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MODULATION OF NUCLEAR RECEPTOR FUNCTION BY INTERACTING PROTEINS

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To my beloved family for all their love and support.

Waffa Yassin Salih Osman

ABSTRACT

Nuclear receptors are a family of transcription factors involved in many biological processes. They represent key potentials as therapeutic targets for several clinical conditions such as diabetes, obesity, cardiovascular disease and cancer, because their activity is modulated by small molecules. Collectively these diseases represent a large market for prescription drugs. The nuclear receptor family consists of 48 members. The nuclear receptors have three conserved domains, the N-terminal transactivation domain, the central DNA binding domain and the C-terminal ligand binding domain. Nuclear receptors are ligand activated transcription factors that are not only regulated by small lipophilic ligands, but also by interaction with coactivators and corepressors as well as other binding proteins. Protein-protein interactions are essential in many biological pathways and are also attractive for drug discovery. In this thesis I elucidate the functional significance of some nuclear receptor interacting proteins. I have investigated the mechanism of the corepressor receptor interacting protein 140 (RIP140) for repression of the glucocorticoid receptor (GR) and liver X receptor (LXR) and the functional significance of the novel interacting proteins polyamine-modulated factor 1 (PMF-1) and germinal center associated nuclear protein (GANP) in glucocorticoid signalling.

RIP140 represses ligand activated nuclear receptors, such as GR and LXR. It is localized in small nuclear foci targeted by a 40 amino acid long sequence. Even though the foci targeting sequence in RIP140 overlaps with the binding site of the corepressor C-terminal binding protein (CtBP), interaction with CtBP is not essential for foci targeting. Upon complex formation of ligand activated GR or LXR with RIP140 the complex redistributes to larger foci distinct from RIP140 foci. The redistribution of RIP140/GR involves RIP140 repression domains and the DNA binding domain of GR. RIP140 repression domains include the C-terminal receptor interacting LXXLL motifs binding to GR and the interaction domain of the corepressor CtBP. The repression of RIP140/LXR involves the integrity of the C-terminal domain of RIP140 including the LXXML motif. Jointly, these results suggested that RIP140 represses in multiple ways including direct binding to ligand activated NR and the formation and redistribution of intranuclear repressive protein complexes.

PMF-1 is a transcription factor induced by polyamines, which are important regulators of cell growth and cell death and are implicated in glucocorticoid induced apoptosis. We identified PMF-1 to functionally interact with RIP140 and GR. PMF-1 represses glucocorticoid induced GR activity and has an intrinsic repression activity, which could contribute to the repressive action. Although a physical interaction was observed between PMF-1 and RIP140, we found that PMF-1 does not further enhance RIP140 repressive effect.

We identified GANP and the GANP splice variant MCM3 associated protein (MCM3AP) to bind to GR ligand binding domain. We found GANP to be a shuttling protein that shuttles between the nucleus and the cytoplasm, and contains nuclear localization and nuclear export signals. GANP and MCM3AP were initially reported as proteins binding to MCM3, which is a member of the MCM protein complex that is involved in initiating of DNA replication. We show that glucocorticoids regulate the chromatin loading of MCM3, inhibit DNA replication and arrest cells in the G_1 phase of the cell cycle. We also show that MCM3AP counteracts the repressive activity of glucocorticoid on DNA replication. In conclusion we suggest that interaction between GR, GANP/MCM3AP and MCM3 could imply a new way for glucocorticoid regulation of cell proliferation.

LIST OF PUBLICATIONS

This thesis is based on the following papers, referred to in the text by their Roman numerals.

I. Hiroshi Tazawa, **Waffa Osman**, Yutaka Shoji, Eckardt Treuter, Jan-Åke Gustafsson and Johanna Zilliacus. (2003). Regulation of subnuclear localization is associated with a mechanism for nuclear receptor corepression by RIP140. *Molecular and Cellular Biology.* **23** (12), 4187-4198.

II. Tomas Jakobsson*, **Waffa Osman***, Jan-Åke Gustafsson, Johanna Zilliacus and Anette Wärnmark. (2007). Molecular basis for repression of liver X receptor-mediated gene transcription by receptor-interacting protein 140. *Biochemical Journal*. **405** (1), 31-39.
*Contributed equally.

III. Yutaka Shoji, **Waffa Osman** and Johanna Zilliacus. (2007). Polyamine-modulated factor 1 represses glucocorticoid receptor activity. *Biochemical and Biophysical Research Communications, in press.*

IV. **Waffa Osman**, Sanna Laine and Johanna Zilliacus. (2006). Functional interaction between the glucocorticoid receptor and GANP/MCM3AP. *Biochemical and Biophysical Research Communications.* **348** (4), 1239-1244.

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LIST OF ABBREVIATIONS

AF	Activation function
AP-1	Activating protein 1
AR	Androgen receptor
CBP	cAMP response element binding protein (CREB)-binding protein
CDK	Cyclin dependent kinase
CoRNR motif	Corepressor-nuclear receptor interaction motif
CtBP	C-terminal binding protein
DBD	DNA binding domain
DDK	Dbf4-dependent kinases
Dex	Dexamethasone
ER	Estrogen receptor
GANP	Germinal center associated nuclear protein
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HRE	Hormone response element
LBD	Ligand binding domain
LXR	Liver X receptor
MCM	Minichromosome maintenance
MCM3AP	MCM3 associated protein
MR	Mineralocorticoid receptor
NCoR	Nuclear receptor corepressor
NES	Nuclear export signal
NF-kB	Nuclear factor-kB
NLS	Nuclear localization signal
NR	Nuclear receptor
Nrf-2	NF-E2 related factor 2
ORC	Origin recognition complex
PMF-1	Polyamine-modulated factor 1
PPAR	Peroxisome proliferator-activated receptor
PR	Progesterone receptor
PRE	Polyamine responsive element
pre-RC	pre-replication complex
RAR	Retinoic acid receptor
RD	Repression domain
RIP140	Receptor interacting protein 140
RNAPII	RNA polymerase II
RXR	Retinoid X receptor
SMRT	Silencing mediator of retinoid and thyroid hormone action protein
SRC-1	Steroid receptor coactivator-1
SSAT	Spermidine/spermine N1-acetyltransferase
TR	Thyroid hormone receptor
VDR	Vitamin D receptor

1 INTRODUCTION

This thesis elucidates the molecular significance of nuclear receptor (NR) interacting proteins receptor interacting protein 140 (RIP140), polyamine-modulated factor 1 (PMF-1) and germinal center associated protein (GANP). In the introduction NRs and especially the glucocorticoid receptor (GR) and liver X receptor (LXR) will be reviewed. Also reviewed in this introduction are polyamines and PMF-1 and DNA replication and GANP.

1.1 NUCLEAR RECEPTORS

The NR super family consists of a large group of transcription factors that are essential for growth, differentiation, metabolism, reproduction and morphogenesis. There are 48 members of the NR super family that are all derived from a common ancestor (Robinson-Rechavi et al. 2003; Germain et al. 2006). Phylogenic analysis has subdivided NRs into six subfamilies based on their sequence homology (Committee 1999). NRs are also subdivided according to their mechanism of action (type I to IV), where they are classified according to their DNA binding domain and dimerization properties. Type I includes GR, estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR) and mineralocorticoid receptor (MR) that are activated by steroid ligands and bind DNA as homodimers. Type II includes LXR, peroxisome proliferator-activated receptor (PPAR), retinoid X receptor (RXR) and retinoic acid receptor (RAR) etc that are activated by non-steroid ligands and activate gene transcription by heterodimerizing with RXR. Type III receptors bind as homodimers to direct repeats and type IV bind extended core sites as monomers. Examples of receptors included in type III and IV receptors are the orphan receptors (Mangelsdorf et al. 1995).

1.1.1 Nuclear receptor action

In the unliganded state, type I NRs (subfamily 3) are located mainly in the cytoplasm, were they are held inactive by the interaction of chaperone proteins including heat shock protein 90 (hsp90), hsp70, p60, p23 and immunophilins (Griekspoor et al. 2007). Upon agonist ligand binding type I NRs dissociate from the chaperones and become

hyperphosphorylated, leading to nuclear localization signal (NLS) exposure and nuclear translocation. In the nucleus the NR will bind as a homodimer to specific palindromic hormone response elements (HRE) found within the promoters of the target genes and recruit coregulators (coactivators and corepressors) that either activate or repress gene transcription (Griekspoor et al. 2007). Type II NRs are already present in the nucleus and heterodimerized with RXR and held inactive by corepressors, however upon ligand binding their transcription is initiated by the dissociation of corepressors and binding of coactivators. During transcriptional activation, NRs will recruit coactivators, which open chromatin and interact with general transcription factors that in turn recognize the RNA polymerase II (RNAPII) and initiate transcription (Figure 1) (McKenna et al. 1999; McKenna et al. 2002).

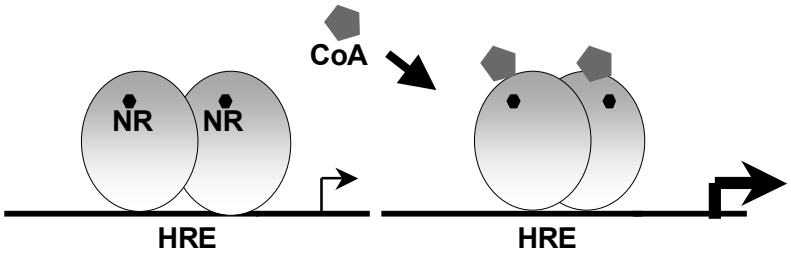

Figure 1. An illustration of agonist activated NR gene transcription and coactivators (CoA) role in enhancing NR transactivation. The black circle depicts the ligand.

NRs can also crosstalk with other transcription factors and thereby interfere with their target gene regulation. The influence of crosstalk by ERs, GR and PPARs on cytokine signalling transduction has been reviewed (Wang et al. 2004). For example the crosstalk between GR and nuclear factor kappa B (NFκB) and activating protein-1 (AP-1) result in the transcriptional repression of their target genes (Liberman et al. 2007). In most cases the cross-talk results in repression, but there is data showing that crosstalk between ER and AP-1 promotes transcription of the collagenase gene on an AP-1 promoter (Bjornstrom et al. 2002).

1.1.2 Intracellular localization of nuclear receptors

Intracellular localization and translocation of proteins to specific compartments is important for the being of living cells. The nuclear envelop of eukaryotic cells separates cytoplasmic and nuclear compartments to allow the movement of macromolecules (such as NRs) to obtain a specific intracellular localization. Intracellular localization is essential for the regulation of protein mechanism. A side from the name, NRs are not always localized in the nucleus before ligand binding, but instead have different cellular distribution. Steroid receptors for instance were first thought to predominantly localize in the cytoplasm in absence of ligand. However today there is strong evidence showing that GR and AR are localized in the cytoplasm, MR is evenly distributed in both nucleus and cytoplasm and ER and PR are localized in the nucleus. Upon ligand induction GR, AR and MR are translocated to the nucleus (Kumar et al. 2006). The cellular distribution of non-steroid binding NRs also differs, whereby the vitamin D receptor (VDR) is mainly localized in the cytoplasm and translocated to the nucleus after ligand induction. While, thyroid hormone receptor (TR), RXR, RAR and LXR are localized in the nucleus both in absence and presence of ligand (Maruvada et al. 2003; Dong et al. 2004; paper II). Interestingly others have identified unliganded TRα and RARα to be localized in the cytoplasm (Braun et al. 2000; Bunn et al. 2001).

Coregulators can also play a role in the distribution of NRs. Studies on TR showed that corepressor nuclear receptor corepressor (NCoR) together with RXR maintained unliganded TRβ in the nucleus (Baumann et al. 2001; Bunn et al. 2001). In absence of ligand, ER and the coactivator steroid receptor coactivator-1 (SRC-1) were evenly distributed in the nucleus. However, in the presence of ligand, ER and SRC-1 translocated to distinct foci in the nucleus (Stenoien et al. 2000). Interestingly this was also observed by us when coexpressing corepressor RIP140 with GR (paper I) and LXR (paper II), and by others for AR/RIP140 coexpression (Carascossa et al. 2006).

1.1.3 Structures and functions of nuclear receptor domains

NRs are modular proteins with four distinct functional and structural domains, comprising an N-terminal A/B domain, a highly conserved DNA binding domain (DBD), a ligand-binding domain (LBD) and a hinge domain (Figure 2) (Germain et al. 2006; Bain et al. 2007). Some NRs also contain a C-terminal F domain with unknown function.

1.1.3.1 N-terminal domain (A/B region)

The N-terminal A/B region has the weakest conservation and varies significantly in length (Aranda et al. 2001; Germain et al. 2006). A/B domains of receptor isoforms generated from a single gene vary also in both size and sequence due to alternative splicing or use of different promoters. This region also undergoes post-translational modification such as phosphorylation by mitogen-activated protein kinase (MAPK) and cyclin-dependent kinase (CDK). It also comprises a constitutively active hormone independent activation function (AF-1) that is cell and promoter specific and important for gene activation and transcription component binding. The AF-1 autonomous activation is observed when placed outside the full length receptor, but in context with the full length receptor, the AF-1 domain is controlled by the LBD. In addition the N-terminal domain interacts with cofactors like the coactivators and other transcription factors and also with its own C-terminal domain.

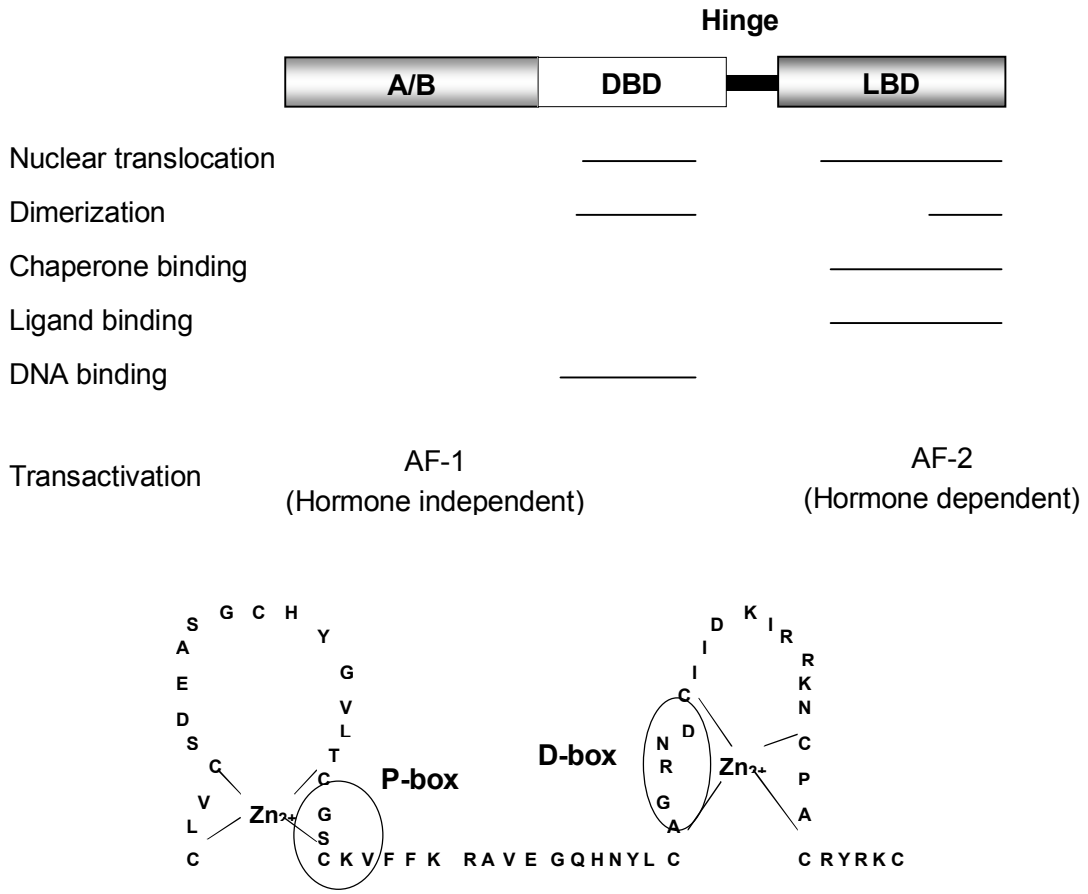

Figure 2. The upper figure illustrates the schematic structural and functional domains of NR. The lower figure shows a magnification of GR DBD with its two zinc fingers, linker region and the P and D boxes.

