C domain, also known as DNA-binding domain (DBD), is highly conserved among NRs. The DBD has a general globular structure where the C-terminal portion of the domain plays a role in receptor dimerization (67). DBD is made of two zinc finger motifs that
recognized specific DNA sequences in the regulatory regions of target genes (6, 68, 69). These specific DNA sequences are known as hormone response elements (HRE). The HREs determine the specificity of NR action on genes. In other words, each NR recognizes its own HRE (see Figure 2.4). HREs are composed of half-sites onto which NRs can bind as monomers, homodimers or heterodimers, depending on the type of NR (70, 71).

The specificity of the binding of NRs to their corresponding HREs depends on the sequence, orientation, spacing of the half sites and on the flanking sequences of the half-sites (69, 72). A half-site is composed of 6bp with a core motif of AGNNCA (69, 71). As shown in figure 2.4, pairs of half-sites, to which homo- and heterodimers bind, have different orientations. They can be either direct repeats, inverted repeats (also known as palindromes, i.e. head to head) and everted repeats (also known as inverted palindromes, i.e. tail to tail) (70). The ER, AR, GR, mineralocorticoid receptor (MR) and PR bind as homodimers and recognize inverted repeats: the ER recognizes the core motif AGGTCA and the AR, GR, MR and PR recognize the core motif AGAACA (see Figure 2.4) (69). The latter was determined through studies of HREs in the viral promoter of the mouse mammary tumor virus (MMTV) (73-76). In this model, it was shown that all three NRs (AR, GR, MR and PR) efficiently and equally activate transcription through the same HRE. Even though one can see the viral efficiency of such a system that utilizes different host signaling pathways, this cannot be the case in vertebrates where androgen, glucocorticoid, mineralocorticoid and progesterone have unique physiological effects (74).
Studies conducted to understand the specific binding of the AR, GR and PR identified HREs that deviated from the common MMTV HRE sequence and showed preferential binding to individual NRs (69, 73, 75-78). As mentioned previously, the DBD domain has two zinc finger motifs. It was shown that the N-terminal zinc finger plays a role in the identification and specific binding of HREs, whereas the second zinc finger (closer to the C-terminal portion of the DBD) plays a role in the affinity of the binding (67). The region containing both zinc fingers is sometimes referred to as the minimal region of the DBD and is highly conserved among NRs (69, 79, 80). However, the rest of the domain is not as well conserved and was recently shown to play an important part in the discrimination of sequences necessary for specific NR binding, demonstrating the importance of half-site flanking sequences (69, 78-80). Interestingly, the newly identified HREs that showed specificity for individual NRs had lower binding affinity versus the common MMTV HRE (69). However, the binding affinity does not hold a linear relationship with transcription activity. Studies with hormone-responsive promoters upstream of a luciferase reporter showed that the newly identified HREs drove greater hormone-dependent transactivation that the common MMTV HRE (69). Therefore, many elements come into play for NR recognition of HREs: slight changes in the sequence of half-sites (sometimes referred to as imperfect response elements), sequence of nucleotides separating the two half-sites and HRE flanking sequences (68, 69, 79-81).
Figure 2.4- Hormone response elements. Schematic view of NRs with LBD and DBD depicted (top portion of the figure). HREs can be either direct repeats, inverted repeats or everted repeats of half sites, as shown in the bottom of the figure with orange arrows. Examples of HREs are given for the ER, AR, GR, MR and PR.

Many studies focus on the identification of NR target genes using genome-wide techniques as expression microarrays and computational models (68, 82-84). In one of these studies, Bourdeau and co-workers identified over 70,000 EREs in the human genome where 17,000 of these were found in regions proximal to known transcriptional start sites (82). Chromatin immunoprecipitation (ChIP) assays have become a strong tool in the search of HREs. By cross-linking cellular extract, ChIP assays are a snap-shot of direct or indirect
protein-DNA interaction at a specific time and place (i.e. temporal and spatial specific) and the immunoprecipitated product can be visualized with either PCR or quantitative PCR (QPCR). Where traditionally ChIP assays were used in the study of transcription factor and coregulator recruitment to specific DNA sequences, their combined application with expression microarrays offer a powerful tool for HRE identification. The ChIP-microarray technique, also referred to as ChIP-chip, is unbiased to the gene promoter regions and reliably identifies cis-regulatory regions. This technique was applied for the identification of ERα binding sites in the human genome by hybridizing ERα ChIP material to microarrays (85). This study demonstrated that the great majority of EREs are not restricted to proximal promoter regions. In order for gene transcription activation in the presence of ligand to occur, it is hypothesized that proteins bound to distal enhancers and proximal promoter regions of a target gene come to close contact due to chromatin looping, and that the proteins track the distance between the two sites (85). A new technique has emerged for the study of enhancer-promoter interaction that utilizes restriction enzymes and ligation in combination with quantitative PCR: chromosome conformation capture (3C) (86). The gene regulated in breast cancer 1 (GREB1) is an highly inducible gene by estrogen and was shown to have three EREs spread over 20kb upstream of the transcriptional start site (87). The application of the 3C technique demonstrated that the physical association between the three EREs occurred only in the presence of estradiol. This new technique was recently upgraded by expanding it to a genome-wide scale by combining it with expression microarrays (88, 89). With this approach, also known as circular chromosome conformation capture
(4C), trans-acting elements (i.e. interaction of regulatory regions between chromosomes) are investigated.

**D domain**

The NR D domain acts as a flexible hinge between the C and E domains and in many cases contains the nuclear localization signal (70). An additional function of the D domain was discovered by experiments conducted by Mader et al in the White laboratory (90). They showed the stabilizing function of the D domain in DNA binding by the DBD of the ERα (90). The amino-terminal region of the D domain was important for the interaction of the DBD with perfectly palindromic EREs and essential for the DBD binding of imperfect EREs (90).

**E domain**

The E domain is also known as the ligand binding domain (LBD). Contrary to DBD, LBD are less conserved permitting ligand specificity (67). As seen in Figure 2.5, the LBD is composed of a series of α-helices with a structure that is often referred to as a three-layered sandwich (8, 18), forming a central core used as the ligand-binding cavity (8, 18). The LBD contains the transactivation domain AF2, also known as ligand-induced activation function domain (8, 63). Depending on the NR, the AF2 was shown to be important for ligand and coregulator interactions (63, 67). Helix 12, which is critical to AF2 domain function, acts as a lid of the ligand-binding cavity (8, 91). As shown in Figure 2.5, in the presence of ligand, the helix 12 moves up into the cavity, stabilizing interaction with the ligand (67). The movement of helix 12 creates a hydrophobic cleft, producing a site for
coregulator recruitment. The changed LBD conformation exposes different surfaces which can interact with a new series of coregulators, resulting in ligand-induced gene expression control (8, 11, 18). The LBD is also responsible for receptor dimerization, and in the case of some NRs, interaction with heat-shock proteins and other proteins that form the pre-activated complex in the absence of ligand (67, 92). The F domain, the least conserved of all domains, is absent in some NRs and no function is attributed to it (3, 18, 63, 93).

Figure 2.5- Ligand binding domain in the absence and presence of ligand. Schematic view of the LBD based on the crystal structure. In the presence of ligand, helix 12, shown in green, enters that ligand-binding cavity to ensure interaction with the ligand. A slight shift in the positioning of the other helices is also observed. This figure is based on published images of crystal structures (65).
2.1.4 Evolution of nuclear receptors

The evolution of steroid NRs is quite fascinating and provides information on why some domains are more conserved than others (in this case, the well conserved DBD versus the more variable LBD). The strong sequence homology and conserved structure of NRs demonstrate common ancestry among NRs dating back over 450 million years (6, 94). Recent phylogenic studies have shown that ancestral steroid NRs were present before the split between deuterostomes and protostomes (see Figure 4) (6, 95). Deuterostomes and protostomes are the major compositions of the bilateria taxon clade (96). These animals are differentiated from other taxonomic clades by having a bilateral symmetry and development from three germ layers (endoderm, mesoderm and ectoderm) (96). The embryonic development differentiates the two clades deuterostomes and protostomes: the blastopore (the opening during embryonic gut formation) first becomes the mouth in protostome (greek word for “first mouth”) and the anus in deuterostomes (greek word for “second mouth”) (96). With these studies, the belief that NRs were specific to vertebrates was refuted by discovering ER orthologs in cephalochordates (close relatives to vertebrates) (see Figure 2.6).

The ERs are believed to be the first NR to evolve (6, 94). The ancestral ER is identified in cephalochordates and differs from the vertebrate ERs in that it did not function in a ligand-dependent fashion. The ancestral ER was able to bind specific HREs and activate transcription, however its LBD did not bind specifically estrogen and did not activate transcription in the presence of ligand. It seemed to have acted constitutively (94). Therefore, the novelty of vertebrate ERs is their structure-function activity relationship. The
relationship between specificity and function brings forth the co-evolution theory, also known as the ligand exploitation theory (6, 7, 94). The evolution and diversification of NRs did not result from simple gene duplications, but also from selection pressures, in this case the presence of ligands and their biosynthesis intermediates (94). Phylogenic studies define six subfamilies of NRs where their diversification follows a two waves gene duplication model (7, 70, 97). The first wave of gene duplication resulted in the appearance of various groups of receptors (ER, VDR, RXR, etc), as for the second wave of gene duplication gave rise to diversification inside each group of receptors (70). Therefore, due to gene duplication, new mutant NRs that diverged from the ER sequence appeared and showed affinity for estrogen biosynthesis intermediates. In other words, previous ligand biosynthesis intermediates became novel ligands for new NRs. This is seen with the C21 estrogen biosynthesis intermediate that became the ligand for a mutant ancestral ER 3-keto steroid receptor, a product of gene duplication (94, 98). C21 is progesterone and 3-keto steroid receptor is the PR. The same process occurred for the PR, GR and MR (94, 98). Therefore, gene duplications and the presence of ligands gave rise to vertebrate NRs that maintain strong structure-function relationship and ensure proper and specific regulation of hormonal response.
Figure 2.6- Evolutionary model of steroid nuclear receptors. Graphical representation of bilateral taxon clan showing the origins of ER, PR and AR. The image is modified from a published figure (6).

2.1.5 Post-translational modification of nuclear receptors

Recent experimental data have shown that NRs are post-translationally modified, adding complexity to our understanding of NR-mediated transcriptional control (99). Post-translational modifications of NRs are phosphorylation, acetylation, ubiquitylation and sumoylation, as illustrated in Figure 2.7 (99, 100). These covalent modifications can greatly change the function of NRs by affecting their stability, localization, target gene specificity, or by altering the interactions with its coregulators (66, 99, 101). An example of this can be seen with the phosphorylation state of NRs. Phosphorylation is the most studied covalent
modification (100). Phosphorylation sites in NRs were identified by radiolabeling and mass spectrometry techniques, and the great majority of these sites are situated in the N-terminal domain of NRs (100). As a general rule, the presence of ligand was shown to increase the level of NR phosphorylation (101). A possible effect of the latter is NR hypersensitivity to its ligand: phosphorylation of the PR residue Serine 294 was shown to augment PR sensitivity to low concentrations of progesterone as was the mutation of the ERα residue Lysine 303 changed the sensitivity of the receptor to levels of estrogen (66, 100).

As seen previously, upon binding of ligand, NRs undergo conformational changes exposing new surfaces of interactions. These new surfaces can now recruit a different selection of coregulators, classically defined as coactivators, by dissociating with corepressors (i.e. coregulators bound in the absence of ligand). Many of these coregulators are responsible for the observed post-translational modifications of NRs, each resulting in a different covalent modification (100-102). The oscillation of coactivator and corepressor recruitment that occurs as a result of presence or absence of ligand, respectively, exemplifies the complexity of NR-mediated transcriptional control and the fine-tuning needed for the proper functioning of the latter. In addition, recent data show that coregulators themselves can be modified post-translationally resulting in more extensive regulation of NR-mediated gene expression (100). Therefore, coregulators play an important role in the proper function of NR-mediation transcriptional control, a subject that will be reviewed in the following section.
Figure 2.7- Post-translational modifications of ER\(\alpha\) and PR. Schematic representation of covalent modifications present in the ER\(\alpha\) and PR. The different colored sections of the NR represent its domains: purple color for the A/B domain, lime green color for the DBD, pink color for the D domain and blue color for the LBD. Two isoforms exist for the PR (PR-B and PR-A), as demonstrated with the start points of each isoform (indicated by a dark blue triangle), PR-B being the full length isoform and PR-A the N-terminal truncated isoform. The different covalent modifications sites are color coded in the figure, as described in the legend: phosphorylation sites are indicated in green, acetylation sites are indicated in purple and sumoylation sites are indicated in pink. In the case of ER\(\alpha\), sites K266 and K268 are both acetylated and sumoylated. The figure is modified from a published image (99).

2.2 Nuclear receptor coregulators

Transcriptional control by NRs is a multistep process that requires the participation of many protein complexes known as coregulators (i.e. coactivators and corepressors) (91). The classical model of NR transcriptional control presents a simplified mechanism of action where in the presence of ligand, coactivators are recruited to NRs and transcription is activated, whereas in the absence of ligand, corepressors are recruited to NRs and transcription is repressed (93, 103). As will be seen in the current section, recent findings are showing a more complex model where the division between coactivators and
corepressors is not as clear as initially thought. More importantly, the ever expanding field of coregulators is painting an image of intricate and temporal interactions among cofactors that result in context specific (i.e. gene and cell type specific) biological responses that can no longer be generalized with an “one size fits all” model.

2.2.1 The role of chromatin in transcriptional initiation

Transcription activation is a multi-step process that requires many cofactors and protein complexes. The TATA box, initiator sequence and transcriptional start site are elements often found in the core promoter (65). Initiation of transcription occurs upon the binding of activators and general transcription factors upstream to the core promoter while RNA polymerase II and its cofactors (together forming the preinitiation complex) are bound to the core promoter (104, 105). In the case of sequence-specific transcription factors, which include NRs, transcription rate is influenced by their ability to interact with the basal transcriptional machinery. This interaction can be either direct or indirect with the help of coregulators (65).

Despite our tendency to view DNA and transcriptional control in a linear fashion, regulation of gene expression is a three dimensional process. Chromatin is highly compacted into nucleosomes (see Figure 2.8). The latter are composed of 147bp of DNA wrapped 1.65 times around a core octamer complex (104, 106). The octamer is composed of two copies of each of the four core histones: H2a, H2b, H3 and H4 (106). Histones core are globular structures with an alpha-helical core arranged in helix-turn-helix (HTH) motifs. These motifs were shown to be responsible for many of the protein-protein and protein-
DNA interactions observed in multi-protein complexes (106, 107). There are 14 contacts points between the DNA and histones, making the nucleosome one of the most stable protein-DNA complexes (104). Despite their great stability, nucleosomes are highly dynamic units.

Nucleosomes provide a higher order of gene expression regulation by limiting access of the transcriptional machinery to promoters and regulatory regions of genes. In order for transcriptional initiation to take place, the compaction of nucleosomes must be loosened. This is observed as the unwinding of DNA around the histone core. Two mechanisms exist to achieve this goal: ATP-dependent remodeling complexes change the position and/or stability of nucleosomes, and histone variant substitutions (106). In the first mechanism, the chromatin-remodeling complexes alter the DNA-histone contacts with the ATP-hydrolysis activity (108). By reducing the DNA-histone contacts, greater accessibility to the nucleosomal DNA is obtained by forming DNA-loops, by sliding the nucleosome to a different position or by completely evicting the histone octamer (104, 108). For the second mechanism to function, histone variants are necessary. Contrary to initial belief (104), there are many variant forms of histones (104). These variants are important for many diverse genomic functions, including regulation of gene expression, DNA repair and mitosis (106, 109). With the exception of H4, core histones have many isotypes that are not splice variants of the same gene product, but rather products of different genes (106). The isotypes are classified in three groups depending on their pattern of expression: (i) replication-dependent, (ii) replication-independent, or (iii) tissue-specific (106, 109). An example of how histone variants play a role in transcriptional control is seen with variant
H3.3 which was shown to imprint an increase in chromatin activity (110). H3.3 is present in both the promoter and coding regions of actively transcribed genes (110). Interestingly, recent findings are showing a pattern of nucleosome density at promoter regions where the former was shown to be lower in coding regions versus non-coding regions (104). These results are an indication of genomic premeditation where the positioning of nucleosomes seems to be embedded in the genomic sequence in order to ensure accessibility of regulation regions to transcription factors.

Similar to NRs, histones are subject to post-translational modifications, and the great majority occur at the N-terminal region of histones (also known as histone tails). Although histone cores are globular, the N-terminal regions of histones are structurally flexible and highly dynamic as their crystal structure presents them as irregular chains (111, 112). Due to their high concentration of lysine and arginine residues, histone tails are also very basic (111), and are modified post-translationally by acetylation, phosphorylation, methylation, ubiquitination and sumoylation (104, 113). These covalent nucleosomal modifications occur in the regulatory regions of genes (upstream region, core promoter and 3’ end of the open reading frame) and are introduced, maintained and reversed by enzymes (106). Histones are acetylated by proteins with histone acetyl-transferase activity (HAT). This reaction can be reversed by histone deacetylases (HDACs). Interestingly, the great majority of NR coactivators are associated with HAT activity. HDACs, and proteins interacting with HDACs, are generally considered to function in corepression (65).
Histone covalent histone modifications are critical for regulation of gene expression. The modification of the histone tails by either acetylation or phosphorylation changes the electrostatic charge on the surface of the nucleosome that results in reorganization of the chromatin. Not only can histone modifications expose regulatory elements for transcription factor and cofactor binding, but their specific sites generate a pattern of modifications known as the “histone code”. The histone code adds a new layer of gene expression regulation: not only are the sites recognized by coregulators, by they are made by coregulators and the coregulators themselves can be modified post-translationally (106). Therefore, the histone code will determine which coregulator is going to be recruited to the regulatory site, and the presence of a specific group of coregulators will determine the combinatorial effect on the biological response.

As it will be seen below, recent discoveries in the field of chromatin structure and dynamics have dramatically modified the classical model of NR-mediated transcriptional control. Even though the latter has greatly increased in complexity, the newly acquired information brings better understanding of hormone-dependent physiological processes and diseases.
Figure 2.8- Chromatin structure. Schematic view of the different organizational structures of a chromatin. A nucleosome consists of a histone octamer wrapped 1.65 times with a DNA segment of 147bp in length. Nucleosomes are often referred to as “beads on a string” due to their appearance when observed with an electron microscope (114).
2.2.2 Coactivators

Coactivators are defined as proteins that activate transcription in the presence of ligand by interacting directly with the NR activation domain (AF-2) and members of the basal transcription machinery (17, 103). NR structural studies performed in the presence of ligand have characterized in detail the conformational changes necessary for NR-activator interaction (115). As mentioned previously, in the absence of ligand, the LBD is in an extended form with helix 12 pointing away from the LBD (8). Helix 12 is an amphipatic helix in which negatively charged residues are exposed on the surface and hydrophobic residues point towards the hydrophobic core of the LBD (65). Therefore, upon ligand binding, helix 12 is pulled in towards the central hydrophobic core, creating a charge clamp between itself and helix 3. The conformational changes of helix 12 have a dual function: stabilizing ligand interaction and exposing a new surface recognized by coactivators (115). The LXXLL (where L is a leucine and X is any a.a.) motif, also known as the NR box, is responsible for the direct interaction between coactivators and agonist-bound NRs in the majority of cases (115, 116). The LXXLL motif, similar to helix 12 of the LBD, is an amphipathic α-helix in which the leucine residues align on one face of the helix, forming a hydrophobic surface (65). The stability of the interaction is due to hydrogen bonds formed between specific residues of the charge clamp and the NR box α-helix: a lysine residue in helix 3 and glutamic acid residue in helix 12 (115).

