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TRANSCRIPTIONAL REGULATION BY THE NUCLEAR RECEPTORS STEROIDOGENIC FACTOR-1 AND LIVER RECEPTOR HOMOLOGUE-1

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ABSTRACT

Steroidogenic Factor-1 (SF-1) and Liver Receptor Homologue-1 (LRH-1) are transcription factors belonging to the nuclear receptor (NR) family. In the adrenal cortex and gonads, SF-1 governs the expression of multiple enzymes and transporters required for converting cholesterol to steroid hormones. LRH-1 is mainly expressed in the enterohepatic system and regulates transcriptions of enzymes and transporters important in the conversion of cholesterol to bile acids. While most NRs bind DNA as either homo- or heterodimers, both SF-1 and LRH-1 interact with their response elements as monomers. On target gene promoters, they recruit coregulators that can either activate or repress transcription. Work presented in this thesis aims at elucidating the molecular mechanisms of SF-1 and LRH-1 actions in transcriptional regulation.

In Paper I we describe how a previously unknown modulator of SF-1 activity, RNF31, is important in DAX-1-dependent repression of SF-1. RNF31 is an E3 ubiquitin ligase and we show that it can monoubiquitinate DAX-1. RNAi-mediated knockdown of RNF31 and presumed loss of DAX-1 ubiquitination leads to increased expression of the SF-1 target genes StAR and aromatase (CYP19). We also show that RNF31 is present on the promoters of StAR and aromatase together with DAX-1 and SMRT and that its presence is SF-1-dependent. In conclusion, RNF31 is shown to be a novel coregulator of SF-1, acting via DAX-1 to repress transcription of StAR and aromatase.

In Paper II we further explore the role of RNF31 and SF-1 in adrenocortical cells. We knock down either RNF31 or SF-1 using RNAi, and use microarray analysis to identify differentially expressed genes. The cells depleted of RNF31 are shown to have significant changes in pathways related to steroidogenesis and cholesterol metabolism and many known SF-1 target genes are among those altered by RNF31 loss. This adds further support to our hypothesis that RNF31 is a coregulator of SF-1 activity. SF-1 depletion alters, besides steroidogenesis, pathways governing cell proliferation and differentiation. Genes involved in TGFβ- and Wnt/β-catenin-signalling are upregulated in response to SF-1 knockdown indicating that SF-1 signalling has repressive effects on these pathways.

In Paper III we show that LRH-1 is involved in transrepression of the hepatic acute phase response. The repression is dependent on agonist activation of LRH-1 and occurs in only a subset of the acute phase proteins. The regulated proteins have in common that the NCoR/HDAC3/GPS2-corepressor complex is recruited to the promoters. Upon ligand-activation, SUMOylated LRH-1 is tethered to and stabilises the corepressor complex, hindering its dissociation from the promoter and thus transcription of the target gene.

In conclusion, the major findings presented in this thesis are the characterisation of RNF31 as a coregulator of SF-1 and steroidogenesis, of GPS2 as a coregulator of SUMOylation-dependent transrepression by LRH-1 and the new, active role of ubiquitin-like modifications in transcriptional regulation by both LRH-1 and SF-1.

LIST OF PUBLICATIONS

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LIST OF ABBREVIATIONS

aa	Amino acid(s)
ACTH	Adrenocorticotropic hormone
AF-1/2	Activation function-1/2
AKR1D1/C4	Aldo-keto reductase family 1, member D1/C4
APC	Adenomatous polyposis coli
BA	Bile acid
BSEP	Bile acid export pump
CA	Cholic acid, 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid
cAMP	cyclic AMP
CDCA	Chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholan-24-oic acid
CDK7	Cyclin-dependent kinase 7
ChIP	Chromatin immunoprecipitation
CoA	Coenzyme A
CREB	cAMP responsive element binding protein
CREM	cAMP responsive element modulator
CTE	C-terminal extension
DAX-1	Dosage-sensitive sex reversal, adrenal hypoplasia congenita critical region on chromosome X, gene 1
DBD	DNA-binding domain
DHEA	Dehydroepiandosterone
EGF	Epidermal growth factor
ER	Oestrogen receptor
ERK1/2	Extracellular signal-regulated kinase
ERR	Oestrogen-related receptor
FTZ-F1	Fushi tarazu factor 1
FXR	Farnesoid X receptor
GnRH	Gonadotropin-releasing hormone
GR	Glucocorticoid receptor
GRIP1	Glutamate receptor interacting protein 1
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HDL-R	High density lipoprotein receptor

HECT	Homologous to the E6-AP carboxyl terminus
HNF4	Hepatocyte nuclear factor 4
HSD	Hydroxysteroid dehydrogenase
I16/IL1β	Interleukin 6/1β
ISG15	Interferon-stimulated gene 15
LBD	Ligand-binding domain
LBP	Ligand-binding pocket
LCoR	Ligand-dependent nuclear receptor corepressor
LDL	Low density lipoprotein
LHβ	Luteinizing hormone β
LRH-1	Liver receptor homologue-1
LUBAC	Linear ubiquitin chain assembly complex
LXR	Liver X receptor
MAPK	Mitogen activated protein kinase
MC2R	Melanocorticoid-2 receptor
NCoR	Nuclear receptor corepressor
NPC-1	Niemann-Pick type C1
Nedd8	Neural precursor cell expressed, developmentally downregulated 8
NR	Nuclear receptor
PA	Phosphatidic acid
PAI-1	Plasminogen activator inhibitor-1
PKA	Protein kinase A
PPAR	Peroxisome-proliferator activated receptor
PTM	Posttranslational modification
PXR	Pregnane X receptor
RBCK1	RanBP-type and C3H4-type zinc finger containing 1
RE	Response element
RIP140	Receptor interacting protein 140 kDa
RNA Pol II	RNA polymerase II
RNF31	Ring finger protein 31
SAA	Serum amyloid A
SCARB1	Scavenger receptor class B1
SCP2	Sterol carrier protein 2

SF-1	Steroidogenic factor-1
SHP	Small heterodimer partner
SMRT	Silencing mediator of retinoic acid and thyroid hormone
SRC	Steroid receptor coactivator
SUMO	Small ubiquitin-related modifier
Tcf4	Transcription factor 4
TGFβ	Transforming growth factor β
TNFα	Tumour necrosis factor α
UBC	Ubiquitin conjugating enzyme
VDR	Vitamin D receptor
Wnt	Wingless-type

1 INTRODUCTION

1.1 NUCLEAR RECEPTORS

The nuclear receptor (NR) family of transcription factors comprises 48 members in humans. The NRs are involved in almost all physiological processes, including development, homeostasis and cell cycle regulation, and dysregulation often causes disease. As many NRs bind small hydrophobic ligands, they are attractive drug targets and today NR-targeting drugs are used to treat such diverse conditions as breast cancer, hypertension and various inflammatory diseases.

The NRs share a modular structure of an N-terminal A/B-domain followed by the DNA-binding domain (DBD), the hinge region, the ligand-binding domain (LBD) and a short C-terminal domain. The A/B region varies in both length and function within the family and sometimes includes a ligand-independent activation domain called Activation Function-1 (AF-1). The DNA-binding domain is the most conserved within the NR family and has two typical zinc-fingers that mediate the binding to specific DNA sequences. The hinge region connects the DBD with the LBD and can contain sites for post-translational modifications that change the behaviour of the receptor. The LBD includes the ligand-binding pocket and the Activation Function-2 (AF-2) coregulator-binding surface (figure 1) (53, 71, 117).

NRs bind as monomers, homo- or heterodimers to direct or inverted repeats or single copies of variations of the DNA sequence 5´-AGGTCA-3', the so-called NR half-site. The spacing between the half-sites governs what NR dimers can bind and the requirement for additional specific bases around the half-site differs depending on the receptor dimer. The DNA-bound receptors then regulate the expression of target genes by recruiting cofactors to the regulatory elements of the genes. The cofactors can either activate by modifying chromatin to an accessible state so that the general transcription machinery can initiate transcription or repress by recruiting corepressors making the chromatin inaccessible (71, 143). An alternative mode of action that has been shown for some of the NRs is association to DNA *in trans* via tethering to other transcription factors. This mechanism is often utilised in crosstalk between different signalling pathways (50).

Ligand-binding infers a conformational change in the structure of the NR, repositioning helix 12 in the LBD, thus making the coactivator-binding surface available and masking the corepressor-binding surface. For some receptors, like the glucocorticoid receptor (GR), ligand binding leads to changed subcellular localisation as the unliganded receptors are tethered to heat-shock proteins in the cytoplasm. When ligand binds, GR dissociates from the heat-shock complex and translocates to the nucleus to exert its transcriptional activities (53).

Even though all human NRs have LBDs, not all have known natural ligands. These receptors are often referred to as orphan receptors. With many crystal structures of NR LBDs being solved during recent years, ligands to previous orphans have sometimes been found within the ligand-binding pocket (LBP), thus adopting the orphan to the

group of receptors with known ligands (117). For NURR-1 (NR4A2) and other members of the NR4 group, however, the LBD structure revealed that the LBP is filled with amino acid side-chain, leaving no room for a ligand to enter (78, 168). NURR-1 is thus a true orphan receptor and seems to rely on ligand-independent mechanisms of regulation.

The NR family has been divided into seven groups according to the evolution of the well conserved DBDs and LBDs (1). The groups turn out to correspond to receptors with functional similarities e.g., the two oestrogen receptor (ER) subtypes (ERα and ERβ) fall into group 3, where the other steroid receptors (androgen receptor, progesterone receptor, GR, mineralocorticoid receptor) also can be found. Apart from the six identified groups there is a seventh group, group 0, containing the atypical receptors that lack either the LBD or DBD, but not necessarily sharing any other close common ancestry. In humans, group 0 contains two receptors, DAX-1 (NR0B1, Dosage sensitive sex reversal, adrenal hypoplasia congenita sensitive region on chromosome X, gene 1) and SHP (NR0B2, short heterodimer partner). These two receptors both lack the DBD of the other NRs.