1.1.3.2 DNA binding domain (C region) and hinge region (D region)

The DBD encompasses a sequence specific DNA recognition domain that structurally consists of two zinc-finger motifs that individually are surrounded by four cysteine residues (Aranda et al. 2001; Bain et al. 2007). It also has two main sequence elements: the P- and D-box (Figure 2). The P box is required for the specific recognition of the response element and the D box is involved in NR dimerization.

NRs promote gene transcription by binding to HREs. There are two HRE consensus half-sites bound by NR DBD, one generally for non-steroid NRs and ER (5´-AGG/TTCA-3´) and the other for the rest of the steroid NRs (5´-AGAACA-3´). The half-sites are arranged in repeats such as direct or palindromic repeats. Generally all steroid hormone receptor (GR, MR, AR, PR, ER) bind as homodimers to palindromic repeats with three nucleotide spacing, while non-steroid NR bind as heterodimers with RXR to direct repeats with different spacing configuration.

The hinge region is a hinge between the DBD and LBD, allowing the DBD and LBD to adopt different conformations without creating a steric hindrance.

1.1.3.3 Ligand binding domain (E region)

The NR-LBD undergoes dimerization and ligand dependent activation, and is the binding region for ligands, chaperones and coregulators (Bain et al. 2007). Also like the A/B domain, NR-LBD has an AF domain (AF-2), which possesses cell and promoter specificity. LBD is flexible and changes conformation dependent on presence or absence of agonist ligand. LBD consists of α-helices and β-sheets that form two imperative sites that regulate its activity, the hydrophobic ligand-binding pocket (LBP) and the binding groove. Binding groove residues within α-helices 3/4/5 and 12 are important for coregulator (coactivator/corepressor) interaction. In absence of ligand, corepressors bind NRs through their corepressor-nuclear receptor interaction motif (CoRNR box), (L/IXXI/VI, where L= leucine, I=Isoleucine, V=Valine and X= any amino acid) forming an extended α-helical domain that docks into the groove created by helices 3/4/5 or 6 (Hu et al. 2001; Nettles et al. 2005).

In presence of agonist ligand binding to the LBP, the LBD will endure conformation changes (from an apo- to a holoform) and make the groove accessible for coactivator binding. This is acquired by helix 12 reorientation and corepressor release to form a new docking site for coactivators, made up of helices 3, 4 and 12. The vital binding of coactivators to NR-LBD is obtained through their NR box motif (LXXLL). There is

also a ligand-activated charge clamp that is specific for the length of coactivators and will enclose the coactivator to stabilize the binding and inhibit the interaction of corepressors (Nagy et al. 2004).

The LBD is a region where chaperone proteins, such as hsp90, bind NRs when they are present in the cytoplasm. Upon ligand binding a NLS in the LBD is exposed to induce the nuclear translocation of the NR. NR-LBD together with NR-DBD also consists of a sequence involved in NR dimerization with the NR partner (Aranda et al. 2001; Kumar et al. 2006).

In this thesis the general focus of my investigations are on NRs GR and LXR that I will describe in more detail below.

1.2 GLUCOCORTICOID RECEPTOR

Glucocorticoids are lipophilic steroid hormones produced in the adrenal cortex through cholesterol metabolism. They regulate numerous physiological processes, such as glucose and mineral homeostasis, development, inflammation, cell growth and differentiation. Levels of circulating glucocorticoids are regulated by adrenocorticotrophic hormone (ACTH), which are largely under the control of the hypothalamic-pituitary-adrenal (HPA) axis. Glucocorticoids secretion is also regulated by neuroendocrine control that includes the circadian rhythm, stress responsiveness and feedback inhibition at both the pituitary and hypothalamus level. When glucocorticoids are secreted into the blood system, they bind plasma-binding proteins that transport them to their target cells, where they dissociate and diffuse through the cell membrane (Newton 2000). In the cell glucocorticoids mediate their cellular action by binding and activating the GR.

GR is a ubiquitously expressed protein at 94kDa, with highest mRNA levels in lung, spleen, brain and liver and come in two isoforms, GRα and GRβ. GRα is the main isoform that most of the experiments are based on whilst the physiological role of GRβ is still unclear. However we know that GRβ and GRα have sequence homology until the last few amino acids of the C-terminal domain and that GRβ binds DNA, but not ligand and therefore is unable to transactivate glucocorticoid responsive genes (Lewis-Tuffin et al. 2006).

Upon glucocorticoid binding GR enters into the nucleus, homodimerizes and binds to glucocorticoid response elements (GRE), which are conserved binding sites for the GR-DBD and are found upstreams of GR activated target genes. The positive regulation of

GR called transactivation involves up-regulation of genes. Active GR also induces transcriptional repression mainly through direct protein-protein interaction with other transcription factors to interfere with their target gene regulation. Examples of transcription factors that cross-talk with GR include the proinflammatory genes AP-1, NF-κB and signal transducers and activators of transcription (STAT) family proteins (Necela et al. 2004).

1.2.1 Biological implications

1.2.1.1 Glucose metabolism

Early observations revealed glucocorticoids to be involved in glucose metabolism, hence the name. In fasting state cortisol induces overall increase or maintenance of blood glucose by stimulating transactivation of genes involved in gluconeogenesis in liver. Glucocorticoids also induces the breakdown through proteolysis of amino acids and fat from peripheral tissues e.g. muscle and adipocytes, to be converted to glucose by hepatic gluconeogenesis. GRE bound GR regulates transcription of hepatic glyconeogenic enzyme genes such as glucose-6-phosphatase (G6Pase) and phosphenolpyruvate carboxykinase (PEPCK) (Friedman et al. 1993; Vander Kooi et al. 2005). PEPCK is a rate-limiting enzyme in liver gluconeogenesis and is activated by GR, chicken ovalbumin upstream promoter transcription factor (COUP-TF) and additional transcription factors (De Martino et al. 2004).

1.2.1.2 Immunosuppressive and anti-inflammatory responses

Protection against infection requires both innate and acquired immunity. Innate immunity is comprised of four types of defence barriers including the inflammatory barrier. Upon infection the inflammatory barrier induces leakage of antibacterial activity and influx of phagocytotic cells into the affected area. Acquired immunity involves the activation and differentiation of the immune system to specifically eliminate non-self antigens. Cytokine proteins are essential in the regulation of the immune response by immune cells and are synthesized and secreted by several cell types. On their target cells cytokines activate the mitogen-activated protein kinase (MAPK) pathway, which triggers the transcription of proinflammatory genes such as AP-1, NF-κB and STAT family proteins. These proinflammatory proteins in turn regulate cytokines, adhesion molecules and lymphocyte activation and proliferation. Glucocorticoids are used in several inflammatory disorders such as rheumatoid

arthritis, asthma and autoimmune diseases (Rhen et al. 2005). They are potent immunosuppressive and anti-inflammatory agents, by inducing transactivation of anti-inflammatory genes like IκB, but also suppressing proinflammatory transcription factors e.g. NF-κB, AP-1 and STAT family proteins by direct protein-protein interaction (Franchimont 2004; Liberman et al. 2007). There are several proposed mechanisms by which GR regulates proinflammatory transcription factors, this includes GR interfering with the activity of proinflammatory transcription factors, so called cross talk, to repress proinflammatory gene transcription (Smoak et al. 2004). Additionally glucocorticoids induce decrease in the number of circulating haematological cells through apoptosis by direct transactivation of genes in both the extrinsic and intrinsic apoptosis pathways. Glucocorticoids also induce inhibition of cell proliferation and apoptosis in nonhaematological cells as discussed below.

1.2.1.3 Side effects

Despite beneficial implication of glucocorticoids in anti-inflammatory and immunosuppressive responses, continued and high doses of glucocorticoid result in extensive side effects. These include hypertension and dyslipidemia of the cardiovascular system, bleeding and pancreatitis of the gastrointestinal tract and a broad immunosuppression of the immune system. Also osteoporosis, cataract of the eye and delayed puberty are all due to prolonged treatment with glucocorticoid (Rhen et al. 2005; Mazziotti et al. 2006).

Another clinical side effect of glucocorticoids is their ability to block cell proliferation, though the exact mechanism is unclear (Newton 2000). Glucocorticoids induce an anti-proliferative action on several cell lines by causing G_1 cell cycle arrest (Sanchez et al. 1993; Frost et al. 1994). Several mechanisms for the G_1 cell cycle arrest have been suggested. In A549 cells, osteosarcoma cells and the rat lung glucocorticoids inhibit the activity of cell cycle kinases and the associated cyclins such as CDK2, CDK4 and cyclin D1, D3 and E (Rogatsky et al. 1997; Corroyer et al. 2002; Greenberg et al. 2002). In the same cells as well as in HT22 and HeLa cells glucocorticoids can induce the levels of cell cycle inhibitors such as $p21^{Waf1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ (Rogatsky et al. 1997; Corroyer et al. 2002; Greenberg et al. 2002). Glucocorticoids have also been shown to regulate factors upstream of the cell cycle regulators and inhibit the G_1/S transition gene regulator, extracellular signal-related kinase (ERK) activation by up regulating the MAPK phosphatase 1 (MKP-1) that dephosphorylated and inactivated ERK (Greenberg et al. 2002; Engelbrecht et al. 2003). In summary the anti-

proliferative action of glucocorticoids is exerted through the GR and involves many different levels.

1.3 LIVER X RECEPTORS

Cholesterol is an essential molecule found abundantly in the cell membrane to modulate the fluidity and permeability of the membrane by interacting with membrane lipids (Maxfield et al. 2005). Cholesterol is a precursor for steroid hormones, such as glucocorticoid and estradiol, bile acids and vitamin D. Cholesterol is synthesized in animal tissues or obtained from diet. Cholesterol is continuously modified to several derivatives including oxysterols, which are oxidized cholesterol. Oxysterols are a large group of molecules that are oxygenized in different carbon groups and are tightly regulated by cytochrome 450 enzymes (Chiang 2002; van Reyk et al. 2006). Oxysterols exert their biological effects by binding LXR. LXRs are generally localized in the nucleus bound to DNA with the heterodimer partner RXR (Moore et al. 2006). Upon oxysterol binding corepressors bound to LXR-RXR heterodimer dissociate and coactivators can bind to activate transcription. There are two subtypes of LXR, LXRα and LXRβ that are generated from two different chromosomes. They also differ in tissue expression, where LXRα is highly expressed in liver, kidney, macrophages etc. and LXRβ is ubiquitously expressed, suggesting regulation of different physiological functions. LXRs are relevant in many aspects of human physiology including cholesterol turnover and transport, lipid metabolism, inflammation and immune system.

1.3.1 Biological implications

1.3.1.1 Cholesterol metabolism

The role of LXR in maintaining cholesterol homoeostasis was the first observed physiological significance of LXR. Studies on LXRα knockout mice showed a distinct increase in cholesteryl ester accumulation in liver after consumption of cholesterol rich diet (Peet et al. 1998). This accumulation was due to the lack of expression of the rate-limiting enzyme in bile-acid synthesis, *CYP7a1*, which is regulated by LXR binding to a LXRE in the promoter. LXR also has an important role in reverse cholesterol transport, which involves the removal of excess cholesterol from the peripheral tissues to the liver (Steffensen et al. 2004; Zelcer et al. 2006). LXR regulates the expression of the membrane transporter ATP-binding cassette (ABC) protein family. LXR ligand

induced ABC proteins are involved (1) in the efflux of cholesterol to lipoproteins for further transport to the liver, (2) in the inability of cholesterol absorption from the intestine and (3) in promoting cholesterol efflux from hepatocytes into the bile duct. LXR also regulates cholesterol metabolism in macrophages found in atherosclerosis lesions. Macrophages phagocyte lipoproteins in such lesions, which may contribute to lipoprotein foam production in macrophages. The accumulation of lipoproteins is repressed by LXR-induced cholesterol efflux by ABC transporters. In addition to the role of LXR in cholesterol efflux, LXR also induces cholesterol transport to the liver by up regulating the expression of cholesterol acceptors, *apoe* genes in macrophages and adipocytes.

1.3.1.2 Lipid and glucose metabolism

LXR is a regulator of lipid metabolism, since studies of LXRα knockout mice revealed decreased expression of the hepatic lipogenesis regulators, sterol response element binding protein-1c (SREBP-1c) and stearoyl CoA desaturase-1 (SCD-1), and an impaired fatty acid synthesis in liver (Repa et al. 2000; Steffensen et al. 2004; Zelcer et al. 2006). SREBP-1c induction by LXR ligand is mediated through an LXRE found upstream of the SREBP gene and results in elevated triglyceride levels that accumulate in liver. Additionally, LXR ligand promotes glucose tolerance in diabetic mice, indicating the role of LXRs in glucose metabolism. This is achieved by down-regulation of gluconeogenesis by repression of expression of phosphenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) as well as induction of glucose transporter 4 (GLUT4). Together this promotes to decreased blood glucose and increased glucose storage in the liver. A role for LXR in regulating energy balance, was identified by observing LXR knockout mice which were resistant to high fat and cholesterol-induced obesity (Juvet et al. 2003; Gerin et al. 2005; Kalaany et al. 2005). This was found to be associated with abnormal high energy consumption, because of uncoupled oxidative phosphorylation and elevated expression of uncoupling proteins (UCP) in muscle and white adipocytes.

1.3.1.3 Immune system

The role of LXR in the immune system is still unclear but there is evidence that LXR represses inflammatory genes up regulated in arteriosclerotic lesions, including cytokines, iNOS and COX2 genes (Zelcer et al. 2006). Mechanistically how this repression is obtained is still not clear, but is believed to not involve inhibition of NFκB

nuclear translocation, DNA binding or IκB degradation. Interestingly, activation of toll like receptors (TLR) by infections repressed expression of LXR regulated genes such as ABC transporters and APOE and resulted in inhibition of cholesterol efflux from macrophages. Additionally, mice lacking LXR are in high risk of a specific intracellular pathogen, which could be due to impaired regulation of anti-apoptotic genes in macrophages.