Coactivators play many roles in the transcriptional activation process, including histone modifications, chromatin remodeling and pre-initiation complex assembly (117). The different steps of transcriptional activation are dependent on the enzyme activity of
coactivators. These enzymatic activities result in a series of post-translational modifications (i.e. phosphorylation, methylation, acetylation, ubiquitylation and sumoylation) of target proteins (117). The acetyltransferase is the most described enzymatic activity in coactivators. Initially, proteins having acetyltransferase activity were described as histone acetyltransferases (HATs) (118). Acetylation of specific lysine residues in histone tails result in a more relaxed chromatin structure, hence permitting access and recruitment of the transcriptional machinery.

Nuclear receptor coactivator-1 (NCoA1, also known as SRC-1), was the first coactivator identified (102). SRC-1 is part of the p160 family of proteins that contains two other members, SRC-2 and SRC-3 (102). All three proteins are homologous, sharing 40% a.a. identity with the N-terminal ends being the most conserved. In addition, they share common domains: NR interacting domain (NID), basic helix-loop-helix (bHLH, responsible for DNA interaction), NR box and two activation domains (AD1 and AD2) (102). Through these domains, and consequent protein interactions, the p160 family proteins were shown to participate in chromatin remodeling and de-condensation (65).

2.2.3 Corepressors

NRs bound to HREs in the absence of ligand interact with corepressors to inhibit basal transcription (65). Corepressors interact with NRs through a corepressor NR box (CoRNR, pronounced “corner”). The CoRNR has a consensus sequence: LXXXI/LXXXI/L where L is leucine, I is isoleucine and X is any a.a. Through this motif, corepressors bind to their docking site on helices 3/5/6 to which they have access to due to the extended
conformation of the LBD in the absence of ligand (119). The conformational change caused by the presence of ligand blocks access of the docking site, resulting in corepressor release followed by coactivator recruitment (91, 119).

Silencing mediator of retinoid and thyroid hormone receptors (SMRT, pronounced “smart”) and nuclear hormone receptor-corepressor (NCoR) were the first corepressors identified (119). SMRT and NCoR are products of two different loci and share 45% a.a. identity (119). They both have transcriptional repression domains (RDs) and nuclear receptor interaction domains (NDs) (119). Contrary to agonist-bound NRs that recruit HATs to activate transcription, NRs in the absence of ligand recruit histone deacetylases (HDACs). One function attributed to HDAC proteins is the removal of acetyl groups from histone tail lysine residues, resulting in tight and compact chromatin structure, hence occluding transcriptional machinery binding sites (118). The RDs in SMRT and NCoR recruit HDACs and other components of the corepressor complex (119). Therefore, SMRT and NCoR can be seen as protein scaffolds to which other corepressors are recruited in order to maintain transcriptional repression (119).

2.2.4 Ligand-dependent corepressors

The initial understanding of NR-mediated transcription control described a simplistic model. It went as follows: corepressors are bound to the NR in the absence of ligand and consequently inhibiting transcription, whereas in the presence of ligand, coactivators are bound to the NR resulting in transcription activation. However, this two-mode model has recently been challenged with the discovery of corepressors that exhibit their
transcriptional corepressor function in the presence of ligand (1, 4, 91, 120, 121). Even though their “raison d’être” is not well understood, they seem to have a fine-tuning function on NR-mediated transcription. This fine-tuning function results either by recruiting proteins responsible for post-translation modifications of histones, or by modulating the activity of coregulators with post-translational modifications. In both cases, the management of the histone code ensures proper transcriptional regulation and suggests a highly dynamic system. Two examples of agonist-bound corepressors are given below.

**RIP140**

Receptor interacting protein 140 (RIP140, also known as NRIP1) was the first agonist-bound corepressor identified (122). RIP140 is expressed in many tissues and was shown to be essential for female fertility, fat accumulation in adipose tissue and energy homeostasis (122-124). It was also shown to interact directly with several NRs, including ERα, PR, GR and VDR (122). Several domains are found in RIP140 and were shown to mediate its transcriptional corepression function by interacting with other members of the corepressor family (122, 125). RIP140 has nine NR boxes with which it interacts with NRs and 4 RDs which can recruit several HDACs (123). Recent studies are showing a more complex transcriptional regulation by RIP140 due to its many post-translational modifications (123, 126, 127). Multiple post-translational modifications were identified in RIP140, including phosphorylation, methylation, acetylation, and most recently sumoylation, all of which were shown to affect its transcriptional corepressor function (126-131). Phosphorylation was shown to increase its transcriptional corepressor activity through
enhanced HDACs recruitment, whereas methylation reduced its corepressor activity (127, 129). This was due to attenuation of HDAC interaction and increased nuclear export. The acetylation state of RIP140 had a dual effect on its corepressor activity. Therefore, RIP140 is a perfect example of transcriptional activity modulation by post-translational modifications.

**LCoR**

The ligand-dependent corepressor LCoR was identified through a yeast two-hybrid screen for which the LBD of ERα was used as bait (1). LCoR is a 47kDa protein that is expressed in a variety of fetal and adult tissues as well in two-cell stage mouse embryos, suggesting functions in embryonic development. Sequence analysis has identified several conserved domains in LCoR: NR box and two C-terminal binding protein (CtBP)-binding domains at the N-terminus end, a helix-turn-helix (HTH) motif and sumoylation site at the C-terminus end (see Figure 2.9). *In vitro* studies have shown the interaction of LCoR with agonist-bound NRs to be dependent on the integrity of its NR box (LXXLL motif). Reporter gene assays showed LCoR-dependent corepression of GR-, PR- and vitamin D receptor (VDR)-mediated gene transcription implicating LCoR in a wide variety of physiological processes (1). Since a great number of corepressors mediate their transcriptional function through HDAC-dependent activity, the HDAC inhibitor TSA was used to investigate the mechanism of action of LCoR (91). Interestingly, results showed an HDAC-dependent and – independent transcriptional function where only the ERα and GR were affected by TSA treatment in COS7 cells (1). The functionality of the two CtBP-binding domains was confirmed through GST-pulldowns that showed a direct binding between LCoR and CtBP1.
Figure 2.9- Schematic representation of LCoR and its domains. LCoR contains a NR box and two CtBP-binding domains at its N-terminal end. LCoR mediates its interaction with NRs through the NR box. Both CtBP-binding domains were shown to bind directly to CtBP1 and deletion of both motifs abolished CtBP-binding. At the C-terminal end of LCoR a HTH motif and a sumoylation site are found. Many transcription factors have a HTH motif and the latter was shown to be responsible for DNA-protein and protein-protein interactions. Sumoylation is a form of post-translational modification and is usually attributed to corepressor function of coregulators.

2.2.5 Cyclical and temporal recruitment of coregulators

Classically, NRs were thought to bind and remain bound to the HREs of their target genes for the duration of the stimulus (132, 133). For proper transcriptional regulation to occur, NRs had to sense the stimulus, respond to the stimulus and sense the end of the stimulus (32). These chronological steps were done with the help of coregulators. With the mounting number of coregulators identified in the human genome, new efforts are being made to characterize the mechanistic nature of their transcriptional action (133, 134). Ten years ago, the Evans group has shown disruption of interaction between agonist-bound ERα and the chromatin due to acetylation (32). This finding prompted many studies investigating
the recruitment of transcriptional cofactors and their coregulators on regulatory regions of
target genes to see if there is a logical stream of events occurring upon hormonal stimulus.

The Brown lab successfully showed with ChIP assays a dynamic recruitment of
cofactors on endogenous estrogen target promoters upon estradiol stimulus (32). For the
duration of the stimulus, they showed ERα and its transcription complex cycled on and off
the regulatory region of estrogen target genes. In 2003, the Gannon group published
ground breaking work in which recruitment of over twelve cofactors on the estrogen target
gene promoter pS2 was investigated by ChIP and reChIP (two consecutive rounds of ChIP
assays that demonstrate the presence of two proteins present at the place and at the same
time) assays (133). They presented a highly dynamic model in which cofactors followed a
strict and sequential recruitment pattern (133, 135). Interestingly, they showed the
recruitment of HDACs during stimulus for which their cycle was off-phase from the ERα
recruitment, suggesting a promoter-clearance function for HDACs by recondensing the
chromatin (135).

The on-off cycling of cofactors was initially suggested to be due to “environmental
assessment” or protein-binding kinetics (32, 135). However, recent studies are suggesting a
more elaborate system where the focus is brought on the transcriptional function of
coregulators that is mediated by post-translational modifications (136). Coregulators are
part of multi-subunit complexes which contain many different, and sometimes opposing,
enzymatic activities (132). As it will be seen in the next few chapters, the combinatorial
effect of protein interactions determines the final coregulator transcriptional output.
Chapter 3

Function of HDAC6 as a cofactor of nuclear receptor coregulator LCoR
Preface

The first paper published from the John White laboratory on LCoR, published in *Molecular Cell* (1), identified the interaction of LCoR with HDAC6 in vitro. The current chapter investigates in greater depth the physiological importance of the interaction between LCoR and HDAC6. A series of kinetic ChIP assays showed corecruitment of HDAC6 and LCoR in a ligand-dependent and partial synchronized fashion on promoters of endogenous estrogen target genes. To further investigate the physiological meaning of LCoR-HDAC6 interaction, siRNA-mediated knockdowns were performed and their effect on ERα-regulated genes was studied. Our data confirm a newly introduced concept in the field of NR-mediated transcriptional control where the recruitment of NR cofactors is context and gene specific.
3.1 Abstract

Ligand-dependent corepressor LCoR was identified as a protein that interacts with the estrogen receptor α (ERα) ligand binding domain in a hormone-dependent manner. LCoR also interacts directly with histone deacetylases (HDACs) 3 and 6. Notably, HDAC6 has emerged as a marker of breast cancer prognosis. However, while HDAC3 is nuclear, HDAC6 is cytoplasmic in many cells. We found that HDAC6 is partially nuclear in estrogen-responsive MCF7 cells, colocalizes with LCoR, represses transactivation of estrogen-inducible reporter genes and augments corepression by LCoR. In contrast, no repression was observed upon HDAC6 expression in COS7 cells, where it is exclusively cytoplasmic. LCoR binds to HDAC6 in vitro via a central domain and repression by LCoR mutants lacking this domain was attenuated. Kinetic chromatin immunoprecipitation (ChIP) assays revealed hormone-dependent recruitment of LCoR to promoters of ERα-induced target genes in synchrony with ERα. HDAC6 was also recruited to these promoters, and reChIP experiments confirmed the co-recruitment of LCoR with ERα and with HDAC6. Remarkably, however, while we find evidence for co-recruitment of LCoR and ERα on genes repressed by the receptor, LCoR and HDAC6 failed to coimmunoprecipitate, suggesting that they are part of distinct complexes on these genes. While siRNA-mediated knockdown of LCoR or HDAC6 augmented expression of an estrogen-sensitive reporter gene in MCF7 cells, unexpectedly their ablation led to reduced expression of some endogenous estrogen target genes. Taken together, these data establish that HDAC6 can function as a cofactor of LCoR, but suggest that they may act to enhance expression of some target genes.
3.2 Introduction

Nuclear receptors are ligand-regulated transcription factors whose activities are controlled by a variety of lipophilic extracellular signals, including steroid and thyroid hormones, metabolites of vitamins A (retinoids) and D (137, 138). DNA-bound nuclear receptors regulate transcription by recruiting complexes of coregulatory proteins, classified as coactivators or corepressors depending on whether they act to stimulate or repress transcription (138-140). Many coactivators interact with receptors through signature LXXLL motifs, known as NR boxes, which are oriented within a hydrophobic pocket of agonist-bound receptor ligand binding domains (LBDs) (19). Several coactivators or their associated cofactors possess histone acetyltransferase (HAT) activity, which essentially caps positively charged lysine residues and loosens their association with DNA, facilitating chromatin remodeling and subsequent access of the transcriptional machinery to promoters.

Nuclear receptor corepressors NCoR and SMRT were isolated as factors that interacted with hormone-free, but not hormone-bound thyroid and retinoid receptors (141, 142). They bind to receptor ligand binding domains through extended LXXXIXXXXL/I motifs known as CoRNR boxes (143, 144) and recruit multi-protein complexes implicated in transcriptional repression and histone deacetylation (138-140, 145-148). Hormone binding induces a conformational change in ligand binding domains that leads to dissociation of NCoR or SMRT. Both corepressors are components of several different complexes containing distinct combinations of ancillary proteins and class I or class II histone deacetylases (HDACs), suggesting that their function depends on cell type, combinations of transcription factors bound to specific promoters, and phase of the cell cycle.
We identified a ligand-dependent corepressor, LCoR, as an NR box-containing protein that interacted with the LBDs of agonist-bound receptors and repressed hormone-dependent transactivation when overexpressed (1). While LCoR interacts with nuclear receptors in essentially the same manner as coactivators, it recruits both HDACs and C-terminal binding proteins (CtBP) corepressors. LCoR interacts directly with class I HDAC3 and class II HDAC6 in vitro, and coimmunoprecipitates with the two proteins from MCF7 cell extracts (1). While HDAC3, like LCoR, is a nuclear protein, the interaction of LCoR with HDAC6 is remarkable as HDAC6 is cytoplasmic in many cells (149). Indeed, HDAC6 has been shown to function as a tubulin deacetylase (150, 151), through an association controlled by a tetradecapeptide motif (152). However, a portion of HDAC6 can be nuclear in some cells. Notably, experiments performed in breast cancer cells have revealed that HDAC6 is an estrogen target gene (153), and that HDAC6 protein is present in the nuclei of normal breast epithelial cells, but is cytoplasmic in adjacent malignant cells (154, 155). Moreover, these studies found that HDAC6 expression levels correlate with better prognosis and response to endocrine therapy in breast cancer (153-155).

Based on the above, we examined the subcellular localization of HDAC6 in estrogen-responsive MCF7 breast cancer cells and its potential role as an LCoR cofactor. We find that HDAC6 is partially nuclear in MCF7 cells, and that LCoR and HDAC6 are recruited together during ERα-dependent gene regulation in MCF7 cells. Remarkably, however, while ablation of LCoR or HDAC6 enhanced estrogen-dependent stimulation of a reporter gene, the effect was not reproduced on endogenous ERα target genes. Rather, the results suggested that the two proteins can act to enhance expression of specific estrogen regulated genes.
3.3 Results

3.3.1 Colocalization of HDAC6 with LCoR in MCF7 cells

Our previous results showed that endogenous LCoR coimmunoprecipitated with endogenous HDACs 3 and 6 from extracts of MCF7 cells (1). However, as HDAC6 is cytoplasmic in many cells, we further investigated the colocalization of LCoR and HDAC6 in MCF7 cells by immunocytochemistry. As expected (1), LCoR was almost exclusively nuclear, as was HDAC3, and there was marked colocalization of the two proteins (Fig. 1a). Moreover, a substantial portion of HDAC6 was nuclear in MCF7 cells, and there was a clear colocalization of nuclear HDAC6 with LCoR (Fig. 1a), substantiating the possibility that the two proteins function together.

3.3.2 Cell-specific repression of hormone-dependent transcativation by HDAC6

The capacity of HDAC6 to function as a (cell-specific) cofactor in LCoR-dependent corepression of estrogen-dependent transactivation was further analyzed in transiently transfected COS7 and MCF7 cells. COS7 cells were chosen for comparison because HDAC6 remains cytoplasmic even when overexpressed in transient expression experiments (Fig. 1b). Coexpression of HDAC6 with LCoR in COS7 cells had no effect on LCoR-dependent corepression (Fig. 1c). As a control for repressive effects of HDAC cotransfection in COS7, we performed a similar coexpression experiment with HDAC3, which unlike HDAC6, is nuclear in a wide variety of cell types (145, 146). HDAC3 repressed ERα-dependent
luciferase expression in COS7 cells on its own (but not that of the internal control), and enhanced transcriptional repression by LCoR (Fig. 1c).

In contrast to the above, HDAC6 partially repressed ERα-dependent transactivation in MCF7 cells on its own, and enhanced corepression by LCoR (Fig. 1d). Note that these transfections were performed with limiting amounts of LCoR and HDAC6, under conditions that repressed estrogen-dependent reporter gene activity without affecting expression from the internal control plasmid. While corepression was apparently further enhanced when cells were cotransfected with larger combined amounts of LCoR and HDAC6 expression vectors, these conditions also affected expression of the β-galactosidase internal control (data not shown).

Consistent with our previous findings that LCoR corepression of ERα-dependent transcription is sensitive to the HDAC inhibitor trichostatin A (TSA) (1), corepression by both LCoR and HDAC6 of ERα transactivation was fully abolished by TSA (Fig. 1e). In contrast, treatment of cotransfected cells with HDAC inhibitor trapoxin (TRAP) only partially abolished corepression (right-hand panel; Fig. 1e), consistent with the resistance of HDAC6 activity to trapoxin (151, 152). Taken together, these data strongly support the idea that HDAC6 can function as a nuclear cofactor of LCoR in MCF7 cells.

3.3.3 Delineation of an HDAC6-interacting domain of LCoR

The domain of interaction of HDAC6 with LCoR was determined by generating a series of GST fusions of C- and N-terminal deletion mutants of LCoR (Fig. 2a), and analyzing the capacity of these mutants to pull down in vitro translated HDAC6. All deletion mutants were
well expressed in bacteria (Fig. 2a). GST pull-down experiments performed with these mutants showed that residues lying between amino acids 203 and 319 in the central portion of LCoR were required for interaction with HDAC6 \textit{in vitro} (Figs. 2b-d).

The role of the HDAC6 interaction domain in corepression by LCoR was analyzed in transfection experiments in MCF7 cells by expression of Flag-tagged wild-type LCoR and the Flag-tagged mutant form lacking amino acids 203-319. Reporter gene experiments showed that corepression by the wild-type and mutant forms of LCoR was similar at low concentrations. However, the mutant exhibited no dose-dependent increase in corepression (Fig. 3a). Western analysis with an anti-Flag antibody showed that the tagged proteins were expressed at similar levels (Fig. 3b). Moreover, the deletion mutant could be detected with an antibody against LCoR (Fig. 3b). In order to verify that the LCoR mutant lacking the HDAC6 domain is still an active protein, a dominant-negative experiment was performed where constant levels of LCoR were cotransfected with greater amounts of the mutant form (Fig. 3c). The coexpression of the mutant LCoR reduced the repression observed with the wild type protein, hence showing competition between the two forms of LCoR.

\subsection*{3.3.4 Hormone-dependent association of LCoR with estrogen-responsive promoters \textit{in vivo}}

To further substantiate the role of HDAC6 as a cofactor of LCoR in transcriptional regulation in MCF7 cells, we performed chromatin immunoprecipitation (ChIP) assays to analyze the recruitment of LCoR and HDAC6 to ER-binding regions of estrogen-inducible
promoters of the pS2, insulin-like growth factor binding protein 4 (IGFBP4), adenosine A1 receptor (ADORA1), and nuclear receptor interacting protein 1 (NRIP1) genes \textit{in vivo}. As we lack an antibody that reliably immunoprecipitates endogenous LCoR, we analyzed recruitment of transiently expressed tagged LCoR to the pS2 promoter with an anti-Flag antibody. Rapid (15min) estradiol-dependent recruitment of ER\(\alpha\) was observed to the ERE region of the pS2 promoter, but not to non-target sequences (Fig. 4a). The kinetics of ER\(\alpha\) recruitment under these conditions is entirely consistent with data reported by other groups (82, 133). The anti-Flag antibody consistently detected recruitment of tagged LCoR by 30min of estradiol treatment to the region of the pS2 ERE, but not non-target DNA (Fig. 4a).

