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JOANNA LEMPIÄINEN

Protein Interactions of the Glucocorticoid and Androgen Receptors

PROTEIN INTERACTIONS OF THE GLUCOCORTICOID AND ANDROGEN RECEPTORS

Joanna Lempiäinen

PROTEIN INTERACTIONS OF THE GLUCOCORTICOID AND ANDROGEN RECEPTORS

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ABSTRACT

The glucocorticoid and androgen receptors (GR and AR) are transcription factors (TFs) that bind to chromatin in order to regulate the expression of genes. The GR mediates the effects of glucocorticoids on metabolism, as well as developmental and immune responses throughout the human body. Synthetic glucocorticoids, such as dexamethasone, are widely prescribed to treat inflammatory conditions and acute lymphoblastic leukemia. The androgen-activated AR mainly regulates the development, maintenance and function of the male reproductive organs but also the development and progression of prostate cancer. Synthetic AR antagonists, antiandrogens, such as enzalutamide, are widely used for the treatment of castration-resistant prostate cancer (CRPC). However, many patients develop enzalutamide-resistance that, in cell models, has been shown to be driven by crosstalk between the GR and the AR. When bound to chromatin, the functions of both the GR and AR are dependent on interactions with coregulator proteins that modulate gene expression through a variety of mechanisms, such as post-translational modification of histones, including monoubiquitination, and chromatin remodeling. Coregulator dysfunction may lead to severe pathologies, and therefore coregulators are emerging as potential drug targets in various diseases. Despite the importance of coregulator interactions in regulating the effects of GR and AR, the protein interactomes of these physiologically important TFs have remained poorly defined. In this thesis, state-of-the-art proteomic methods, proximity-dependent biotin identification (BioID) and chromatin immunoprecipitation coupled with selective isolation of chromatin associated proteins (ChIP-SICAP), were utilized to map the protein interactions of the GR and the AR. Protein interactomes of agonist-bound GR and AR contain both coactivators (factors that enhance transcription) and corepressors (factors that inhibit transcription) with many interactions being shared between the receptors. Furthermore, antagonist-bound GR and AR, and a DNA-binding -deficient GR exhibit an impaired ability to interact with coregulators. These proteomics methods were employed in parallel with genome-wide methods, chromatin immunoprecipitation sequencing (ChIP-seq) and assay for transposase-accessible

chromatin sequencing (ATAC-seq), to explore how the post-translational modification of GR with a small ubiquitin-related modifier (SUMO) changes its effects on chromatin. The SUMOylation-deficient GR interacts more efficiently with chromatin remodelers and is more effective at opening closed chromatin sites than its wild-type counterpart. In addition, ChIP-seq and whole transcriptome sequencing (RNA-seq) were utilized to define the role of BCL6 corepressor (BCOR), one of the novel AR-interacting proteins found in this work, in AR signaling in castration-resistant prostate cancer (CRPC) cells. BCOR is recruited to AR chromatin-binding sites and regulates AR target gene expression in CRPC cells in part via regulating monoubiquitination of histone H2A at lysine 119 (H2AK119ub1). Importantly, BCOR depletion attenuates the proliferation and induces the apoptosis of CRPC cells. Taken together, the findings of this thesis contribute to clarifying the role of coregulators and post-translational modifications in nuclear receptor (NR) function. The protein interactomes of GR and AR discovered in this thesis represent a valuable resource in the NR field. The novel interactors may have previously unrecognized roles in NR function, and they may provide potential drug targets in inflammatory conditions, acute lymphoblastic leukemia and prostate cancer.