1.4 GENE TRANSCRIPTION

A characteristic range of genes are expressed due to biochemical and cellular properties of the cell and are altered during different states of the cell. The general transcription machinery mediates transcriptional expression of genes. The machinery is recruited to a core promoter, which consists of a TATA box motif and is found upstreams of target genes. The machinery is either assembled in a sequential manner by initial binding of the general transcription factors (GTFs) to the core promoter and then the binding of RNA polymerase II (RNAPII) to the GTFs or as a GTFs-RNAPII holoenzyme to form the pre-initiation complex. A variety of different compositions of GTFs have been suggested, but the textbook version includes the initial binding of the transcription factor IID (TFIID) protein complex to the TATA box. The TFIID complex consists of a TATA binding protein (TBP) subunit that binds the TATA box of the target gene and together with the rest of the TFIID complex is essential for the recruitment of the transcriptional machinery. The TFIIA is recruited by TFIID to dissociate negative regulators and stabilize TBP-DNA binding. The RNAPII holoenzyme including TFIIB, TFIIE, TFIIF binds TFIID. TFIIE recruits TFIIH to the pre-initiation complex, which has helicase activity that promotes DNA melting and unwinding during elongation. The pre-initiation complex is complete after the binding of regulatory factors termed mediators (see below), resulting in phosphorylation of the C-terminal domain of the large subunit of RNAPII (Veenstra et al. 2001; Orphanides et al. 2002). This will induce the dissociation of RNAPII from the pre-initiation complex at the transcription start site and the recruitment and binding of transcription elongation factors to pursue transcription.

1.5 TRANSCRIPTIONAL REGULATION BY NUCLEAR RECEPTORS

The mechanism of transcriptional regulation by NRs requires the recruitment of the general transcription machinery. Although some evidence suggested a direct binding of the NRs to the machinery early speculations predicted a role for coregulators in NR action. A large number of coregulators have been identified and the models for transcriptional regulation have become more complicated. In general, coregulators are classified as either modifiers of the chromosomal components through reversibly stabilizing or destabilizing chromatin or functioning as bridging factors between NRs and the basal transcription machinery. Coregulators are divided into two groups, the coactivators that facilitate NRs in activating transcription and corepressors which are recruited for repression of gene transcription (Smith et al. 2004).

1.5.1 Transcriptional activation

The initial evidence for the existence of NR coactivators arose from the observation of transcriptional interference (squelching), by which one receptor inhibits another receptor when competing for the same essential cofactor (Meyer et al. 1989). The majority of coactivators interact in an agonist dependent manner with the AF-2 in the LBD containing coregulator binding grooves, through their NR box motifs.

Due to the discovery of receptor activating proteins with varied characteristics, certain criteria were suggested to define coactivators. (1) Coactivators should interact with activation domains of receptors in a ligand-specific manner, (2) thereby enhancing activation function of receptors and (3) relieving squelching. Coactivators can be divided in to three families: the histone acetyltransferase (HAT) family, the SWI/SNF family and the TRAP/SMCC/Mediator family.

In order to initiate gene transcription, chromatin structure surrounding the promoter region needs to be recognized. The HAT family proteins are considered to be among the first recruited to the ligand-activated NR. The steroid receptor coactivator (SRC) gene family was the first cloned and characterized coactivator family for NRs. It contains the homologous members SRC-1 (NCoA-1), SRC-2 (GRIP1, TIF2, NCoA-2) and SRC3 (p/CIP, RAC3, ACTR, AIB1, TRAM-1). All three SRC members share a common domain structure with a highly conserved basic helix-loop-helix-Per/Arnt/Sim (bHLH-PAS) domain and two activation domains in the N-terminal domain, a NR box

that binds NR AF-2 domain in the center and a HAT activity mapped to the C-terminal domain of SRC-1 and SRC-3 (Xu et al. 2003). However the C-terminal HAT activity is weak and is enhanced by the binding of cointegrators cAMP response element binding protein (CREB)-binding protein (CBP) or its homolog p300 and their interacting protein, CBP/p300-associated factor (pCAF) (McManus et al. 2001). All three proteins are acetyltransferases that interact and acetylate both free histones and nucleosome histones with substrate preferences for histones H3 and H4.

Additional chromatin modification involves an ATP dependent chromatin remodelling, for chromatin structure recognition and destabilization of histone-DNA contact. Such coactivators include yeast SWI/SNF and mammalian BRG1 complexes, which directly or indirectly remove or alter repressive chromatin structure. This is essential for NR transcriptional activation as observed by Fryer et al who studied the ability for GR to activate transcription from a mouse mammary tumour virus (MMTV) promoter and showed that interaction between a BRG1-containing complex and GR was required when promoter was stably integrated into the chromosomal DNA, but not when promoter was assessed in transiently transfected cells (Fryer et al. 1998).

NR sequential model of transcriptional initiation suggests that subsequent to acetylation by the HAT protein complex, the protein complex is dissociated. After HAT complex dissociation NRs will recruit and bind the mediator complex that in turn will recruit the RNAPII holoenzyme to initiate gene transcription. Biochemical purification strategies purified TR-associated proteins (TRAPs), which interacted with TR in a ligand dependent manner (Ito et al. 2001). In addition, vitamin D receptor (VDR) interacting proteins (DRIPs) were also isolated using VDR in a similar purification assay, and modestly enhances the activity of VDR in a ligand-dependent manner. Generally both TRAP and DRIP are multi-protein complexes with at least nine, and up to 16 proteins, that interact with NR AF-2 domains. The TRAP/DRIP complex is also very similar to the yeast mediator (MED) complex. The MED complex forms together with suppressor of RNA polymerase (SRB) proteins the SRB/MED containing cofactor (SMCC) complex, and associates with the large subunit of RNAPII (Ito et al. 2001). Interestingly, other human MED-like, SRB interacting complexes, such as activator-recruited cofactor (ARC) are similar in composition to TRAP/DRIP complexes, suggesting that TRAP/DRIP complexes potentially recruit RNAPII to target promoters (Naar et al. 1999). Thus, TRAP/DRIP complexes are believed to function as bridging factors by connecting NR with the basal transcription machinery, but additional functions for these complexes must be considered (Lewis et al. 2003).

1.5.2 Transcriptional repression

In addition to NRs functioning as activators of gene transcription, they are also able to repress transcription by binding to corepressors. Corepressors negatively regulate gene transcription by repressing NRs in their apo-form or steroid receptors in an antagonist liganded manner. They repress by binding the coregulator binding groove via their CoRNR boxes, which are similar to LXXLL motifs and thereby prohibiting binding of coactivators. Steroid receptor antagonist ligands induce unique helix 12 conformation that favour corepressor binding but are distinct from conformations in absence of ligand or in response to agonist ligand binding.

Contrary to coactivators, corepressors recruit histone deacetylase (HDAC) to reverse the acetylation of histones and thus repress transcription. Such corepressors are NCoR and silencing mediator for retinoid and thyroid hormone receptor (SMRT). SMRT (TRAC2) and NCoR (RIP-13) were the first corepressors identified for NRs. They are encoded by two distinct loci, but have very similar structural identity in the N- and C-terminal domains. In the N-terminal there are three to four distinct transcriptional repression domains (RDs), which function as docking surfaces for corepressor complexes including HDACs and Sin3. The recruitment of HDACs and their supporter proteins result in inhibition of transcription by modification of the chromatin. HDAC3 for example directly interacts with SMRT/NCoR and switches on the enzymatic activity of deacetylase to repress transcription. This recruitment will result in the removal of acetyl groups from the histones and the condensation of chromatin and inactivation of gene transcription. SMRT and NCoR also make direct contact with the basal transcriptional machinery. The C-terminal of SMRT and NCoR are composed of two and three CoRNR boxes respectively, that are involved in NR/corepressor specificity. Collectively this evidence shows that SMRT and NCoR function as intermediates that tether together NRs, through their C-terminal, and HDACs, through their N-terminal (Privalsky 2004).

However, in addition to the above-mentioned classical corepressors, there are a couple of corepressors that bind agonist liganded NRs through their LXXLL motif, such as ligand-dependent corepressor (LCoR) and RIP140 both functioning as molecular scaffolds for repression proteins (White et al. 2004). In this thesis I investigated the mechanism of corepressor RIP140 for corepression of GR and LXR.

1.6 RECEPTOR INTERACTING PROTEIN 140

RIP140 was initially identified as a coregulator of ER in breast cancer cell lines. RIP140 transactivated ER by binding ER AF-2 domain and enhancing its activity in presence of estrogen (Cavailles et al. 1995). Later, subsequent work defined RIP140 as a corepressor of agonist liganded NRs. RIP140 has been shown to interact with a number of NRs, including ER, GR, RAR, RXR, VDR, TR, AR, PPAR, LXRα and β, and orphan receptors steroidogenic factor 1 (SF-1) and testicular receptor 2 (TR-2) (L'Horset et al. 1996; Ikonen et al. 1997; Masuyama et al. 1997; Lee et al. 1998; Miyata et al. 1998; Treuter et al. 1998; Subramaniam et al. 1999; Wiebel et al. 1999; Windahl et al. 1999; Sugawara et al. 2001; Mellgren et al. 2003; Albers et al. 2006). RIP140 also interacts with transcriptional factors such as the aryl hydrocarbon receptor and c-jun, and the adapter protein 14-3-3 (Kumar et al. 1999; Zilliacus et al. 2001; Teyssier et al. 2003). The human RIP140 is mapped to chromosome 21q11.2 and is ubiquitously expressed as a 140kDa large protein (Cavailles et al. 1995). RIP140 mRNA levels are upregulated by estrogen and retinoid in breast cancer cells, by androgens in prostate cancer cells and by estrogen-related receptor α (ERRα) during adipogenesis (Kerley et al. 2001; Augereau et al. 2006; Carascossa et al. 2006; Nichol et al. 2006).

1.6.1 RIP140 repression domains and NR box motifs

RIP140 was first proposed to repress NR action by competing with the binding of SRC coactivators and cointegrators p300/CBP to the liganded receptor (Treuter et al. 1998). But then, RIP140 was shown to have an intrinsic repression activity mediated by binding of HDACs and the corepressor C-terminal binding protein (CtBP) (Wei et al. 2000; Vo et al. 2001). HDAC represses transcription by deacetylating histone groups to induce compact chromatin and strong histone-DNA interaction (de Ruijter et al. 2003). CtBP is a member of large histone modification complex and functions as a corepressor through unclear mechanisms (Turner et al. 2001).

1.6.1.1 RIP140 intrinsic repression

We mapped RIP140 domains containing an intrinsic repression activity (Figure 4). This was analyzed by fusing RIP140 to GAL4 DBD to study the induced repression of a reporter gene regulated by GAL4 binding sites. We identified several repression

domains (RD), the N-terminal (aa 1-472) and middle and C-terminal domain (aa 431-1158) of RIP140 had strong intrinsic repression activity. Weak intrinsic repression was also mediated by the short N-terminal (aa 1-281) and C-terminal domains (aa 747-1158) (paper I). We also examined the significance of CtBP binding for RIP140 intrinsic repression, since both N-terminal and middle and C-terminal domains of RIP140 contain a previously identified binding site for CtBP (PIDLS) (Vo et al. 2001). Interestingly we found that mutation of the CtBP binding site did not affect RIP140 intrinsic repression activity, which indicated that CtBP is dispensable for the intrinsic repression activity of RIP140. In continuance others classified four RDs in RIP140, using a slightly different assay in which GAL4 DBD fused RIP140 inhibits the transactivation activity of LexA-fused VP16. They showed that two fragments, N-terminal (aa 1-528) and C-terminal (aa 535-1158) of RIP140 contained intrinsic repression activity. Further analysis of the N- and C-terminal showed intrinsic repression in N-terminal residues 78-333 and 410-700 and in C-terminal residues 737-885 and 1118-1158, identifying four intrinsic RDs in RIP140, RD1-4 (Christian et al. 2004). Residues 78-303 have earlier been reported to bind class I HDACs (Wei et al. 2000). Further studies identified the region 27-199 as the main interaction site for both HDAC class I and II (Castet et al. 2004). However treatment with the HDAC inhibitor trichostatin A (TSA) had no effect on the intrinsic repression activity on full length RIP140 (Castet et al. 2004; Christian et al. 2004). In contrast, the HDAC inhibitor could reverse the repression activity of RD2 and to some extent RD3 and RD4 (Christian et al. 2004).

Studies on CtBP binding sites showed that in addition to the first identified CtBP binding site (PIDLS) additional sites where found. The sites PIDLS and PINLS in RD2 and VRLDS in RD4 were shown to bind CtBP. However the sites PIDLS and PINLS were the main mediators of the repressive effect (Christian et al. 2004). Confirming our data, the mutation of the CtBP binding sites in the full length RIP140 did not have a major effect on the intrinsic repression activity (Castet et al. 2004). The repression activity by different RDs was observed to vary in different cell types. Especially RD4 was less active in ovarian cells compared to kidney cells (Christian et al. 2004).

In summary RIP140 contains several intrinsic repression domains. CtBP can mediate the repression of RD2, whereas HDAC activity contributes to the repression by RD2, 3 and 4. However neither CtBP nor HDAC are indispensable for the intrinsic repression activity of the full length RIP140.

1.6.1.2 Corepression by RIP140

We performed corepression studies to analyse the mechanism of how RIP140 represses the receptor mediated transactivation. This was performed by analysing the effect of RIP140 on a HRE regulated reporter gene in the presence of the receptor. We found that the C-terminal domain of RIP140 (aa 747-1158) is important for corepression of GR (paper I) and LXR (paper II). Corepression of GR is mainly mediated by the C-terminal (aa 747-1158) but the best corepression through RIP140 is in residues 431-1158 that also contains the CtBP binding site. Deletion of the CtBP binding site reduced the repression. Analysis of the role of the two NR boxes (NR8 and 9) and the NR-box like motif (NR10) in the C-terminal for the repression, showed that mutation of NR8 alone or in combination had the most apparent effect on RIP140 corepression of GR activity. In summary, RIP140 corepression of GR requires CtBP binding and the C-terminal NR8 (paper I).

RIP140 repressive action on LXR activity mainly resides in the C-terminal domain (aa 747-1158). The short N-terminal (aa 1-281) and middle part (aa 431-745) have also repressive potential on LXR mediated transcription. Interestingly, in contrast to GR, the NR-box like motif (NR10) in the C-terminal is important for the corepression activity. This was observed using RIP140 C-terminal NR box mutants, where mutation of NR10 alone or in combination with the C-terminal NR boxes had significantly reduced repressive effect of the C-terminus. Studies on the relevance of the different fragments of the C-terminal domain of RIP140 showed that deletion of residues 747-862 also reduced repression, indicating that both NR10 and the integrity of the C-terminal domain of RIP140 are needed for full repressive activity (Paper II).

RIP140 was shown to corepress ER activity using both N-terminal (aa 1-528) and C-terminal domains (aa 535-1158) (Christian et al. 2004). Mutation of the CtBP binding site and CtBP null cells showed that CtBP binding to RIP140 was not necessary for RIP140 corepression of ERα activity. Studies using HDAC inhibitor showed that HDAC enzymatic activity is not required for RIP140 repressive action on ER transcription (Castet et al. 2004).

RIP140 was found to be a strong repressor of ligand induced AR activity. Further mapping of the repressive domains in RIP140, showed RIP140 C-terminal residues 917 to 1158 exhibited strong repression activity. Mutation of the CtBP binding sites on RIP140 had no effect on RIP140 repression on AR, however inhibition of HDAC activity demonstrated reduced repression by RIP140 on ligand induced AR (Carascossa et al. 2006).