The recruitment of HDAC6 to the pS2 promoter followed a similar, but not identical pattern to that of LCoR; for example, unlike LCoR, HDAC6 did not dissociate from the pS2 promoter after 60min of estradiol treatment (Fig. 4a). As others have shown that HDAC6 can function as a regulator of the histone acetyltransferase p300 (156), we analyzed p300 recruitment to pS2, and found that it was recruited rapidly but lacked the clear cyclical pattern of LCoR. Overlapping patterns of corecruitment of ER\(\alpha\), LCoR and HDAC6 were also observed to ER binding regions of promoters of genes encoding IGFBP4, ADORA and NRIP1 (82) (Figs. 4b-d). Note that we consistently observed binding of ER\(\alpha\) and NRIP1 promoters in the absence of estradiol (Figs. 4c and d), a phenomenon that has been observed by others on estrogen-inducible promoters (82).
We also analyzed binding of ERα, LCoR and HDAC6 to regulatory regions of the gene encoding bone morphogenic protein 7 (BMP7), previously identified as being downregulated by estrogen (157). A peak of LCoR recruitment to the BMP7 promoter occurred 30 min after addition of estradiol (Fig 5a), similar to the kinetics of recruitment to estrogen-inducible genes. However, we observed a largely estrogen-independent association of HDAC6 with the BMP7 promoter.

The binding of p300 to promoters complicates interpretation of experiments, as HDAC6 could be recruited to the promoters in association with either p300 or LCoR. Therefore, to determine whether LCoR and HDAC6 are co-recruited to promoters in vivo, we performed a series of reChIP experiments on all promoters analyzed. Experiments were performed from extracts of MCF7 cells treated with estradiol for 30 min, a time corresponding to peak LCoR recruitment to all promoters analyzed. Extracts were immunoprecipitated with an anti-FLAG antibody, followed by a second round of immunoprecipitation with either anti-ERα or anti-HDAC6 antibodies. The observed coimmunoprecipitation of LCoR with ERα or HDAC6 confirms their co-association with the ERE regions of estrogen-inducible pS2, ADORA1, IGFBP4 and NRIP1 promoters (Fig. 5c). Remarkably, however, while ERα and LCoR were recruited together on the BMP7 promoter, we found no evidence for co-recruitment of LCoR and HDAC6 (Fig. 5c), a result that was reproduced several times. Note that all reChIP experiments presented in figure 5 were performed on the same sets of extracts. Thus, while LCoR and HDAC6 are present on The BMP7 promoter, they appear to be associated with distinct complexes.
3.3.5 Effects of ablation of LCoR or HDAC6 expression in MCF7 cells on estrogen-regulated gene transcription

To determine the functional significance of association of LCoR and HDAC6 with ERα target genes, we performed knockdown experiments with siRNAs targeting LCoR or HDAC6 (Fig. 6a). Knockdown of LCoR or HDAC6 augmented both basal and hormone-stimulated expression from an estrogen-sensitive reporter gene. Essentially identical results were obtained in several independent sets of siRNA transfections. Note that the elevated luciferase expression seen in the absence of estradiol is consistent with the dose-dependent inhibition of basal expression from estrogen-sensitive promoter/reporters observed upon LCoR overexpression [14]. These data suggest that LCoR and HDAC6 can function as attenuators of (hormone-regulated) expression of estrogen target genes.

The effects of LCoR and HDAC6 ablation in MCF7 cells on regulation of endogenous estrogen target genes were also examined (Fig. 7). Genes analyzed included those tested in ChIP assays in Fig. 5, along with several other direct target genes identified in recent microarray studies in MCF-7 cells (158). Knockdowns generally led to unexpected and gene-specific changes in gene expression. In contrast to data obtained in repeated experiments with an estrogen-sensitive reporter gene (Fig. 6), ablation of either LCoR or HDAC6 expression did not augment estrogen-stimulated expression of any of the genes tested (Fig. 7). Rather, knockdown of LCoR either did not affect expression (pS2, NRIP1, GREB1, SGK3; Figs. 7A, D, F, G) or markedly reduced estrogen-induced transcription of the IGFBP4, ADORA1 and CYP26B1 genes (Figs. 7B, C and E). In addition, combined knockdown of LCoR and HDAC6 led to reduced estrogen-dependent stimulation of the GREB1 gene even though
individual knockdowns had no substantial effect on hormone-regulated expression of these genes (Figs. 7B). A similar effect of double knockdown was observed on the SGK3 gene (Fig. 7C), although it do not quite reach statistical significance. These effects, seen in multiple biological replicates, are in striking contrast to the enhanced reporter gene expression seen above after LCoR or HDAC6 knockdown, and are not consistent with the two proteins serving corepressor functions on the genes affected. These results suggest an unexpected role of LCoR in activation of a subset of estrogen-stimulated genes. In contrast, combined ablation of LCoR and HDAC6 augmented estrogen-stimulated expression of the NRIP1 gene (Fig. 7D).

Consistent with previous reports (22, 159-162) estrogen repressed expression of the BMP7 gene (Fig. 7H), and the gene encoding the r7as-associated protein keratin 4 (KRT4; Fig. 7I). While ablation of LCoR had no substantial effect on BMP7 mRNA levels, knockdown of HDAC6 or together with LCoR reduced BMP7 expression in the absence of estrogen (Fig. 7H). In addition, knockdown of LCoR or HDAC6 individually or together markedly reduced basal expression of KRT4, but had no substantial effects on estrogen-repressed expression of the gene.

Taken together, the colocalization, direct association and corecruitment of LCoR and HDAC6, along with results of knockdown of LCoR or HDAC6 on expression of an estrogen-sensitive reporter gene are consistent with HDAC6 functioning as a cofactor of LCoR in transcriptional corepression. However, analysis of the effects of knockdowns on endogenous estrogen target genes suggest that the two proteins function independently on
some genes and reveal a potential roles of both LCoR and HDAC6 in enhancing expression of specific genes.

3.4 Discussion

This study has analyzed the recruitment of corepressor LCoR and associated HDAC6 to estrogen-regulated genes in MCF7 cells. Both proteins are widely expressed in adult organisms; HDAC6 is present in mouse oocytes and zygotes (163), and LCoR is expressed as early as the 2-cell stage of mouse embryonic development (1). HDAC6 likely plays numerous biochemical roles during development and in the adult. Cytoplasmic HDAC6 is best known for its function as a tubulin deacetylase (150). Remarkably, however, HDAC6 knockout mice are viable and exhibit hyperacetylated tubulin in most tissues while demonstrating apparently normal development (164), suggesting that other HDACs can substitute for some cytoplasmic and nuclear functions of HDAC6.

Evidence accumulated to date has suggested that HDAC6 can either suppress or promote tumorigenesis, and that its precise function may depend on its subcellular localization. Immunohistochemical analyses showed that a portion of HDAC6 was nuclear in normal breast epithelial cells, but entirely cytoplasmic in adjacent breast tumor cells, suggesting that nuclear localization of HDAC6 is at least partly dependent on the state of differentiation of cells (154). This notion is supported by the observation that transfer of a portion of HDAC6 from the cytoplasm to the nucleus accompanied the induced differentiation and cell cycle arrest of the mouse B16 melanoma line (165). MCF7 cells
express both ERα and the PR and are estrogen-dependent for growth, consistent with a relatively well-differentiated phenotype. HDAC6 expression is induced by estradiol in MCF7 and other breast cancer cells, and its level of expression correlates with a better prognosis and response to endocrine therapy (153-155). In addition, patients with ER-positive breast tumours who received tamoxifen as adjuvant therapy for two years have better prognosis and survival rate when tumours expressed HDAC6 (153). Moreover, inhibition of HDAC6 enhanced HSP90-mediated maturation of MMP-2, which was associated with increased breast cancer cell invasion in an in vitro model (163). However, other studies have shown that cytoplasmic HDAC6 may enhance cell motility and thus metastases, and that inhibition of the tubulin acetylation activity of HDAC6 in multiple myeloma may have therapeutic potential (166, 167).

Our previous work showed that LCoR interacted with HDAC6 in vitro and coimmunoprecipitated with HDAC6 from MCF7 breast cancer cell extracts (1). However, given several studies showing the cytoplasmic location and function of HDAC6 (168), as well as its emergence as a prognostic marker of breast cancer, we were interested in examining its potential function as an LCoR cofactor more closely. We found that a substantial portion of HDAC6 was nuclear in MCF7 cells. Its function as an LCoR cofactor was supported by the finding that its coexpression with ERα repressed estradiol-dependent transactivation in reporter gene assays, and that it augmented the repressive effect of coexpressed LCoR. This effect was cell-specific as HDAC6 was entirely cytoplasmic when expressed in COS7 cells and did not enhance corepression by overexpressed LCoR. In contrast, HDAC3, which is a class I HDAC and a nuclear protein, strongly repressed hormone-dependent transcription in COS7
cells. Given its estrogen-dependent expression (153-155), our results raise the possibility that HDAC6 may function with LCoR on some genes as part of a feedback loop to regulate estrogen-dependent gene regulation in breast cancer cells.

The notion that HDAC6 can function in transcriptional repression is supported by studies showing that HDAC6 contributed to SUMO (small ubiquitin-related modifier)-dependent repression of p300 HAT activity (156). P300 is a component of HAT complexes recruited by nuclear receptors, including ERα, during transcriptional activation (133, 139, 140). A role for HDAC6 in transcriptional repression is also supported by studies showing that it can act as a cofactor of the repressor Runx2 in osteoblastic cells (169). However, HDAC6 is also associated with the promoter of the c-jun gene, whose transcription is inhibited by treatment with TSA, suggesting that HDAC6 may contribute to activation of c-jun expression (170).

Kinetic ChIP assays investigating the association of cofactors with estrogen target promoters have shown recruitment to be dynamic following a specific sequential order (135, 171). We found above that estrogen-induced recruitment of LCoR to the well-characterized pS2 promoter peaked at 30-45 min. Notably in this regard, others have found in MCF7 cells that estrogen-dependent recruitment of NR box-containing corepressor NRIP1 (RIP140) to the pS2 promoter also peaked at 30-45 min (172), raising the possibility of functional redundancy between the two corepressors. Like LCoR, overexpression of NRIP1 represses estrogen-dependent gene expression in transient expression studies (4). Similar to NRIP1 and LCoR, recruitment of corepressors NCoR and SMRT in the presence of estrogen was also observed on the pS2 promoter (173, 174). Moreover, association of
HDACs with the pS2 promoter in the presence of estrogen has been documented (133). These studies demonstrated estrogen-dependent recruitment of HDACs 1 and 7, which appeared to act redundantly.

Knockdown of LCoR or HDAC6 expression in MCF7 cells augmented basal and estrogen-stimulated expression of an ERE-containing reporter gene, consistent with the results of our transient expression studies, and supporting their potential roles as attenuators of ERα-dependent transactivation. However, the effects of ablation of LCoR or HDAC6 on endogenous ERα target genes were distinct and gene-specific. Loss of LCoR and/or HDAC6 had no effect on estrogen-regulated expression of the pS2 gene, for example. We speculate that the loss of LCoR and associated cofactor function in regulation of estrogen target genes in MCF7 cells can be compensated for by other corepressors recruited in the presence of hormone such as NRIP1 or ZNF366 (120). It is important to note that while knockdown of NRIP1 in MCF-7 cells augmented estrogen-stimulated expression of a transiently expressed reporter plasmid (175), NRIP1 ablation had no effect on regulation of a number of endogenous estrogen target genes in another study (85).

Unexpectedly, the results of LCoR or HDAC6 ablation provide evidence for potential roles of these proteins in maintenance of gene expression. LCoR knockdown abolished or reduced expression of IGFBP4, ADORA1 and CYP26B1 genes stimulated by estradiol, and ablation of LCoR or HDAC6 diminished basal expression of the KRT4 and BMP7 genes. While these findings point to roles for LCoR and/or HDAC6 in control of (estrogen-regulated) gene expression, none is consistent with their function as corepressors on the genes affected. The effects of LCoR ablation on endogenous estrogen-regulated gene expression are also in
contrast to observations in the accompanying manuscript that LCoR knockdown generally augmented progesterone receptor-stimulated expression of endogenous target genes (176).

It is important to note that, while the nature of effects of LCoR or HDAC6 ablation on endogenous gene regulation was unexpected, the results are consistent with data in the literature on roles in gene activation of factors generally associated with gene repression (136). For example, knockdown of NRIP1 in human embryonal carcinoma cells diminished ligand-dependent activation of a subset of retinoic acid-inducible target genes (121). In addition, a number of studies have shown that pharmacological inhibition of HDAC activity leads to activation and repression of roughly equal numbers of genes, providing evidence for a role of HDACs in both gene activation and repression (136). Recruitment of LCoR and HDAC6 to some estrogen-regulated promoters may be necessary for direct or indirect regulation of post-translational modifications of non-histone proteins associated with the dynamics of gene activation (136).

In summary, our results provide evidence that HDAC6 can function as a cofactor of LCoR, and show that LCoR and HDAC6 are co-recruited to promoters regulated by estradiol. While transient expression experiments suggest that LCoR and HDAC6 can function as corepressors, results of gene knockdown experiments indicate that the proteins individually or together are required for maintenance of expression of a subset of estrogen target genes.
3.5 Experimental procedures

**Antibodies**

A rabbit polyclonal antipeptide antibody was raised against LCoR amino acids 20-36 (QDPSQPNSTKNQSLPKA) fused to keyhole limpet hemocyanin, and purified over a peptide affinity column (Bethyl Laboratories, Montgomery TX, USA). Goat polyclonal HDAC3 (sc-8138), goat polyclonal HDAC6 (sc-5253), rabbit polyclonal HDAC6 (sc-11420), rabbit polyclonal ERα (sc-543), protein A-agarose (sc-2001) and protein G PLUS-agarose (sc-2002) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal p300 (ab3425) was from Abcam Inc (Cambridge, MA, USA). Cy3-donkey polyclonal α-goat (705-165-147) and Cy2-goat polyclonal α-rabbit (711-225-152), Cy3-donkey polyclonal α-rabbit (711-165-152), Cy2-donkey polyclonal α-mouse (715-225-150) were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Mouse monoclonal α-Flag M2 (F3165), and α-FLAG M2 HRP-conjugate (A8592), monoclonal α-rabbit HRP conjugate (A2074), rabbit polyclonal α-goat HRP conjugate (A5420) were from Sigma (St. Louis, MO, USA).

**Recombinant plasmids**

PSG5/LCoR, Flag-HDAC6/pcDNA3, HA-HDAC3/pCDNA3.1, Flag-LCoR/pcDNA3.1 have been described (1). Flag-LCoRΔHDAC6/pcDNA3.1 was made using QuikChange Mutagenesis Kit (cat# 200518, Stratagene, La Jolla, CA, USA) as per manufacturer’s instructions. Primers were designed to delete amino acids 203-319 from LCoR. The new construct was sequenced to confirm proper deletion and Western blot was performed to show equal level of expression when compared to wild type LCoR.
Cell culture and transfections

All cells were cultured under the recommended conditions. For immunocytochemistry, COS7 cells grown on collagen IV-treated microscope slides in 6-well plates in DMEM, supplemented with 10% FBS were transfected in medium without serum with 12.5μl of Lipofectamine 2000 (Invitrogen, Burlington Ont, Canada.) containing 1 μg each of pSG5/LCoR and HA-Flag-HDAC6/pcDNA3. Medium was replaced 24h after transfection and cells were prepared for immunocytochemistry after 48h as described below. For analysis of the effects of HDACs 3 or 6 on LCoR corepression, COS7 cells (60-70% confluent) grown in DMEM without phenol red, supplemented with 10% FBS on 6-well plates were transfected in minimal medium without serum with Lipofectamine 2000 (5μl) with 100ng of ERα expression vector, 250ng of ERE3-TATA-pXP2 reporter plasmid and 250ng of internal control vector pCMV-βgal. Quantities of expression vectors (LCoR/pSG5, HA-HDAC3/pCDNA3.1, Flag-HDAC6/pcDNA3, Flag-LCoR/pcDNA3.1 and Flag-LCoRΔHDAC6/pcDNA3.1) used are indicated in figures or corresponding figure legends. Medium was replaced 18 hr after transfection with medium containing charcoal-stripped serum and estradiol (10nM) for 30hr. MCF7 cells grown in 6-well plates were transfected similarly. MCF7 cells were also grown in 24-well plates and were transfected using a 1/5th scale. TSA and Trapoxin were added to 500nM and 50nM, respectively, as indicated. Cells were harvested in 250 μl of reporter lysis buffer (Promega). Note that the transfection conditions above were chosen because the amounts of HDAC and LCoR expression vectors
used led to selective repression of ERα-dependent transactivation without affecting expression of the β-galactosidase internal control.

**Immunocytochemistry**

Cells were cultivated on collagen IV-treated microscope slides in 6-well plates, fixed with 2% paraformaldehyde for 15 min at room temperature, washed (3X) with 1xPBS, and permeabilized with 0.2% Triton X100/5% BSA/10% horse serum in PBS. MCF7 cells were then incubated with α-LCoR (1:500), and goat polyclonal antibodies against HDAC6 or Bmi1 (1:50) in buffer B (0.2% Triton X100/5% BSA in PBS), for 1h at room temperature. Cells were washed (3x) with PBS, and incubated with goat anti-rabbit-Cy2 and donkey anti-goat Cy3 (1:300) in buffer B for 1h at room temperature. Transiently transfected COS7 cells were incubated with α-LCoR (1:500), and anti-FLAG (1:300) to detect Flag-HDAC6. Cells were washed (3x) with PBS, and incubated with Cy3-donkey polyclonal α-rabbit (1:300), Cy2-donkey polyclonal α-mouse (1:400) in buffer B for 1h at room temperature. Slides were mounted with Immuno-Fluore Mounting Medium (ICN, Aurora, Ohio) and visualized using a Zeiss LSM 510 confocal microscope at 63x magnification.

**Western blotting**

The following primary (1st) antibodies (Abs) were used: LCoR (GenWay Biotech, cat. no. 18-003-44018), Flag (Santa Cruz Biotechnology, sc-807). The following secondary (2nd)
Ab was purchased from Santa Cruz Biotechnology: goat anti-rabbit (catalog no. sc-2004). Western blot was performed as previously described (177) using MCF7 cells extracts. Cells were grown in 10 cm dishes (70% confluent) and transiently transected with 500ng of Flag-tagged LCoR. 30h later, cells were harvested.

_Chromatin immunoprecipitation (ChIP) and reChIP assays_

ChIP and reChIP assays were performed as previously described (178) in MCF7 cells. Cells were grown in 10 cm dishes (70% confluent) and transiently transected with 500ng of Flag-tagged LCoR. Following the transfection, cells were starved for two days in DMEM-phenol free and FBS free media and treated with 2.5µM α-amanitin (Sigma, A2263) for 2h prior to hormone treatment in order to properly synchronize cells. Cells were collected and cofactor recruitment was evaluated on promoter regions containing EREs of estrogen target genes. Immunoprecipitations were performed with the following antibodies: ERα (sc-543), HDAC6 (Upstate, 07-732), Flag (OctA- Probe, sc-807) and p300 (sc-8981). Protein A Agarose (sc-2001) was used for the immunoprecipitation and normal Rabbit IgG (sc-2027) for background control. Primer sequences used are the following: pS2 promoter forward 5’-CTCTCACTATGAATCCTTTGCAG-3’, reverse 5’-AGATAACATTTGCCAACAGAGGAGCC-3’, non-targeting pS2 forward 5’-CAGCCCCCAAGAAGTCCAG-3’, reverse 5’-TGACGACGGTAGGTAGCAGACCTT -3’, ADORA1 promoter forward 5’-CAGAAGCTCTGTTGGCCATG-3’, reverse 5’-ATCGGGCTTTGACGTGGT-3’, ADORA1 non-targeting forward 5’-TAGAATCCACTAGTCCACCTT -3’, reverse 5’-TCACTTGCTGTTACACTTTACCCCTC -3’, IGFBP4 promoter forward 5’-
CTTCTTTGCTGCAAGTCCC-3’, reverse 5’-ATGGCCTTCCATGCTACAAG-3’, IGFBP4 non-targeting forward 5’-GCCAGGGACCGGTATAAAAG -3’, reverse 5’- GACGTAGCGGGGGAAGTTAG -3’, NRIP1 promoter forward 5’-GATGCAGATTGGCTGACAGA-3’, reverse 5’- CCCACCCCCAATTCTATCT-3’, NRIP1 non-targeting forward 5’- GCGAGGGGAGGGACTGGG -3’, reverse 5’- ATGTCTCGAGGCTGACTTT -3’, BMP7 promoter forward 5’-TGCAGACGACGAAAAATCAG-3’, reverse 5’-AGGGGTGGGAGGGTAGATG -3’, BMP7 non-targeting forward 5’-CGCTATCAGTCACCCCATTT -3’, reverse 5’-CGAAAGGCTTTGAGATTGC-3’.