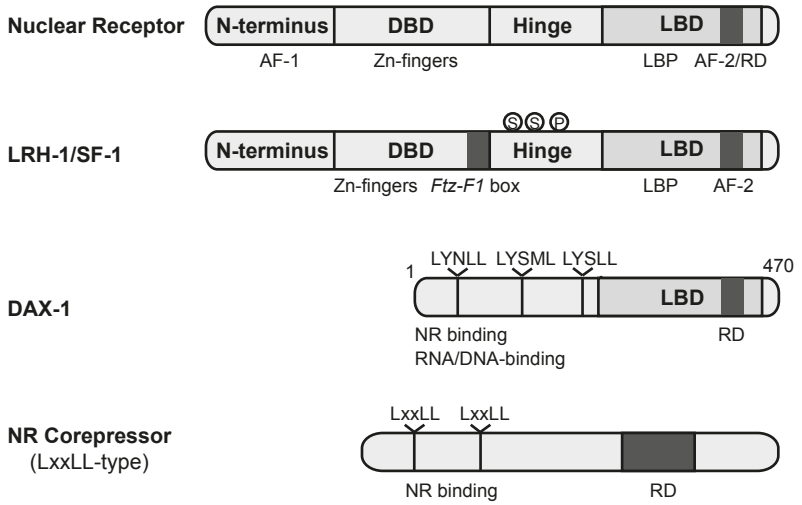

Figure 1. *Domain structure of a canonical nuclear receptor, of LRH-1/SF-1, of DAX-1 and of a ligand-dependent NR corepressor. AF: Activation function, S: SUMOylation site, P: Phosphorylation site, RD: Repression domain.*

1.2 NR5A RECEPTORS

In humans, the NR5 subgroup consists of only two receptors, NR5A1 or Steroidogenic Factor-1 (SF-1) and NR5A2 or Liver Receptor Homologue-1 (LRH-1). The NR5A group is also referred to as *Ftz-F1* due to the fact that its first discovered member *Ftz-F1* was identified as an activator of the *fushi tarazu (ftz)* gene in *Drosophila*(97). Like humans, rodents and flies have two NR5A receptors while *C. elegans* has one and teleost fish like zebrafish has four. It is believed that the two human NR5A receptors are paralogues that arose in a genome-amplification event early in vertebrate evolution (94).

1.2.1 Expression

1.2.1.1 NR5A1

The mammalian NR5A1 receptor SF-1 was discovered by two groups working in parallel. Morohashi *et al* purified bovine SF-1 from adrenal gland extracts (124) while Lala *et al* managed to isolate mouse SF-1 from the mouse adrenal cell line Y1 (95). From the start it was clear that SF-1 was involved in transcriptional regulation of steroid hormone production, as the main sites of SF-1 expression were in the tissues in the hypothalamic-pituitary-adrenal/gonadal axis. SF-1 is found in all the three layers of the adrenal cortex as well as in the steroidogenic cell types of the gonads including testicular sertoli and leydig cells and ovarian interstitium, granulosa and theca and corpus luteum cells (72, 122, 124, 125, 141). SF-1 is also expressed in the pituitary gonadotrophs (9, 77, 130), in the ventromedial hypothalamus (169) and in the spleen (126, 141).

During fetal development SF-1 expression appears in the urogenital ridge as one of the first signs of the developing adrenal/gonadal primordium (64, 65, 73, 123). SF-1 is then expressed continuously in the fetal adrenal zone and in the developing testis and human ovary but disappears during the development of the rodent ovary only to reappear after birth (64, 73, 123). Characterization of SF-1 knockout mice showed adrenal and gonadal agenesis, further highlighting the importance of SF-1 in the development of these organs. The SF-1 knockout mice all develop as phenotypical females, even those with XY chromosomes (111, 148).

1.2.1.2 NR5A2

The first mammalian NR5A2 receptor to be cloned was mouse LRH-1 (10). Subsequent studies revealed LRH-1 to be expressed in the adult tissues of the enterohepatic system including liver, intestine and exocrine pancreas, and in ovary, pre-adipocytes, adrenal and testis (41). LRH-1 knockout mice die early during development (12, 132) and investigations have shown that LRH-1 is expressed in murine embryonic stem cells and is important for maintaining their pluripotency (54).

1.2.2 Structural characteristics and ligands

The NR5A receptor joins a small subgroup of NRs including also the ERR2 (NR3B2) (47) and NGFI (NR4A1) (118) that bind as monomers to DNA. In absence of dimerisation partners these receptors seem to employ an attenuated mode of DNA-binding, adding the requirement of three 5'-bases to the canonical six-bases NR half-site. Interestingly, the main fold of the monomeric NR DBD closely resembles that of dimerizing NRs but it extends the DNA contact using a C-terminal extension (CTE) that contacts the minor groove of the DNA. In the NR5A receptors, the CTE includes an FTZ-F1-box (figure 1B), which folds into an α-helix that has contacts with both the minor groove of the DNA and the core DBD of the receptor. Interestingly, the F1-box also seems to contain a protein-protein interaction surface that can recruit specific coactivators e.g., MBF1 (156) that increases the transcriptional activity of the receptor, possibly by mediating interaction with neighbouring transcription factors (108, 154).

Up until recently SF-1 and LRH-1 were considered orphan receptors and the first structure of an NR5A LBD (mLRH-1) gave evidence of a large but empty LBP while the H12 of the LBD was in "active" conformation with the coactivator-binding surface exposed (146). This led to the hypothesis that the NR5A NRs were constitutively active and relied on regulatory processes such as post-translational modifications instead of ligands. However, later structure determinations of the human and mouse SF-1 and human LRH-1 LBDs showed bacterial phospholipids (originating from the production of the protein in *E. coli*) inside the LBP, indicating that the NR5As might not be true orphans after all (93). A later study showed that the bacterial lipids could be exchanged for mammalian phospholipids (145). There are indications of sphingosine acting as a natural antagonist ligand to SF-1 and an increased intracellular cAMP-level (an inducer of many SF-1-dependent target genes) has been shown to increase the catabolism of sphingosine possibly allowing natural agonists to bind to SF-1 (162). Sphingosine can induce the dissociation of a coactivator complex from SF-1 on the CYP17 target gene promoter, further indicating its antagonistic effect (31). Phosphatidic acid (PA) binds SF-1 in the H295R adrenocortical carcinoma cell line and PA increases transcription of SF-1 target genes (106) indicating that it may be a natural agonist of SF-1. All in all, evidence seems to suggest that multiple natural SF-1 ligands may exist and modulate SF-1 activity together with coregulator availability and post-translational modifications. However, the role of sphingosine, phosphatidic acid and other suggested SF-1 ligands needs to be further confirmed and tested *in vivo* before any final conclusion can be reached. Synthetic ligands, including both agonists (170) and inverse agonists (33), have been characterised for both SF-1 and LRH-1 opening up the possibility of treatment of diseases related to NR5A activity.

1.2.3 Transcriptional mechanisms

As discussed above, NR5A receptors bind as monomers to DNA. Like other transcription factors they are then reliant on a set of coregulators to execute the desired transcriptional response. The response may vary depending on tissue and cell type as each target tissue has a distinct set of regulated genes. This is achieved through multiple levels of control: first the state of the chromatin around the target gene and its regulatory elements. Hypermethylated DNA packs tightly and is largely inaccessible to transcription factors leading to efficient silencing of the hypermethylated gene. Methylation also governs promoter usage in cases where a gene has multiple promoters active in different cell types. Second, transcription factor availability and activity determines target gene expression. Third, the set of coregulators available in the cell determines the outcome of transcription factor binding. If, for example, high levels of a corepressor are present in the cell this will prevent the transcription factor from activating its target genes while, if levels are low, activation may be achieved.

The transcriptional process is also highly dynamic and directional in the sense that events need to take place in a certain order to achieve successful transcription. For NRs such as the oestrogen receptor (ER) it has been shown that association with the promoter is cyclic upon activation with a first round of binding that remodels nucleosomes at the promoter to allow for transcription. This round occurs within 20 min of activation and is unproductive in terms of transcribed RNA. The promoter is

then cleared and a second round of ER-binding initiates the first productive transcription round, with RNA Pol II being recruited and able to read through the gene. The productive cycle approximately 40 min after induction can be followed by monitoring activating histone modifications such as H3K14-acetylation. At the end of the cycle, corepressor complexes are recruited that change the chromatin environment to a more repressed state thus clearing the promoter. A second cycle of productive transcription is then initiated and peaks about 100 min after oestrogen treatment, ending again in recruitment of corepressors that silence the promoter by changing histone modifications and nucleosome positioning (62, 119).

Most work regarding the transcriptional mechanisms of the NR5A receptors have used transient transfections and promoter reporter assays. These assays do not fully capture the dynamics of the transcriptional process and thus the knowledge about the actual events on the NR5A target promoters is limited. Two studies of SF-1 transcriptional dynamics report SF-1 cycling in response to increased intracellular cAMP-levels. Winnay and Hammer studied the transcriptional events on the MC2R promoter in the mouse Y1 adrenocortical cell line stably overexpresssing HA-tagged SF-1. Using time-resolved chromatin immunoprecipitation (ChIP) assays, they show two cycles of SF-1 promoter binding dependent on PKA and MAPK activation and phosphorylation of SF-1 Ser203. SF-1 binds the promoter together with the coactivator GCN5 and members of the SRC-family, histone H4 acetylation increases and RNA Pol II is recruited. During the intermediate cycles, histone deacetylases are recruited and RNA Pol II is lost (172). Dammer *et al* performed a similar study on the CYP17 promoter using the human adrenocortical cell line NCI-H295R and endogenous proteins. The Dammer study confirms many of the conclusions from the Winnay study regarding the cycling, timing, histone modifications, and also shows the recruitment of corepressors such as NCoR and RIP140 during the silent cycles (31). Winnay *et al* attribute most of the activation of SF-1 to the increased phosphorylation of Ser203 in response to activation of the kinase ERK1/2 (MAPK). However, since the paper was published the increase of the possible SF-1 agonist phosphatidic acid and decrease of the antagonist sphingosine in response to raised intracellular cAMP-levels have been described (106, 162). Thus, it is not implausible that ligand activation also plays a role in the cycling of SF-1 on the MC2R promoter. In the paper by Dammer *et al* it is shown that increased levels of sphingosine can disrupt the formation of the SF-1/GCN5/SRC-1 transcriptional activator complex on the CYP17 promoter. To our knowledge no kinetic studies of LRH-1 transcription have yet been made.

1.2.4 Post-translational modifications and transcription

1.2.4.1 SF-1

SF-1 is involved in the regulation of genes governed by pituitary gonadotropes. These peptide hormones bind to cell surface receptors and activate intracellular signalling pathways like PKA and MAPK. These pathways can use post-translational modifications of effector proteins like SF-1 to govern transcription. Serine203 in the hinge region of SF-1 is a site for regulatory phosphorylation and in response to cAMP-stimuli SF-1 phosphorylation increases. Phosphorylation is required for maximal activation of some target genes and P-Ser203 increases the affinity of SF-1 for the

coregulators GRIP1 (activator) and SMRT (repressor). PKA is the most well known kinase activated by cAMP and SF-1 does have a PKA-consensus site. However, PKA does not phosphorylate SF-1; instead the MAP Kinase ERK1/2 is able to induce Ser203 phosphorylation *in vivo* and MAPK inhibitors reduce the transcriptional activity of SF-1 in cellular assays (63). More recent work has identified Cyclin dependent kinase-7 (CDK7) as another kinase that can phosphorylate Ser203. CDK7 is a part of the TFIIH complex; i.e. the part of the general transcriptional machinery that can phosphorylate the C-terminal domain of RNA Pol II and is thus present on actively transcribed promoters. Mutations in the LBP of SF-1 thought to disrupt its ability to bind ligands inhibit the ability of CDK7 to phosphorylate Ser203 (105). Possibly, LBP mutations decrease the recruitment of SF-1 to promoters, thus preventing the interaction between CDK7 (TFIIH) and SF-1 (105).