RIP140 corepresses RAR/RXR activity in a retinoic acid induced manner, however repression is impaired at increasing levels of HDAC inhibitor, suggesting a role for HDAC in RAR/RXR repression by RIP140 (Wei et al. 2000). RIP140 interacts with and corepresses retinoic acid induced RAR/RXR by the C-terminal NR-box like motif (NR10). Retinoic acid induces a complex formation between RIP140 and HDAC to the RAR/RXR dimer, through the RIP140 NR-box like motif (NR10) (Wei et al. 2001). HDAC mediated repression of RIP140 on RAR/RXR is regulated by posttranslational modification such as phosphorylation, methylation and conjugation. Phosphorylation and conjugation with pyridoxal 5´phosphate (PLP), the active form of vitamin B6, enhanced HDAC binding to RIP140 and thereby RIP140 corepression of RAR/RXR activity (Gupta et al. 2005; Huq et al. 2007). In contrast methylation of RIP140 reduces binding of HDAC and RIP140 repressive action on RAR/RXR activity (Mostaqul Huq et al. 2006).

In summary corepression of GR, LXR, AR and RAR/RXR is mainly mediated by the C-terminal domain of RIP140. RIP140 repression of ligand-activated GR is partially mediated by the recruitment of CtBP, while the corepression of RAR/RXR and AR is partially mediated by the enzymatic activity of HDAC.

1.6.1.3 RIP140 NR box motifs

RIP140 consists of nine NR box motifs and one C-terminal NR-box like motif (NR10) (Heery et al. 1997) (Figure 4). Different NR-boxes have different affinity for different NRs. We have identified the NR box 8 of RIP140 to be important in repressing GR activity, but others detected using a mammalian two hybrid assay that GR-LBD strongly interacted with RIP140 NR box 6 (Hu et al. 2006; paper I). Others have also found TRβ to have preference for NR boxes 3, 5 and 8, ERα and RARα for NR box 6 and MR for NR boxes 4 and 6 (Heery et al. 2001; Moore et al. 2004; Hultman et al. 2005). Interestingly we found the NR-box like motif (NR10) is required for the binding of RIP140 to LXRβ, whereas LXRα requires additional elements for strong binding (paper II). Moreover, we found that the NR-box like motif (NR10) and the integrity of the whole C-terminal domain are needed for RIP140 repression of LXR activity. The NR-box like motif (NR10) was also observed by others to be involved in repression of RAR/RXR activity (Wei et al. 2001).

1.6.2 RIP140 posttranslational modification

Interesting evidence implies that posttranslational modification of RIP140 such as phosphorylation and acetylation are essential for controlling RIP140 activity. Phosphorylation of RIP140 was first observed by Zilliacus et al when showing that ubiquitous factor 14-3-3 interaction with RIP140 was impaired after alkaline phosphatase treatment of RIP140 (Zilliacus et al. 2001). Phosphorylation of RIP140 was later identified on nine serine and two threonine residues (Huq et al. 2005). RIP140 phosphorylation by mitogen-activated protein kinase (MAPK) on threonine residues 202 and 207 enhanced RIP140 repression partially by regulating HDAC3 interaction (Gupta et al. 2005). RIP140 acetylation by CBP at lysine 446 has been found to inhibit CtBP interaction (Vo et al. 2001). Recently additional acetylation sites have been identified at the N-terminal and central region of RIP140, which regulate RIP140 nuclear translocation (Huq et al. 2005). RIP140 is also methylated by protein arginine methyltransferase 1 at several residues (Mostaqul Huq et al. 2006). Methylation of RIP140 suppresses RIP140 repression by preventing HDAC3 binding to RIP140 and decreasing RIP140 nuclear translocation. RIP140 can also be conjugated by pyridoxal 5´phosphate (PLP), which is the biological active form of vitamin B6 (Huq et al. 2007). PLP functions as a cofactor in amino acid metabolism and is a modulator of steroid hormone receptor function (Allgood et al. 1992). PLP conjugation results in enhanced RIP140 repression of RARβ transcription by increased HDAC3 interaction (Huq et al. 2007).

1.6.3 RIP140 intracellular localization and NR redistribution

Studies on the cellular compartmentalization of proteins entail a lot about the function of proteins. We found that RIP140 is localized in the nucleus in distinct small foci and we mapped the foci targeting domain to a 40 amino acid domain (aa 431-472). This domain overlaps with CtBP binding site, but we showed that CtBP binding to RIP140 is not required for RIP140 foci distribution. Interestingly, when coexpressed with GR, RIP140 and GR translocated to distinct large foci, in presence of GR ligand (paper I). Coexpression of RIP140 and LXR in absence of LXR ligand moved RIP140 from small foci to an even localization with LXR. Upon ligand binding RIP140 and LXR obtained similar intracellular redistribution as the GR/RIP140 complex (paper II). Others have also identified related redistribution of RIP140 with AR in presence of

ligand (Carascossa et al. 2006). The significance of this redistribution is still unclear, however it may imply a novel repression mechanism for RIP140.

1.6.4 RIP140 biological significance

RIP140 knockout (RIPKO) mice demonstrated the physiological relevance of RIP140 in female fertility and fat accumulation (White et al. 2000; Leonardsson et al. 2004). Depletion of RIP140 results in oocyte ovulation defects and female infertility and in fat depletion, increased energy dissipation, mitochondrial uncoupling and reduced lipogenesis. The role of RIP140 in energy homeostasis is mediated by regulating genes involved in energy storage and expenditure. RIPKO mice had high energy expenditure, high expression of uncoupling protein 1 (Ucp1) and carnitine palmitoyltransferase 1b (Cpt1b), and high levels of total fatty acid oxidation (Christian et al. 2005). Powelak et al recently identified RIP140 as a repressor of carbohydrate and fatty catabolism in RIPKO mice. They showed that these mice exhibited higher glucose tolerance and insulin responsiveness when fed a high fat diet. This was due to RIP140 repressive action of genes involved in cellular respiration, citric acid cycling, glycolysis and hexose uptake (Powelka et al. 2006).

1.7 POLYAMINES AND POLYAMINE-MODULATED FACTOR 1

Among the NR interacting proteins studied in this thesis is the polyamine regulated protein PMF-1. Polyamines, putrescine, spermidine and spermine, are polycationic compounds present in millimolar concentrations in living cells and are essential for cell growth and differentiation (Igarashi et al. 2000; Thomas et al. 2001). Their intracellular levels are regulated by synthesis, degradation, uptake and excretion (Thomas et al. 2003; Gerner et al. 2004). In the cell, positively charged polyamines are metabolized via a highly regulated pathway and interact electrostatically with negatively charged DNA, RNA and proteins. On DNA they increase DNA melting temperature, stabilize the DNA structure and induce conformational changes to DNA. PMF-1 is a cofactor regulated by polyamines and is expressed in many human tissues like the heart, skeletal muscle, kidney and liver. The mouse PMF-1 (mPMF-1) shares almost 80% homology with human PMF-1 (hPMF-1) and encodes for two alternatively spliced mRNAs, PMF-1 long (mPMF-1L) and PMF-1 short (mPMF-1S) (Wang et al. 2001). Polyamines are reported to induce PMF-1 heterodimerization with NF-E2 related factor 2 (Nrf-2) and

binding to polyamine responsive element (PRE) to stimulate spermidine/spermine N^1-acetyltransferase (SSAT) gene transcription (Wang et al. 1999; Wang et al. 2001; Wang et al. 2001). SSAT is a rate-limiting enzyme in the catabolic pathway of polyamine metabolism, catalyzing acetylation of spermidine/spermine for later cleavage by polyamine oxidase enzymes. Identification of other PMF-1 interacting proteins and existence of other genes that have functional PREs are currently being elucidated. The human homologue to subunit 7 of the Arabidopsis COP9 signalosome, CSN7, functionally interact with PMF-1 and competes with it for binding to Nrf-2 (Wang et al. 2002). COP9 signalosomes are highly conserved proteins and involved in intracellular signalling and transcriptional control, suggesting PMF-1/CSN7 interaction to have a potential effect in PRE-regulated SSAT gene transcription. Polyamine regulated eukaryotic initiation factor 4E binding protein 1 (4E-BP1) gene has a PRE at the 5´flanking region, where PMF-1/Nrf-2 heterodimer binds in a polyamine dependent manner. The role of 4E-BP1 in the regulating protein translation in a polyamine dependent manner, implies a role for PMF-1 in protein translation to regulate cellular growth and differentiation (Stephenson et al. 2006). PMF-1 is also suggested to be a member of a kinetochore associated multiprotein complex, to mediate chromosomal segregation during mitosis (Obuse et al. 2004; Meraldi et al. 2006). Together, some physiological aspects and mechanisms of PMF-1 have been presented, but still much more remains unclear.

1.8 DNA REPLICATION

In my thesis I have studied the protein GANP, which is involved in DNA replication.

1.8.1 Cell cycle

Eukaryotic cells undergo a particular sequence of events (cell cycle) starting from the "birth" until their division into two cells. The cell cycle is divided into four phases, the G_1, S, G_2 and M phase. The mammalian cell cycle consists of two gap phases (G) in which cells remain in resting state. G_1 is the entry point into the cell cycle and is the only phase regulated by growth factors. In S phase DNA is replicated, in G_2 cells prepare for division and M phase cells will undergo mitosis. Some cells normally remain in a quiescent state for their whole life, but external stimulation can cause them to reenter into the cell cycle. There are three checkpoints in the cell cycle to ensure normal cell cycle progression. The first one in late G_1, where if passed the cell can

progress through the rest of the cell cycle and duplicate. The second check point is the S phase, where damaged DNA can be repaired and the last check point is in the transition between G_2 and M phase, where cells undergo a last check up to ensure that they have fully replicated and undamaged DNA. The progression throughout the cell cycle is regulated by various molecules e.g. cyclins, CDK and Dbf4-dependent kinases (DDK). The length of time for the mammalian cell cycle varies depending on the cell type, but in average takes about 24 hours (Harper et al. 2005).

1.8.2 G_1 phase and DNA replication initiation

DNA replication occurs once per cell cycle and is initiated at defined sequences called origins of replication. Here, initiator proteins will form a pre-replication complex (pre-RC) and unwind the double helix to make the DNA accessible for the DNA replication machinery. DNA replication is a bidirectional process, where two replication forks are formed at each origin and involves DNA polymerase and its accessory proteins, which are enzymes capable of replicating DNA.

The pre-RC is assembled in the G_1 phase to the origin. The pre-RC include origin recognition complex (ORC), cyclins Cdc6 and Cdt1 and minichromosome maintenance (MCM) proteins (Figure 3). This complex will limit the replication to once per cell cycle and initiate DNA replication (Lei et al. 2001).

ORC is a multi-subunit complex that distinctly recognizes origins of replication and is capable of initiating DNA replication. All eukaryotic ORCs bind DNA in presence of ATP and this ATP dependence is essential for Cdc6 origin binding. There are four identified human ORC subunits (ORC2-5), all of which are involved in DNA replication. Human ORC have also been involved in interacting with HAT complex to make the origin site accessible to replication. However, ORC function is not limited to replication initiation but has also been implicated in heterochromatin assembly, histone modification, chromosome condensation and transcriptional silencing.

Cdc6 is recruited by ORC to bind chromatin in an ATP dependent manner during G_1-phase and is in turn essential for MCM chromatin loading. At the entry of cells into S-phase, Cdc6 N-terminal becomes phosphorylated by CDK promoting the exposure of a nuclear export signal (NES) at the C-terminus and translocation of Cdc6 to the cytoplasm. Cdt1 is part of the pre-RC and binds the ORC prior to the binding of Cdc6 to the chromatin, but its molecular contribution is unclear (Da-Silva et al. 2007).

MCM proteins are paralogues that share a role in DNA replication, by being a part of the pre-RC and limiting the replication to once per cell cycle. The MCM-complex is recruited by the ORC, Cdc6 and Cdt1 to form the pre-RC in late G_1 phase, where after a series of events take place to convert the MCM-complex to an active helicase and to initiate replication initiation. More recently the role of MCM protein in replication elongation, transcription and chromatin remodelling has also been proposed. The family of MCM proteins consist of six highly conserved subunits (MCM2-7), only found in eukaryotes, that associate in a hexamer with a 1:1:1:1:1:1 stoichiometry. They are a distinct subgroup of the AAA ATPase family that have several cellular functions and generally form large ATP-dependent heterohexamer complexes. MCM proteins have also MCM boxes, zinc finger binding motifs and NLS, which are all important for MCM protein function. MCM proteins are constantly located in the nucleus surrounding the chromatin. During G_1/S interface MCM proteins assemble onto specific replication origin sites scattered around the chromatin by ORC, Cdc6 and Cdt1 recruitment.

To initiate replication, CDK and DDK control the origin firing. CDK is associated with several components of the pre-RC and directly interact and activate ORC and Cdc6, indicating their relevance in replication initiation. DDK requires the association of MCM proteins to the chromatin. The phosphorylated MCM proteins recruit in turn Cdc45, which interact with chromatin bound MCM proteins and initiate DNA replication by unwinding DNA and recruiting DNA polymerase.

Together kinases CDK and DDK assemble during S-phase to trigger specific steps in the transition to replication, especially those leading to the Cdc45 binding to chromatin and DNA unwinding to achieve origin firing (Lei et al. 2001).

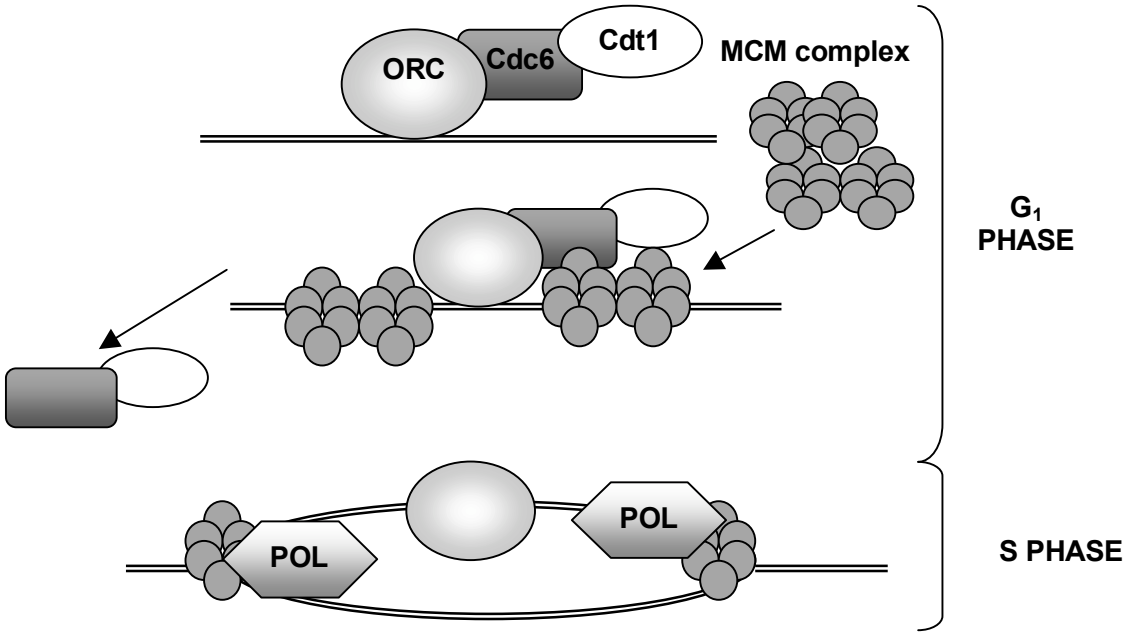

Figure 3. Schematic illustration of the initiation of DNA replication, including DNA polymerase (POL).