**SiRNA knockdowns**

SiRNAs were purchased from Thermo Scientific Dharmacon (Lafayette, CO, USA). The following ON-TARGETplus SMART pool siRNA were used: LCoR (L-026303-00), HDAC6 (L-003499-00) and non-targeting (D-001818-10). siRNAs were resuspended per manufacturer’s instructions. Transfections were done in 6-well plates as described previously. Lipofectamine 2000 (10 ul) was used as the transfection reagent. DMEM phenol-free with 10% stripped FBS was added 12h after transfection. For Western blot analysis, cells were collected 48h after transfection. Luciferase reporter assays after siRNA knockdowns were performed as follows: 100ng of ERα expression vector and 250ng of ERE3-TATA-pXP2 vector were transfected with the corresponding siRNA. DMEM phenol-free with 10% stripped FBS was added 12h after transfection. Estradiol (10nM) was added 36h after transfection and cells were collected 24h later. Luciferase activity was measured as previously described.
Cells were grown in 100-mm dishes. Media was replaced with charcoal-stripped medium containing ligand. Total RNA was extracted with TRIZOL reagent. cDNA synthesis was performed with iScript cDNA Synthesis Kit (Bio-Rad, Hercules, Ca, USA) according to the instructions of the manufacturer. MiniOpticon Real-Time PCR System with iQ SYBR Green Supermix (Bio-Rad) were used for qRT-PCR expression analysis of target genes. Program used is as follows: (1) incubate at 94°C for 60sec, (2) incubate at 95°C for 20sec, (3) incubate at 60°C for 30sec (decrease temperature by one degree per cycle), (4) incubate at 72°C for 30sec, (5) plate read, (6) repeat from step 2 five more times, (7) incubate at 95°C for 20sec, (8) incubate at 57.5°C for 30sec, (9) incubate at 72°C for 30sec, (10) plate read, (11) repeat from step 7 thirty-five more times, (12) perform melting curve and end. Results were normalized to β-actin mRNA expression. The following primers were used: pS2 forward 5’- ACCATGGAGACAAGGTGAT- 3’, pS2 reverse 5’- AAATTCACACTCTTCTCTG- 3’; GREB1 forward 5’- CCACAAAGGGTGTCCTCCAGAA- 3’, GREB1 reverse 5’- CACTGGCTTGCCCTGATATT- 3’; SGK3 forward 5’- CAAAAGAGGTCCACCACCA- 3’, SGK3 reverse 5’- TGTCGAAGTTCTGATATCTCTC- 3’; CYP26B1 forward 5’- ACATCCACCGCAACAAGC- 3’, CYP26B1 reverse 5’- GGATCTGCGGCAGGTAACTCT- 3’; BMP7 forward 5’- GTGTACGAGCTTCGTAACC- 3’, BMP7 reverse 5’- GCAGGAAGAGATCCGATTCC- 3’; KRT4 forward 5’- GCCGCAATGACTTTGTGGT- 3’, KRT4 reverse 5’- CCTCCACTCCACCTTTGTC- 3’; β-actin forward 5’- GCCATGGGTCAGAAGGATTCC- 3’, β-actin
reverse 5' - GCTGGGTTGTTAAGGTCTC - 3'. ADORA1 forward 5' - GACCTACTTCCACACCTGCCTCA - 3', ADORA1 reverse 5' - CCAGCCAAACATAGGGGTCAGT - 3', IGFBP4 forward 5' - GGGGGCAAGATGAAGGTCAT - 3', IGFBP4 reverse 5' - CGGTCCACACACCAGCAGTAA - 3', NRIP1 forward 5' - GTGATTCCAGGATGGTTTG - 3', NRIP1 reverse 5' - ATGGTTTTAATAAAGGATGATGC - 3'.
3.6 Acknowledgments

We are grateful to Dr. M. Yoshida (RIKEN, Japan) for trapoxin, and to Jacynthe Laliberté for technical assistance with confocal microscopy. A special thank you goes to Drs. Myles Brown (Harvard Medical School, USA) for providing locations of EREs and ER regulated enhancers. This work was supported by grants MT-11704 from the Canadian Institutes of Health Research (CIHR) to J.H.W, and grant from the National Cancer Institute of Canada to X.J.Y. I.F. was supported by postdoctoral fellowships from l’Association pour la Recherche sur le Cancer (l’ARC) and the CIHR. A.P. was supported by studentships from the CIHR and the Montreal Centre for Experimental Therapeutics in Cancer (MCETC). L.E.T.-M. was supported by Fonds de Recherche en Santé du Québec (FRSQ). X.J.Y. is the holder of a CIHR scholarship. J.H.W. and S.M. are Chercheurs Boursier National of the Fonds de recherché en Santé du Québec (FRSQ).
3.7 Figures

Figure 3.1-Colocalization and coexpression of HDAC6 and LCoR.

A. Colocalization of endogenous HDAC3 and endogenous LCoR (1st row) or endogenous HDAC6 and endogenous LCoR (2nd row) by confocal microscopy (see Experimental Procedures for details). B. Colocalization of transiently expressed HDAC6 and LCoR in COS7 cells by confocal microscopy. Overexpressed HDAC6 is exclusively cytoplasmic in COS7 cells. LCoR (nuclear) was detected with Cy3-conjugated antibody and HA-Flag-HDAC6 (cytoplasmic) with Cy2-conjugated antibody. C. Contribution of HDAC6 to LCoR corepression in COS7 cells. COS7 cells were transfected with expression vectors for ERα, LCoR and HDAC6, as indicated (E2, 10nM). Coexpression of HDAC3 with LCoR and ERα was used as a positive control for HDAC-specific repression. D. Contribution of HDAC6 to LCoR corepression in MCF7 cells. MCF7 cells were transfected with expression vectors for ERα, LCoR and HDAC6, as indicated (E2, 10nM). E. Effect of HDAC inhibitor trichostatin A (TSA; 500nM) or trapoxin (TRAP; 50nM) on repression by LCoR and HDAC6 in MCF7 cells. For C-E, data are averages three or more independent experiments, and error bars represent the standard error of the mean; *, P < 0.05 for results of corresponding overexpression (LCoR, HDAC3 or HDAC6) versus empty vector control.
Figure 3.1
**Figure 3.2- Delineation of the domain of interaction of LCoR with HDAC6.**

A. C- and N-terminal mutants of LCoR fused to GST used in this study. The results of GST pull-down assays with *in vitro*-translated HDAC6 are summarized in the middle with SDS-PAGE analyses confirming the expression of GST-LCoR fusions. Note that the C-terminal deletion mutant truncated at 169 was expressed at similar levels to other mutants (not shown). B and C. GST pull-down analyses of the interaction of *in vitro*-translated HDAC6 with C- and N-terminal LCoR mutants presented in A. The 146KDa band corresponding to HDAC6 is indicated. D. Schematic representation of the domain structure of LCoR based on present results and those of reference 14. The NR box (LXXLL motif) required for interaction with nuclear receptors is indicated, as are the two motifs (1 and 2) required for interaction with CtBPcorepressors. The central domain required for interaction with HDAC6 is indicated, as is the C-terminal helix-turn-helix (HTH) domain.
Figure 3.2
**Figure 3.3 - Analysis of the function of the HDAC6-interacting domain of LCoR.**

A. Dose-response curves of Flag-LCoR or Flag-LCoR$\Delta$HDAC6 in MCF7 cells treated with E2 (10nM). MCF7 cells were transfected with expression vectors for ER$\alpha$ and Flag-LCoR or Flag-LCoR$\Delta$HDAC6 (as indicated). Data is shown as relative luciferase units (RLU). B. Western blot of MCF7 extracts expressing Flag-LCoR or Flag-LCoR$\Delta$HDAC6 probed for LCoR (1$^{\text{st}}$ row) or Flag (2$^{\text{nd}}$ row). GAPDH used as loading control (3$^{\text{rd}}$ row). C. A dominant negative experiment in MCF7 cells treated with E2 (10nM). Cells were transfected with expression vectors for ER$\alpha$ and Flag-LCoR alone (200 ng), or ER$\alpha$ and Flag-LCoR (200ng) along with increasing amounts of Flag-LCoR$\Delta$HDAC6 (200, 400 and 800ng), as indicated. Data is shown as relative luciferase units (RLU). Data are averages three or more independent experiments, error bars represent the standard error of the mean; *, P < 0.05 for results of corresponding expression (Flag-LCoR or Flag-LCoR$\Delta$HDAC6) versus empty vector control.
Figure 3.3
Figure 3.4- Kinetic chromatin immunoprecipitation (ChIP) assays of estrogen-induced target genes.

MCF7 cells synchronized for 2h with α-amanitin (2.5μM) and treated with E2 (10nM) were collected at 15min increments and immunoprecipitated with IgG, or antibodies against ERα, Flag, HDAC6 or p300, as indicated. A-D. Kinetic ChIP assays on the pS2, IGFBP4, ADORA1 and NRIP1 promoters. Graphical representations of promoters indicate location of ERE sequence, the ER-binding region amplified by PCR, and non-target sequences analyzed. Note that the region of the pS2 promoter amplified lies immediately adjacent to the ERE and is identical to that amplified by others (133, 158) in analysis of estrogen regulation of the promoter.
Figure 3.4
Figure 3.5- Chromatin immunoprecipitation assays of estrogen-repressed target gene

BMP7.

A. MCF7 cells synchronized for 2h with α-amanitin (2.5μM) and treated with E2 (10nM) were collected at 15min increments and immunoprecipitated with IgG, or antibodies against ERα, Flag, or HDAC6, as indicated. Graphical representations of promoter indicate location of enhancer sequences, the region amplified by PCR, and the non-target sequences amplified. B. Re-chromatin immunoprecipitation (reChIP) assays. MCF7 cells treated with E2 (10nM) for 30min and immunoprecipitated with Flag. Second round of immunoprecipitations with IgG, ERα or HDAC6 were performed as indicated. The promoters of the pS2, ADORA1, IGFBP4, NRIP1 and BMP7 genes were investigated.
**Figure 3.6- SiRNA knockdown of LCoR and HDAC6 expression.**

A. Western blot of MCF7 extracts. Cells were transfected with corresponding siRNA (scrambled, LCoR or HDAC6) for 48h and cells were harvested. GAPDH expression was used as control. B. *Luciferase* assay analyzing the effects of knockdowns on estrogen-regulated reporter expression. An ERα expression vector and ERE3-TATA-pXP2 reporter plasmid were transfected along with scrambled, LCoR or HDAC6 siRNA. After 24h of treatment with DMSO or E2 (10nM), cells were harvested and *luciferase* activity was measured. Data is shown as relative *luciferase* units (RLU). Data are averages three or more independent experiments, error bars represent the standard error of the mean; *, P < 0.05 for results of specific knockdown (LCoR and HDAC6) versus results with scrambled siRNA.
Figure 3.6
**Figure 3.7- Effects of LCoR and HDAC6 ablation in MCF7 cells on regulation of endogenous estrogen target genes.**

Cells were transfected with corresponding siRNA (scrambled, LCoR, HDAC6 or LCoR and HDAC6) for 36h, then treated with vehicle (DMSO) or E2 (10nM) for 24h. QRT-PCR was performed on A. pS2, B. IGFBP4, C. ADORA1, D. NRIP1, E. CYP26B1 and F. GREB1, G. SGK3, H. BMP7, I. KRT4 genes. β-actin was used as internal control. Results are shown as fold induction. Data are averages three or more independent experiments, error bars represent the standard error of the mean; *, P < 0.05 for results of specific knockdown (LCoR, HDAC6 or LCoR and HDAC6) versus results with scrambled siRNA.
Figure 3.7
Ligand-dependent corepressor LCoR is a attenuator of progesterone-regulated gene expression
LCoR contains two adjacent CtBP domains on its N-terminus extremity and was shown to interact in vitro with CtBP1. This chapter investigates the contribution of CtBP1 to the nuclear receptor corepressor function of LCoR in both MCF7 and T47D breast cancer cells in presence of either estradiol or progesterone. CtBP1 was shown to contribute to the repression observed with LCoR on NR-mediated transcription control through a series of transient transfections in which both LCoR and CtBP1 were overexpressed. Further, ChIP assays showed corecruitment of both proteins on promoter regions of endogenous ERα- and PR-induced target genes. The effects of LCOR on gene expression of estrogen and progesterone target genes were investigated through siRNA-mediated knockdowns. These experiments showed LCoR to be an important cofactor in the regulation of progesterone target genes.
4.1 Abstract

Ligand-dependent corepressor LCoR interacts with the progesterone receptor (PR) and estrogen receptor ERα in the presence of hormone. LCoR contains tandem N-terminal PXDLS motifs that recruit C-terminal binding protein (CtBP) corepressors, as well as a C-terminal helix-turn-helix (HTH) domain. Here, we analyzed the function of these domains in coregulation of PR- and ERα-regulated gene expression. LCoR and CtBP1 colocalize in nuclear bodies that also contain CtBP-interacting protein CtIP, and polycomb group repressor complex marker BMI1. Coexpression of CtBP1 in MCF7 or T47D breast cancer cells augmented corepression by LCoR, while coexpression of CtIP did not, consistent with direct interaction of LCoR with CtBP1, but not CtIP. The N-terminal region containing the PXDLS motifs is necessary and sufficient for CTBP1 recruitment and essential for full corepression. However, LCoR function was also strongly dependent on the HTH domain, as its deletion completely abolished corepression. LCoR, CtBP and CtIP were recruited to endogenous PR- and ERα-stimulated genes in a hormone-dependent manner. Similarly, LCoR was recruited to estrogen-repressed genes, whereas hormone treatment reduced CtBP1 binding. SiRNA-mediated knockdown of LCoR or CtBP1 augmented expression of progesterone- and estrogen-stimulated reporter genes, as well as endogenous progesterone-stimulated target genes. In contrast their ablation had gene-specific effects on ERα-regulated transcription that generally led to reduced gene expression. Taken together, these results show that multiple domains contribute to LCoR function. They also reveal a role for LCoR and CtBP1 as attenuators of progesterone-regulated transcription, but suggest that LCoR and CtBP1 can act to enhance transcription of some genes.
4.2 Introduction

The progesterone receptor (PR) and estrogen receptor α (ERα) are members of the nuclear receptor (NR) superfamily of hormone-regulated transcription factors, whose functions have been implicated in a broad range of physiological responses (29, 179-184). During activation of gene transcription, agonist-bound NRs recruit so-called coactivators such as p160 proteins (133, 138, 179, 185). Coactivators act in part by functioning as histone acetyltransferases (HATs) or by recruiting HAT activity to target promoters. Notably, screens designed to identify coactivators revealed that agonist-bound NRs also recruit proteins with corepressor properties, such as nuclear receptor interacting protein 1 (RIP140/NRIP1) (4, 126, 175, 186) or LCoR (1, 4). We identified LCoR as a protein that interacted with the estrogen-bound ligand binding domain of ERα. Transiently expressed LCoR repressed hormone-dependent transactivation by a range of nuclear receptors. This included particularly efficacious repression of progesterone-stimulated transcription. LCoR transcripts are detectable as early as the 2-cell stage of embryonic development, and the protein is robustly expressed in numerous fetal and adult tissues, including progesterone and estrogen target tissues such as breast, endometrium and placenta (1).

LCoR acts as a scaffold for both histone deacetylases (HDACs) and C-terminal binding protein (CtBP) corepressors, and functions by both HDAC-dependent and –independent mechanisms in a receptor-specific manner. For example, while the HDAC inhibitor trichostatin A (TSA) abolished LCoR-dependent corepression of estrogen-stimulated reporter gene expression, PR-stimulated expression was largely resistant to TSA (1). LCoR contains two adjacent sequences, P/VXDLXXK/R or P/VXDLXXK, near its N-terminus that
correspond to extended PXDLS consensus motifs recognized by CtBPs (126). CtBP1 was identified as a protein that bound the C-terminal region of human adenoviral E1A (187) and is the founding member of a well-conserved family of proteins (188-190). Highly homologous CtBP2 was identified from EST databases (191). Remarkably, CtBPs bind NAD(H), and are related to 2-hydroxy acid dehydrogenases, and CtBP1 has weak dehydrogenase activity. In addition, dinucleotide binding stimulates protein oligomerization activity and corepression.

CtBPs interact directly with several transcriptional coregulatory proteins, many of which share the PXDLS motifs described above (126, 188, 192). For example, a screen for CtBP cofactors identified CtBP interacting protein (CtIP) (193), which also binds BRCA1 and retinoblastoma gene product (Rb) tumor suppressor proteins (194, 195). Like LCoR, CtBPs can function by HDAC-dependent or –independent mechanisms depending on the promoter tested (188). They are components of several multi-subunit assemblies, including polycomb repressor PRC1 complexes (196-198). Targeted ablation of CtBP1 or 2 expression in mice revealed that the two proteins play important and overlapping roles in mouse development (190, 199).

In addition to its N-terminal CtBP-interaction motifs and a central HDAC-binding domain (200), LCoR contains a C-terminal helix-turn-helix (HTH) domain which is homologous to motifs encoded by the Eip93F and MBLK-1 genes of Drosophila, and Honeybee (Apis mellifera) (201), respectively. The LCoR HTH domain also bears 35% homology to pipsqueak motifs (PSQ). PSQ motifs are repeated four times in the DNA binding region of the Drosophila pipsqueak transcription factor, which plays a role in gene silencing (202).
Multiple repeats of the domain are required for PSQ DNA binding (202), and mutation of one of the two HTH motifs in the MBLK-1 gene strongly reduced site-specific DNA binding (201). The PSQ domain is homologous to unique motifs found in a number of prokaryotic and eukaryotic proteins that interact with DNA, such as recombinases (107, 202), raising the possibility that LCoR itself may interact with DNA. Other studies have shown that HTH domains can function in protein-protein interactions, where the HTH motif, combined with other domains, can induce formation of multi-subunit complexes (203). Such proteins with multi-domains can act as scaffolds between the basal transcription machinery and transcription factors (203).

The accompanying paper showed that LCoR recruits histone deacetylase 6 (HDAC6) through a central domain (200). In this study, we have analyzed the roles of domains controlling recruitment of CtBPs and the C-terminal HTH motif in corepression by LCoR. We were primarily interested in determining the roles of these domains in corepression of the PR, as our previous work showed that the efficacious corepression of PR-driven gene reporter gene expression by LCoR appeared to be largely insensitive to HDAC inhibition (1). We find that both the PXDLS motifs and the HTH domain are required for corepression of both the PR and ERα, as disruption of either region markedly attenuated LCoR function. LCoR colocalizes with CtBP1 and CtIP in nuclear foci and CtBP1 is co-recruited with LCoR to PR and ERα target genes in a hormone-dependent manner. Ablation of LCoR and/or CtBP1 enhanced progesterone-stimulated gene expression in T47D breast cancer cells. In contrast, loss of LCoR or CtBP1 had gene-specific effects on ERα-regulated genes and generally led to reduced target gene expression.
4.3 Results

4.3.1 Association of LCoR with CtBP1 and CtIP

Colocalization of LCoR with CtBP1 in MCF7 cells was confirmed by immunocytochemical analyses (Fig. 1A). Both proteins were broadly distributed in the nucleus and were also concentrated in discrete nuclear bodies. A similar colocalization of CtBP2 and LCoR was also observed (data not shown). Given the extensive overlap of CtBPs and LCoR, we determined whether LCoR colocalized with CtIP, which was identified as a CtBP-interacting protein containing an extended PXDLS motif (193). Similar to results obtained with CtBP, CtIP and LCoR showed strongly overlapping patterns of expression concentrated in discrete nuclear bodies (Fig. 1B). In addition, endogenous LCoR coimmunoprecipitates with antibodies directed against either CtBPs or CtIP (Fig. 1C).