While phosphorylation increases SF-1 activity, SUMOylation has been shown to do the opposite. SUMOylation occurs in the hinge-region of SF-1, on K119 and K194, thus in close proximity to the phosphorylation site (23, 92). Actually, SUMOylation limits the phosphorylation both in pure *in vitro* (18) and in cell based assays (175). Only small portions of the total cellular SF-1 content are SUMOylated and how this translates to efficient repression has been debated. *In vitro* assays suggest that SUMOylated SF-1 has decreased affinity for a subset of target genes with non-canonical response elements (18). Another hypothesis postulates that SUMOylated transcription factors are sequestered into distinct nuclear bodies where they are unable to bind DNA and activate transcription. Indeed, SUMOylated SF-1 has been shown to relocate to distinct speckles (23, 98). Sequestering could potentially explain how SUMOylation inhibits phosphorylation *in vivo*. If SF-1 phosphorylation primarily occurs due to CDK7 on the promoter of actively transcribed genes, exclusion from DNA-binding via SUMO-dependent sequestering would lead to decreased phosphorylation. If SUMOylation takes place on or near the promoters of target genes those promoters with high affinity also for SUMO-SF-1 could possibly retain the protein until a SUMO-peptidase removes the PTM, while those promoters with weak affinity would lose the SUMO-SF-1 to nuclear speckles. This would result in subsets of SUMO-sensitive and -insensitive genes as described by Campbell *et al* (18). To date, it is not known what cellular signals induce SUMOylation of SF-1. Such knowledge is necessary to allow full interpretation of the functional consequences of SF-1 SUMOylation.

The SUMO-relative ubiquitin is best known for its ability to regulate the degradation of proteins by targeting them for destruction by the proteasome. SF-1 has been shown to be ubiquitinated and the proteasome inhibitor MG132 actually abolishes GnRH induced SF-1 cycling on and off the LHβ-promoter in the LβT2 cell line (165). MG132 also inhibits RNA Pol II cycling in the same way showing that proteasomal activity is important for proper transcription of SF-1 target genes. In a recent paper it is shown that ubiquitination of SF-1 is phosphorylation-dependent and occurs at K119, the lysine that is also SUMOylated (112). One could speculate that SF-1 is phosphorylated on the promoter during the active phase of the transcription cycles and that the phosphorylated SF-1 has increased affinity for the yet undefined E3 ubiquitin ligase that polyubiquitinates it. Polyubiquitination then attracts the proteasome, which

clears the promoter for another round of transcription, or for silencing if the activating signal is gone.

SF-1 is also a target for acetylation by the coactivator protein GCN5 (80) and by p300 (22). cAMP induces the p300 mediated acetylation and increases the DNA-binding capacity of SF-1. The site for acetylation is located within the *Ftz-F1* box in the DBD and acetylation seems to increase DNA-binding (22).

1.2.4.2 LRH-1

LRH-1 is phosphorylated in the hinge region in a similar manner as SF-1. The acceptor serine residues differ between the proteins however, with Ser238 and Ser243 reported for LRH-1. These residues are not conserved in the SF-1 sequence. Interestingly it seems that the MAPK pathway is responsible also for LRH-1 phosphorylation and P-LRH-1 is also activated compared to LRH-1, similar to SF-1 (99). The detailed consequences of LRH-1 phosphorylation are not completely understood but P-LRH-1 synergizes more effectively with GATA transcription factors leading to greater activation of the CYP19 gene in breast cancer cells (13). The increased synergy could perhaps be valid also in other promoter contexts but this remains to be proven.

LRH-1 is also SUMOylated resulting in transcriptional repression. SUMOylated LRH-1 is sequestered into nuclear PML-bodies where it is retained from activating transcription. Induction of cAMP-signalling changes the dynamics of LRH-1 recruitment to PML-bodies indicating that activating signals alter the SUMOylation status of LRH-1 (20). Raised cAMP-levels also change the expression of enzymes in the SUMOylation pathway. The E2 conjugating enzyme UBC9 and the E3 ligases of the PIAS family are reduced by cAMP while the SUMO-specific protease SENP2 expression is increased, indicating reduced SUMOylation of target proteins (174). The SUMO-sites of LRH-1 are located in the hinge region and it is the same sites as used in SF-1 SUMOylation.

1.3 DAX-1

The NR0B receptor subfamily has two members in humans, DAX-1 (NR0B1) and SHP (NR0B2). They share a unique domain structure within the human NR family as they lack the DBD and retain only the LBD. In the N-terminus they both carry LxxLL-motives, also called NR-boxes, often found in coactivators of NR transcription. The NR-box binds to the surface exposed on the LBD of ligand-activated NRs, the so-called AF-2 domain (figure 1) (82, 83, 155, 178). Both SHP and DAX-1 have mainly been described as repressors of transcription. The lack of DBD and the presence of NR-boxes makes the NR0B receptors more functionally similar to coregulators than conventional NRs and they join a small but important class of corepressors including RIP140 and LCoR that interact with agonist-activated NRs (figure 1) (58). In addition to being regarded as corepressors, DAX-1 and SHP could be viewed as transrepressive NRs that have lost the ability to bind DNA. They are not the only NRs that act *in trans* on the promoter of target genes. In fact, repression by tethering to another transcription factor seems to be a common way for NRs to interfere in other signalling pathways. For

example, in inhibiting the expression of pro-inflammatory factors, the glucocorticoid receptor can bind to both NF-κB and AP1 transcription factors to block coactivator recruitment. Similar, but SUMO-dependent, pathways are employed by PPARγ and the LXRs in inhibiting pro-inflammatory gene expression (50).

DAX-1 was first identified as the cause of two X-linked developmental disorders, adrenal hypoplasia congenita (AHC) (176) and XY-sex reversal (8). AHC is connected to mutations of DAX-1 (128) while sex reversal is caused by a duplication of the NR0B1 gene on the X chromosome (8). DAX-1 is functionally closely connected to SF-1 and they have remarkably similar expression patterns in the hypothalamic-pituitary-adrenal/gonadal axis (74, 75). Transcriptional repression by DAX-1 has been attributed to two different mechanisms: first, by direct interaction with target gene promoters via the unique N-terminal domain (115, 177) and second, by interaction with the coactivator binding surface of target NRs as described above. Specific stem-loop (hairpin) structures of DNA at the promoters are required for direct interaction between DAX-1 and DNA and have been proposed to exist in the StAR and DAX-1 promoters (115, 177), but it remains to be proven if such structures are actually formed *in vivo*. RNA, on the other hand, readily forms stem-loop structures and DAX-1 has been reported to bind to RNA (96). Transrepression of NRs by DAX-1 works at two levels. Interaction with the AF-2 surface on the target receptor blocks recruitment of coactivators in a "repression by exclusion" mechanism. The LBD of DAX-1 can additionally recruit corepressors such as Alien (5) and NCoR (29) and probably other yet unknown factors such as histone deacetylases (HDACs) and methyltransferases that are known to work together with SHP (40, 51), DAX-1's closest relative.

A crystal structure of the LBD of DAX-1 in complex with the LBD of LRH-1 suggested a heterotrimeric complex containing two DAX-1 molecules and one LRH-1. The LBPs of both DAX-1 molecules in this structure were filled with amino acid side chains leaving only 80 $Å^3$ compared to the 830 $Å^3$ of the LRH-1 LBP. 80 $Å^3$ is too small to accommodate a ligand making it probable that DAX-1 is truly ligand-independent in its actions. In the complex, a helix in the DAX-1 LBD interacted with LRH-1. This mode of binding was somewhat surprising and may not be biologically relevant, as the crystallised proteins did not contain the N-terminal part of DAX-1. However, the authors report that the DAX-1 LBD actually has a higher affinity for LRH-1 than its N-terminus, LxxLL-containing part *in vitro*, giving an indication that this binding mode may be correct (147). These data are contradictory to previous yeast-two hybrid data showing that the DAX-1 N-terminus is indispensible for interaction with NRs (155, 178). Future experiments will have to determine which model is most accurate *in vivo*.

In light of the apparent ligand-independence of DAX-1, surprisingly little is known about alternative modes of regulation such as post-translational modification. In contrast to SHP where phosphorylation and ubiquitination (120) have been described to influence the transcriptional activity no such studies concerning DAX-1 have, to our knowledge, yet been published. This could be due to the lack of good, commercially available, antibodies specific to DAX-1 making this research technically challenging.

1.4 STEROIDOGENESIS AND SF-1

The biosynthesis of steroid hormones from cholesterol is a multistep process catalysed by members of the cytochrome P450 (CYP450) and hydroxysteroid dehydrogenase (HSD) enzyme families (figure 2). The process also depends on cholesterol transporter proteins for substrate availability. Two rate-limiting steps govern steroid synthesis in all steroidogenic tissues: the availability of cholesterol in the inner mitochondrial membrane controlled by the steroidogenic acute regulatory protein (StAR) and the conversion of cholesterol to pregnenolone catalysed by the CYP11A enzyme. StAR is responsible for the fast changes in synthesis rate while CYP11A-concentration sets the maximum synthesis rate in the long term. After cholesterol is converted to pregnenolone, pregnenolone is turned into either progesterone, by 3βHSD, or to 17α-Hydroxypregnenolone, by CYP17. CYP17 can convert 17α-hydroxypregnenolone to dehydroepiandosterone (DHEA), an androgen secreted by the adrenal zona reticularis. CYP17 also turns progesterone into 17α-hydroxyprogesterone and further to androstenedione, which is the precursor to the sex steroids produced in the gonads. CYP17 is not expressed in the adrenal zona glomerulosa of humans. The lack of CYP17 and expression of CYP11B2 allows for the production of the mineralocorticoid aldosterone. Progesterone or 17α-hydroxyprogesterone is converted to 11-deoxycorticosterone or 11-deoxycortisol by CYP21 and then to corticosterone or cortisol by CYP11B1. Cortisol is the main glucocorticoid synthesised by the human adrenal cortex and the main production site is the zona fasciculata. In the gonads, androstenedione can be aromatised into oestrone by CYP19 (aromatase) and then converted to oestradiol by 17HSD1, or 17HSD3 can convert androstenedione to testosterone, which can be aromatised by CYP19 to estradiol (121, 135).

Adrenal steroidogenesis is mainly under the control of the peptide hormones angiotensin II and adrenocorticotropic releasing hormone (ACTH). A diverse set of other factors, such as epidermal growth factor (EGF), basic fibroblast growth factor, calcium ion influx and Wnt/β-catenin signalling, also contribute but to a lesser extent. Angiotensin II is important for regulating blood pressure and sodium/potassium balance of the body and in the adrenal it mainly affects the mineralocorticoid production of the zona glomerulosa. ACTH is released from the pituitary gland and affects steroid synthesis in the whole adrenal cortex, but is perhaps most important in the zona fasciculata and zona reticularis (153).