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TIIVISTELMÄ

Glukokortikoidi- ja androgeenireseptori (GR ja AR) ovat transkriptiotekijöitä, jotka säätelevät geenien ilmentymistä. GR säätelee muun muassa immuunipuolustuksen toimintaa ja elimistön aineenvaihduntaa sitoessaan glukokortikoideja. Synteettisiä GR:n agonisteja (aktivoiva ligandi), kuten deksametasonia, käytetään tulehdussairauksien ja akuutin lymfoblastisen leukemian hoidossa. AR puolestaan välittää miessukupuolihormonien, androgeenien, vaikutukset soluihin ja on keskeinen säätelijäproteiini sekä normaalissa eturauhasen kehityksessä että eturauhassyövän muodostumisessa ja etenemisessä. Synteettisiä AR antagonisteja (inaktivoiva ligandi), kuten entsalutamidia, käytetään hillitsemään eturauhassyövän kasvua ja etenemistä. Aktivoituessaan nämä reseptorit sitoutuvat kromatiiniin (perimään) ja vuorovaikuttavat muiden proteiinien, kuten transkriptiotekijöiden ja niiden aktiivisuutta säätelevien tekijöiden, koregulaattorien kanssa. Koregulaattorit säätelevät geenien luentaa muun muassa vaikuttamalla histonien posttranslationaalisiin muokkauksiin, kuten monoubikitinaatioon, tai muokkaamalla kromatiinin avoimuutta. Koregulaattorit ovat potentiaalisia lääkekohteita, sillä ne toimivat viallisesti monissa sairauksissa, kuten syövässä. Koregulaattorien tärkeydestä huolimatta niiden vuorovaikutukset GR:n ja AR:n kanssa on huonosti kartoitettu. Tässä väitöskirjassa sovellettiin äskettäin kehitettyjä proteomiikan menetelmiä (BioID ja ChIP-SICAP) GR:n ja AR:n proteiinivuorovaikutusten kartoittamiseksi. Ensimmäisessä osatyössä osoitettiin, että agonistiin sitoutuneet reseptorit vuorovaikuttavat niin koaktivaattorien (geenejä aktivoivien tekijöiden) kuin korepressorien (geenejä passivoivien tekijöiden) kanssa, ja että em. reseptoreilla on paljolti päällekkäinen vuorovaikutusprofiili. Antagonistin sitoneet reseptorit ja DNA:han sitoutumaton GR-mutantti vuorovaikuttivat puolestaan vähemmän koregulaattoreiden kanssa. Nämä tulokset tuovat uutta tietoa lääkeaineiden vaikutuksesta GR:n ja AR:n proteiinivuorovaikutuksiin. Kartoitetut proteiinivuorovaikutukset sisältävät myös tekijöitä, joiden ei ole aiemmin tiedetty säätelevän näiden reseptorien toimintaa. Toisessa osatyössä äskettäin kehitettyjä proteomiikan menetelmiä käytettiin genominlaajuisten tehosekvensointi-menetelmien (ChIP-seq ja ATAC-seq) rinnalla selvittämään kuinka

posttranslationaalinen SUMO (small ubiquitin-related modifier) -muokkaus vaikuttaa GR:n vuorovaikutuksiin koregulaattorien kanssa ja sen aktiivisuuteen kromatiinilla. Tutkimuksissa selvisi, että SUMOyloimaton GR-mutantti pystyy paremmin vuorovaikuttamaan kromatiinin avoimuutta säätelevien koregulaattoreiden kanssa ja se on kykeneväisempi avaamaan suljettua kromatiinia kuin SUMOyloituva GR. Nämä tulokset tuovat uutta tietoa posttranslationaalisten muokkausten vaikutuksesta transkriptiotekijöiden toimintaan. Kolmannessa osatyössä selvitettiin genominlaajuisia tekniikoita (ChIP-seq ja RNA-seq) käyttäen tässä tutkimuksessa uutena löytyneen AR:n koregulaattorin, BCOR:in, roolia AR:n signaalinvälityksessä kastraatioresistenteissä eturauhassyöpäsoluissa. Hormonin sitonut AR kutsuu BCOR:n kromatiinille säätelemään satojen geenien ilmentymistä. Osaa näistä geeneistä BCOR passivoi ylläpitämällä histoni H2A:n lysiini-119:n monoubikitinaatiota, eli säätelee geenien ilmentymistä epigeneettisesti. Monet em. geeneistä ovat hyvin tärkeitä eturauhassyöpäsolujen kasvun säätelijöitä. BCOR:n poistaminen vähensikin kastraatioresistenttien eturauhassyöpäsolujen kasvua ja laukaisi niiden ohjelmoidun solukuoleman. Tulosten perusteella BCOR vaikuttaa mahdolliselta eturauhassyövän uudelta lääkeaineiden vaikutuskohteelta. Tämä väitöskirja tuo uutta tietoa koregulaattorien toiminnasta GR:n ja AR:n säätelijöinä. GR:lle ja AR:lle kartoitetut proteiinivuorovaikutukset sisältävät koregulaattoreita, joiden ei ole aiemmin tiedetty säätelevän näiden reseptorien toimintaa. Nämä aiemmin tuntemattomat koregulaattorit ovat potentiaalisia uusia lääkekohteita tulehdussairauksien, akuutin lymfoblastisen leukemian ja eturauhassyövän hoidossa.