Other studies have shown that CtIP interacts directly with the retinoblastoma gene product (193), and have linked CtBP1 and Rb to polycomb group repressor complexes (197, 204). PRC1 complexes form large foci containing numerous factors, including BMI1 polycomb ring finger oncogene (BMI1), visible as discrete nuclear structures (205). Indeed, we found that BMI1 and LCoR coimmunoprecipitated, and colocalized in nuclear bodies (Supplemental Fig. 1). Taken together, these studies show that LCoR extensively colocalizes with CtBP1 and CtIP in the nucleus, including in PRC1 complexes.

The specificity of the interactions of LCoR with CtBP1 were further analyzed in vitro using glutathione-S-transferase (GST) fused to a series of C-terminal deletion mutants of LCoR, or LCoR mutant m1m2, which lacks the tandem PXDLS motifs (Fig. 2A). CtBP1 bound to full length LCoR (Fig. 2B, top) and all C-terminal deletion mutants (Fig. 2B, bottom), but
not to LCoR m1m2 (Fig. 2A). Moreover, tagged LCoR mutated in either one of the two PXDLS motifs coimmunoprecipitated with endogenous CtBPs from extracts of MCF7 cells (m1, a.a. 64-70 deleted; m2, a.a. 82-88 deleted; Fig. 2C, bottom panel). In contrast, no coimmunoprecipitation was observed in cells expressing the m1m2 derivative lacking both sites (Fig. 2C, bottom), although the wild-type and m1m2 proteins were expressed at similar levels (Fig. 2D). Taken together, these results show that the integrity of at least one of the two PXDLS motifs of LCoR is required for binding to CtBP1.

We also analyzed the association of LCoR with CtIP by GST pull-down. The coimmunoprecipitation of CtIP and LCoR is remarkable given that CtIP and LCoR interact with CtBPs through common motifs. However, no evidence was found for LCoR binding directly to CtIP in vitro (Fig. 2E), suggesting that their association in cells is indirect.

**4.3.2 CtBP1 but not CtIP augments LCoR-dependent corepression**

The function of CtBP1 and CtIP as corepressors of progesterone- or estrogen-regulated gene expression in the absence or presence of LCoR was analyzed in MCF7 (Fig. 3) and T47D (Supplemental Fig. 2) breast cancer cells. Neither protein repressed (hormone-dependent) gene expression in MCF7 cells in the absence of LCoR when coexpressed in dose-response experiments with either the PR or ERα (Figs. 3A-D). On the other hand, cotransfection of a limiting amount (200ng) of a CtBP1 expression vector (Figs. 3E and F) significantly augmented LCoR-dependent corepression, whereas coexpression of CtIP had no effect on LCoR function (Figs. 3G and H). This is consistent with the direct interaction of CtBP1, but not CtIP, with LCoR observed above. In agreement with a role of CtBP recruitment in LCoR
function, corepression of progesterone- or estrogen-induced gene expression by the m1m2 mutant of LCoR lacking the CtBP binding motifs was markedly attenuated (Figs. 3I and J). Similar results were obtained in T47D cells (Supplemental Fig. 2).

4.3.3 CtBP1 is co-recruited with LCoR to PR- and ERα-stimulated target genes in the presence of hormone

The binding of LCoR and CtBP1 to hormone-responsive promoters was also analyzed by chromatin immunoprecipitation (ChIP) assay. As we do not have an antibody that immunoprecipitates endogenous LCoR efficiently, LCoR was expressed as a FLAG-tagged protein in these experiments. Treatment of T47D or MCF7 cells with hormone induced PR binding to the progesterone response element (PRE) of the insulin-like growth factor binding protein 1 (IGFBP1) promoter (Figs. 4A-C, left-hand side). Similarly, LCoR, CtBP1 as well as CtIP were recruited to the promoter under the same conditions in both cell lines. Non-target controls showed that the binding of cofactors and nuclear receptors was specific for their corresponding hormone response elements (Figs 4A, B). In re-ChIP experiments, proteins from progesterone-treated T47D cells were immunoprecipitated with an anti-FLAG antibody to concentrate LCoR, and were re-immunoprecipitated with antibodies against either the PR or CtBP1 (Fig. 4C), which confirmed that LCoR is recruited to the same promoters in vivo as the PR and CtBP1. Similar results were obtained when recruitment of ERα, LCoR, CtBP1 and CtIP to the estrogen-responsive trefoil factor 1 (pS2) promoter was analyzed in MCF7 cells (Fig. 4A right-hand side, Figs. 4D and E). Note that essentially identical results were obtained in multiple biological replicates with both cell lines.
The recruitment of CtIP is intriguing, given its indirect association with LCoR and the lack of effect of CtIP on hormone-dependent transactivation, either in the presence or absence of LCoR (Fig. 3). The data raise the possibility that at least a portion of CtIP may be recruited to hormone-responsive promoters through its colocalization with LCoR in PRC1 complexes. Consistent with this hypothesis, we found that PRC1 marker BMI1 was also recruited to the pS2 promoter in the presence of estradiol (Supplemental Fig. 1).

4.3.4 Deletion of the HTH domain of LCoR abolishes corepressor function

Function of the C-terminal HTH motif of LCoR in corepression of progesterone- and estrogen-regulated gene expression was analyzed by deletion of the domain (LCoRΔHTH; Fig. 5A), and comparing the function of the resulting mutant to full length LCoR. Western blotting and ChIP assays showed that the ΔHTH mutant was expressed at similar levels in T47D cells as full-length LCoR, and that it was recruited to the IGFBP1 promoter in the presence of progesterone (Figs. 5B and C). Remarkably, however, the LCoRΔHTH protein was essentially devoid of corepressor activity on either progesterone- or estrogen-responsive promoters in transient expression experiments (Figs. 5D and E). In similar studies (not shown), corepressor activity of the LCoRΔHTH mutant was even more attenuated than that of LCoR derivative m1m2 lacking PXDLS motifs, identifying the HTH region as being critical to LCoR corepressor function.
4.3.5 Function of LCoR and CtBP1 as attenuators of progesterone-regulated gene expression

To further address the function of LCoR and CtBP1 in regulating hormone-dependent gene transcription, we knocked down expression of both proteins using siRNAs in T47D and MCF7 cells. Knockdown of cyclophilin B was used as control for off-target effects of siRNA in these studies (Fig. 6A-C). Loss of either LCoR or CtBP1 augmented progesterone-induced reporter gene expression in T47D cells (Fig. 6D) and estrogen-stimulated reporter expression in MCF7 cells (Fig. 6E), consistent with a role in corepression of hormone-inducible gene transcription.

We extended this analysis to the regulation in T47D cells of progesterone target genes encoding IGFBP1, mucin 1 (MUC1) and FK506 binding protein 5 (FKBP51) (181, 206, 207) (Fig. 7). Knockdown of LCoR markedly enhanced progesterone-stimulated expression of the IGFBP1 gene (Fig. 7A). Unexpectedly, ablation of CtBP1 expression alone or in combination with LCoR slightly attenuated basal and hormone-induced expression of the gene. In contrast, loss of LCoR or CtBP1 individually or in combination substantially augmented progesterone-stimulated expression of the MUC1 and FKBP51 genes (Figs 7B and C). Taken together, the data in Figs. 6 and 7 provide evidence for roles of LCoR and CtBP1 as hormone-recruited attenuators of progesterone-regulated gene transcription. However, they also suggest that CtBP1 function may enhance the expression of some genes, similar to what was observed below in an analysis of estrogen-regulated gene expression.
4.3.6 Ablation of LCoR or CtBP1 diminishes expression of estrogen target genes in a gene-specific manner

The effects of LCoR and/or CtBP1 knockdown on expression of a series of ERα target genes (82) were examined in MCF7 cells. Unlike the general stimulatory effect on progesterone-induced gene expression, knockdown of LCoR elicited distinct and gene-specific effects on estrogen target gene regulation. Notably, loss of CtBP1 but not LCoR attenuated estrogen-induced pS2 transcription (Fig. 8A). This effect of CtBP1 ablation is in agreement with previous studies showing that CtBP1 or CtBP2 knockdown attenuated estrogen-stimulated pS2 expression in MCF7 cells (208), and is consistent with the reduced expression of the IGFBP1 gene seen above in CtBP1-deficient cells. While loss of CtBP1 had no effect on regulation of the GREB1 gene (gene regulated by estrogen in breast cancer; Fig. 8B), its knockdown attenuated both basal and estrogen-regulated expression of the serum/glucocorticoids-regulated kinase 3 (SGK3) and cytochrome P450, family 26, subfamily b, polypeptide 1 (CYP26B1) genes (Figs. 8C and D). Knockdown of LCoR had no effect on basal or estrogen-induced expression of GREB1 or SGK3, whereas estrogen-induced expression of CYP26B1 was eliminated (Fig. 8D), mimicking the effect of CtBP1 ablation. These results are remarkable given the augmented progesterone-stimulated gene transcription seen in LCoR-deficient cells.

Given the unexpected effects of knockdowns on estrogen-induced expression, we also analyzed the potential roles of LCoR and CtBP1 on genes whose transcription is repressed by estrogen (82). Loss of CtBP1, but not LCoR, attenuated basal expression of the bone morphogenetic protein 7 (BMP7) gene (Fig. 8E), whereas loss or either protein attenuated
basal expression of the keratin 4 (KRT4) gene (Fig. 8F). Effects of ablation on estrogen-repressed transcription were variable, with no change in estrogen-repressed BMP7 transcription, and a slight increase in expression of the KRT4 gene. In general, these effects are not consistent with CtBP1 or LCoR functioning as corepressors on these genes.

We further analyzed the molecular basis of these results by performing ChIP assays on the BMP7 promoter, where the ERα binding region has been identified (162). Remarkably, we found that whereas LCoR was strongly recruited to the promoter after 30 min of estrogen treatment, CtBP1 partially dissociated from the promoter, indicating that the two factors were functioning independently (Fig. 8G). ReChIP analysis found evidence for corecruitment of LCoR with ERα but not CtBP1 on the BMP7 promoter after 30 min of estrogen treatment (not shown). These results are consistent with an effect of CtBP1 ablation on basal, but not estrogen-regulated expression of the BMP7 gene.

4.4 Discussion

We have analyzed the roles of LCoR and its cofactor CtBP1 in coregulation of progesterone and estrogen receptor-regulated gene expression. Our previous findings showed that LCoR was a particularly efficacious inhibitor of progesterone-regulated reporter gene expression and that corepression was largely resistant to HDAC inhibitor TSA (1). This study showed that CtBP1 did not repress hormone-regulated gene expression on its own in transient expression studies, but did contribute to corepression by LCoR. GST pull-down experiments and coimmunoprecipitations with a series of LCoR mutants suggested that the N-terminal region of LCoR containing the tandem PXDLS motifs was necessary and
sufficient for interactions of CtBP1 with LCoR. Other experiments showed that deletion of the C-terminal HTH domain severely abrogated corepression, although this domain had no apparent role in CtBP1 recruitment. The HTH domain is distinct from the central region of LCoR identified in the accompanying manuscript as being required for interaction with HDAC6. Together, these findings emphasize that LCoR is a multi-domain protein that exerts its coregulator function through diverse mechanisms. SiRNA-mediated knockdown of LCoR expression established its role in attenuation of progesterone-regulated gene transcription, as hormone-stimulated expression was enhanced on all three PR target genes tested in cells lacking LCoR.

The function of LCoR in corepression of PR-stimulated gene expression may be of considerable physiological importance. Progesterone signaling is essential for normal development and homeostasis of a number of physiological processes including female sexual behavior, ovulation, protection against seizures, maintenance of quiescence of the uterus during pregnancy, induction of germ cell maturation and oocyte maturation (29, 182, 183). Progestins and anti-progestins are used clinically in contraception, hormone replacement therapy, induction of labor, treatment of endometriosis and endometrial cancer (209). Moreover, expression of the PR along with ERα and human epidermal growth factor receptor 2 (HER2) are used as predictive markers for breast cancer therapy (210).

There is considerable overlap in the expression patterns of LCoR and the PR. LCoR is widely expressed throughout development and in the adult (1). In tissue blots, we observed highest expression of LCoR in the placenta, along with robust expression in several fetal tissues. Notably, the placenta is a site of progesterone biosynthesis and in situ hybridization
analysis of near-term placenta (1) revealed that LCoR mRNAs were predominantly expressed in the syncytiotrophoblast layer of terminally differentiated cells, a site of PR expression and signaling (211, 212). The syncytiotrophoblast layer acts as a barrier between maternal circulation and the fetus, and its function is critical for controlling maternal signals that modulate fetal metabolism and development (213).

Compared to the substantial increases observed in progesterone-stimulated gene expression, the effects of LCoR or CtBP1 ablation on estrogen target genes were distinct and gene specific. We observed no effect of LCoR knockdown on expression of three of four estrogen-stimulated genes studied. This may reflect a redundancy in corepressor function on the genes tested. For example, knockdown in MCF7 cells of NRIP1, another corepressor recruited in the presence of hormone, had gene-specific effects on estrogen-dependent transactivation (131). LCoR ablation did augment both basal and hormone-stimulated expression of an estrogen-sensitive reporter gene, pointing to a potential role as an attenuator of ERα signaling. Remarkably, however, its knockdown blocked estrogen-stimulated expression of CYP26B1. This may reflect its function as a cofactor of CtBP1 on the CYP26B1 promoter, as ablation of CtBP1 also abolished estrogen-induced transcription. Notably, we found in the accompanying manuscript that knockdown of HDAC6 had no effect on CYP26B1 regulation. A diminution in estrogen-stimulated gene expression in the absence of CtBP1 was also observed on two of three other estrogen-stimulated genes tested. While these results were unexpected, they are consistent with other findings that knockdown of CtBP1 led to reduced expression of the ATP-binding cassette, sub-family B (MDR/TAP) member 1 (MDR1) gene (214).
We also found that LCoR and CtBP1 are corecruited to the progesterone-stimulated IGFBP1 gene and the ERα target gene pS2. This behavior is in apparent contrast to the results of Stossi et al, who found estrogen-induced dissociation of CtBP1 from the pS2 promoter (208). They found that CtBP1 controlled estrogen-mediated gene repression, and that CtBP1 was recruited to repressed genes in the presence of estradiol. On the other hand, our observations are entirely consistent with other findings (173) in which peak recruitment of CtBP1 to the pS2 promoter was observed after 30min of estradiol treatment.

CtBP1 and LCoR were extensively colocalized in the nucleus, including in pronounced foci. This pattern was similar to the colocalization of LCoR with CtIP and PRC1 marker BMI1, and strongly suggests that a substantial portion of CtBP1-bound LCoR is associated with PRC1 complexes. We found no evidence for direct interaction of LCoR with CtIP (Fig. 2) or BMI1 (unpublished data) by GST pull-down. However, their indirect association is supported by coimmunoprecipitation of LCoR with both proteins and their hormone-dependent recruitment to estrogen or progesterone-regulated genes. Polycomb group proteins and complexes, including PRC1, form nuclear foci visualized as PcG bodies (215). Immunocytochemical studies have estimated these bodies to measure between 0.2 and 1.5µm and to vary greatly in composition, suggesting that they represent foci with numerous independently functioning transcriptional regulatory complexes (196, 216).

Over thirty transcription factors have been shown to interact with CtBP1 (217). Recent studies characterized a CtBP-corepressor complex which contained a great number of proteins with opposing enzymatic activities. This included histone modifying enzymes (217) and other coregulatory proteins that can either activate or repress transcription depending
on the context. Additionally, LCoR was identified as one of the components of a CtBP-corepressor complex purified from nuclear extracts of HeLa cells (218). Taken together, these studies suggest that LCoR is associated with several distinct multi-subunit transcriptional regulatory complexes, hence implying the importance of LCoR in transcriptional control.

4.5 Experimental procedures

Antibodies

A rabbit polyclonal antipeptide antibody was raised against LCoR a.a 20-36 (QDPSQPNSTKNQSLPKA) fused to keyhole limpet hemocyanin, and purified over a peptide affinity column (Bethyl Laboratories, Montgomery TX). Rabbit polyclonal α-CtBP (sc-11390), goat polyclonal α-CtBP1 (sc-5963), goat polyclonal α-CtIP (sc-5970), goat polyclonal α-Bmi1 (sc-8906), rabbit polyclonal α-Bmi1 (sc-10745), mouse monoclonal GAPDH (sc-69778), rabbit polyclonal ERα (sc-543), rabbit polyclonal OCTA-Probe (sc-807), goat anti-mouse IgG (sc-2005), goat anti-rabbit IgG (sc-2004), normal mouse IgG (sc-2025), normal rabbit IgG (sc-2027) and protein A-agarose (sc-2001) and protein G Plus-agarose (sc-2002), were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cy3-donkey polyclonal α-goat (705-165-147) and Cy2-goat polyclonal α-rabbit (711-225-152), Cy3-donkey polyclonal α-rabbit (711-165-152), Cy2-donkey polyclonal α-mouse (715-225-150) were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Mouse monoclonal α-Flag M2 (F3165), and α-FLAG M2 HRP-conjugate (A-8592), monoclonal α-rabbit HRP conjugate (A2074), rabbit
polyclonal α-goat HRP conjugate (A5420) were from Sigma (St. Louis, MO). Rabbit polyclonal CtBP1 (07-306) was purchased from Millipore (Temecula, CA, USA). Rabbit polyclonal LCoR (18-003-44018) was purchased from GenWay Biotech (San Diego, CA, USA). Rabbit polyclonal PR (ab68195) and rabbit polyclonal CtIP (ab70163) were purchased from Abcam (Cambridge, MA, USA).

Recombinant plasmids

PSG5/LCoR, Flag-LCoR/pcDNA3.1 and LCoR derivatives mutagenized in the CtBP binding motifs, PLDLTVR (LCoR a.a. 64-70; m1) and VLDLSTK (LCoR a.a. 82-88; m2) and the double mutant (m1m2) have been described (3). cDNAs mutated in the CtBP binding motifs were subcloned downstream of Flag in pCDNA3.1. Flag-LCoRΔHTH/pcDNA3.1 was made using QuikChange Mutagenesis Kit (Stratagene, cat# 200518, La Jolla, CA, USA) as per manufacturer’s instructions. Primers were designed to delete amino acids 350-395 from LCoR. The new construct was sequenced to confirm proper deletion LCoR.

Cell culture and transfections

All cells were cultured under the recommended conditions. For immunocytochemistry, MCF7 cells grown on collagen IV-treated microscope slides in 6-well plates in DMEM supplemented with 10% FBS. Cells were prepared for immunocytochemistry as described below. For immunoprecipitation of tagged proteins, MCF-7 cells in 100mm dishes were transfected with 10 µl of lipofectamine containing 10 µg of pSG5 vectors containing Flag-LCoR, Flag-m1, Flag-m2 or Flag-m1+2. For analysis of the
effects of CtBP1 and CtIP on LCoR corepression, MCF7 cells (60-70% confluent) grown in DMEM without phenol red, supplemented with 10% FBS on 6-well plates. Cells were transfected in medium without serum (Opti-MEM (Invitrogen, Burlington, Ontario, Canada) with lipofectamine 2000 (Invitrogen, Burlington, Ontario, Canada). The amounts of expression vectors are as follows: 100ng of ERα or hPR expression vectors (as indicated), 100ng of LCoR/pcDNA3.1, 250ng of ERE3-TATA/pXP2 or pGRE5/pXP2 reporter plasmid, 250ng of internal control vector pCMV-βgal. Quantities of expression vectors used are indicated in figures or corresponding figure legends. Medium was replaced 18 hr after transfection with medium containing charcoal-stripped serum and estradiol (10nM) for 30hr. Medium was replaced 18 hr after transfection by a medium containing charcoal-stripped serum and ligand (10nM) for 30hr, as indicated. For luciferase reporter assay, cells were harvested in 250 μl of reporter lysis buffer (Promega).