ACTH binds to the melanocorticoid 2 receptor (MC2R) expressed on the surface of adrenal cells through which it activates adenylate cyclase that converts ATP to cAMP. SF-1 controls the expression of MC2R (17) and can thus attenuate the hormone response elicited by the cell. Raised intracellular cAMP levels activate protein kinase A (PKA) that can phosphorylate numerous intracellular targets including the CRE-binding proteins. Phosphorylated CREB/CREM can activate many of the same genes as SF-1. Activation of PKA does not affect SF-1 directly but, as mentioned previously, raised cAMP-levels induce catabolism of the SF-1 antagonist sphingosine and biosynthesis of the putative SF-1 agonist phosphatidic acid (106, 162)

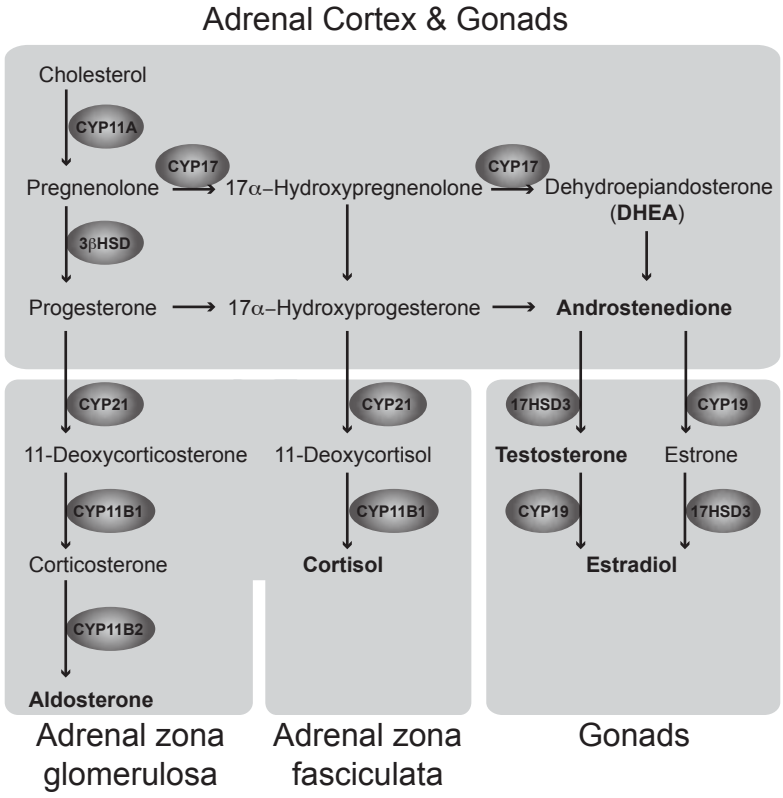

Figure 2. *Schematic figure showing the main steps of steroid synthesis in the adrenal glands and gonads. All enzymes in the figure have been described as SF-1 target genes (67).*

SF-1 target genes in the steroidogenic tissues include most of the proteins needed for the production of steroid hormones from cholesterol, indicating that SF-1 is a master regulator of steroidogenesis. It acts from the level of cholesterol import via the HDL-R (SRB1) (19) through intracellular transport of cholesterol by SCP2 (109) and NPC-1 (48) and mitochondrial import via StAR to the level of conversion of cholesterol to steroid hormones by the cytochrome P450 enzymes including CYP11, CYP17, CYP19 and CYP21 and 3β-HSD (7, 43, 102, 113, 127).

Among the SF-1 target genes is DAX-1 (16). As mentioned, DAX-1 is a repressor of SF-1 dependent transcription (79) and forms a local negative feedback loop where high SF-1 activity increases DAX-1 levels causing repression of SF-1. When cAMP-levels rise, DAX-1 levels drop involving a not fully characterised mechanism dependent on kinase activity and transcriptional regulation of DAX-1. As DAX-1 levels drop and the raised cAMP-levels induce steroidogenesis, glucocorticoid levels rise. The glucocorticoids activate GR in the adrenal that activates transcription of DAX-1, which can then again repress steroidogenesis through interaction with SF-1 (55).

Analogous to its role in the adrenal, SF-1 also governs steroidogenesis in testes by activating the transcription of steroidogenic enzymes and cholesterol transporters. No feedback mechanism resembling that of the glucocorticoid induction of DAX-1 has been described in the testes but as GR and the androgen receptor bind to the same response elements (34) it is possible that increased androgen synthesis leads to

increased expression of DAX-1 through an androgen receptor dependent mechanism. This, however, remains to be tested *in vivo*.

1.5 SF-1 AND CANCER

The development of the adrenal cortex is complex and the importance of SF-1 in this process is highlighted by the lack of adrenal glands and gonads in SF-1 knockout mice (148). After birth the fetal zone of the human adrenal cortex undergoes massive apoptosis and simultaneously the adult definitive zones of the adrenal cortex proliferate and migrate to form the adult adrenal cortex. The steroid hormone production profile is also different between the fetal and adult cortex with the fetal cortex producing high levels of DHEA while the adult cortex produces higher levels of mineralocorticoids and glucocorticoids (84). Adrenocortical carcinoma is a rare disease but it has severe consequences for the patients with high mortality rates and no efficient treatment. In children, adrenocortical carcinoma is most common during the first five years of life. The incidence of childhood adrenocortical carcinomas is highest in southern Brazil, in a population that has a low penetrance germline p53 mutation (139, 142). Interestingly, SF-1 was found to be overexpressed in these tumours (42, 138). In an effort to investigate whether SF-1 had influence on the pathogenesis of childhood adrenal carcinomas, an adrenal cell line overexpressing SF-1 was created. Increased SF-1 dosage did increase the proliferation rate of the cells, and genes involved in cell cycle regulation were shown to be altered (38). SF-1 inverse agonists could decrease proliferation (37) but the suggested natural SF-1 antagonist sphingosine (38) had no effect on the SF-1 overexpressing cell line. The steroid secretion profile of the SF-1 overexpression cell line was also changed and became more "fetal-like" with high levels of DHEA and lesser amounts of cortisol and aldosterone (38). It has been suggested that childhood adrenocortical tumours arise when the fetal zone fails to regress properly (171); the fact that high SF-1 dosage induces a fetal-type secretion pattern as well as increased proliferation seems to support this hypothesis.

In adults, there are less clear data regarding SF-1 expression during adrenocortical carcinogenesis. SF-1 overexpression does not seem to be a common theme (4) but a recent, large study showed that SF-1 expression has diagnostic value in determining whether a tumour is of adrenal origin or not. This is often difficult since adrenal tumours are heterogeneous and metastases from cancers of other origins are often located in the adrenal. Of the samples tested, 98% (158 of 161) of the adrenal tumours expressed SF-1 while none of the others (0 of 73) expressed SF-1. Interestingly, there was a negative correlation between survival rate and level of SF-1 expression in the adrenal carcinomas indicating that SF-1 could be of importance also for cancer progression in adults (151).

1.6 CHOLESTEROL METABOLISM AND LRH-1

Maintenance of cholesterol homeostasis is accomplished by balancing cholesterol synthesis, uptake and excretion. The conversion of hydrophobic cholesterol into amphipatic bile acids is the main pathway for cholesterol excretion. Bile acids are primarily synthesised in the hepatocytes from where they are transported into the gall

bladder and stored until, upon food intake, they are excreted into the intestinal tract where they are essential for lipid and fat-soluble vitamin uptake. 95% of all bile acids are recycled, some through passive uptake in the upper intestine but most are reabsorbed in the ileum and returned to the hepatocytes through the portal vein. About 5% escape the enterohepatic circulation during each cycle representing approximately 0.2-0.6 g each day in humans (24).

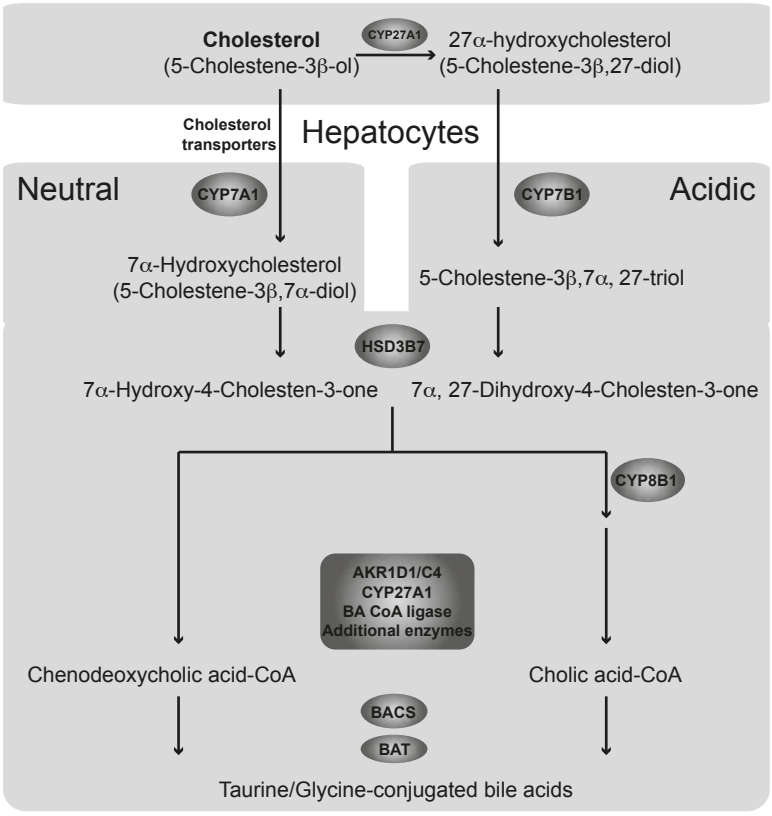

Figure 3. *Schematic figure of bile acid synthesis. CYP7A1, CYP8B1 and cholesterol transporters are among the known LRH-1 regulated genes.*

The conversion of cholesterol to bile acids is a multistep process (figure 3) that starts with the action of the rate-limiting enzyme CYP7A1 that turns cholesterol into 7α-hydroxycholesterol. The sterol ring is then further modified by HSD3B7 to 7α-hydroxy-4-cholesten-3-one then by either AKR1D1 followed by AKR1C4 to 5β-cholestane-3α,7α-diol or by CYP8B1 to 7α,12α-dihydroxy-4-cholesten-3-one followed by AKR1D4 and AKR1C4 to 5β-cholestane-3α,7α,12α-triol. The side-chain is shortened by the actions of CYP27A1 and additional enzymes to produce Coenzyme A(CoA)-conjugated cholic acid (CoA-CA, 3α,7α,12α-trihydroxy-5β-cholan-24-oic-CoA) if CYP8B1 was used or CoA-conjugated chenodeoxycholic acid (CoA-CDCA, 3α,7α-dihydroxy-5β-cholan-24-oic-CoA) otherwise (144). An alternative pathway, sometimes called the acidic pathway, bypasses the need of CYP7A1 and starts with producing 27-hydroxycholesterol by the action of CYP27A1 and then uses CYP7B1 to produce 7α-hydroxylated intermediates that pass into the classical pathway at the level of HSD3B7. The acidic pathway is responsible for about 25% of all bile acid synthesis (103, 144, 158). CoA-cholic acid and CoA-chenodoxycholic acid are conjugated to taurine or glycine before they are passed into the gall bladder. The primary bile acids CA and CDCA are processed by microbes in

the gut to form the secondary bile acids deoxycholic acid (3α, 12α-dihydroxy-5β-cholan-24-oic acid, from CA) and lithocholic acid (3α-hydroxy-5β-cholan-24-oic acid, from CDCA). The cycling bile acids are a mixture of freshly made primary and recycled secondary bile acids. CA and CDCA are the main primary bile acids in humans but in rodents CDCA is turned into muricholic acid (3α,6α,7β-trihydroxy-5β-cholanoic acid) (103, 158).