1.9 GERMINAL CENTER-ASSOCIATED NUCLEAR PROTEIN

GANP is a 210kDa large protein that is expressed in various kinds of cells such as the heart, brain, lung, placenta, liver, skeletal muscle, kidney and pancreas. Furthermore it is found in the immune system in the spleen, lymph nodes and thymus (Abe et al. 2000; Kuwahara et al. 2000). One highly studied area of the immune system is the antigen-driven B-cell. These B-cells are derived from immunization with T cell-dependent antigens. The B-cells develop into large centroblasts after immigrating into germinal center dark zone. Here they rapidly proliferate for clonal expansion and further differentiate into small centrocytes that abruptly stop proliferating. One protein denoted in regulating B-cell transition is GANP, since it is up regulated in centrocytes of the germinal center area suggesting it to be involved in cell progression (Abe et al. 2000; Kuwahara et al. 2000). Recently studies showed increased expression of GANP in human B-cell lymphomas and that abnormal levels of GANP induce malignant B-cells (Fujimura et al. 2005).

GANP is a large protein and several functional domains have been implicated. A region in the N-terminal domain of GANP is similar to the DNA primase component p49 and has DNA primase activity that is stimulated by the T cell dependent antigen (Kuwahara

et al. 2001). The middle domain of GANP has homology to yeast SAC3 protein that is associated with the nuclear pore complex and functions in mRNA export (Lei et al. 2003). SAC3 is associated via the Sus1 protein with the SAGA histone acetylase complex (Rodriguez-Navarro et al. 2004). The SAGA complex and acetylation in general has been shown to be an important regulator of gene transcription. C-terminal domain of GANP is also shared by an 80kDa large protein, MCM3 associated protein (MCM3AP), which is a RNA splicing variant of GANP (Takei et al. 1998; Abe et al. 2000). MCM3AP was originally identified in a yeast two-hybrid screen as a protein interacting with MCM3 (Takei et al. 1998). MCM3AP has been shown to acetylate MCM3 and inhibit initiation of DNA replication via the interaction with MCM3 (Takei et al. 2001; Takei et al. 2002).

2 AIMS OF THE THESIS

Protein-protein interaction studies have identified several NR interacting proteins that can modulate NR signalling. The aim of this thesis is to characterize three nuclear receptor interacting proteins: RIP140, PMF-1 and GANP and their role in hormone signalling.

The specific aims of the thesis are to:

RIP140

- Characterize functional regulation of GR and LXR signalling by RIP140.
- Study the role of intranuclear localization in corepression.
- Study the repressive action of RIP140 domains.

PMF-1

- Characterize the interaction between GR, RIP140 and PMF-1.
- Elucidate the significance of PMF-1 interaction with GR and RIP140 on GR activity.

GANP

- Characterize the interaction between GR and GANP.
- Study the functional significance of the interaction in glucocorticoid signalling.
- Study the effect of glucocorticoids on DNA replication and cell cycle progression.

3 RESULTS AND DISCUSSION

Protein-protein interactions are essential in many biological pathways and are also attractive for drug discovery. We performed yeast two-hybrid screens to identify novel NR interacting proteins and identified RIP140, PMF-1 and GANP. Together in these papers we have defined the significance of the receptor-protein interactions.

3.1 Paper I and II: MOLECULAR BASIS OF REPRESSION BY RIP140 ON GR AND LXR MEDIATED GENE TRANSCRIPTION

NR mediated gene transcription involves the recruitment of a large number of coregulators. RIP140 is a coregulator that interacts and inhibits many ligand activated NRs. Such NRs include GR and LXR, which may recruit RIP140 to regulate glucose metabolism and energy homeostasis, respectively. In paper I and II, I have characterized the molecular mechanism of repression by RIP140 on GR and LXR activity.

RIP140 is localized in small foci in the nucleus

We studied the cellular localization of RIP140, and found it localized in small foci in the nucleus. When analyzing colocalization of RIP140 with previously described nuclear domains, no colocalization of RIP140 with promyelocytic leukemia (PML) nuclear bodies, that consist of PML proteins involved in transcriptional regulation was revealed (Zhong et al. 2000). However a partial colocalization of RIP140 with SMRT was observed. This indicated that RIP140 foci are to some extent related to MAD bodies that include SMRT proteins, but the MAD bodies are larger in size compared to RIP140 foci. Therefore the localization of RIP140 in small foci is distinct from other nuclear domains. In continuance we have mapped the foci targeting domain to a 40 amino acid sequence in RIP140, aa 431-472 (Figure 4). The foci targeting domain overlapped with the binding sequence for the corepressor CtBP. Previous studies had shown that a protein phosphatase 1 regulator, NIPP1, is targeted to nuclear domains called speckles through interaction with a splicing factor that is localized in speckles, suggesting that the subnuclear targeting domain can function by protein-protein interaction (Jagiello et al. 2000). But although we found that the foci-targeting domain

overlapped the CtBP binding domain, interaction with CtBP was not necessary for foci localization (paper I).

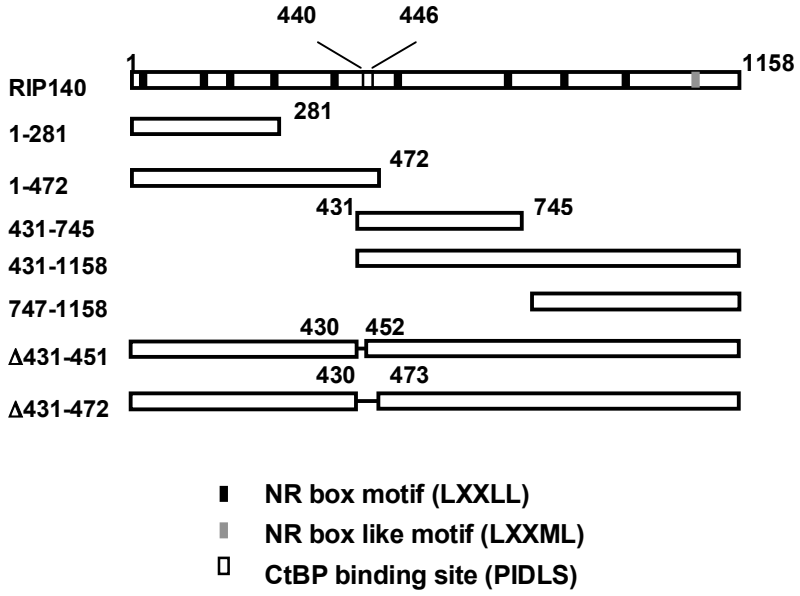

Figure 4. Schematic representation of RIP140.

RIP140 contains intrinsic repression domains

When characterizing the intrinsic repression activity we fused GAL4 DBD to RIP140 short N-terminal (aa 1-281), N-terminal (aa 1-472), middle and C-terminal (aa 431-1158) and C-terminal (aa 747-1158) (Figure 4). We found that aa 1-472 and aa 431-1158 of RIP140 had the same intrinsic repression as the RIP140 wild type. But, aa 1-281 and aa 747-1158 had weak intrinsic repression. Since both aa 1-472 and aa 431-1158 contain a CtBP binding site and RIP140 foci-targeting domain, we investigated the relevance of these domains in RIP140 intrinsic repression. The RIP140 deletion mutants RIPΔ431-451, a CtBP none binding mutant, and RIPΔ431-472, a mutant which is not targeted to foci, are fully able to repress. Together, RIP140 intrinsic repression activity involved RIP140 N-terminal and C-terminal domains and is dispensable from CtBP binding and RIP140 foci targeting (paper I).

RIP140 interacts with GR and LXR

It is previously understood that RIP140 interacts and represses several NRs. Here we elucidated the specific RIP140 determinants for the repressive effect on GR and LXR

subtypes α and β. We found that RIP140 interacts with GR and LXR subtypes in a ligand dependent manner.

When analyzing GR interaction with RIP140, we found endogenous RIP140 to interact with GR. We also found GR-LBD to interact with all domains of RIP140 presented above, except for the middle domain (aa 431-745) (paper I).

We performed a yeast-two hybrid screen using ligand activated LXR as bait and found RIP140 to interact with LXRα and β. The interaction was then verified and the results showed that the binding of LXRα to RIP140 is only somewhat enhanced by ligand, whereas LXRβ interaction is strongly ligand regulated. RIP140 C-terminal (aa 560-1158) could bind the LBD of both LXR subtypes, but in addition bound weakly to LXRα N-terminal. When mapping binding sites for LXR on RIP140 C-terminal, we could show that LXR subtypes had different preferences for RIP140 C-terminal domain. LXRβ only requires RIP140 NR-box like motif (NR10) for interaction with RIP140, while LXRα needs more than one NR box to obtain sufficient binding to RIP140. In summary, RIP140 interaction with LXR subtypes mainly requires LXR-LBD, but also LXRα N-terminal had a weak interaction. In addition, the LXR subtypes have different preference for RIP140 C-terminal domain (paper II).

RIP140 corepresses GR and LXR

To understand the mechanism of corepression by RIP140 on GR and LXR, the effect of different fragments of RIP140 on GR and LXR transactivation was studied. We found that it is mainly the C-terminal domain (747-1158) of RIP140 that repress GR. Although to get the best repression, RIP140 domain aa 431-1158, also containing the CtBP binding and foci-targeting domains, was needed. Mapping the relevance of CtBP binding and foci-targeting using deletion mutants, showed that CtBP binding, but not the foci targeting domain, contributed to the repression. Since that C-terminal domain was a powerful repressor alone, we wanted to study the significance of the two NR boxes (NR8 and 9) and NR-box like motif (NR10) in RIP140 repression by using different mutations of the NR-boxes. This showed that mutation of all motifs reduced RIP140 repression activity, but it was mutation of NR8 alone or in combination that had the most apparent effect. In summary, RIP140 corepression of GR requires CtBP binding and the C-terminal NR8 (paper I).

To gain molecular insight into the repressive effect of RIP140 on LXR, deletion mutants were used showing that RIP140 repressive action mainly resides in the C-terminal domain (aa 747-1158). Also the short N-terminal (aa 1-281) and middle part (aa 431-745) have repressive potential on LXR medicated transcription. This is similar to GR repression by RIP140. But in contrast to GR that requires NR8 for full repression, repression of LXR is mediated by the NR-box like (NR10) motif and the integrity of the C-terminal domain. This was observed using RIP140 C-terminal NR box mutants, where mutation of NR10 alone or in combination with NR8 and/or NR9 had significantly reduced repressive effect. However similar mutations of NR8 and NR9 had no effect on repression by RIP140 C-terminal. In addition studies on the relevance of the different fragments of the C-terminal domain of RIP140 showed that fragments containing NR10 repressed well, and mutants not containing NR10 had no repression activity. Also deletion of residues 747-862 gave reduced repression, showing that both NR10 and the integrity of the C-terminal domain of RIP140 are needed for full repressive activity (paper II).

GR, LXR and ER subnuclear distribution in presence of ligand

NR subnuclear localization is regulated by ligand as well as formation of protein complexes. Analysis of the subnuclear localization of the GR, LXR subtypes and ERα in presence and absence of agonist ligand was performed. The result showed that nuclear localization of both LXR subtypes is ligand independent. Mapping of the different domains showed that neither N-terminal nor LBD are needed for LXR nuclear localization. This suggests a possible role for the DBD and hinge region in nuclear localization, which supports a recently identified NLS in hinge region (Prufer et al. 2007). Additionally, we found that the N-terminally deleted LXR was distributed in foci in the nucleus, however the significance of this distribution is unclear (paper II). ERα was localized in the nucleus independent of ligand. ERα foci distribution in presence of ligand has been reported by other groups (Htun et al. 1999), however we observed no ligand induced change in the nuclear distribution of ERα (our unpublished data). Studies on the nuclear localization of GR, showed all GR domain; DBD, LBD and DBD-LBD are evenly distributed in the nucleus in presence of ligand, however a weak cytoplasmic localization of GR-LBD was also observed (paper I).

Redistribution of RIP140 with ligand activated GR, LXR and ER

Thereafter we analyzed the intranuclear localization of the repressive complex between RIP140 and GR, LXR and ER, respectively. We found that upon ligand binding, GR and RIP140 are redistributed to large nuclear domains distinct from the RIP140 foci. The redistribution requires regions of RIP140 with a corepressor activity as well as GR DBD (paper I). RIP140 coexpression with LXR in absence of ligand showed redistribution of RIP140 from small foci to even redistribution and upon ligand binding both LXR and RIP140 redistributed to large foci similar to GR and RIP140. This suggests that RIP140/LXR colocalize independent of ligand and that ligand only changes RIP140/LXR complex subnuclear localization. We also found that LXR-LBD is necessary for colocalization whereas helix 12 in LXR-LBD is not required for RIP140 colocalization. This is interesting, since it is reported that coactivators require NR helix 12 to bind NRs, whereas the helix 12 is not required for the corepressors NCoR and SMRT (Nagy et al. 2004). These results suggest that RIP140 does not require NR helix 12 to interact with LXR (paper II). In finding that the colocalization of RIP140 and LXRα and β was independent of the presence of helix 12 we wondered whether this was specific for LXR. To understand this, colocalization between RIP140 and full length ERα (ERαWT, aa 1-596) or ERα helix 12 mutant (ERαΔH12, aa 1-536) was studied. RIP140 and ERαWT colocalized in the presence of ligand in diffuse large nuclear domains in 50% of the cells studied (Figure 5A, panels a-f, our unpublished data), In contrast, no colocalization of RIP140 with ERαΔH12 in nuclear domains was observed (Figure 5B, panels g-i), indicating that the very C-terminal part of ERα contributes to the subnuclear localization of the RIP140/ERα complex (our unpublished data). In summary, the results on the role of helix 12 in LXR and ERα for colocalization, might suggest that the RIP140 protein complexes formed with the two receptors differ because of RIP140 differences in binding the receptor subfamilies.

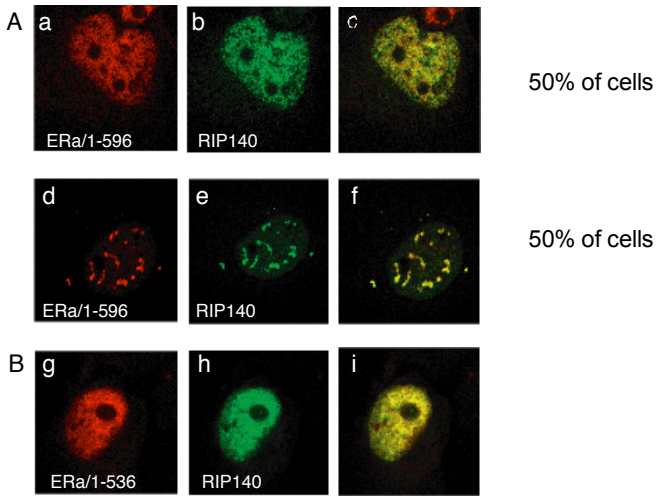

Figure 5. Intracellular colocalization of ERα and RIP140. COS-7 cells were transfected with ERα/1-596 (ERα WT) or ERα/1-536 (ERα ΔH12) and GFP-RIP140. The cells were treated with 10^{-8} M E_2 overnight before visualization of the expressed proteins using confocal microscopy. Panels a, d and g show the localization of ERα WT or ΔH12 proteins in red and panels b, e and h show the localization of RIP140 in green in the same cells. Panels c, f and i show the merged image, where colocalization of proteins is in yellow. RIP140 and wild type ERα colocalized in diffuse large nuclear domains in 50% of the cells (panels d-f) and were evenly distributed in 50% of the cells (panels a-c). RIP140 and ERα ΔH12 were evenly distributed in 100% of the cells studied (panels g-i).