**Immunocytochemistry and immunoprecipitations**

Cells were cultivated on collagen IV-treated microscope slides in 6-well plates, fixed with 2% paraformaldehyde for 15 min at room temperature, washed (3X) with PBS, and permeabilized with 0.2% Triton X100/5% BSA/10% horse serum in PBS. MCF-7 cells were then incubated with α-LCoR (1:500), and goat polyclonal antibodies against CtBP1, CtIP, or Bmi1 (1:50) in buffer B (0.2% Triton X100/5% BSA in PBS), for 1h at room temperature. Cells were washed (3x) with PBS, and incubated with goat anti-rabbit-Cy2 and donkey anti-goat Cy3 (1:300) in buffer B for 1h at room temperature. Slides were mounted with Immuno-
Fluore Mounting Medium (ICN, Aurora, Ohio) and visualized using a Zeiss LSM 510 confocal microscope.

For immunoprecipitation of endogenous CtBP, CtIP, or Bmi1, MCF-7 cells in 150 mm dishes were lysed 3 min at 4°C in 1 ml of LB (150 mM NaCl/10 mM Tris-HCl pH 7.4/0.2 mM Na orthovanadate/1 mM EDTA/1mM EGTA/1% Triton-100X/0.5% IGEPAL CA-630/protease inhibitor cocktail; Boehringer-Mannheim, Laval, Qc). Cell debris were pelleted by centrifugation (14,000 rpm, 5min), and proteins immunoprecipitated with 4 µg of αCtBP or αCtIP or polyclonal rabbit αBMI1 or control rabbit or goat IgG at 4°C overnight followed by 2 hours incubation at 4°C with protein A agarose (for αCtBP, αBmi1, control rabbit IgG) or protein A+G agarose (for αCtIP or control goat IgG). Beads were washed (3x) with LB. Bound immunocomplexes were boiled in Laemmli buffer, separated by 10 % SDS/PAGE, and blotted on PVDF membrane with α-LCoR (1/1000), α-CtBP1, α-CtBP2, α-CtIP, or α-BMI1 (1:100), and detected by enhanced chemiluminescence (NEN Life Science Products, Boston, MA). For immunoprecipitation of tagged proteins, transfected MCF-7 cells were lysed 30min at 4°C in 1ml of LB, 48h after transfection. Supernatants were cleared, incubated overnight with 4 µg of αCtBP or α-Flag M2 antibody followed by 2 hours incubation with protein-A agarose or protein A+G agarose beads respectively. Beads were washed (3x) with LB and Western blotted as above. Dilutions of specific antibodies used for Western blotting were: α-CtBP1, (1:100), α- Flag M2-peroxidase (1:100).
**Western blotting**

Western blot was performed as previously described (177) using MCF-7 cell extracts. Cells were grown in 10 cm dishes (70% confluent) and transiently transfected with 500ng of Flag-tagged full length of mutated LCoR. Cells were harvested 30h later.

**Chromatin immunoprecipitation (ChIP) Assays and reChIP assays**

ChIP and reChIP assays were performed as previously described (178) in MCF7 and T47D cells. Cells were grown in 10 cm dishes (70% confluent) and were transiently transfected with 500ng of Flag-tagged LCoR (or Flag-tagged LCoRΔHTH as indicated). Following the transfection, cells were starved for two days in DMEM-phenol free and FBS free media and treated with 2.5µM α-amanitin (Sigma, A2263) for 2h prior to hormone treatment in order to properly synchronize cells. Cells were collected and cofactor recruitment was evaluated on promoter regions containing either ERE or PRE (as indicated) of target genes. For ChIP primer sequences, please refer to Table A1 of supplemental data.

**SiRNA Knockdowns**

SiRNAs were purchased from Thermo Scientific Dharmacon (Lafayette, CO, USA). The following ON-TARGETplus SMART pool siRNA were used: LCoR (L-026303-00), CtBP1 (L-008609-00), CyPB (D-001820-10) and non-targeting (D-001818-10). siRNAs were resuspended per manufacturer’s instructions. Transfections were done in 6-well plates as described previously. Lipofectamine 2000 (10 ul) was used as the transfection reagent.
DMEM phenol-free with 10% stripped FBS was added 12h after transfection. For Western blot analysis, cells were collected 48h after transfection. Luciferase reporter assays after siRNA knockdowns were performed as follows: 100ng of ERα expression vector and 250ng of ERE3-TATA-pXP2 vector were transfected with the corresponding siRNA. DMEM phenol-free with 10% stripped FBS was added 12h after transfection. Ligand was added 36h after transfection and cells were collected 24h later. Luciferase activity was measured as previously described.

**RNA isolation, cDNA synthesis and quantitative real-time polymerase chain reaction (qRT-PCR)**

Cells were grown in 100-mm dishes. Media was replaced with charcoal-stripped medium containing ligand. Total RNA was extracted with TRIZOL reagent. cDNA synthesis was performed with iScript cDNA Synthesis Kit (Bio-Rad, Hercules, Ca, USA) according to the instructions of the manufacturer. MiniOpticon Real-Time PCR System with iQ SYBR Green Supermix (Bio-Rad) were used for qRT-PCR expression analysis of target genes. Program used is as follows: 1- incubate at 94°C for 60sec, 2- incubate at 95°C for 20sec, 3- incubate at 60°C for 30sec (decrease temperature by one degree per cycle), 4- incubate at 72°C for 30sec, 5- plate read, 6- repeat from step 2 five more times, 7- incubate at 95°C for 20sec, 8- incubate at 57.5°C for 30sec, 9- incubate at 72°C for 30sec, 10- plate read, 11- repeat from step 7 thirty-five more times, 12- perform melting curve and end. Results were normalized to β-actin mRNA expression. For qRT-PCR primer sequences, please refer to Table A2 of supplemental data.
4.6 Acknowledgments

We are grateful to Jacynthe Laliberté for technical assistance with confocal microscopy. A special thank you to Dr. Myles Brown (Harvard Medical School, USA) for providing locations of EREs and ER-regulated enhancers. This work was supported by grant MT-11704 from the Canadian Institutes of Health Research (CIHR) to J.H.W. A.P. was supported by studentships from the CIHR and the Montreal Centre for Experimental Therapeutics in Cancer (MCETC). I.F. was supported by postdoctoral fellowships from l’Association pour la Recherche sur le Cancer (l’ARC) and the CIHR. L.E.T.-M. was supported by Fonds de Recherche en Santé du Québec (FRSQ). J.H.W. and S.M. are holders of FRSQ Chercheur-Boursier National scholarships.

4.7 Figures

Figure 4.1- Association of endogenous LCoR with endogenous CtBP1 and CtIP

A and B. Confocal microscopic analysis of the subcellular colocalization of LCoR with CtBP1 (A) and CtIP (B) by immunocytochemistry (see Experimental Procedures for details). C. Analysis of the association of LCoR with CtBP1 and CtIP by coimmunoprecipitation (Co-IP). Extracts of MCF7 cells were immunoprecipitated (IP) with antibodies against CtBP1 or CtIP as indicated, and probed by western blotting for enrichment of target proteins in immunoprecipitates. Immunoprecipitates were also probed for the CoIP of LCoR.
Figure 4.1
Figure 4.2- Direct association of LCoR with CtBP1 but not CtIP.

A. A schematic representation of the primary structure of LCoR, along with LCoR mutated in the PXDLS motifs that bind CtBPs (m1m2) and C-terminal deletion mutants of LCoR are shown. B. GST pull-down analysis of the interaction of CtBP1 present in MCF7 cell extracts (MCF7 extr.) with GST fusions of wild-type LCoR or mutants described in A. CtBP1 bound to fusion proteins was detected by western (W) blotting. C. Mutation of both CtBP binding sites of LCoR disrupts its interaction with CtBPs in MCF7 cell extracts. MCF7 cells were transfected with Flag-tagged wild-type LCoR or tagged LCoR mutated in one (m1 or m2) or both (m1m2) CtBP binding sites, as indicated. Top panel: extracts and immunoprecipitations with anti-Flag antibody of transfected MCF7 cells showing that tagged proteins are expressed at similar levels in all cases. Middle panel: control immunoprecipitation with anti-CtBP1 antibody and western blot showing that CtBP1 is expressed at similar levels in all cases. Bottom panel: Coimmunoprecipitation of tagged LCoR derivatives from extracts of transfected MCF7 cells. D. Western blot of MCF7 extracts expressing Flag-LCoR or Flag-m1m2 blotted for Flag (1st row) or GAPDH (2nd row), used as loading control. E. GST-pulldowns showing no direct binding between LCoR and CtIP. Binding of CtBP1 to GST-LCoR fusions was used as positive control.
**Figure 4.3- Roles of CtBP1 and CtIP in LCoR-dependent corepression in MCF7 cells.**

Cells were transiently transfected with expression vectors for either PR (100ng) or ERα (100ng) and their corresponding reporter plasmids (250ng) for 18h. Media was then changed and cells were treated with vehicle or hormone for 30h. A and B. Dose-response curves analyzing the effects of CtBP1 on reporter gene expression in cells treated with P4 (10nM; A) or E2 (10nM; B). Increasing amounts of CtBP1 were transfected (200, 400 and 600ng). C and D. Dose-response curves analyzing the effects of CtIP on reporter gene expression in cells treated with P4 (C) or E2 (D). Increasing amounts of CtIP1 were transfected (200, 400 and 600ng). E and F. Analysis of the effects of coexpression of LCoR and CtBP1 on hormone-dependent gene expression. Cells were transiently transfected with either vector alone, LCoR alone (100ng), CtBP1 alone (200ng) or with both LCoR and CtBP1, and treated with P4 (E) or E2 (F). *, P < 0.05 for results of LCoR and CtBP1 coexpression versus LCoR expression alone. G and H. Analysis of the effects of coexpression of LCoR and CtIP on hormone-dependent gene expression. Cells were transiently transfected with either vector alone, LCoR alone, CtIP1 alone or with both LCoR and CtIP1 and treated with P4 (G) or E2 (H). I and J. Dose response curves of either LCoR or m1m2 in cells treated with P4 (I) or E2 (J). Increasing amounts of wild-type or mutant LCoR were transfected (200, 400 and 600ng). *, P < 0.05 for results of corresponding wild-type LCoR versus mutant form m1m2.
Figure 4.3
MCF7 and T47D cells were transiently transfected with Flag-tagged LCoR, synchronized for 2h with α-amanitin (2.5µM) and treated with P4 (10nM) for 45min or E2 (10nM) for 30min, as indicated. Cell extracts were collected and immunoprecipitated with IgG, or antibodies against PR, ERα, Flag, CtBP1 or CtIP, as indicated. A. Schematic representation of the progesterone-sensitive IGFBP1 promoter (left hand side) and estrogen-stimulated pS2 promoter (right hand side). The HRE, transcription start site, and both PCR-amplified sequences (target and non-target control regions) are depicted. B. ChIP assay of factor binding to the IGFBP1 promoter in MCF7 cells (left hand side) and T47D cells (right hand side). Results of semi-quantitative and qPCR analyses are presented. Note that no signal was detected by qPCR in the non-target controls. C. ReChIP assay in extracts of T47D cells treated with P4, and immunoprecipitated with Flag. A second round of immunoprecipitations with IgG, PR or CtBP1 was performed, as indicated. D. ChIP assays of factor binding to the pS2 promoter in MCF7 cells treated with E2. E. ReChIP assay in extracts of MCF7 cells treated with E2 and immunoprecipitated with Flag. A second round of immunoprecipitations with IgG, ERα or CtBP1 was performed, as indicated.
Figure 4.4
Figure 4.5- SiRNA knockdown of LCoR and CtBP1 expression in MCF7 and T47D cells.

A. Schematic representation of full-length LCoR (upper panel) and a deletion mutant lacking the HTH domain (lower panel; LCoRΔHTH) in which a.a. 350-395 were deleted, leaving the C-terminal portion of the protein (395-433 a.a.) intact. B. LCoR and LCoRΔHTH are expressed equally in T47D cells. Western blot of T47D cell extracts expressing Flag-LCoR or Flag-LCoRΔHTH blotted for Flag (1st row) or loading control GAPDH (2nd row). C. LCoRΔHTH is recruited to the progesterone target gene encoding IGFBP1. ChIP assays in T47D cells treated with P4 (10nM) for 45min and immunoprecipitated with Flag. Upper panel shows schematic view of IGFBP1 promoter. D and E. Deletion of HTH domain of LCoR abolishes corepressor function. Dose response curves were performed analyzing corepression in the presence of increasing amounts of LCoR or LCoRΔHTH expression vectors (0, 100, 400 and 600ng, as indicated). T47D cells were treated with P4 (10nM; panel D) and MCF7 cells were treated with E2 (10nM; panel E). Cells were transiently transfected with expression vectors of either PR (100ng) or ERα (100ng) and their corresponding reporter plasmid (250ng) for 18h. Media was then changed and cells were treated for 30h.
Figure 4.5
Figure 4.6 - SiRNA knockdown of LCoR and CtBP1 expression in MCF7 and T47D cells.

A, B and C. Western blot of MCF7 and T47D extracts. Cells were transfected for 48h with pools of scrambled siRNAs (Scr.) or siRNAs targeting LCoR (A) or CtBP1 (B), as well as siRNAs targeting CyPB (C) to control for off-target effects. GAPDH expression was used as a control. Densities of bands on Western blots, scanned with Bio-Rad Gel Doc XR System and analyzed with Quantity One, are presented below images of blots. D and E. Luciferase reporter assays in siRNA-transfected cells. A PR expression vector was transfected along with scrambled, LCoR, CtBP1 or CyPB siRNAs (D), or an ERα expression vector was transfected along with scrambled, LCoR, CtBP1 or CyPB siRNAs (E). After 24h of treatment with P4 (10nM; D) or E2 (10nM; E), cells were harvested and luciferase activity was measured. Data is shown as relative luciferase units (RLU). Data are averages three or more independent experiments, and error bars represent the standard error of the mean; *, P < 0.05 for results of specific knockdown (LCoR, CtBP1 or CyPB) versus results with scrambled siRNA.
Figure 4.6
Figure 4.7 - Effects of LCoR and CtBP1 ablation in T47D cells on regulation of endogenous PR target genes.

Cells were transfected with corresponding siRNAs (scrambled, CyPB, LCoR, or CtBP1, or LCoR and CtBP1) for 36h, then treated with vehicle (EtOH) or P4 (10nM) for 24h. QRT-PCR was performed to analyze regulated expression of IGFBP1 (A), MUC1 (B), or FKBP51 (C), and β-actin expression was used as an internal control. Results are shown as fold induction. Data are averages of three or more independent experiments. Error bars represent the standard error of the mean; *, P < 0.05 for results of specific knockdown (LCoR, CtBP1 or LCoR and CtBP1) versus scrambled results with scrambled siRNA.
Figure 4.7
Figure 4.8- Effects of LCoR and/or CtBP1 ablation in MCF7 cells on regulation of endogenous estrogen target genes.

Cells were transfected with corresponding siRNAs (scrambled, CyPB, LCoR, or CtBP1, or both LCoR and CtBP1) for 36h, then treated with vehicle (DMSO) or E2 (10nM) for 24h. QRT-PCR was performed to analyze regulated expression of pS2 (A), GREB1 (B), SGK3 (C), CYP26B1 (D), BMP7 (E), or KRT4 (F), and β-actin was used as an internal control. Results are shown as fold induction. Data are averages three or more independent experiments. Error bars represent the standard error of the mean; *, P < 0.05 for results of specific knockdown (LCoR, CtBP1 or both LCoR and CtBP1) versus scrambled results. G. ChIP assays analyzing factor binding to the BMP7 promoter (upper panel) in MCF7 cells treated with E2 (10nM). Cell extracts were collected and immunoprecipitated with IgG, or antibodies against ERα, Flag or CtBP1, as indicated. Results of semi-quantitative and qPCR analyses are presented. Note that no signal was detected by qPCR in the non-target controls.
Figure 4.8
Chapter 5

Domain analysis of LCoR
Preface

In chapters 3 and 4, LCoR domains interacting with cofactors HDAC6 and CtBP, as well as the HTH, motif were characterized and their contribution to transcriptional corepression observed with LCoR in NR-mediated transcription was analyzed. The current chapter is a continuation of this work where further analyses of these domains were performed. Furthermore, preliminary data investigating the function of LCoR in histone code modifications is presented. Our results underline the importance of in depth analysis of domains of coregulatory proteins that will permit better understanding of their multifaceted effects on NR-mediated transcriptional control.
5.1 Abstract

Ligand dependent corepressor LCoR interacts directly with nuclear receptors (NRs) and was shown through siRNA-mediated knockdowns to regulate endogenous estrogen and progesterone target genes in a promoter-specific manner. LCoR contains many well defined protein-protein interaction domains, notably its HDAC6 interaction domain, two extended PXDLS motifs that bind CtBPs and a C-terminal helix-turn-helix (HTH) motif, all of which were shown to contribute to its corepressor function. Here, further analyses of these domains were conducted by site-directed mutagenesis. The central region of LCoR was shown to contain multiple histone deacetylase (HDAC) binding domains. The deletion of this central region resulted in significant reduction of transcriptional corepression of ERα-regulated transcription and transcriptional activation in the case of PR-regulated gene expression. Both PXDLS motifs and HTH motif contribute individually to the corepressor function of LCoR. However, the combined deletion of both motifs resulted unexpectedly in corepression equivalent to that of full length LCoR, indicating the possibility of a loss of interaction between LCoR and a protein complex containing transcriptional activation properties. Further analysis showed the importance of adjacent a.a. residues of the PXDLS sites in LCoR transcriptional corepression. With the emergence of multiple histone modification complexes containing LCoR interacting proteins, the participation of LCoR in histone code modification was investigated. Chromatin immunoprecipitation (ChIP) assays showed co-recruitment of LCoR and members of the CtBP-corepressor complex to promoter regions of endogenous ERα-stimulated genes in a hormone-dependent manner. Taken together, these results indicate a dual function of LCoR as a ligand-dependent modulator of
NR-dependent transcriptional control: a direct function where LCoR acts as a cofactor scaffold directly recruited to NR, or indirect as a member of histone-modifier complexes.

5.2 Introduction

Ligand-dependent corepressor LCoR was identified through a yeast two-hybrid screen in which the ligand binding domain (LBD) of estrogen receptor alpha (ERα) was used as bait (1). Overexpression studies showed LCoR as a corepressor of NR-mediated transcription activation in the presence of ligand (1). LCoR contains many conserved motifs and protein-interacting domains. We have recently defined a domain in LCoR between a.a. 203 and 319 that interacts directly with HDAC6 (200). This interaction is unexpected as LCoR is a nuclear protein and HDAC6 is cytoplasmic in a great number of cell types. Ablation of the HDAC6 interaction domain in LCoR significantly reduced transcriptional corepression of ERα-induced transcription. To further study the mechanistic nature of LCoR and HDAC6 interaction, kinetic chromatin immunoprecipitation (ChIP) assays were performed on regulatory regions of ERα target genes, revealing a ligand-dependent corecruitment of factors in a partially synchronized fashion (200). However, siRNA-mediated ablation of HDAC6 and/or LCoR resulted in little or no effect on ERα-induced gene transcription on the genes tested. This observation gives rise to different mechanistic models for the action of LCoR-HDAC6 complex on NR-mediated transcription control: (a) the regulatory functions of LCoR-HDAC6 complex are redundant with other corepressor complexes, (b) the regulatory functions of LCoR-HDAC6 complex are promoter-specific, or (c) that LCoR is a component of
multiple protein complexes containing other cofactors in addition to HDAC6 resulting in context-specific regulation of NR-mediated transcription control.

Our investigation of C-terminal binding protein (CtBP) domains, also known as PXDLS motifs, revealed a more straightforward mechanistic function (176). The tandem PXDLS motifs lie at the N-terminus of the protein (LCoR a.a 64-70; m1 and a.a. 82-88; m2) with which C-terminal binding protein 1 (CtBP1) and C-terminal binding protein 2 (CtBP2) interact (1, 176). CtBP1 was shown to contribute to transcriptional corepression by LCoR of both ERα- and PR-regulated reporter gene expression assays. The function of LCoR and CtBP1 as attenuators of endogenous progesterone-regulated gene expression was determined with siRNA-mediated knockdowns. Ablation of LCoR and/or CtBP1 significantly reduced transcriptional corepression of endogenous progesterone target gene expression (176). However, similar to siRNA-mediated knockdowns of HDAC6, CtBP1 knockdowns had gene-specific effects on ERα-regulated transcription.