Bile acids function as signalling molecules by binding both to the G-protein coupled receptor TGR5 and to the nuclear receptors FXRα, PXR and VDR. FXRα is often considered the master regulator of bile acid homeostasis as it acts on multiple levels to ensure that correct levels of circulating bile acids are maintained. In the liver, raised BA levels induce FXR transcriptional activity on the SHP (NR0B1) promoter. Raised levels of SHP, in turn, repress the expression of the rate-limiting BA synthesis enzyme CYP7A1, a mechanism that also involves GPS2 (150). Both HNF4α (30, 100) and LRH-1 (32, 52, 110) have been implicated in the bile acid induced repression of CYP7A1 and both can bind to the same region in the CYP7A1 promoter. However, recent publications report that two strains of liver specific LRH-1 knockout mice surprisingly did not have altered levels of CYP7A1 (116) and retained the ability of BA repression of CYP7A1(101) indicating that LRH-1 may play a minor or redundant role in CYP7A1 regulation in mice. The observation that levels of HNF4α correlate with CYP7A1 levels in human liver supports this notion (2). Further experiments are needed to reconcile the conflicting data regarding the effect of LRH-1 on CYP7A1.

The liver-specific LRH-1 knockout mice did however show a drastic decrease of CYP8B1 expression indicating that LRH-1 may be crucial for determining the composition of the bile acid pool. Other genes important for BA synthesis and transport, including SHP, CYP27A1, SCARB1 and sodium-dependent bile acid transporter, are also affected by LRH-1 knockout emphasising that LRH-1 does indeed have an important role in BA metabolism even though it may play a smaller role in CYP7A1 regulation than previously believed. Interestingly, even though CDCA is a more potent agonist than CA for FXR in many assays (14, 114, 133, 166), CA seems to induce a subset of FXR target genes more efficiently than CDCA. This was demonstrated by the use of CYP8B1 knockout mice. They lost some of the ability of BA-mediated repression of CYP7A1 causing an increase in the total BA pool in the animals. It was also shown that dietary cholate more efficiently than dietary chenodeoxycholate restored the BA pool size to normal levels. Hepatic SHP levels were reduced in the CYP8B1 knockouts while other FXR targets like BSEP were unchanged and cell-based promoter assays showed that SHP responded more efficiently to CA than CDCA (107). If CA is indeed a more potent FXR agonist than CDCA for a subset of genes, then the altering levels of CYP8B1 could lead to changes in this CA-sensitive subset of FXR target genes giving LRH-1 an additional role in the regulation of cholesterol/bile acid metabolism (101).

The BA induced SHP-dependent repression of CYP7A1 and BA synthesis is not the only mechanism in place to control the rate of BA formation. BA concentration in the ileum regulates the activity of locally expressed FXRα that induces the expression of fibroblast growth factor (FGF) 15 (mice) / 19 (humans). FGF15/19 then circulates to the hepatocytes where it binds to the membrane receptor FGFR4 and apparently

induces SHP-independent repression of BA synthesis (68, 76). The SHP-independence is strengthened by the observation that dietary BAs, but not synthetic FXR ligands, can induce repression of CYP7A1 in SHP knockout mice (88, 167). However, the FGF15/19 pathway may not be completely SHP-independent as activation of FGF4R by FGF15/19 results in increased ERK1/2 kinase activity. ERK1/2 phosphorylates SHP on Ser26 and that inhibits targeting for degradation by ubiquitination on Lys122/123. The stability of SHP thus increases, as does the intracellular SHP pool (120). The contribution of this mechanism to the overall FGF15/19 repression of BA synthesis has yet to be worked out and harmonised with the SHP knockout data.

1.7 LRH-1 IN INFLAMMATION AND CANCER

Apart from regulating genes involved in cholesterol and lipid homeostasis, LRH-1 is also known for its role in governing cell proliferation. Through interactions with the Wnt/β-catenin pathway, LRH-1 has been shown to increase levels of cyclin D1 and cyclin E1 leading to a progression of the cell through the cell cycle. The mechanism of regulation differs for the two cyclins: on the cyclin E1 promoter LRH-1 interacts directly with a DNA RE to activate transcription and β-catenin can further enhance transcription by binding to LRH-1. On the cyclin D1 promoter LRH-1 activation is independent of the LRH-1 DBD and only requires the C-terminus to function. Here, LRH-1 is tethered to the DNA-bound Tcf4/β-catenin complex, acting in a sense as a transcriptional coactivator in this crosstalk mechanism. The interaction between the β-catenin pathway and LRH-1 was shown to be important in the renewal of intestinal crypts (12). Aberrant Wnt/β-catenin signalling is known to be of great importance in colon cancer (25) and mice with LRH-1 haploinsufficiency are less sensitive to carcinogen-induced intestinal tumour formation than wild-type mice. When heterozygous LRH-1 knockout mice were crossed with mice heterozygous for a mutation in the APC gene ($APC^{min/+}$ mice, a common colon cancer model), the resulting $APC^{min/+}$/LRH-$1^{+/-}$ mice developed fewer tumours compared to the $APC^{min/+}$ mice. However, when intestinal tumours were assayed for LRH-1 content they showed a reduction compared to normal tissue, which was linked to increased inflammation and TNFα-levels in the tumours (152). What this implies for the role of LRH-1 in intestinal tumours awaits clarification.

Inflammation in the intestine is connected to cancer but also to the pathogenesis of Crohn's disease and ulcerative colitis. LRH-1 haploinsufficient mice are sensitive to induced inflammation in the intestine through a mechanism involving alteration of local glucocorticoid production. Levels of CYP11A1 and CYP11B1 enzymes important for cortisol and corticosterone production are reduced in both LRH-1 haploinsufficient and in intestinal conditional LRH-1 knockout mice. Human patients suffering from inflammatory bowel disease showed decreased levels of LRH-1, CYP11A1 and CYP11B1. The reduction was correlated to an increased inflammation strengthening the hypothesis that LRH-1 may be acting inhibitory in this inflammatory process (28). LRH-1 has also been implicated to have an inhibitory effect on the hepatic acute phase response (APR). Through crosstalk with C/EBP, LRH-1 inhibits the expression of acute phase proteins such as C-reactive protein, haptoglobin and serum amyloid A. The mechanism probably involves tethering of LRH-1 to DNA-bound C/EBP and recruitment of additional, yet unknown, factors (164). LRH-1 further inhibits APR

through induction of the interleukin-1 receptor antagonist (163). Taken together, the data from the intestine and liver indicates that LRH-1 is an important suppressor of inflammatory processes that could be of use in treating conditions such as inflammatory bowel disease and artherosclerosis.

CYP11A1 and CYP11B1 are known SF-1 targets in the adrenal and gonads. As SF-1 and LRH-1 have the capacity to bind the same response elements, it is not surprising that LRH-1 could regulate the expression of CYP11A1 and CYP11B1 in the intestine (28). The ability of LRH-1 and SF-1 to regulate common target genes possibly has consequence for the development and maintenance of breast cancer as well. In normal adipose tissue aromatase is controlled by a promoter called 1.4 (180) but in breast cancer tissue the promoter usage changes and one of the implicated promoters is PII (3), containing the SF-1 RE (113). LRH-1 has been shown to induce the expression of aromatase in breast tumour tissue or the surrounding adipose tissue by binding to PII (26, 27, 181). Interestingly, LRH-1 expression is induced by prostaglandin E2 excreted by breast tumours. This stimulates LRH-1 and thus aromatase expression and oestrogen production in the surrounding adipose tissue. The increased oestrogen concentration further stimulates tumour growth and prostaglandin E2 production and a feed-forward loop is created (181). This mechanism is of extra importance in postmenopausal patients as their main site of oestrogen synthesis driving the progression of hormone-dependent breast cancer is the adipose tissue. Adding to the feed-forward loop is the reciprocal regulation between LRH-1 and ERα in breast cancer cells where LRH-1 can induce ERα expression (157) and ERα can induce LRH-1 (6). Increase of LRH-1 could potentially also drive the expression of the other steroidogenic enzymes required for oestrogen production as these enzymes are SF-1 target genes in other tissues and thus contain LRH-1 binding sites. It has also been shown that LRH-1 can increase breast cancer cell invasiveness by changing the actin cytoskeleton and E-cadherin-dependent adherens junctions in both ERα-positive and negative cells (21). In light of these data, LRH-1 antagonists could constitute a novel strategy to treat breast cancer in the future.

1.8 UBIQUITINATION AND SUMOYLATION

Posttranslational modification of proteins by ubiquitination was first discovered as a “kiss of death” as proteasome mediated degradation depends on labelling targets with ubiquitin chains. This mechanism earned the discoverers the Nobel Prize in Chemistry in 2004. Since its discovery ubiquitination has turned out to be a much more diverse signal and different types of ubiquitin chains, monoubiquitination and mulitmonoubiquitination have now been described. These are not recognised by the proteasome but have other regulatory functions (89).

Attaching the 9 kDa protein ubiquitin to a substrate is a three step process (figure 4) initiated by the E1 ubiquitin activating enzyme that catalyses the formation of a thioester bond between its active site cysteine and the N-terminus of ubiquitin. The activated ubiquitin is then transferred to the E2 conjugating enzyme. Multiple E2s exist in the human genome and they seem to have some specificity in regard to which E3 they can deliver ubiquitin to. The last step, where ubiquitin is attached to a lysine residue (or in infrequent cases the N-terminus) on the substrate protein, is carried out by

the E3 ubiquitin ligase. The E3 ligase confers the substrate specificity in the process and hundreds of putative ligases have been identified in the human genome. There are different classes of E3 ligases, the biochemically best characterised being the HECT proteins. They have a cysteine residue in the active site to which the activated ubiquitin is delivered from the E2 before being transferred to the substrate protein. Another class of E3 ubiquitin ligases, the so-called ring-finger proteins, seems to bring the target lysine and the E2-attached ubiquitin into a favourable position for the transfer to occur but are not believed to participate directly in the process (89, 140).