In summary the results show that the formation of a repressing complex between GR and LXR and RIP140 correlates with the relocalization of both proteins within the nucleus to specific large nuclear domains. Collectively we suggest that RIP140 represses in multiple mechanisms, including forming a repressive complex and intranuclear redistribution.

3.2 PAPER III: POLYAMINE-MODULATED FACTOR 1 REPRESSES GR ACTIVITY

To identify novel RIP140 interacting proteins we performed a yeast two-hybrid screen and found PMF-1 to interact with RIP140. Mouse PMF-1 undergoes splicing to give form to PMF-1L and PMF-1S. Here we have focused on PMF-1L and found a CoRNR box motif, reported to be important for corepressor binding to NRs. We verified the interaction between PMF-1 and RIP140 and found PMF-1 to interact with

RIP140 N- and C-terminal domains. We also found PMF-1L to interact with GR. Verification of the functional role of this interaction showed that PMF-1 represses ligand induced GR activity of GRE-regulated reporter gene. Additionally we found that PMF-1 contains an intrinsic repression activity, which may be involved in GR repression. Since we have documented that RIP140 functions as a corepressor for GR activity, we suggested that a coexpression of RIP140 and PMF-1 might further stimulate repression of GR activity. Nonetheless, results suggested that PMF-1 does not modify RIP140 corepression of GR activity. Clarification of the role of PMF-1 in affecting RIP140 intrinsic repression showed that it did not alter the RIP140 repression activity. This suggests that although PMF-1 interacts with RIP140, it will not change the repression activity of RIP140 on GR, but may in some other context modulate RIP140 function.

Interestingly it is reported that the PMF-1 regulated gene SSAT is upregulated in glucocorticoid-induced apoptosis, suggesting a role for GR/PMF-1 interaction. Additionally, PMF-1 partner Nrf-2 is involved in regulating the antioxidant defence, which is also regulated by GR. This suggests that a role for PMF-1 in coregulating antioxidant defence by Nrf-2 and GR.

In summary the versatility of PMF-1 in interacting with several different proteins and functioning as a activator (SSAT), a repressor (GR) and a member of a corepressor complex (RIP140), implies that PMF-1 may have a broader function in regulating genes.

3.3 PAPER IV: GR AND GANP INTERACTION REGULATES DNA REPLICATION

To identify novel GR-LBD interacting proteins, we performed a yeast two hybrid screen using a HeLa cell library. We found a C-terminal fragment of GANP to interact with GR-LBD. We verified the interaction and found both GANP and its splice variant MCM3AP to interact with GR-LBD. We also found that the interaction between GR and MCM3AP is hormone independent, since the interaction was observed both in presence and absence of GR ligand. As both GANP and MCM3AP are known for their role in regulating DNA replication, their functional interaction with GR classify them as a new class of NR interacting proteins involved in DNA replication. The C-terminal domain of GANP and MCM3AP that binds to GR-LBD has four LXXLL motifs that usually are involved in coregulator binding to NRs, suggesting that these motifs in

GANP/MCM3AP could be involved in the interaction with GR. Classification of the cellular localization of a protein is important for characterizing its function and signalling pattern. We established that full length GANP was localized in small distinct cytoplasmic foci and MCM3AP was evenly localized in the cytoplasm. The N-terminal domain of GANP (GANPN) was localized in the nucleus and middle domain of GANP (GANPM) was localized in the cytoplasm in larger foci than full length GANP. To determine if full length GANP, MCM3AP and GANP domains GANPN and GANPM, are targets of the nuclear exporter protein, CRM1, we transfected COS-7 cells with the different proteins and treated the cells with CRM1 inhibitor, leptomycin B (LMB). This resulted in that full length GANP and MCM3AP were translocated into the nucleus and GANPN and GANPM had an unchanged location. This indicated that GANPM lacked a nuclear export signal (NES) and since GANPN was already expressed in the nucleus it was not affected by LMB. In summary full length GANP has two NLS, one in the N-terminal and the other in the C-terminal domain, and also a NES in the C-terminal domain (Figure 6). The GANP cytoplasmic foci distribution prompted use to ask if GANP is localized in the cytoplasmic organelles such as the mitochondria or lysosomes. We found that GANP was not colocalized with either mitochondria or lysosomes, indicating that GANP foci are distinct. These results characterize GANP as a shuttling protein that can shuttle between the nucleus and the cytoplasm. Additionally the foci localization was distinct from mitochondria and lysosome localization.

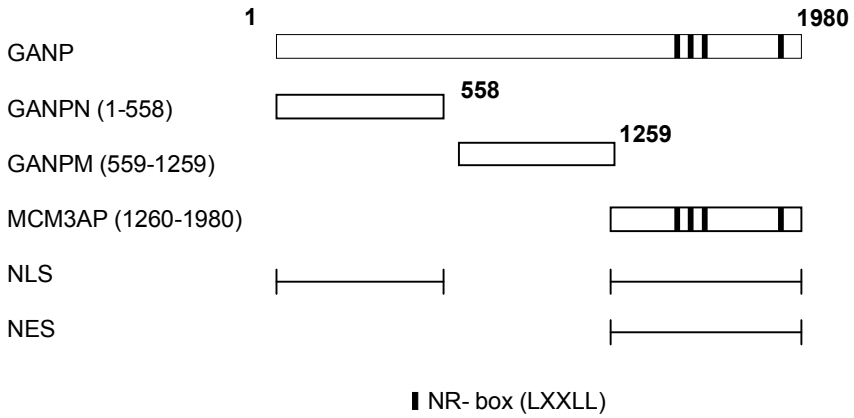

Figure 6. Schematic representation of GANP and GANP domains.

GANP and MCM3AP interact with MCM3, a member of MCM complex that is necessary for initiation of DNA replication. For MCM initiation of replication it needs to associate to the origins of replication in the chromatin. The observed interaction between GR and GANP/MCM3AP inspired us to study the connection between the

antiproliferative effect of glucocorticoids and DNA replication. We examined the role of glucocorticoids in cell cycle distribution, and found that G1 synchronized cells stimulated to reenter the cell cycle, stayed in G1 phase after GR ligand treatment compared to untreated cells, indicating that glucocorticoid promote G1 cell cycle arrest (Figure 7, our unpublished data).

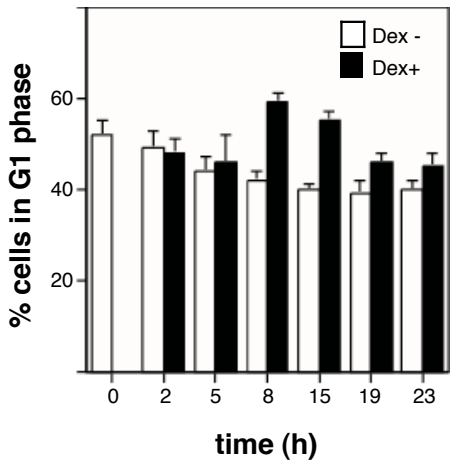

Figure 7. HeLa cells expressing endogenous GR were studied in presence and absence of GR ligand dexamethasone (Dex) at different time points. HeLa cells were synchronized by 48h serum starvation, then stimulated to reenter the cell cycle with 10% serum in presence (+) or absence (-) of Dex, and collected at the indicated time points. The DNA content of the cells was determined by FACS analysis after propodium iodide staining. The % of cells in G1 phase is shown. Black bar: in presence of GR ligand (Dex) and white bar: in absence of Dex.

We then studied the effect of glucocorticoids on DNA replication more directly, and found that the replication was inhibited in the presence of GR ligand (paper IV). Furthermore, when determining if the identified interaction between GANP/MCM3AP and GR have implication in the regulation of DNA replication by glucocorticoids, we found that in MCM3AP transfected cells glucocorticoids did not inhibit proliferation (paper IV).

To date the function of the interaction between GANP/MCM3AP and MCM3 is unclear, but from the data presented above a complex between GR, GANP/MCM3AP and MCM3, could be involved in GRs repressive action on replication. By studying the levels of MCM3 bound to chromatin in absence and presence of GR ligand we found that GR ligand induced an earlier chromatin binding of MCM3 than in untreated cells, indicating that regulation of DNA replication by glucocorticoids can be observed at the level of the formation of the pre-RC (Figure 8, our unpublished data).

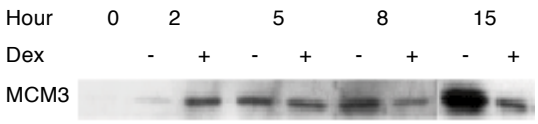

Figure 8. HeLa cells expressing GR were treated with or without GR ligand (Dex) in a time-dependent manner to study the effect of GR on MCM3 chromatin loading. HeLa cells were synchronized and stimulated to reenter the cell cycle as in figure 7. Chromatin binding of MCM3 at different time points after stimulation to enter the cell cycle in the presence and absence of Dex was determined by analyzing the chromatin fraction by SDS-PAGE and Western blotting using MCM3 antibody.

4 CONCLUSIONS

4.1 MOLECULAR BASIS OF REPRESSION BY RIP140 ON GR AND LXR TRANSCRIPTION (PAPER I AND II)

Based on results from papers I and II, we identify repression domains in RIP140 and show that ligand activated GR or LXR interact with RIP140 and together are redistributed to distinct structural domains in the nucleus.

- We mapped a RIP140 40 amino acid domain (aa 431-472) involved in targeting RIP140 to small foci in the nucleus.
- RIP140 foci distribution was distinct, since it did not colocalize with promyelocytic leukemia (PML) bodies, but partially colocalized with SMRT-MAD bodies.
- The 40 amino acid foci targeting domain overlaps with a CtBP binding site, but CtBP binding is not required for RIP140 foci distribution.
- RIP140 intrinsic repression involves both N-terminal and C-terminal domains of RIP140 and neither the CtBP binding site nor foci targeting domain are necessary.
- GR-LBD is required for RIP140 interaction. Several domains of RIP140 interact with GR except for the middle domain of RIP140 (aa 431-745).
- Ligand activated LXR interaction with RIP140 mainly resides in the LBD, but LXRα N-terminal also has potential to bind RIP140.
- Differential preference for RIP140 C-terminal domain by LXR subtypes was apparent since LXRβ only required NR10 to acquire sufficient RIP140 binding, while LXRα needed more than one NR box motif.
- RIP140 repression of ligand induced GR activity involves CtBP binding as well as RIP140 C-terminal NR-box motifs.
- The integrity of RIP140 C-terminal domain and NR10 are needed in mediating LXR transcriptional repression.
- Neither the LXR N-terminal nor LBD is required for LXR nuclear localization.
- Ligand induced redistribution of RIP140 and GR requires regions within RIP140 with a corepressor activity and the GR DBD.

- In absence of ligand RIP140 is redistributed from small foci to even distribution when coexpressed with LXR. Upon ligand binding RIP and LXR are redistributed to distinct intranuclear foci.
- LXR-LBD is essential for LXR colocalization with RIP140, while helix 12 in the LBD is not necessary for LXR/ RIP140 colocalization.

4.2 POLYAMINE-MODULATED FACTOR 1 REPRESSES GR ACTIVITY (PAPER III)

Paper III describes the novel functional interaction of PMF-1 with RIP140 and GR.

- Yeast two-hybrid screening was performed to identify novel RIP140 interacting proteins and found PMF-1L to interact specifically with RIP140.
- Both N- and C-terminal domains of RIP140 interact with PMF-1L.
- GR interacts with PMF-1L.
- PMF-1L represses ligand induced GR transactivation.
- PMF-1L has an intrinsic repression activity.
- PMF-1 has no effect on the RIP140 intrinsic repression or on its corepression of GR activity.

4.3 GR AND GANP INTERACTION REGULATES DNA REPLICATION (PAPER IV)

In paper IV the functional interaction of two novel proteins GANP and MCM3AP with GR is described.

- The interaction of GANP and MCM3AP C-terminal domain with GR-LBD was identified.
- GANP/MCM3AP are both cytoplasmic proteins. GANP is localized in small distinct foci and MCM3AP is evenly distributed in the cytoplasm.
- Inhibition of nuclear exporter resulted in nuclear translocation of GANP and MCM3AP, indicating that GANP have NLS and NES, which may characterize GANP as a shuttling protein.
- We found that glucocorticoids repress DNA replication and mediate G_1 cell cycle arrest, to facilitate the antiproliferative effect of glucocorticoids.
- We show that MCM3AP counteracted the negative effect of glucocorticoids on DNA replication.

- The chromatin loading of MCM proteins is essential for progression and the initiation of DNA replication in a time dependent manner. GR ligand induced an earlier chromatin loading of MCM3 than control, indicating that glucocorticoid regulation of replication could be detected in early G_1, during that assembly of the pre-RC.

5 CONCLUDING REMARKS

To understanding a disease phenotype, a molecular based approach is essential in unravelling the functional mechanisms of disease pathways. Research in the molecular mechanisms involves genomic and proteomic studies of disease phenotypes. In earlier days little was known on the role of proteins in drug therapy, even though most drugs target proteins. Human genome sequencing however provided new impact on drug target discovery and validation. Screening for novel molecules and targets to understand hormonal signalling have recently received increasing attention, this has given better understanding of classical hormone regulated NR signalling and identified potentially novel mechanisms for hormone signalling. Protein-protein interaction studies with NRs are essential in understand NRs biological pathways and potential significance in drug discovery. In this thesis I have studied three NR interacting proteins RIP140, PMF-1 and GANP. Collectively I believe that the molecular approach elucidated in this thesis shows novel roles for GR signalling in glucose metabolism and cancer development (i.e. DNA replication, apoptosis and antioxidant defence). In addition I also suggest a common repressive action by LXR and RIP140 on energy homeostasis.

Glucocorticoids increase blood glucose during stress, when returning to normal homeostasis RIP140 may repress GR regulated genes involved in gluconeogenesis in the liver. In addition I suggest a novel role for GR in repressing DNA replication by inducing early chromatin binding of MCM3. This repressive action of GR on DNA replication we found to be counteracted by MCM3AP, signifying that GR and MCM3AP may have a common mechanism of repression. I also suggest a novel role for GR in apoptosis and antioxidant defence together with PMF-1, because there are two common genes regulated by both glucocorticoid and polyamine signalling. In this thesis I have also studied the molecular mechanisms of RIP140 repression on LXR that could have implications in LXR physiological control of energy and glucose homeostasis, since both RIP140 and LXR regulate genes involved in energy and glucose homeostasis include uncoupling protein 1 (Ucp1) and glucose transporter 4 (GLUT4).

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7 REFERENCES

Abe, E., Kuwahara, K., Yoshida, M., Suzuki, M., Terasaki, H., Matsuo, Y., Takahashi, E. I. and Sakaguchi, N. (2000). Structure, expression, and chromosomal localization of the human gene encoding a germinal center-associated nuclear protein (GANP) that associates with MCM3 involved in the initiation of DNA replication. *Gene* **255**, 219-27.

Albers, M., Blume, B., Schlueter, T., Wright, M. B., Kober, I., Kremoser, C., Deuschle, U. and Koegl, M. (2006). A novel principle for partial agonism of liver X receptor ligands. Competitive recruitment of activators and repressors. *J Biol Chem* **281**, 4920-30.