At its C-terminal end, LCoR has a pipsqueak-like domain, also known as helix-turn-helix (HTH) motif which is homologous to a domain identified in the DNA binding region of the Drosophila pipsqueak transcription factor (202). The HTH domain is well conserved among transcription factors and is known for DNA-protein and protein-protein interactions (203). The ablation of the HTH motif in LCoR abolished corepression of both ERα- and PR-mediated transcriptional transactivation, indicating a fundamental role for this motif in the corepressor function of LCoR.
The gene-specific effects of LCoR and its cofactors on NR-mediated gene transcription motivated a detailed investigation of the function of LCoR domains in NR-mediated transcription regulation. A series of truncated forms of LCoR were made and their effects on ERα-induced transcription were studied. Greater emphasis was brought on the PXDLS motifs in our investigation due to recent studies identifying LCoR as a member of the CtBP-corepressor complex (218, 219). The latter comprises protein members responsible for both gene targeting and histone modifications that permits proper repression of gene expression observed with CtBPs. To further our understanding of the function of LCoR in this corepressor complex, kinetic ChIP assays of LCoR and other complex members were performed on a selection of promoter regions of endogenous estrogen target genes. In whole, the results of these experiments offer preliminary data essential for a more in-depth study of LCoR-CtBP interaction and the function of LCoR as a member of a histone modification complex.

5.3 Results and Discussion

5.3.1 Analysis of LCoR domains and their effects on NR-mediated transcriptional control

LCoR contains several conserved motifs and protein-interacting domains. At its N-terminus are found an NR-box, a motif through which coregulators interact with the AF-2 domain of agonist-bound NRs, and two tandem PXDLS repeats through which LCoR interacts with CtBPs. LCoR interacts with both class I and class II HDACs, including HDACs 3 and 6,
with its central region. Finally, the C-terminus region of LCoR contains a HTH motif. All of these motifs were shown to contribute to the transcriptional corepression observed with LCoR.

In order to further investigate the role of these domains in the transcriptional regulation by the corepressor of LCoR, additional LCoR deletion mutants were made (see Figure 5.1A). The central domain of LCoR is responsible for HDAC interactions. The importance of HDAC interaction with LCoR has been previously shown where treatment of MCF7 breast cancer cells with trichostatin A (TSA) resulted in reduced transcriptional corepression (1, 200). Interestingly, this effect is NR specific (1). In the case of ERα-induced transcription, LCoR corepression activity was lost when cells were treated with TSA. However, in the case of PR-induced transcription, the treatment of MCF7 cells with TSA did not affect dose-dependent corepression, indicating a specific role for LCoR-HDAC interactions in estrogen-regulated gene expression. This was further demonstrated with the mutant LCoRΔHDACs where the whole central region responsible for direct interaction between LCoR and HDAC was deleted (see figures 5.1 B and C). In the case of PR-induced transcription, overexpression of LCoRΔHDACs modestly activated transcription (see figure 5.1C). In contrast, LCoRΔHDACs corepressed ERα-induced transcription but at a significantly reduced level when compared to corepression by full-length LCoR (see Figure 5.1B).

The maintenance of corepressor activity observed with mutant LCoRΔHDACs in ERα-mediated transactivation, in combination with our previous HDAC-dependent corepression of ERα-induced transcription data, indicates that LCoR maintains some HDAC-recruitment
activity independent of the central HDAC interaction domain. Notably, CtBP corepressors are known to interact with HDAC proteins (218, 220). In fact, depending on the context of the promoter, the corepressor activity of CtBPs can be either HDAC-dependent or – independent (194, 220-222). Hence, corepressors can recruit HDAC activity by either interacting directly or indirectly with HDACs. Oncogene Evi-1 is an example of a CtBP-interacting protein with HDAC-dependent activity (220, 223). When interacting with CtBPs, Evi-1 represses transforming growth factor-β-responsive genes. However, in the presence of TSA, gene repression is relieved (220, 223). Therefore, the HDAC-dependent activity of LCoR is due to both direct interaction (i.e. HDAC interaction with the central HDAC binding domain of LCoR) and indirect interaction (i.e. HDAC interaction with CtBPs bound to N-terminus PXDLS motifs of LCoR).

The mutants m1m2 (LCoR a.a 64-70 and a.a 82-88 deleted) and LCoRΔHTH (LCoR a.a 319-395 deleted) were previously described (1, 176). Since both CtBP-interacting domains and the HTH motif contributed to transcriptional repression, we wanted to see if the ablation of all three regions (defined as mutant m1m2ΔHTH in Figure 5.1) would result in transcriptional activaton. To our surprise, m1m2ΔHTH retained transcriptional corepressor activity equivalent to that of full-length LCoR (see Figure 5.1B and C). These unexpected results suggest a functional interaction between the CtBP-binding region and the HTH motif of LCoR. The CtBP-binding region may function in transcriptional repression in the presence of the HTH but in transactivation in its absence, as seen with the LCoRΔHTH mutant. In fact, there are several studies reporting CtBPs functioning as transcriptional activators in a gene-specific manner (189, 224, 225). In Drosophila, a selection of Wnt target genes was
repressed in the absence of Wnt stimulation (225). The gene-specific transcriptional activity of CtBPs (i.e. coactivator or corepressor) may depend on its association with factors that activate transcription and on post-translational modifications of the gene regulatory region and its associated factors (189). All of this suggests the importance of understanding the function of the HTH motif and further functional analysis of these mutants is necessary for our understanding of the transcriptional corepressor function of LCoR.

5.3.2 Effect of a lysine residue adjacent to a PXDLS motif on LCoR corepressor activity

Recent studies on CtBP binding properties have shown the importance of adjacent sequences to the highly conserved PXDLS motif (226). Substitution experiments of either N- or C-terminal residues of PXDLS motif in adenovirus 12 E1A greatly affected the binding affinity of CtBP (226). In addition, a specific lysine found 2 a.a. from the C-terminus end of the PXDLS motif is an acetylation site. The lysine is acetylated by histone acetyltransferase (HAT) members (p300, P/CAF and GEN5) and in some cases was shown to affect CtBP binding affinity (226, 227). There is discussion in the field as to whether acetylation of this lysine residue reduces CtBP binding or whether it modifies the corepressor function of the protein interacting with CtBPs (227, 228). Therefore, post-translational modifications of proteins interacting with CtBPs may alter their affinity for CtBPs, hence affecting their transcriptional corepressor function.

Each of the CtBP binding sites in LCoR has a lysine residue at the C-terminus end of the motif (see Table 5.1). In order to study their effect on LCoR corepression of NR-
mediated transcriptional control, point mutations of the lysine residue at the m1 site (the N-terminal motif of the two tandem PXDLS repeats) were made. A glutamine residue is neutral and of similar size and will imitate the effect of acetylation of wild type LCoR. On the other hand, an arginine residue is strongly positive and its guanidium group is not a substrate for acetylation. With an arginine substitution we would expect an increase binding of CtBP to LCoR which would result in increased transcriptional corepression.

A series of deletion mutants were made and their effect on ERα-induced transcription in MCF7 cells was investigated (figure 5.2). Wild-type LCoR, as expected, represses transactivation in a dose-response manner. The mutant lacking both CtBP-binding sites, m1m2, also represses transactivation, but in a significantly reduced manner. Similarly, the mutations of individual PXDLS motifs, m1 for the N-terminal motif and m2 for the C-terminal motif functioned as attenuated corepressors. The glutamine substitution mutant (Q1), as expected, has similar transcriptional corepression capabilities to wild type LCoR. However, the arginine mutant (R1) did not exhibit similar dose-responsive corepression. Contrary to our expectations, its corepression activity was significantly reduced. The inconsistency of these results with published data may be due to many factors (226). First, the study of lysine residue acetylation by Molloy et al was performed on synthetic peptides which by themselves may not represent the effect of acetylation on CtBP-binding by an intact protein (226). Second, the reported effect of lysine acetylation on CtBP-binding affinity is very subtle, with 1.4 to 1.9 increased CtBP-binding in the absence of acetylation, as shown by Molloy et al, while a different group showed no effect of lysine acetylation on CtBP binding (226, 228).
In the case of LCoR, the substitution of the lysine residue by arginine had a pronounced effect. Whether this is due to changes of acetylation status or a steric effect of the insertion of a larger amino acid remains to be determined. By looking at the reporter assay in Figure 5.2, mutant Q1 shares the same level of transactivation as m1m2, hinting at a global loss of CtBP-binding. Given that both CtBP-binding sites are relatively close to each other, the arginine substitution might alter the structure resulting in diminished CtBP binding. Taken together, these experiments show the importance of CtBP interaction. Further studies are necessary to determine the post-translational modifications of LCoR as these might have important effects on its corepressor activity.

5.3.3 LCoR as a member of the CtBP corepressor complex

Recent studies are showing the importance of post-translational modifications in transcription control and how these modifications can result to context-specific activity of transcription cofactors (189, 229, 230). These modifications can alter either histone tails or cofactors. Over the years, new advances in molecular biology techniques have permitted a clearer view of the mechanistic nature of cofactor action during transcriptional activation and repression (229). It is now known that coregulators function as components of multi-protein complexes (101, 132, 229). Moreover, the composition of these complexes is apparently fluid. More precisely, the composition of coregulators complexes is very dynamic and each coregulator can be a member of many different protein complexes, hence explaining the multi-faceted transcriptional action of a single cofactor (132, 189, 229).
Our previous studies have shown the action of LCoR on transcriptional control of endogenous gene expression to be gene-specific, as is the case with many coregulators (17, 136, 231). This would be consistent with LCoR being a component of a multi-subunit protein complex. In fact, a recent study identified LCoR as a member of the CtBP corepressor complex (218). Interestingly, the same complex contains a histone methyltransferase, G9a, and a histone demethyltransferase, LSD1, which are both known for their gene repression function (189). The complex also contains several HDACs (218).

To investigate whether LCoR is part of this complex during ERα-induced transcription, a series of kinetic ChIP assays on endogenous promoters of estrogen target genes were performed (see Figure 5.3). The corecruitment of ERα, LCoR, CtBP1, LSD1, CoREST, G9a was studied. CoREST, a protein initially identified as a corepressor to the REST transcription factor, forms a complex with HDAC1/2 and binds LSD1 (232). When LSD1 is bound to CoREST, the former has the ability to demethylate H3K9 (232-234). The CoREST-LSD1 target genes are hypoacetylated, suggesting a role in transcription repression (235). Since LSD1 and G9a methylate and demethylate histone 3 lysine 9 (H3K9) and histone 3 lysine 4 (H3K4), respectively, the methylation status of these two methylation sites were investigated as well.

ChIP assays were performed on promoter regions of pS2, IGFBP4, ADORA1 and NRIP1. All four genes show variability in the temporal recruitment of cofactors. For all four promoters, ERα, Flag-LCoR and CtBP1 show strong corecruitment after 30min of estradiol treatment, as shown previously. At two promoters G9a is absent at this time point: pS2 and
IGFBP4 (Figure 5.3 A and B). For both of these promoters, the overall level of H3K4me2 is quite strong when compared to H3K4me1, indicating a lack of need for G9a methylase activity. However, promoters ADORA1 and NRIP1 (Figure 5.3 C and D) show strong recruitment of G9a in the 15 to 30min interval of estradiol treatment. This results in lower levels of H3K4me1 and higher levels of H3K4me2 after 60min of estradiol treatment, indicating the importance of H3K4me2 in the later phase of ERα cycling. For pS2, IGFBP4 and ADORA1 promoters (Figure 5.3 A-C), the level of H3K9me2 is low, suggesting that this post-translational modification signal is not important for ERα-mediated transcription control of these genes. The lack of synchronized recruitment of LSD1 and coREST at these promoters further supports this observation.

Overall, these results support the highly dynamic temporal and spatial cofactor corecruitment necessary for proper gene expression. More importantly, the differences in histone methylation highlight the variability in epigenetic modifications occurring on different promoters. The lack of synchronized recruitment of cofactors may be an indication of the participation of multiple protein complexes which share members (229). Therefore, the lack of synchronized recruitment may be a technical artifact arising from different pools of cofactor present at the same place and at the same time.

5.4 Conclusions

LCoR is a modulator of NR-mediated gene expression that requires interaction of other cofactors for its transcriptional corepressor function. We have shown through use of
deletion mutants crosstalk between the CtBP-binding domains and HTH motif, indicative of the complexity of gene expression regulation by LCoR. Point mutation experiments of a hypothetical acetylation site, a lysine residue in proximity to CtBP-binding sites substituted with an arginine residue, also significantly affected LCoR coregulation. The strong effect of this point mutation suggests post-translational modifications of LCoR, in addition to interaction with cofactors, by which its transcriptional corepressor activity is modulated. In fact, many recent coregulator studies have shown exactly this; i.e. depending on the post-translational modification state, a coregulator can be either a transcriptional corepressor or a coactivator. Not only do these modifications alter the coregulator itself, but they can as well alter the composition of the protein complex with which it interacts. This can be seen as a multi-layered control system where each element individually, or in combination, may affect the final transcriptional output: repression or activation. Therefore, the next phase in the field of coregulators is identifying the post-translational modifications of coregulators and how each of these may, or may not, affect their transcriptional activity.

5.5 Experimental procedures

Antibodies

A rabbit polyclonal ERα (sc-543), rabbit polyclonal OCTA-Probe (sc-807), normal mouse IgG (sc-2025), normal rabbit IgG (sc-2027) and protein A-agarose (sc-2001) and protein G Plus-agarose (sc-2002), were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal CtBP1 (07-306), mouse monoclonal LSD1 (05-939), rabbit polyclonal CoREST (07-
rabbit polyclonal H3K4me1 (07-436), rabbit polyclonal H3K4me2 (07-030) and rabbit polyclonal H3K9me2 (07-441) were purchased from Millipore (Temecula, CA, USA). Rabbit polyclonal G9a (G6919) was purchased from Sigma (St-louis, MO, USA).

**Recombinant plasmids**

Flag-LCoR/pcDNA3.1 and LCoR derivatives mutagenized in the CtBP binding motifs, PLDLTVR (LCoR a.a. 64-70; m1) and VLDLSTK (LCoR a.a. 82-88; m2), the double mutant (m1m2), and LCoR lacking the HTH motif (LCoR a.a. 350-395; LCoRΔHTH) have been previously described (1, 176). LCoR lacking the central HDAC binding domain (LCoR a.a. 100-319; LCoRΔHDACs), and single site mutants Q1, R1) are described in Table 1. All mutants were obtained using QuikChange Mutagenesis Kit (Stratagene, cat# 200518, La Jolla, CA, USA) as per manufacturer’s instructions. The new constructs were sequenced to confirm proper sequence deletions in LCoR.

**Cell culture and transfections**

All cells were cultured under the recommended conditions. For analysis of the effects of LCoR variants, MCF7 cells (60-70% confluent) were grown in DMEM without phenol red, supplemented with 10% FBS on 6-well plates. Cells were transfected in medium without serum (Opti-MEM (Invitrogen, Burlington, Ontario, Canada) with lipofectamine 2000 (Invitrogen, Burlington, Ontario, Canada). The amounts of expression vectors are as follows: 100ng of ERα or hPR expression vectors (as indicated), 250ng of ERE3-TATA/pXP2 or pGRE5/pXP2 reporter plasmid, 250ng of internal control vector pCMV-βgal. Quantities of expression vectors used are indicated in figures or corresponding figure legends. Medium
was replaced 18 hr after transfection with medium containing charcoal-stripped serum and estradiol (10nM) for 30hr. For *Luciferase* reporter assay, cells were harvested in 250 µl of reporter lysis buffer (Promega).

Chromatin immunoprecipitation (ChIP) Assays and reChIP assays

ChIP and reChIP assays were performed as previously described (178) in MCF7 and T47D cells. Cells were grown in 10 cm dishes (70% confluent) and were transiently transfected with 500ng of Flag-tagged LCoR. Following the transfection, cells were starved for two days in DMEM-phenol free and FBS free media and treated with 2.5µM α-amanitin (Sigma, A2263) for 2h prior to hormone treatment in order to properly synchronize cells. Cells were collected and cofactor recruitment was evaluated on promoter regions containing ERE of estrogen target genes. For ChIP primers sequences are the following: *pS2* promoter forward 5’- CTCTCAGCTATGAATCTCAGCAGCAG-3’, reverse 5’-AGATAAACATTGCAAGGAGCG-3’, non-targeting *pS2* forward 5’- CAGCCCCAAGACTTCAG-3’, reverse 5’-TGAGCAGGTTGTCAGCAGCAGCAG-3’, *ADORA1* promoter forward 5’- CAGAAGCTCTGTTGGGCAAG-3’, reverse 5’-ATGGGCTCTTGACGCTGTGT-3’, *ADORA1* non-targeting forward 5’- TAGAATCCACTAGTCCACCT-3’, reverse 5’-TCACTTGTGCTTACTACCTTGCTTT-3’, *IGFBP4* promoter forward 5’- CTTTCTTGTGCTTACTACCTTGCTTT-3’, reverse 5’-ATGGGCTCTTGACTGCTGCTGTGT-3’, *IGFBP4* non-targeting forward 5’- GCCAGGGACCGGTATAAAG-3’, reverse 5’-GACGTAGCGGGGAGGTTAG-3’, *NRIP1* promoter forward 5’-GATGCAGATTGGCTGACAGG-3’, reverse 5’-CCCACCCCCAATTTCTATCT-3’, *NRIP1* non-targeting forward 5’-GCCAGGGGAGGACTGGG-3’, reverse 5’-ATGTCTGCGGCTGACTTT-3’.
5.6 Figures

Figure 5.1- Analysis of LCoR domains and their effects on NR-mediated transcriptional control.