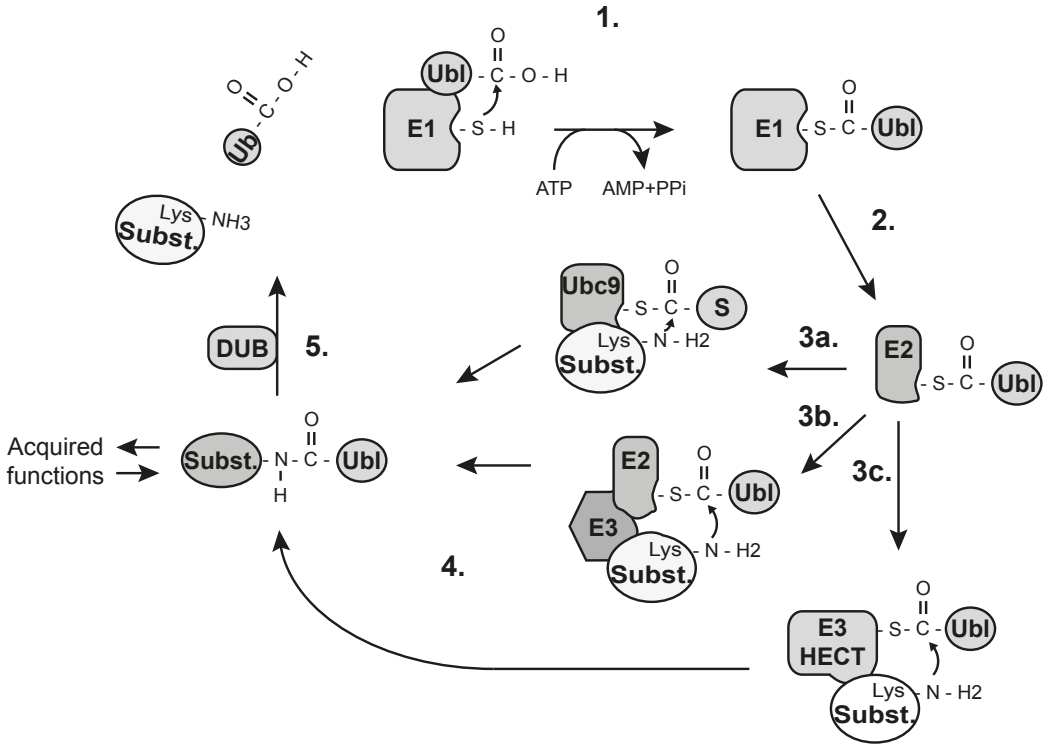

Figure 4. *Post-translational modification of a substrate protein with ubiquitin-like protein.* ***1****. Ubl is activated and attached via a thiolester bond to the E1 enzyme in an ATP-dependent process.* ***2.*** *The activated Ubl is transferred to the E2 conjugating enzyme.* ***3a.*** *In the case of SUMO, the E2 can interact directly with certain substrates and transfer SUMO to a lysine residue in a SUMO-consensus motif on the substrate protein.* ***3b.*** *Most Ubls must, and SUMO can, use an E3 ligase to transfer Ubl to a lysine residue on a substrate protein.* ***3c.*** *HECT-domain ubiquitin ligases has an active site cysteine used in the process of attaching ubiquitin to substrate proteins.* ***4.*** *Ubl-modified substrates have acquired functions compared to the unmodified protein. Ubls can both create and mask interaction surfaces thus changing the interactome of the substrate protein.* ***5.*** *Deconjugating enzymes (DUB in figure) can reverse modification with Ubl, returning substrate to its ground state.*

A whole class of ubiquitin-like post-translational modifications was found following the initial discovery of ubiquitin. These include SUMO, ISG15 and Nedd8. Ubiquitin-like modification of target proteins follows a similar mechanism as ubiquitination (figure 4), involving the same type of enzymes. However, they all have

their separate E1, E2 and E3s. SUMOylation is the best-characterised ubiquitin-like modification and has been shown to have functions in e.g. transcription, nuclear transport and DNA-repair (46, 89). There are four different SUMO-proteins in the human genome, SUMO-1, 2, 3 and 4. Whether SUMO-4 functions as a protein modification or not is debated (57, 131). SUMO-2 and 3 are very similar and seem to be interchangeable while SUMO-1 has more distinct functions. In contrast to the ubiquitination system, where tens of E2s and hundreds of E3s have been identified, only one SUMO-E2 (Ubc9) and a few E3s (including PIAS and RanBP2) are known today. SUMOylation can occur with only the action of the E1 and E2 SUMOylation enzymes, even though an E3 is thought to make conjugation more efficient. The reason appears to be that the SUMO consensus site ψKX(D/E) (where ψ is a large, hydrophobic aa) interacts with Ubc9 (149). Thus some of the substrate specificity is conferred by the E2 in the SUMOylation reaction. SUMOylation also occurs at non-consensus sites and in those cases the E3 is solely responsible for specificity. For nuclear receptors, SUMOylation is generally linked to decreased transcriptional activity via sequestering to distinct nuclear bodies as described for e.g., LRH-1 (20) or increased transrepression as is the case for e.g., PPARγ (134) and LXR (49), constituting a passive and active method of repression respectively (161).

1.9 RNF31

Ring finger protein 31 (RNF31, PAUL, ZIBRA, HOIP) was first described for its expression in skeletal muscle where it interacts with the muscle specific kinase (MuSK) (15) and for its expression in breast cancer cells (159). RNF31 belongs to the ring-in between-ring (RBR) family of E3 ubiquitin ligases. The RBR domain has a three partite composition of an N-terminal ring domain followed by an IBR (in between rings) and a C-terminal atypical ring domain. The RBR protein family has 15 members in humans and is the least understood domain in terms of both structure and function among the ubiquitin ligases (39). RNF31 also contain a putative ubiquitin binding domain (UBA) and three C-terminal zinc fingers of a type implicated in ubiquitin binding (15).

RNF31 is best known for its participation in the complex LUBAC where it catalyzes the formation of linear ubiquitin chains together with the RBR-protein RBCK-1 (RNF54, HOIL1, XAP3) (91, 160). The LUBAC complex was shown to mediate degradation of conventional protein kinase C (cPKC) family members (129). The first report of linear ubiquitin chains formed in cells, however, came with the demonstration that LUBAC attaches linear polyubiquitin chains to NEMO in response to TNFα- and IL-1β-stimulation. NEMO is part of the complex retaining NF-κB in the cytoplasm in absence of stimulatory signals. Upon stimulation, Tokunaga *et al* showed that LUBAC induces linear polyubiquitination of a lysine residue within NEMO. The chain serves as a scaffold for recruiting TAB2/TAK-1-containing complex, ultimately resulting in K48-linked polyubiquitination and proteasomal degradation of IKK. Loss of IKK releases NF-κB to translocate to the nucleus and activate target gene transcription (160). The pathway seems to serve as an alternative to the previously described mechanism where K63-linked polyubiquitination of NEMO by the Ubc13/Uev1A/TRAF2/5 ubiquitin E2/E3 complex induces the same process as the linear polyubiquitination of NEMO (60). The mechanism was confirmed and

elaborated when the recruitment of LUBAC to the TNF-receptor complex, via interaction with a ubiquitin chain, leading up to linear ubiquitination of NEMO was described (61). It also seems that RNF31 (HOIP) plays a role in CD40-dependent activation of NF-κB pathways as RNF31 copurifies with the CD40 in CH12.LX cells (69). Whether or not HOIL is involved in this mechanism remains to be elucidated. Despite the first implications of linear ubiquitination in degradation of a model substrate it seems that, in contexts of endogenous pathways LUBAC does not mediate degradation but instead induces other regulatory functions. RNF54 (HOIL-1) has also been described to act as a ubiquitin ligase for substrates on its own (173) and also to have functions in the nucleus where it can regulate the expression of ERα (59).

1.10 GPS2

G-protein pathway supressor-2 (GPS2) is a transcriptional coregulator with the capacity to engage in both activation and repression of target genes in a context dependent way. As an integral part of the NCoR/HDAC3/TBL1/TBLR1 corepressor complex it is recruited to promoters of repressed genes, both via nuclear receptors and other transcription factors like JNK (179). GPS2 also interacts with the histone acetyl transferase (HAT) p300 to activate both p53 and papillomavirus E2 protein dependent transcription (136, 137). NRs have been shown to interact with both corepressor and coactivator GPS2-containing complexes. In cholesterol metabolism, GPS2 recruits the NCoR-complex to the CYP7A1 promoter via interaction with SHP and promoter-bound LRH-1, thus repressing the gene. However, it also interacts with activated FXR on an enhancer element in the CYP8B1 bringing in p300 and probably connecting it to the proximal promoter through p300 interacting with HNF4 (150). A similar mechanism seems to take place on the cholesterol transporter ABCG1-promoter. GPS2 mediates the contact between enhancer-bound LXR/RXR dimer with LXR/RXR bound to response elements in the proximal promoter. The recruitment of HATs and histone demethylases is dependent on GPS2 in this system and the mechanism sets the highly inducible ABCG1 apart from the more constitutively expressed ABCA1 gene. On ABCA1, GPS2 binds to LXR/RXR together with the NCoR corepressor complex, thus repressing instead of activating (81). It is still unknown what signals govern the recruitment of either coactivator bound or corepressor bound GPS2 to a gene. The observation that the same proteins can utilise both the coactivator and corepressor functions of GPS2 suggests that post-translational modifications might be involved. Alternatively, unknown adaptor proteins could mediate the effect.

2 AIMS OF STUDY

The aim of this study has been to increase the overall knowledge about how the NR5A receptors regulate transcription with special focus on the molecular mechanisms used at the promoters. Specifically we aimed at

I. Understanding the role of RNF31 and DAX-1 in SF-1-dependent transcriptional regulation (Paper I & II)

II. Understanding how SF-1 impacts on the transcriptome of the adrenocortical cell line NCI-H295R (Paper II)

III. Identifying possible crosstalk between NR5A receptors and non-NR signalling pathways (Paper II & III)

IV. Understanding how ubiquitin-like post-translational modifications influence the signalling of the NR5A receptors (Paper I, II & III)

3 RESULTS

3.1 E3 UBIQUITIN LIGASE RNF31 COOPERATES WITH DAX-1 IN TRANSCRIPTIONAL REPRESSION OF STEROIDOGENESIS (PAPER I)

The antagonistic relationship of SF-1 and DAX-1 in regulating the expression of genes in the pathway converting cholesterol to steroid hormones is well documented but many of the molecular details remain elusive. In this paper we describe how the E3 ubiquitin ligase RNF31 contributes to the repressive potential of DAX-1, thus participating in the regulation of SF-1 transcriptional activity.