Allgood, V. E. and Cidlowski, J. A. (1992). Vitamin B6 modulates transcriptional activation by multiple members of the steroid hormone receptor superfamily. *J Biol Chem* **267**, 3819-24.

Aranda, A. and Pascual, A. (2001). Nuclear hormone receptors and gene expression. *Physiol Rev* **81**, 1269-304.

Augereau, P., Badia, E., Fuentes, M., Rabenoelina, F., Corniou, M., Derocq, D., Balaguer, P. and Cavailles, V. (2006). Transcriptional regulation of the human NRIP1/RIP140 gene by estrogen is modulated by dioxin signalling. *Mol Pharmacol* **69**, 1338-46.

Bain, D. L., Heneghan, A. F., Connaghan-Jones, K. D. and Miura, M. T. (2007). Nuclear receptor structure: implications for function. *Annu Rev Physiol* **69**, 201-20.

Baumann, C. T., Maruvada, P., Hager, G. L. and Yen, P. M. (2001). Nuclear cytoplasmic shuttling by thyroid hormone receptors. multiple protein interactions are required for nuclear retention. *J Biol Chem* **276**, 11237-45.

Bjornstrom, L. and Sjoberg, M. (2002). Mutations in the estrogen receptor DNA-binding domain discriminate between the classical mechanism of action and cross-talk with Stat5b and activating protein 1 (AP-1). *J Biol Chem* **277**, 48479-83.

Braun, K. W., Tribley, W. A., Griswold, M. D. and Kim, K. H. (2000). Follicle-stimulating hormone inhibits all-trans-retinoic acid-induced retinoic acid receptor alpha nuclear localization and transcriptional activation in mouse Sertoli cell lines. *J Biol Chem* **275**, 4145-51.

Bunn, C. F., Neidig, J. A., Freidinger, K. E., Stankiewicz, T. A., Weaver, B. S., McGrew, J. and Allison, L. A. (2001). Nucleocytoplasmic shuttling of the thyroid hormone receptor alpha. *Mol Endocrinol* **15**, 512-33.

Carascossa, S., Gobinet, J., Georget, V., Lucas, A., Badia, E., Castet, A., White, R., Nicolas, J. C., Cavailles, V. and Jalaguier, S. (2006). Receptor-interacting protein 140 is a repressor of the androgen receptor activity. *Mol Endocrinol* **20**, 1506-18.

Castet, A., Boulahtouf, A., Versini, G., Bonnet, S., Augereau, P., Vignon, F., Khochbin, S., Jalaguier, S. and Cavailles, V. (2004). Multiple domains of the Receptor-Interacting Protein 140 contribute to transcription inhibition. *Nucleic Acids Res* **32**, 1957-66.

Cavailles, V., Dauvois, S., L'Horset, F., Lopez, G., Hoare, S., Kushner, P. J. and Parker, M. G. (1995). Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. *Embo J* **14**, 3741-51.

Chiang, J. Y. (2002). Bile acid regulation of gene expression: roles of nuclear hormone receptors. *Endocr Rev* **23**, 443-63.

Christian, M., Kiskinis, E., Debevec, D., Leonardsson, G., White, R. and Parker, M. G. (2005). RIP140-targeted repression of gene expression in adipocytes. *Mol Cell Biol* **25**, 9383-91.

Christian, M., Tullet, J. M. and Parker, M. G. (2004). Characterization of four autonomous repression domains in the corepressor receptor interacting protein 140. *J Biol Chem* **279**, 15645-51.

Committee, N. R. N. (1999). A unified nomenclature system for the nuclear receptor superfamily. *Cell* **97**, 161-3.

Corroyer, S., Schittny, J. C., Djonov, V., Burri, P. H. and Clement, A. (2002). Impairment of rat postnatal lung alveolar development by glucocorticoids: involvement

of the p21CIP1 and p27KIP1 cyclin-dependent kinase inhibitors. *Pediatr Res* **51**, 169-76.
Da-Silva, L. F. and Duncker, B. P. (2007). ORC function in late G1: maintaining the license for DNA replication. *Cell Cycle* **6**, 128-30.
De Martino, M. U., Alesci, S., Chrousos, G. P. and Kino, T. (2004). Interaction of the glucocorticoid receptor and the chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII): implications for the actions of glucocorticoids on glucose, lipoprotein, and xenobiotic metabolism. *Ann N Y Acad Sci* **1024**, 72-85.
de Ruijter, A. J., van Gennip, A. H., Caron, H. N., Kemp, S. and van Kuilenburg, A. B. (2003). Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem J* **370**, 737-49.
Dong, S., Stenoien, D. L., Qiu, J., Mancini, M. A. and Tweardy, D. J. (2004). Reduced intranuclear mobility of APL fusion proteins accompanies their mislocalization and results in sequestration and decreased mobility of retinoid X receptor alpha. *Mol Cell Biol* **24**, 4465-75.
Engelbrecht, Y., de Wet, H., Horsch, K., Langeveldt, C. R., Hough, F. S. and Hulley, P. A. (2003). Glucocorticoids induce rapid up-regulation of mitogen-activated protein kinase phosphatase-1 and dephosphorylation of extracellular signal-regulated kinase and impair proliferation in human and mouse osteoblast cell lines. *Endocrinology* **144**, 412-22.
Franchimont, D. (2004). Overview of the actions of glucocorticoids on the immune response: a good model to characterize new pathways of immunosuppression for new treatment strategies. *Ann N Y Acad Sci* **1024**, 124-37.
Friedman, J. E., Yun, J. S., Patel, Y. M., McGrane, M. M. and Hanson, R. W. (1993). Glucocorticoids regulate the induction of phosphoenolpyruvate carboxykinase (GTP) gene transcription during diabetes. *J Biol Chem* **268**, 12952-7.
Frost, G. H., Rhee, K., Ma, T. and Thompson, E. A. (1994). Expression of c-Myc in glucocorticoid-treated fibroblastic cells. *J Steroid Biochem Mol Biol* **50**, 109-19.
Fryer, C. J. and Archer, T. K. (1998). Chromatin remodelling by the glucocorticoid receptor requires the BRG1 complex. *Nature* **393**, 88-91.
Fujimura, S., Xing, Y., Takeya, M., Yamashita, Y., Ohshima, K., Kuwahara, K. and Sakaguchi, N. (2005). Increased expression of germinal center-associated nuclear protein RNA-primase is associated with lymphomagenesis. *Cancer Res* **65**, 5925-34.
Gerin, I., Dolinsky, V. W., Shackman, J. G., Kennedy, R. T., Chiang, S. H., Burant, C. F., Steffensen, K. R., Gustafsson, J. A. and MacDougald, O. A. (2005). LXRbeta is required for adipocyte growth, glucose homeostasis, and beta cell function. *J Biol Chem* **280**, 23024-31.
Germain, P., Staels, B., Dacquet, C., Spedding, M. and Laudet, V. (2006). Overview of nomenclature of nuclear receptors. *Pharmacol Rev* **58**, 685-704.
Gerner, E. W. and Meyskens, F. L., Jr. (2004). Polyamines and cancer: old molecules, new understanding. *Nat Rev Cancer* **4**, 781-92.
Greenberg, A. K., Hu, J., Basu, S., Hay, J., Reibman, J., Yie, T. A., Tchou-Wong, K. M., Rom, W. N. and Lee, T. C. (2002). Glucocorticoids inhibit lung cancer cell growth through both the extracellular signal-related kinase pathway and cell cycle regulators. *Am J Respir Cell Mol Biol* **27**, 320-8.
Griekspoor, A., Zwart, W., Neefjes, J. and Michalides, R. (2007). Visualizing the action of steroid hormone receptors in living cells. *Nucl Recept Signal* **5**, e003.
Gupta, P., Huq, M. D., Khan, S. A., Tsai, N. P. and Wei, L. N. (2005). Regulation of co-repressive activity of and HDAC recruitment to RIP140 by site-specific phosphorylation. *Mol Cell Proteomics* **4**, 1776-84.
Harper, J. V. and Brooks, G. (2005). The mammalian cell cycle: an overview. *Methods Mol Biol* **296**, 113-53.
Heery, D. M., Hoare, S., Hussain, S., Parker, M. G. and Sheppard, H. (2001). Core LXXLL motif sequences in CREB-binding protein, SRC1, and RIP140 define affinity and selectivity for steroid and retinoid receptors. *J Biol Chem* **276**, 6695-702.
Heery, D. M., Kalkhoven, E., Hoare, S. and Parker, M. G. (1997). A signature motif in transcriptional co-activators mediates binding to nuclear receptors. *Nature* **387**, 733-6.

Htun, H., Holth, L. T., Walker, D., Davie, J. R. and Hager, G. L. (1999). Direct visualization of the human estrogen receptor alpha reveals a role for ligand in the nuclear distribution of the receptor. *Mol Biol Cell* **10**, 471-86.
Hu, X. and Funder, J. W. (2006). The evolution of mineralocorticoid receptors. *Mol Endocrinol* **20**, 1471-8.
Hu, X., Li, Y. and Lazar, M. A. (2001). Determinants of CoRNR-dependent repression complex assembly on nuclear hormone receptors. *Mol Cell Biol* **21**, 1747-58.
Hultman, M. L., Krasnoperova, N. V., Li, S., Du, S., Xia, C., Dietz, J. D., Lala, D. S., Welsch, D. J. and Hu, X. (2005). The ligand-dependent interaction of mineralocorticoid receptor with coactivator and corepressor peptides suggests multiple activation mechanisms. *Mol Endocrinol* **19**, 1460-73.
Huq, M. D., Khan, S. A., Park, S. W. and Wei, L. N. (2005). Mapping of phosphorylation sites of nuclear corepressor receptor interacting protein 140 by liquid chromatography-tandem mass spectroscopy. *Proteomics* **5**, 2157-66.
Huq, M. D., Tsai, N. P., Lin, Y. P., Higgins, L. and Wei, L. N. (2007). Vitamin B6 conjugation to nuclear corepressor RIP140 and its role in gene regulation. *Nat Chem Biol* **3**, 161-5.
Huq, M. D. and Wei, L. N. (2005). Post-translational modification of nuclear co-repressor receptor-interacting protein 140 by acetylation. *Mol Cell Proteomics* **4**, 975-83.
Igarashi, K. and Kashiwagi, K. (2000). Polyamines: mysterious modulators of cellular functions. *Biochem Biophys Res Commun* **271**, 559-64.
Ikonen, T., Palvimo, J. J. and Janne, O. A. (1997). Interaction between the amino- and carboxyl-terminal regions of the rat androgen receptor modulates transcriptional activity and is influenced by nuclear receptor coactivators. *J Biol Chem* **272**, 29821-8.
Ito, M. and Roeder, R. G. (2001). The TRAP/SMCC/Mediator complex and thyroid hormone receptor function. *Trends Endocrinol Metab* **12**, 127-34.
Jagiello, I., Van Eynde, A., Vulsteke, V., Beullens, M., Boudrez, A., Keppens, S., Stalmans, W. and Bollen, M. (2000). Nuclear and subnuclear targeting sequences of the protein phosphatase-1 regulator NIPP1. *J Cell Sci* **113 Pt 21**, 3761-8.
Juvet, L. K., Andresen, S. M., Schuster, G. U., Dalen, K. T., Tobin, K. A., Hollung, K., Haugen, F., Jacinto, S., Ulven, S. M., Bamberg, K., Gustafsson, J. A. and Nebb, H. I. (2003). On the role of liver X receptors in lipid accumulation in adipocytes. *Mol Endocrinol* **17**, 172-82.
Kalaany, N. Y., Gauthier, K. C., Zavacki, A. M., Mammen, P. P., Kitazume, T., Peterson, J. A., Horton, J. D., Garry, D. J., Bianco, A. C. and Mangelsdorf, D. J. (2005). LXRs regulate the balance between fat storage and oxidation. *Cell Metab* **1**, 231-44.
Kerley, J. S., Olsen, S. L., Freemantle, S. J. and Spinella, M. J. (2001). Transcriptional activation of the nuclear receptor corepressor RIP140 by retinoic acid: a potential negative-feedback regulatory mechanism. *Biochem Biophys Res Commun* **285**, 969-75.
Kumar, M. B., Tarpey, R. W. and Perdew, G. H. (1999). Differential recruitment of coactivator RIP140 by Ah and estrogen receptors. Absence of a role for LXXLL motifs. *J Biol Chem* **274**, 22155-64.
Kumar, S., Saradhi, M., Chaturvedi, N. K. and Tyagi, R. K. (2006). Intracellular localization and nucleocytoplasmic trafficking of steroid receptors: an overview. *Mol Cell Endocrinol* **246**, 147-56.
Kuwahara, K., Tomiyasu, S., Fujimura, S., Nomura, K., Xing, Y., Nishiyama, N., Ogawa, M., Imajoh-Ohmi, S., Izuta, S. and Sakaguchi, N. (2001). Germinal center-associated nuclear protein (GANP) has a phosphorylation-dependent DNA-primase activity that is up-regulated in germinal center regions. *Proc Natl Acad Sci U S A* **98**, 10279-83.
Kuwahara, K., Yoshida, M., Kondo, E., Sakata, A., Watanabe, Y., Abe, E., Kouno, Y., Tomiyasu, S., Fujimura, S., Tokuhisa, T., Kimura, H., Ezaki, T. and Sakaguchi, N. (2000). A novel nuclear phosphoprotein, GANP, is up-regulated in centrocytes of the germinal center and associated with MCM3, a protein essential for DNA replication. *Blood* **95**, 2321-8.