A. Schematic representation of full-length and truncated forms of LCoR. For mutant m1m2, tandem PXDLS repeats (LCoR a.a. 64-70; m1 and LCoR a.a 82-88; m2) were deleted. For mutant LCoRΔHTH, HTH motif (LCoR a.a. 350-395) were deleted. The double mutant m1m2ΔHTH was PXDLS tandem repeats and HTH motif deleted. The mutant LCoRΔHDACs has the central region (LCoR a.a. 100-319) deleted. All other areas are left intact, including the C-terminal region for the HTH motif mutants. B and C. Domain analysis of LCoR in ERα-induced (B) and PR-induced (C) transcription. Dose response curves were performed analyzing corepression in the presence of increasing amounts of full-length LCoR, m1m2, LCoRΔHTH, m1m2ΔHTH or LCoRΔHDACs expression vectors (0, 100, 400 and 600ng, as indicated). MCF7 cells were treated with E2 (10nM; panel B) and P4 (10nM; panel C). Cells were transiently transfected with expression vectors of either ERα (100ng) or PR (100ng) and their corresponding reporter plasmid (250ng) for 18h. Media was then changed and cells were treated for 30h. Results are presented as relative luciferase units (RLU). *, P < 0.05 for results corresponding to full-length LCoR versus truncated forms of LCoR.
Figure 5.1
Table 5.1 - Sequence of tandem PXDLS motifs

Description of the a.a. sequences of the PXDLZ motifs in full-length and mutant forms of LCoR. Site m1 defines the first PXDLS motif (LCoR a.a. 64-70) and site m2 defines the second motif (LCoR a.a. 82-88). LCoR describes the full-length form of LCoR (wild type), m1m2 describes a truncated form where both PXDLS tandem repeats have been deleted, m1 describes the deletion of only the first of the two PXDLS repeats, m2 describes the deletion of only the second PXDLS repeat, Q1 is a point mutation of a lysine (K) in the first PXDLS repeat that was replaced by a glutamine (Q; indicated in bold red in a light blue highlighted box) and R1 is a point mutation of a lysine (K) in the first PXDLS repeat that was replaced by a arginine (R; indicated in bold red in a light blue highlighted box).
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Table 5.1
Figure 5.2 - Effect of an adjacent lysine residue to a PXDLS motif of LCoR on ERα-induced transcription

Lysine residue adjacent to the first PXDLS motif in LCoR influences ERα-induced transcription. Dose response curves were performed analyzing corepression in the presence of increasing amounts of full-length LCoR, m1m2, m1, m2, Q1 or R1 expression vectors (0, 250, 500 and 750ng, as indicated). MCF7 cells were treated with E2 (10nM). Cells were transiently transfected with expression vector of ERα (100ng) and its reporter plasmid (250ng) for 18h. Media was then changed and cells were treated for 30h. Results are presented as relative luciferase units (RLU). *, P < 0.05 for results corresponding to full-length LCoR versus mutated forms of LCoR.
Figure 5.2
**Figure 5.3- Kinetic chromatin immunoprecipitation (ChIP) assays of estrogen-induced target genes.**

MCF7 cells synchronized for 2h with α-amanitin (2.5µM), treated with E2 (10nM) were collected at 15min increments and immunoprecipitated with IgG, or antibodies against ERα, Flag, CtBP1, LSD1, CoREST, G9a, H3K4me1, H3K4me2 or H3K9me2, as indicated. A-D. Kinetic ChIP assays on the pS2 (A), IGFBP4 (B), ADORA1 (C) and NRIP1 (D) promoters. Graphical representations of promoters indicate location of ERE sequence, the ER-binding region amplified by PCR, and non-target sequences analyzed. Note that the region of the pS2 promoter amplified lies immediately adjacent to the ERE and is identical to that amplified by others (133, 158) in analysis of estrogen regulation of the promoter.
Figure 5.3
Chapter 6

General Discussion
Coregulators, sometimes referred to as “master genes” (236), are at the center of the transcriptional control program and affect a variety of cellular functions, including growth, differentiation and metabolism. To date, there are approximately 300 identified coregulators (229) indicating their importance in modulating transcription and more importantly, the mechanistic complexity with which NR mediate gene expression. In addition, a great number of pathologies are due to disruptions (i.e. over- or underexpression and gene polymorphism) of coregulator function (229). An example of this is endocrine-related cancers where coregulators are overexpressed in 38% of breast cancers and 43% of prostate cancers (229). The broad implication of coregulators in human diseases demands our better understanding of their function.

The ligand-dependent corepressor LCoR was identified several years ago in the John White laboratory through a yeast two-hybrid screen for which the LBD of ERα was used as bait (1). LCoR is a 47kDa protein that is expressed in a variety of fetal and adult tissues as well in two-cell stage mouse embryos, suggesting functions in embryonic development. LCoR is part of a special class of corepressors which are recruited to agonist-bound NRs. In the traditional NR-mediated transcriptional control model, the LBD helix 12 is in an extended conformation in the absence of ligand, hence exposing the CoRNR box to which corepressors are recruited (91). In the presence of ligand, the LBD changed conformation by repositioning helix 12 which in turn exposed new sequences that favored NR box interaction typically found in coactivators (91). However, LCoR, like other ligand-dependent corepressors, is an exception to this model. In vitro studies have shown the interaction of LCoR with agonist-bound NRs is dependent on the integrity of its NR box (LXXLL motif). This
was further supported by reporter gene assays in COS7 cells comparing full-length LCoR and mutant LCoR (modification of NR box sequence from LSKLL to LSKAA) which revealed a loss of repression in the absence of the NR box motif (1). Reporter gene assays showed LCoR-dependent corepression of GR-, PR- and vitamin D receptor (VDR)-mediated gene transcription implicating LCoR in a wide variety of physiological processes (1). Since several corepressors mediate their transcriptional function through HDAC-dependent activity, the HDAC inhibitor TSA was used to investigate the mechanism of action of LCoR (91). Interestingly, results showed HDAC-dependent and –independent modes of action where only the ERα and GR were affected by TSA treatment in COS7 cells (1). In addition to HDAC, the transcriptional repression function of ligand-dependent corepressors was shown to depend on their interaction with CtBP1 (91). This was also shown to be the case with LCoR which, through sequence analysis, was shown to contain two tandem consensus CtBP-binding motifs at its N-terminal end(1).

Therefore, initial in vitro studies characterized LCoR as an agonist-bound NR corepressor that might require interaction with HDACs and CtBP1 for its corepressor activity. However, the role of LCoR in regulating endogenous NR receptor target genes remained unknown. Since HDAC-dependent activity was shown to be necessary for ERα-mediated transcription control, and PR-transactivation was strongly repressed by LCoR overexpression, the focus of the next phase of experiments was on transcriptional control mediated by these two NRs. With this, the question addressed in this thesis was the following: what is the role of LCoR in the control of endogenous estrogen and progesterone target gene expression?
In order to tackle this problem, further analysis of LCoR interactions with HDACs and CtBP1 was necessary. Even though GST-pulldowns and coIPs gave evidence for HDAC and CtBP1 interaction with LCoR (1), the effects of these interactions on NR-mediated transactivation was unknown. Through reporter gene assays with overexpression of LCoR and truncated LCoR mutants lacking the interacting domains, we have shown that both HDAC6 and CtBP1 contribute to transcriptional corepression observed with LCoR (176, 200). To find out whether these cofactors are recruited to regulatory regions of endogenous ERα and PR target genes, the ChIP and reChIP assays techniques were applied. The latter have shown a ligand-dependent recruitment of LCoR, HDAC6, CtBP1 and corresponding NR in a somewhat synchronized fashion (176, 200). SiRNA-mediated gene silencing of LCoR, HDAC6 and CtBP1 in combination with reporter gene assay showed a transcriptional derepression in ERα- and PR-mediated gene transcription, confirming the transcriptional corepressor function of these coregulators (176, 200). However, the investigation of the effects of siRNA-mediated knockdowns on endogenous gene expression did not paint a clear picture. In fact, LCoR, HDAC6 and CtBP1 knockdowns had different effects on endogenous gene expression, where in some cases LCoR seemed to have a transcriptional coactivator function, as seen with estrogen-regulated genes KRT4 and CYP26B1, for example (176). However, in the case of progesterone target genes, LCoR siRNA-mediated knockdown elevated PR-regulated transactivation, suggesting an important function for LCoR in progesterone physiology.

Therefore, these studies have shown for the first time LCoR to be a bona fide NR coregulator through its ligand-dependent recruitment to endogenous NR target genes in
cyclical and orderly fashion. However, the effects of LCoR siRNA-mediated knockdowns on endogenous estrogen target genes were gene-specific, which brings forth the following question: what is the mechanistic nature of LCoR action that results in promoter-specific transcriptional control?

The first thing to consider in order to find an answer to this question is whether or not reporter gene assays of transiently expressed proteins provide an accurate image of transcription control. Effects of siRNA-mediated knockdowns on reporter gene expression were inconsistent with the results of RT-PCR analysis of endogenous target genes. Many reports have already pointed out the limitations of reporter gene assays because of their lack of in vivo context (66, 100). Because of protein overexpression and the use of compact promoters with multiple tandem HREs, reporter recombinants are not an accurate reflection of the structures of endogenous promoters.

Post-translational modifications of chromatin as well as transcription factors and their ancillary proteins have become a major focus in the field of transcription regulation. Histone modifications have profound effects on chromatin structure of transcription regulatory regions. For example, it was only until recently that histone methyl marks were believed to be permanent marks used by the chromatin for long-term epigenetic memory. However, with the discovery of proteins with methyltransferase and demethylase activity, methyl marks were shown to be highly dynamic, and in combination with acetylation, sumoylation, ubiquitination and phosphorylation, form the histone code. The combinatorial effect of these post-translational marks on the chromatin determines the transcriptional
output, where each mark is the result of different environmental or physiological cues and can signal for the recruitment of various coregulator complexes (132). In addition, not only are coregulators the effectors of post-translational modifications, but they are also targets of post-translational modifications (230). In other words, post-translational modifications are believed to be the reason behind context-specific effects of coregulators.

Many examples where the context of the regulatory region affects the transcriptional function have been shown. LSD1, a histone demethylase that specifically demethylates mono- and dimethylated H3K4, was shown to repress gene expression by maintaining H3K4 in a demethylated state (235). However, AR-mediated gene activation of the prostate-specific antigen (PSA) gene was dependent of LSD1 demethylation of H3K9 (237). This is also true for histone methyltransferase G9a, a H3K9 methyltransferase responsible for mono- and dimethylation of Lys-9 of histone H3 in euchromatin (218). In the case of the E-cadherin promoter (218), G9a was shown to repress its transcription, but activate transcription of pS2 and PSA genes (238).

As mentioned previously, NR coregulators are themselves targets of post-translational modifications which modify their transcriptional activity by regulating their interaction with other cofactors, cellular localization and conformation (101). A perfect example of such transcriptional activity modulation is seen with ligand-dependent corepressor RIP140. Multiple post-translational modifications were identified in RIP140, including phosphorylation, methylation, acetylation, and most recently sumoylation, all of which were shown to affect its transcriptional corepressor function (126-130).
Phosphorylation was shown to increase its transcriptional corepressor activity through enhanced HDAC recruitment, whereas methylation reduced its corepressor activity (127, 129). This was due to attenuation of HDAC interactions and increased nuclear export. The acetylation state of RIP140 had a dual effect on its corepressor activity. RIP140 has seven acetylation sites, and the overall hyperacetylated state was shown to enhance its corepressor activity. However, the acetylation of a specific lysine situated in the CtBP-binding domain reduced the interaction between RIP140 and CtBP which resulted in diminished transcriptional repression (126).

Mechanistic studies have revealed a context-specific transcriptional corepressor function for LCoR which can be due to several factors, including its recruitment to only a specific selection of NR target genes, its participation in several coregulator complexes with different enzymatic activities and to its post-translational modification state. Many experiments are necessary to discern between these different possibilities of mechanistic transcriptional control.

The importance of Lys82 in the transcriptional corepressor function of LCoR, shown with a mutated form of LCoR where the lysine residue was substituted with an arginine, in addition to the crosstalk between the CtBP-binding sites and HTH motif, demonstrate the need to identify post-translational modifications present in LCoR. In silico studies using SUMOplot™ prediction tool by ABGENT (http://www.abgent.com/tools/sumoplot) identified eight possible sumoylation sites in LCoR (see Figure 6.1 A). Sumoylation sites are a minimal consensus motif ψKXE, where ψ is a large hydrophobic residue and X is any residue
Sumoylation modifications are of great interest since they are associated with transcriptional repression (239-241). Of the eight possible sites, only one scored above 0.90 suggesting the K414 residue with the sequence VKIE to be the most plausible sumoylation site (see Figure 6.1 B). Interestingly, the sumoylation site is situated at the C-terminal end of LCoR, right after the HTH motif. Therefore, one cannot rule out the possibility of crosstalk between these two domains. In order to determine whether K414 is an actual sumoylation site, sumoylation assays and mass spectrometry analysis need to be performed (242, 243).

In 2007, the Pandey group analyzed the human genome for phosphorylation sites by applying electron transfer dissociation tandem mass spectrometry (244). In their study, they identified two phosphorylation sites in LCoR, residues Ser\(^{359}\) and Ser\(^{363}\) (see Figure 6.2). This is of great interest because phosphorylation modifications are considered as the first step of post-translational modifications in response to environmental and physiological cues (230). More importantly, crosstalk between phosphorylation and acetylation marks are reported (230, 245). In the case of LCoR, this is very interesting since both phosphorylation sites are situated in the HTH motif which was shown to mediate the transcriptional corepressor function of LCoR.

Therefore, there are many candidate post-translational modifications to be studied in LCoR. The best way to achieve this study would be mass spectrometry analysis of LCoR. Recent advancements have made mass spectrometry the tool of choice for detecting post-translational modifications (246). In fact, large-scale analyses are now possible, including
the screening of protein complexes, which can give a general overview of the possible post-
translation modifications in a multi-subunit coregulator complex.

Figure 6.1- Sumoylation site in LCoR. A. SUMOplot™ Prediction analysis of LCoR a.a. sequence. The in silico analysis has identified eight possible sumoylation sites in LCoR, with the most probable site being residue K414 with a score of 0.93. B. Schematic representation of LCoR with the sumoylation site indicated. The minimal consensus sequence ΨKXE is VKIE in LCoR.
Figure 6.2- Phosphorylation sites in LCoR. Mass spectrometry studies have identified two possible phosphorylation sites in LCoR, Ser$^{359}$ and Ser$^{363}$. Both sites are situated in the HTH domain of LCoR.

To further our understanding of context-specific action of LCoR, genome-wide studies should be performed. First, it would be interesting to see whether LCoR affects the expression of genes implicated in specific cellular functions (i.e. growth, differentiation, metabolism, etc), or a wide range of NR target genes. The effect of LCoR expression on transcriptional control can be studied with microarray expression profiling. In the case of ERα-induced transcription, mRNA of MCF7 cells treated with or without estradiol will be extracted to compare the gene expressions in the presence or absence (LCoR siRNA-mediated knockdown) of LCoR. The same can be done for PR-induced transcription in T47D cells, which are known for strong PR expression. These experiments are going to provide important information on the broadness of LCoR effect on NR-mediated gene expression. However, it will be difficult to distinguish between direct and indirect transcriptional effects of LCoR on specific genes. To achieve this, Chip-chip technology can be applied, where chromatin-bound LCoR immunoprecipitated material is hybridized to microarray chip. This
technique will identify all LCoR binding sites in the genome. The combination of ChIP-chip data for ERα and polymerase II (in phosphorylated form) with LCoR Chip-chip data will help discriminate between active and inactive transcriptional sites in MCF7 cells treated with estradiol. In order to have a generalized view of coregulator complex function, the same can be done for CtBP1 and HDAC6, or any other cofactor of interest, in combination with histone tail post-translational modifications.

In conclusion, LCoR was shown to contribute to ERα- and PR-induced transcription. Its transcriptional corepressor function is highly modulated and its transcriptional action is gene-specific. In order to have a better understanding of the mechanistic nature of LCoR’s transcriptional action, further studies investigating its post-translational modifications are needed. In addition, genome-wide studies will identify gene targets of LCoR transcriptional control.
Chapter 7

Conclusions
LCoR is part of an unique class of corepressors that are recruited to agonist-bound NRs. Since the discovery of LCoR some 10 years ago through a series of in vitro studies, LCoR was characterized as a NR corepressor. The question remained: what is the role of LCoR in the control of endogenous estrogen and progesterone target gene expression? This became the focus of this thesis and to answer this question, the interaction of LCoR with HDAC6 and CtBP1 was investigated.

Many molecular biology techniques were applied and permitted to characterize LCoR as a bonafied NR coregulator. In vitro studies have previously shown LCoR to interact directly with known corepressors, including HDAC6 and CtBP1. Reporter gene assays have shown the contributions of HDAC6 and CtBP1 in NR-mediated transcription control in the presence of LCoR. ChIP and reChIP assays permitted the investigation of LCoR recruitment to regulatory regions of endogenous ERα and PR target genes. In addtition, ChIP assays have shown the recruitment of LCoR to target genes to be somewhat synchronized with its cofactors, CtBP1 and HDAC6. In order to explore the mechanistic nature of NR-mediated transcription repression by LCoR and its cofactors, siRNA-mediated knockdowns of these were performed and the various effects on gene regulation were observed.

Even though knockdowns of LCoR and its cofactors gave rise to unexpected results, they are a clear demonstration of a new developing theme in the field of NR-mediated transcription control: context- and gene-specific effects of coregulators. Since the discovery of proteins with methylase and demethylase enzymatic activities, post-translational histone modifications have been identified and their impact on transcription control are
investigated. In addition, the combinatory effects of histone methylation and demethylation with other post-translational modifications (i.e. acetylation, ubiquitination, phosphorylation, etc) are painting a complex picture in which all of these post-translational modifications impact the different stages transcription regulation, including transcription activation/deactivation, cofactor recruitment and the transcriptional action of cofactors. Therefore, where initially post-translation modifications were thought to have exclusive effects on histones (known as the histone code) and control the chromatin structure, new evidence is characterizing post-translational modifications as a protein code by which all proteins are affected by these. This creates a highly dynamic environment where proteins initially characterized as corepressors, depending on the context, can in fact be coactivators, for example.

The NR classical model that once described transcriptional activation as bi-modal (i.e. transcriptional activation/repression with the presence or absence of ligand, respectively), does not apply anymore. And this is also the case with coactivators and corepressors: post-translational modifications are shown to modulate their action by either attenuating or enhancing their enzymatic activity or by modulating their interaction with other cofactors. Therefore, in the case of LCoR where siRNA-mediated knockdowns have shown a gene-specific control of endogeneous ERα and PR target genes, a more precise definition of LCoR is a coregulator of NR-dependent gene transcription where post-translational modifications may affect its action on transcription regulations.
In conclusion, LCoR was shown for the first to be recruited to regulatory regions of endogenous estrogen and progesterone target genes. The mechanistic nature of LCoR corepressor function was investigated with siRNA-mediated knockdowns which revealed a gene-specific mode of action. The multiple domains and motifs found in LCoR were analyzed in great detail and were shown to contribute to the transcriptional corepressor function of LCoR. Post-translational modifications present in LCoR need to be identified and characterized in their role in transcriptional control to be able to understand the promoter-specific nature of LCoR corepressor function.

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Appendix I

Chapter 4 Supplemental Material
Appendix Figure 1- Association of LCoR with PRC1 complex marker BMI1.

A. Confocal microscopic analysis of the subcellular colocalization of LCoR with BMI1 by immunocytochemistry (see Experimental Procedures for details). B. Analysis of the association of LCoR with BMI1 by coimmunoprecipitation (Co-IP). Extracts of MCF7 cells were immunoprecipitated (IP) with antibodies against BMI1, and probed by Western blotting for enrichment of target proteins in immunoprecipitates. Immunoprecipitates were also probed for the coimmunoprecipitation of LCoR. C. ChIP assays in extracts of MCF7 cells treated with E2 for 30 min. (10nM). The pS2 promoter region is depicted (upper panel). Cell extracts were collected and immunoprecipitated with IgG, or antibody against BMI1.
Figure A1
Appendix figure 2- Roles of CtBP1 and CtIP in LCoR-dependent corepression in T47D cells.

Cells were transiently transfected with expression vectors for either PR (100ng) or ERα (100ng) and their corresponding reporter plasmids (250ng) for 18h. Media was then changed and cells were treated with vehicle or hormone for 30h. A and B. Dose-response curves analyzing the effects of CtBP1 on reporter gene expression in cells treated with P4 (10nM; A) or E2 (10nM; B). Increasing amounts of CtBP1 were transfected (200, 400 and 600ng). C and D. Dose-response curves analyzing the effects of CtIP on reporter gene expression in cells treated with P4 (C) or E2 (D). Increasing amounts of CtIP1 were transfected (200, 400 and 600ng). E and F. Analysis of the effects of coexpression of LCoR and CtBP1 on hormone-dependent gene expression. Cells were transiently transfected with either vector alone, LCoR alone (100ng), CtBP1 alone (200ng) or with both LCoR and CtBP1, and treated with P4 (E) or E2 (F). *, P < 0.05 for results of LCoR and CtBP1 coexpression versus empty vector control. G and H. Analysis of the effects of coexpression of LCoR and CtIP on hormone-dependent gene expression. Cells were transiently transfected with either vector alone, LCoR alone, CtIP1 alone or with both LCoR and CtIP1 and treated with P4 (G) or E2 (H). I and J. Dose response curves of either LCoR or m1m2 in cells treated with P4 (I) or E2 (J). Increasing amounts of wild-type or mutant LCoR were transfected (200, 400 and 600ng). *, P < 0.05 for results of corresponding wild-type LCoR versus mutant form m1m2.
Figure A2
### Appendix table 1 - ChIP and reChIP primer sequences.

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### Appendix table 2- QRT-PCR primer sequences.

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