Using the yeast-two-hybrid system we identified RNF31 as a DAX-1 interacting protein. With deletion constructs we could define the interacting surfaces, where aa 193-670 in RNF31 and the N-terminal aa 2-199 in DAX-1 were indispensible for the interaction. Coimmunoprecipitation confirmed the interaction in mammalian cells and GST-pull down assays using *in vitro* translated and purified protein indicated that the interaction was direct and not mediated by any bridging factors. The interaction between DAX-1 and RNF31 also seemed unique to the NR0B class of NRs as SHP, the other mammalian NR0B family member, was the only other NR shown to interact with RNF31 of the panel tested in the yeast-two-hybrid assay.

We went on to investigate the expression pattern of RNF31 in DAX-1 and SF-1 target tissues and using immunohistochemistry and *in situ* hybridisations we could show that RNF31 is indeed coexpressed with DAX-1 in the adrenal cortex and in testis. The coexpression was particularly clear in the outer adrenal zone, zona glomerulosa. Northern blot analysis of a panel of human tissues showed that RNF31 has a wider expression pattern than that described in the literature for DAX-1. Apart from the steroidogenic tissues testis and ovary, RNF31 was also found in heart, placenta, liver, skeletal muscle, pancreas, prostate, intestine and leukocyte. This was also reflected when whole cell extracts from a selection of cell lines were analysed for RNF31 expression using western blot. RNF31 was found in endometrial, kidney, adrenal, breast, colon, ovary and prostate cancer cell lines. Localization studies revealed that RNF31, as DAX-1, is a shuttling protein present in both nucleus and cytoplasm and that DAX-1 and RNF31 can colocalise in the nuclei of mammalian cells.

Domain analysis showed that RNF31 contains multiple zinc-fingers of a type that may bind ubiquitin, a central ubiquitin-interacting domain (UBA) and a C-terminal E3 ligase domain of the ring-in between-ring (RBR) type. This domain structure indicated RNF31 to be connected to the ubiquitination pathway. The RBR family of E3 ligases is the least characterised with regard to both mechanism of action and the regulatory outcome of substrate ubiquitination. We could show that DAX-1 can indeed act as a substrate for RNF31-dependent ubiquitination and that the E3 ligase domain is indispensible for this action. It seems that RNF31 mainly catalyses the attachment of monoubiquitin and not ubiquitin chains to DAX-1 and this is also indicated by the observation that coexpression of DAX-1 and RNF31 seems to stabilise rather than degrade DAX-1.

Monoubiquitination is indicative of a regulatory function of RNF31 on DAX-1 action. Intriguingly we could see that RNAi mediated knockdown of RNF31 in the adrenal cell line NCI-H295R increased the expression of StAR and aromatase, two steroidogenic genes known to be under control of SF-1/DAX-1. This was evident at both protein and mRNA levels and also supported by experiments employing luciferase reporter constructs containing the proximal promoters of either StAR or aromatase.

To further investigate the effect of RNF31 on SF-1/DAX-1 dependent transcription we performed ChIP assays in NCI-H295R cells treated with RNAi to deplete either SF-1 or RNF31. We could show that SF-1, DAX-1, RNF31 and the corepressor SMRT cooccupy StAR and aromatase promoters under basal condition. The occupancy is dependent on SF-1 as depletion of SF-1 leads to loss of DAX-1, RNF31 and SMRT while leaving CREM/CREB and C/EBPβ unchanged. Knocking down RNF31, on the other hand, leaves SF-1, CREM/CREB and C/EBPβ unchanged but leads to loss of DAX-1 and SMRT and recruitment of SRC2, CBP and RNA polymerase II. This is in accordance with the increased expression of both StAR and aromatase following RNF31 knockdown.

The data lead us to formulate a hypothesis where RNF31 together with SMRT and DAX-1 are part of a corepressor complex present on the StAR and aromatase promoter in an SF-1 dependent way. As complex integrity seems reliant on the presence of RNF31, we speculate that monoubiquitination of DAX-1 is important for the interactions keeping the complex intact. Upon activation, the complex, by unknown mechanisms, dissociates from the promoter giving way for coactivators like SRC2 and the general transcription machinery including RNA polymerase II.

In conclusion RNF31 is established as a coregulator of DAX-1 and SF-1 dependent regulation of StAR and aromatase expression.

3.2 GENOME-WIDE EFFECTS OF RNAi-MEDIATED KNOCKDOWN OF STEROIDOGENIC FACTOR-1 AND RNF31 IN ADRENOCORTICAL CELLS (PAPER II)

In the second paper we investigate the genome-wide effects of both RNF31 and SF-1 on adrenocortical cell line NCI-H295R. Using the RNAi system developed in Paper I we took advantage of the microarray technology to analyse the changes in the transcriptome following knockdown of either RNF31 or SF-1. We also investigate the effects of cAMP-treatment of the cells and use the data to compare with the RNF31 and SF-1 outcomes.

Comparisons of the expressed genes between control-treated and RNF31 RNAi-treated H295R cells show an enrichment of genes in pathways related to steroid hormone synthesis and cholesterol metabolism. Several genes in the steroidogenic pathways are upregulated following RNF31 depletion, showing that the mechanisms of RNF31 regulation of SF-1 may be valid for a larger set of target genes than only StAR and aromatase that were identified in Paper I. On the other hand it is apparent that RNF31 has SF-1 independent effects as well, since the overlap between the RNF31 and

SF-1 samples is relatively limited. However, the data establishes RNF31 as a modulator of steroidogenesis in human adrenocortical cells.

Analysis of the differentially expressed genes following SF-1 knockdown revealed diverse responses with regard to the affected pathways. Most adrenal target genes described to date are involved in the steroidogenic pathway and although genes in this pathway are indeed changed by SF-1 depletion, genes in pathways governing cell proliferation, differentiation and in the TGFβ- and Wnt/β-catenin signalling pathways are also enriched in the list of differentially expressed genes as analysed by the online resource DAVID (35, 70).

Genes in the TGFβ and Wnt/β-catenin pathways were upregulated upon depletion of SF-1. Interestingly, TGFβ-signalling has previously been shown to act inhibitory on SF-1 expression and steroidogenesis in the adrenal (36, 104) and Wnt/β-catenin can activate SF-1-dependent transcription (56, 87). What we saw was the opposite, i.e. SF-1 influences the TGFβ and Wnt-pathways. The effect was inhibitory on these pathways as removal of SF-1 upregulates their gene expression. This indicates that SF-1 may have transrepressive effects, something that to our knowledge has not been described before. Whether these effects come from direct actions of SF-1 on the promoters of the affected genes or indirectly via other regulatory factors remains to be elucidated.

The insulin-related growth factor II (IGF2) was downregulated upon SF-1 knock down. IGFII is known to be important in driving the proliferation of the fetal adrenal cortex and of adrenocortical carcinomas (44, 90). This implies that inhibition of SF-1 activity may be beneficial in the treatment of adrenocortical carcinomas.

In conclusion, RNF31 appears to be a central modulator of steroidogenesis in adrenal cells and SF-1 is shown to have effects on pathways beyond steroidogenesis.

3.3 GPS2-DEPENDENT CO-REPRESSOR/SUMO PATHWAYS GOVERN ANTI-INFLAMMATORY ACTIONS OF LRH-1 AND LXRβ IN THE HEPATIC ACUTE PHASE RESPONSE (PAPER III)

Paper III deals with the transrepressive actions of the NR5A protein LRH-1 and the LXRs in hepatic acute phase response (hAPR), the immunological process where the serum level of acute phase proteins (APP) rises following infection or inflammation. The APPs are produced in hepatocytes and include proteins such as Haptoglobin, Serum Amyloid A (SAA), Fibrinogen β and Plasminogen Activator Inhibitor-1 (PAI-1). The production of these APPs is stimulated by addition of IL-1β and IL6 to either primary human hepatocytes or the hepatocellular carcinoma cell lines Huh7 (human) or HepG2 (mouse).

We could show that pretreatment with either the LXR agonist GW3965 or the LRH-1 agonist GW8470 inhibited the IL-1β/IL6-dependent induction of mRNA of a subset of the APPs in cells in culture. This subset included Haptoglobin and SAA but not PAI-1. Interestingly, LRH-1 and LXRβ were recruited to the promoters of Haptoglobin and SAA but not to PAI-1. The LRH-1/LXR-positive promoters were also

positive for NCoR, HDAC3 and GPS2, while PAI-1 instead of NCoR recruited the corepressor SMRT. In absence of agonist ligand neither LRH-1 nor LXRs were found on any APP gene promoters, but the corepressors NCoR, HDAC3 and GPS2 in the case of SAA/Haptoglobin and SMRT/HDAC3 in the case of PAI-1 were recruited. These corepressors dissociated upon IL-1β/IL6 treatment, unless the cells were pretreated with GW3965 or GW8470 in which case LXRs or LRH-1 respectively, were recruited and the corepressor complex stayed on the promoter as a consequence.

It had previously been shown that transrepression of inflammatory pathways in macrophages by PPARγ and LXRs is dependent on SUMOylation of the NRs using SUMO-2/3. LRH-1, on the other hand was known to be SUMOylated by SUMO-1 on K224 and SUMOylation lead to repression of LRH-1 target gene expression. We could show that SUMO-2/3 was indispensible for LXR transrepression of APR while SUMO-1 on K224 was indispensible for LRH-1 transrepression. SUMO-1 knockout mice displayed an attenuated inflammatory response when treated with LPS and less LRH-1 was recruited to the promoters of Haptoglobin and SAA in the livers of SUMO-1 knockouts as compared to wild-type mice. Mutational analysis in cell lines showed that the K224 of LRH-1 was required for the transrepression to take place since a K224R mutant was not recruited to the Haptoglobin or SAA promoter even after GW8470 treatment.

An important question is how SUMOylation of the NRs is able to redirect the proteins from DNA-binding *in cis* to indirect association to transrepression target genes. We speculated that there must be a SUMO-sensor present in the corepressor complex. One such candidate is GPS2 and we could show that GPS2 can indeed bind SUMO alone and SUMOylated LRH-1 and LXR and that deleting the domains presumably responsible for SUMO-binding reduced the possibility of transrepression.

All in all, the experiments lead up to a model where GPS2 acts as an anchor between the SUMOylated NR (LXRβ or LRH-1), the NCoR-HDAC3 complex and the DNA-bound transcription factor to be repressed. The binding of SUMOylated receptor inhibits cytokine-induced dissociation of the complex, thus inhibiting the hepatic acute phase response.