L'Horset, F., Dauvois, S., Heery, D. M., Cavailles, V. and Parker, M. G. (1996). RIP-140 interacts with multiple nuclear receptors by means of two distinct sites. *Mol Cell Biol* **16**, 6029-36.
Lee, C. H., Chinpaisal, C. and Wei, L. N. (1998). Cloning and characterization of mouse RIP140, a corepressor for nuclear orphan receptor TR2. *Mol Cell Biol* **18**, 6745-55.
Lei, E. P., Stern, C. A., Fahrenkrog, B., Krebber, H., Moy, T. I., Aebi, U. and Silver, P. A. (2003). Sac3 is an mRNA export factor that localizes to cytoplasmic fibrils of nuclear pore complex. *Mol Biol Cell* **14**, 836-47.
Lei, M. and Tye, B. K. (2001). Initiating DNA synthesis: from recruiting to activating the MCM complex. *J Cell Sci* **114**, 1447-54.
Leonardsson, G., Steel, J. H., Christian, M., Pocock, V., Milligan, S., Bell, J., So, P. W., Medina-Gomez, G., Vidal-Puig, A., White, R. and Parker, M. G. (2004). Nuclear receptor corepressor RIP140 regulates fat accumulation. *Proc Natl Acad Sci U S A* **101**, 8437-42.
Lewis, B. A. and Reinberg, D. (2003). The mediator coactivator complex: functional and physical roles in transcriptional regulation. *J Cell Sci* **116**, 3667-75.
Lewis-Tuffin, L. J. and Cidlowski, J. A. (2006). The physiology of human glucocorticoid receptor beta (hGRbeta) and glucocorticoid resistance. *Ann N Y Acad Sci* **1069**, 1-9.
Liberman, A. C., Druker, J., Perone, M. J. and Arzt, E. (2007). Glucocorticoids in the regulation of transcription factors that control cytokine synthesis. *Cytokine Growth Factor Rev* **18**, 45-56.
Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P. and Evans, R. M. (1995). The nuclear receptor superfamily: the second decade. *Cell* **83**, 835-9.
Maruvada, P., Baumann, C. T., Hager, G. L. and Yen, P. M. (2003). Dynamic shuttling and intranuclear mobility of nuclear hormone receptors. *J Biol Chem* **278**, 12425-32.
Masuyama, H., Brownfield, C. M., St-Arnaud, R. and MacDonald, P. N. (1997). Evidence for ligand-dependent intramolecular folding of the AF-2 domain in vitamin D receptor-activated transcription and coactivator interaction. *Mol Endocrinol* **11**, 1507-17.
Maxfield, F. R. and Tabas, I. (2005). Role of cholesterol and lipid organization in disease. *Nature* **438**, 612-21.
Mazziotti, G., Angeli, A., Bilezikian, J. P., Canalis, E. and Giustina, A. (2006). Glucocorticoid-induced osteoporosis: an update. *Trends Endocrinol Metab* **17**, 144-9.
McKenna, N. J., Lanz, R. B. and O'Malley, B. W. (1999). Nuclear receptor coregulators: cellular and molecular biology. *Endocr Rev* **20**, 321-44.
McKenna, N. J. and O'Malley, B. W. (2002). Combinatorial control of gene expression by nuclear receptors and coregulators. *Cell* **108**, 465-74.
McManus, K. J. and Hendzel, M. J. (2001). CBP, a transcriptional coactivator and acetyltransferase. *Biochem Cell Biol* **79**, 253-66.
Mellgren, G., Borud, B., Hoang, T., Yri, O. E., Fladeby, C., Lien, E. A. and Lund, J. (2003). Characterization of receptor-interacting protein RIP140 in the regulation of SF-1 responsive target genes. *Mol Cell Endocrinol* **203**, 91-103.
Meraldi, P., McAinsh, A. D., Rheinbay, E. and Sorger, P. K. (2006). Phylogenetic and structural analysis of centromeric DNA and kinetochore proteins. *Genome Biol* **7**, R23.
Meyer, M. E., Gronemeyer, H., Turcotte, B., Bocquel, M. T., Tasset, D. and Chambon, P. (1989). Steroid hormone receptors compete for factors that mediate their enhancer function. *Cell* **57**, 433-42.
Miyata, K. S., McCaw, S. E., Meertens, L. M., Patel, H. V., Rachubinski, R. A. and Capone, J. P. (1998). Receptor-interacting protein 140 interacts with and inhibits transactivation by, peroxisome proliferator-activated receptor alpha and liver-X-receptor alpha. *Mol Cell Endocrinol* **146**, 69-76.
Moore, D. D., Kato, S., Xie, W., Mangelsdorf, D. J., Schmidt, D. R., Xiao, R. and Kliewer, S. A. (2006). International Union of Pharmacology. LXII. The NR1H and NR1I receptors: constitutive androstane receptor, pregnene X receptor, farnesoid X

receptor alpha, farnesoid X receptor beta, liver X receptor alpha, liver X receptor beta, and vitamin D receptor. *Pharmacol Rev* **58**, 742-59.
Moore, J. M., Galicia, S. J., McReynolds, A. C., Nguyen, N. H., Scanlan, T. S. and Guy, R. K. (2004). Quantitative proteomics of the thyroid hormone receptor-coregulator interactions. *J Biol Chem* **279**, 27584-90.
Mostaqul Huq, M. D., Gupta, P., Tsai, N. P., White, R., Parker, M. G. and Wei, L. N. (2006). Suppression of receptor interacting protein 140 repressive activity by protein arginine methylation. *Embo J* **25**, 5094-104.
Naar, A. M., Beaurang, P. A., Zhou, S., Abraham, S., Solomon, W. and Tjian, R. (1999). Composite co-activator ARC mediates chromatin-directed transcriptional activation. *Nature* **398**, 828-32.
Nagy, L. and Schwabe, J. W. (2004). Mechanism of the nuclear receptor molecular switch. *Trends Biochem Sci* **29**, 317-24.
Necela, B. M. and Cidlowski, J. A. (2004). Mechanisms of glucocorticoid receptor action in noninflammatory and inflammatory cells. *Proc Am Thorac Soc* **1**, 239-46.
Nettles, K. W. and Greene, G. L. (2005). Ligand control of coregulator recruitment to nuclear receptors. *Annu Rev Physiol* **67**, 309-33.
Newton, R. (2000). Molecular mechanisms of glucocorticoid action: what is important? *Thorax* **55**, 603-13.
Nichol, D., Christian, M., Steel, J. H., White, R. and Parker, M. G. (2006). RIP140 expression is stimulated by estrogen-related receptor alpha during adipogenesis. *J Biol Chem* **281**, 32140-7.
Obuse, C., Iwasaki, O., Kiyomitsu, T., Goshima, G., Toyoda, Y. and Yanagida, M. (2004). A conserved Mis12 centromere complex is linked to heterochromatic HP1 and outer kinetochore protein Zwint-1. *Nat Cell Biol* **6**, 1135-41.
Orphanides, G. and Reinberg, D. (2002). A unified theory of gene expression. *Cell* **108**, 439-51.
Peet, D. J., Turley, S. D., Ma, W., Janowski, B. A., Lobaccaro, J. M., Hammer, R. E. and Mangelsdorf, D. J. (1998). Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. *Cell* **93**, 693-704.
Powelka, A. M., Seth, A., Virbasius, J. V., Kiskinis, E., Nicoloro, S. M., Guilherme, A., Tang, X., Straubhaar, J., Cherniack, A. D., Parker, M. G. and Czech, M. P. (2006). Suppression of oxidative metabolism and mitochondrial biogenesis by the transcriptional corepressor RIP140 in mouse adipocytes. *J Clin Invest* **116**, 125-36.
Privalsky, M. L. (2004). The role of corepressors in transcriptional regulation by nuclear hormone receptors. *Annu Rev Physiol* **66**, 315-60.
Prufer, K. and Boudreaux, J. (2007). Nuclear localization of liver X receptor alpha and beta is differentially regulated. *J Cell Biochem* **100**, 69-85.
Repa, J. J., Liang, G., Ou, J., Bashmakov, Y., Lobaccaro, J. M., Shimomura, I., Shan, B., Brown, M. S., Goldstein, J. L. and Mangelsdorf, D. J. (2000). Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes Dev* **14**, 2819-30.
Rhen, T. and Cidlowski, J. A. (2005). Antiinflammatory action of glucocorticoids--new mechanisms for old drugs. *N Engl J Med* **353**, 1711-23.
Robinson-Rechavi, M., Escriva Garcia, H. and Laudet, V. (2003). The nuclear receptor superfamily. *J Cell Sci* **116**, 585-6.
Rodriguez-Navarro, S., Fischer, T., Luo, M. J., Antunez, O., Brettschneider, S., Lechner, J., Perez-Ortin, J. E., Reed, R. and Hurt, E. (2004). Sus1, a functional component of the SAGA histone acetylase complex and the nuclear pore-associated mRNA export machinery. *Cell* **116**, 75-86.
Rogatsky, I., Trowbridge, J. M. and Garabedian, M. J. (1997). Glucocorticoid receptor-mediated cell cycle arrest is achieved through distinct cell-specific transcriptional regulatory mechanisms. *Mol Cell Biol* **17**, 3181-93.
Sanchez, I., Goya, L., Vallerga, A. K. and Firestone, G. L. (1993). Glucocorticoids reversibly arrest rat hepatoma cell growth by inducing an early G1 block in cell cycle progression. *Cell Growth Differ* **4**, 215-25.
Smith, C. L. and O'Malley, B. W. (2004). Coregulator function: a key to understanding tissue specificity of selective receptor modulators. *Endocr Rev* **25**, 45-71.

Smoak, K. A. and Cidlowski, J. A. (2004). Mechanisms of glucocorticoid receptor signaling during inflammation. *Mech Ageing Dev* **125**, 697-706.
Steffensen, K. R. and Gustafsson, J. A. (2004). Putative metabolic effects of the liver X receptor (LXR). *Diabetes* **53 Suppl 1**, S36-42.
Stenoien, D. L., Mancini, M. G., Patel, K., Allegretto, E. A., Smith, C. L. and Mancini, M. A. (2000). Subnuclear trafficking of estrogen receptor-alpha and steroid receptor coactivator-1. *Mol Endocrinol* **14**, 518-34.
Stephenson, A. H. and Seidel, E. R. (2006). Analysis of the interactions of Nrf-2, PMF-1, and CSN-7 with the 5'-flanking sequence of the mouse 4E-BP1 gene. *Life Sci* **79**, 1221-7.
Subramaniam, N., Treuter, E. and Okret, S. (1999). Receptor interacting protein RIP140 inhibits both positive and negative gene regulation by glucocorticoids. *J Biol Chem* **274**, 18121-7.
Sugawara, T., Abe, S., Sakuragi, N., Fujimoto, Y., Nomura, E., Fujieda, K., Saito, M. and Fujimoto, S. (2001). RIP 140 modulates transcription of the steroidogenic acute regulatory protein gene through interactions with both SF-1 and DAX-1. *Endocrinology* **142**, 3570-7.
Takei, Y., Assenberg, M., Tsujimoto, G. and Laskey, R. (2002). The MCM3 acetylase MCM3AP inhibits initiation, but not elongation, of DNA replication via interaction with MCM3. *J Biol Chem* **277**, 43121-5.
Takei, Y., Swietlik, M., Tanoue, A., Tsujimoto, G., Kouzarides, T. and Laskey, R. (2001). MCM3AP, a novel acetyltransferase that acetylates replication protein MCM3. *EMBO Rep* **2**, 119-23.
Takei, Y. and Tsujimoto, G. (1998). Identification of a novel MCM3-associated protein that facilitates MCM3 nuclear localization. *J Biol Chem* **273**, 22177-80.
Teyssier, C., Belguise, K., Galtier, F., Cavailles, V. and Chalbos, D. (2003). Receptor-interacting protein 140 binds c-Jun and inhibits estradiol-induced activator protein-1 activity by reversing glucocorticoid receptor-interacting protein 1 effect. *Mol Endocrinol* **17**, 287-99.
Thomas, T. and Thomas, T. J. (2001). Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. *Cell Mol Life Sci* **58**, 244-58.
Thomas, T. and Thomas, T. J. (2003). Polyamine metabolism and cancer. *J Cell Mol Med* **7**, 113-26.
Treuter, E., Albrektsen, T., Johansson, L., Leers, J. and Gustafsson, J. A. (1998). A regulatory role for RIP140 in nuclear receptor activation. *Mol Endocrinol* **12**, 864-81.
Turner, J. and Crossley, M. (2001). The CtBP family: enigmatic and enzymatic transcriptional co-repressors. *Bioessays* **23**, 683-90.
van Reyk, D. M., Brown, A. J., Hult'en, L. M., Dean, R. T. and Jessup, W. (2006). Oxysterols in biological systems: sources, metabolism and pathophysiological relevance. *Redox Rep* **11**, 255-62.
Vander Kooi, B. T., Onuma, H., Oeser, J. K., Svitek, C. A., Allen, S. R., Vander Kooi, C. W., Chazin, W. J. and O'Brien, R. M. (2005). The glucose-6-phosphatase catalytic subunit gene promoter contains both positive and negative glucocorticoid response elements. *Mol Endocrinol* **19**, 3001-22.
Veenstra, G. J. and Wolffe, A. P. (2001). Gene-selective developmental roles of general transcription factors. *Trends Biochem Sci* **26**, 665-71.
Vo, N., Fjeld, C. and Goodman, R. H. (2001). Acetylation of nuclear hormone receptor-interacting protein RIP140 regulates binding of the transcriptional corepressor CtBP. *Mol Cell Biol* **21**, 6181-8.
Wang, L., Zhang, X., Farrar, W. L. and Yang, X. (2004). Transcriptional crosstalk between nuclear receptors and cytokine signal transduction pathways in immunity. *Cell Mol Immunol* **1**, 416-24.
Wang, Y., Devereux, W., Stewart, T. M. and Casero, R. A., Jr. (1999). Cloning and characterization of human polyamine-modulated factor-1, a transcriptional cofactor that regulates the transcription of the spermidine/spermine N(1)-acetyltransferase gene. *J Biol Chem* **274**, 22095-101.
Wang, Y., Devereux, W., Stewart, T. M. and Casero, R. A., Jr. (2001). Characterization of the interaction between the transcription factors human polyamine modulated factor (PMF-1) and NF-E2-related factor 2 (Nrf-2) in the transcriptional

regulation of the spermidine/spermine N1-acetyltransferase (SSAT) gene. *Biochem J* **355**, 45-9.
Wang, Y., Devereux, W., Stewart, T. M. and Casero, R. A., Jr. (2002). Polyamine-modulated factor 1 binds to the human homologue of the 7a subunit of the Arabidopsis COP9 signalosome: implications in gene expression. *Biochem J* **366**, 79-86.
Wang, Y., Devereux, W., Woster, P. M. and Casero, R. A., Jr. (2001). Cloning and characterization of the mouse polyamine-modulated factor-1 (mPMF-1) gene: an alternatively spliced homologue of the human transcription factor. *Biochem J* **359**, 387-92.
Wei, L. N., Farooqui, M. and Hu, X. (2001). Ligand-dependent formation of retinoid receptors, receptor-interacting protein 140 (RIP140), and histone deacetylase complex is mediated by a novel receptor-interacting motif of RIP140. *J Biol Chem* **276**, 16107-12.
Wei, L. N., Hu, X., Chandra, D., Seto, E. and Farooqui, M. (2000). Receptor-interacting protein 140 directly recruits histone deacetylases for gene silencing. *J Biol Chem* **275**, 40782-7.
White, J. H., Fernandes, I., Mader, S. and Yang, X. J. (2004). Corepressor recruitment by agonist-bound nuclear receptors. *Vitam Horm* **68**, 123-43.
White, R., Leonardsson, G., Rosewell, I., Ann Jacobs, M., Milligan, S. and Parker, M. (2000). The nuclear receptor co-repressor nrip1 (RIP140) is essential for female fertility. *Nat Med* **6**, 1368-74.
Wiebel, F. F., Steffensen, K. R., Treuter, E., Feltkamp, D. and Gustafsson, J. A. (1999). Ligand-independent coregulator recruitment by the triply activatable OR1/retinoid X receptor-alpha nuclear receptor heterodimer. *Mol Endocrinol* **13**, 1105-18.
Windahl, S. H., Treuter, E., Ford, J., Zilliacus, J., Gustafsson, J. A. and McEwan, I. J. (1999). The nuclear-receptor interacting protein (RIP) 140 binds to the human glucocorticoid receptor and modulates hormone-dependent transactivation. *J Steroid Biochem Mol Biol* **71**, 93-102.
Xu, J. and Li, Q. (2003). Review of the in vivo functions of the p160 steroid receptor coactivator family. *Mol Endocrinol* **17**, 1681-92.
Zelcer, N. and Tontonoz, P. (2006). Liver X receptors as integrators of metabolic and inflammatory signaling. *J Clin Invest* **116**, 607-14.
Zhong, S., Salomoni, P. and Pandolfi, P. P. (2000). The transcriptional role of PML and the nuclear body. *Nat Cell Biol* **2**, E85-90.
Zilliacus, J., Holter, E., Wakui, H., Tazawa, H., Treuter, E. and Gustafsson, J. A. (2001). Regulation of glucocorticoid receptor activity by 14--3-3-dependent intracellular relocalization of the corepressor RIP140. *Mol Endocrinol* **15**, 501-11.