4 DISCUSSION

In Paper I we identify a new coregulatory factor in SF-1 transcriptional regulation. RNF31 is recruited to the promoters of StAR and CYP19 together with DAX-1 and SMRT. Loss of RNF31 through siRNA depletion leads to dissociation of DAX-1 and SMRT from the promoters and decreased transcription of the target genes. It will be interesting to see if future research can map the DAX-1/SMRT/RNF31-complex in the cycling of SF-1 on the promoters. As DAX-1 has long been considered one of the most important SF-1 interacting proteins it is surprising that so little still is known about the mechanisms that direct their interactions. Assays like the ChIP assay will be of importance in determining whether DAX-1 binds directly to stem-loop structure on the DNA or by associating to other DNA-bound transcription factors. It seems that it would require the action of energy-dependent enzymes to create DNA stem-loop structures as such structures would induce torsional tension and break the complementary interaction between bases while replacing them with imperfect base pairing. Thus, the free energy change (ΔG) of that process is unlikely to be negative and it must require energy input. To our knowledge no enzyme that carries out this process has yet been described. Our data from the StAR promoter, which is one of the promoters that has been suggested to bind DAX-1 directly, does not support a direct binding model of DAX-1 as SF-1 depletion leads to dissociation of DAX-1 from the promoter. However, the possibility still exists that DAX-1 is associated to both DNA and SF-1, perhaps interacting with SF-1 via the repression helix described in the DAX-1 LRH-1 crystal structure and that the cooperativity is needed for efficient binding.

In Paper II we start to characterise the effects of both SF-1 and RNF31 on a genome-wide level. Depletion of RNF31 is shown to have a significant effect on multiple enzymes and transporter proteins in the steroidogenic pathway, strengthening our hypothesis that RNF31 is indeed important for andrenocortical hormone production. The overlap with the genes affected by SF-1 depletion is not huge, but among the steroidogenic genes it seems sufficient to claim that RNF31 may well play an important role on multiple SF-1 target genes. As many of the same enzymes are utilised in the gonads and as RNF31/SF-1 and DAX-1 are coexpressed in at least testicular Leydig cells, we assume that this may very well be the case there too, but this remains for future experiments to confirm. We would of course have liked to include DAX-1 depleted cells in this study. Unfortunately, and despite intense efforts, we could not identify any siRNA oligos that could knock down DAX-1 sufficiently for this experiment.

As previously described, RNF31 is known to be part of a complex called LUBAC that targets NEMO with linear polyubiquitin chains. It is thus obvious that RNF31 has targets in multiple pathways. The lack of overlap with the gene set in the SF-1 depleted samples is in that sense not surprising. Although we did not find a clear connection with the NF-κB signalling in our bioinformatic analysis, it would be of interest to dig deeper into the material to see if RNF31 and NF-κB signalling interact in this adrenal cell line. Another possibility is that LUBAC is actually involved in the regulation of DAX-1 and SF-1 signalling. We did try to include RNF54 (the other protein in the LUBAC complex) in the ubiquitination assays in Paper I but were unable to see any

additional effect. It is possible that the endogenous levels of RNF54 are sufficient for ubiquitinating DAX-1. However, the interaction with RNF31 is supposedly dependent on the UBA domain of RNF31 (91) and deletion of this domain did not abolish ubiquitination of DAX-1, indicating that RNF31 may be acting on its own in this context.

SF-1 plays important roles during the development of both gonads and adrenals and therefore it is not surprising to find that SF-1 does affect pathways and genes not directly linked to steroidogenesis. Effects on cell proliferation have been described before in the efforts to elucidate the functional consequences of SF-1 overexpression in childhood adrenocortical carcinoma (38). Our microarrays corroborate the finding that SF-1 increases proliferation rates, as depletion leads to less cells in S-phase and more in G1/G0 in flow cytometry analysis. The microarrays identify a number of genes and pathways that could be of consequence for this effect and also a few that counteract it. When we first saw the connection to TGFβ signalling, with a number of proteins in the pathway being upregulated upon depletion of SF-1, including TGFβ2, we thought that this might be the cause of the decreased cell growth. TGFβ is known to act antiproliferatively in normal cells and we thus tested the effect of TGFβ2 on the NCI-H295R adrenocortical cell line. However, it turns out that TGFβ clearly increases proliferation in the NCI-H295R cell line (as does TGFβ1, A.E. unpublished results). This is not uncommon in cancer cells but does not offer us any further clues as to why proliferation actually decreases upon depletion of SF-1. The IGFII gene is one candidate; it has been shown that IGFII increases proliferation in adrenocortical cells (44) and IGFII is downregulated in response to SF-1 depletion. IGFII is under normal conditions mainly expressed by the fetal adrenal and not by the adult adrenal cortex. The fetal adrenal is proliferative compared to the adult adrenal cortex and that a cancer would have a more fetal phenotype is not surprising. Adrenocortical carcinomas often overexpress IGFII and a reduction of IGFII activity could be beneficial in treating the cancer (90). Conceptually, antagonizing SF-1 could thus be beneficial. It is clear, however, that further investigations are necessary to clarify the role of SF-1 in adrenal cell proliferation.

The microarray data in Paper II indicate that SF-1 has repressive effects on both TGFβ- and Wnt/β-catenin-signalling. Many NRs use tethering to repress transcription in crosstalk mechanisms. For GR, PPAR and LXR this has been described in anti-inflammatory pathways (50) and in Paper III we describe how both LXRβ and LRH-1 utilize SUMO-dependent tethering in repressing the hepatic acute phase response. LRH-1 and SF-1 often employ similar mechanisms and it would not be surprising if SF-1 has the capacity to transrepress via promoter tethering as well. LRH-1 has been shown to affect Wnt/β-catenin-signalling via assumed tethering to β-catenin/TCF4 in intestinal cells (12). The effect of LRH-1 on Wnt is activating, however, and thus the opposite of what we observe for SF-1. One way to determine the target transcriptome of SF-1 with reasonable certainty would be to perform chromatin immunoprecipitation of SF-1 coupled to deep sequencing of the isolated DNA fragments. That would generate a list of genomic SF-1 binding sites. The overlap with the microarray data from paper II would determine the direct targets of SF-1 in the H295R cell line in an unbiased way. One could further analyse the enriched DNA motives present at the binding sites. This would presumably indicate the SFRE as the most significant site but

could also reveal if SF-1 is involved in tethering with other transcription factors, as their REs would then show up as significantly enriched.

Paper III identifies proteins in the hepatic acute phase response to be targets of LRH-1 dependent transrepression. It also identifies a specific function of SUMOylated LRH-1 where transcriptional repression is not accomplished by simple sequestering from the target gene. Instead, SUMOylated LRH-1 seems to gain access to target genes by interaction with other DNA-bound transcription factors *in trans* on promoters lacking classical LRH-1 response elements. The same is true for LXRβ, which loses the requirement for its heterodimer partner RXR in the transrepression pathway. The requirement for agonist activation of LRH-1 to achieve the transrepression is somewhat puzzling. Ligand-activation is known to induce transcription of target genes and in the case of the specific LRH-1 agonist, transcription of the LRH-1 target SHP has been shown to increase after treatment. However, the transrepression requires SUMOylation, a modification known to repress target gene transcription. How does the cell distinguish between which genes are to be repressed and which ones are to be induced? The interactions with other cofactors, in this case the GPS2/NCoR/HDAC3 complex is probably of importance. It may be that a small, but significant, pool of LRH-1 is SUMOylated and upon cytokine stimulation, this pool is redirected by unknown mechanisms from the PML bodies to the C/EBPβ REs in some of the hepatic acute phase response proteins. Interestingly, both TNFα and IL-1β have been shown to upregulate LRH-1 mRNA and protein in mouse liver and human HepG2 cells (11). An increased pool of LRH-1 might increase levels, but not necessarily relative levels, of SUMOylated LRH-1 leading to a "surplus" that could be utilised in the transrepression we describe. This response would be tissue specific as TNFα causes downregulation of LRH-1 mRNA in the intestine of mice (152). Other possibilities include that cytokine stimuli could increase SUMOylation of LRH-1. Further investigation will be needed to elucidate the exact mechanisms.

5 CONCLUSIONS

With regards to the aims, the main conclusions of this thesis can be summarised as follows:

I. RNF31 emerges as an important regulator of steroidogenesis through its coregulatory functions in SF-1-dependent transcription. RNF31 can ubiquitinate the corepressor DAX-1 and is critical for the formation of a corepressor complex containing RNF31, DAX-1 and SMRT associated with SF-1 on StAR, aromatase and probably other steroidogenic target gene promoters.

II. SF-1 has profound effects on the transcriptome of the adrenocortical carcinoma cell line NCI-H295R. Pathways involved in steroidogenesis, differentiation and cell proliferation are affected. The IGFII gene known to be of importance in adrenocortical carcinoma is downregulated, outlining a possible beneficial effect of silencing SF-1 activity in treatment of adrenocortical carcinoma.

III. SUMOylation of LRH-1 leads to retention of a corepressor complex on a subset of inflammatory genes, thus fine-tuning the transcriptional output of cytokine signalling. For SF-1, possible crosstalk with the TGFβ and Wnt/β-catenin signalling was identified and SF-1 is implicated to have repressive effects.

IV. SUMOylation was shown to be an integral part of active repression on crosstalk target genes for LRH-1. In that context, SUMOylation is important for the retention of repressor complex on the promoters by interaction of the SUMOylated receptor with an adaptor protein, possibly GPS2. For SF-1, ubiquitination was shown to be of importance for repression, as DAX-1-mediated repression requires the E3 ubiquitin ligase (and actally also ubiquitin-interacting protein) RNF31. A common theme for both SF-1 and LRH-1 regulation emerges where ubiquitin-like modifications are required for correct assembly of repression complexes on target gene promoters.

6 FUTURE PERSPECTIVES

Work in this thesis adds new players to be considered in future research, with RNF31 in the case of SF-1 and GPS2 in the case of LRH-1. It also identifies a new active role for SUMOylated LRH-1 in transcriptional crosstalk and regulation of transrepressed genes. SUMOylation has emerged as a common theme in transcriptional repression working at many levels to assure that the correct transcriptional output is achieved (45). For SF-1 we have identified genes and pathways that could be targets for crosstalk or transrepression adding new directions for future research. The development of genome-wide methods such as ChIP-sequencing and RNA-sequencing allows unbiased discovery of new target genes and promise further advances in the NR5A field in the future. The advances in technology and accumulated knowledge also provide a good basis for exploring the roles of NR5A receptors in novel systems. LRH-1 is known to be expressed in embryonic stem cells (54) where it, together with DAX-1, regulates the expression of factors important for maintaining pluripotency (54, 85, 86). In fact, both LRH-1 and SF-1 can induce pluripotency from somatic cells (66) indicating future possible roles of NR5A receptors in regenerative medicine. The newly discovered possibility of ligand-modulation of the NR5A opens up opportunities for the pharmacological industry of finding new drugs against e.g. metabolic and hormonal disorders as well as cancer, adding extra interest to future advances in the field.

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