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LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications:

I **Lempiäinen JK**, Niskanen EA, Vuoti KM, Lampinen RE, Göös H, Varjosalo M, Palvimo JJ. Agonist-specific protein interactomes of glucocorticoid and androgen receptor as revealed by proximity mapping. *Molecular and Cellular Proteomics 16(8): 1462-1474, 2017.*

II Paakinaho V*, **Lempiäinen JK***, Sigismondo G, Niskanen EA, Malinen M, Jääskeläinen T, Varjosalo M, Krijgsveld J, Palvimo JJ. SUMOylation regulates the protein network and chromatin accessibility at glucocorticoid receptor-binding sites. *Submitted*

III **Lempiäinen JK**, A.B.M. Manjur K, Malinen M, Ketola K, Niskanen EA, Palvimo JJ. BCOR-coupled H2A monoubiquitination represses a subset of androgen receptor target genes regulating prostate cancer proliferation. *Oncogene 39(11): 2391-2407, 2020.*

* Equal contribution.

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APPENDIX: ORIGINAL PUBLICATIONS (I-III)

ABBREVIATIONS

A549	cell line derived from lung cancer adenocarcinoma
AF	activation function
AP-MS	affinity purification coupled with mass spectrometry
AR	androgen receptor
ARE	androgen response element
ATAC-seq	assay for transposase-accessible chromatin sequencing
B-ALL	B cell acute lymphoblastic leukemia
BAF	BRG1- or BRM-associated factor
BCOR	BCL6 corepressor
BET	bromodomain and extraterminal
BioID	proximity-dependent biotin identification
BRD	bromodomain
CHD	chromodomain
ChIP	chromatin immunoprecipitation
ChIP-seq	chromatin immunoprecipitation sequencing
ChIP-SICAP	chromatin immunoprecipitation coupled with selective isolation of chromatin-associated proteins
co-IP	co-immunoprecipitation
cPRC1	canonical type 1 polycomb repressive complex
CRPC	castration-resistant prostate cancer
CTD	C-terminal domain
DBD	DNA-binding domain
Dex	dexamethasone
EGFP	enhanced green fluorescent protein
ER	estrogen receptor
FRAP	fluorescence recovery after photobleaching
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HAT	histone acetyltransferase

HDAC	histone deacetylase
HDM	histone demethylase
HEK293	cell line derived from human embryonic kidney
HMT	histone methyltransferase
HSP	heat-shock protein
ISWI	imitation switch
LBD	ligand-binding domain
LNCaP	cell line derived from prostate cancer lymph node metastasis
MLL	mixed-lineage leukemia
MMTV	mouse mammary tumor virus
MR	mineralocorticoid receptor
MS	mass spectrometry
ncPRC1	non-canonical type 1 polycomb repressive complex
NR	nuclear receptor
NTD	N-terminal domain
PC	prostate cancer
PHD	plant homeodomain
PR	progesterone receptor
PRC1	type 1 polycomb repressive complex
PRC2	type 2 polycomb repressive complex
PTM	post-translational modification
RNA-seq	whole transcriptome sequencing
RNA Pol II	RNA polymerase II
SMT	single-molecule tracking
SUMO	small ubiquitin-like modifier
SR	steroid receptor
SRC	steroid receptor coactivator
SWI/SNF	switch/sucrose non-fermentable
TAU	transactivation unit
TAD	topologically-associated domain
TF	transcription factor
VCaP	cell line derived from prostate cancer lumbar vertebrae metastasis
Y2H	yeast two-hybrid

1 INTRODUCTION

The glucocorticoid receptor (GR) and the androgen receptor (AR) are transcription factors (TFs) belonging to the steroid receptor (SR) family that bind to specific DNA sequences on chromatin to regulate the expression of target genes. The GR mediates the effects of glucocorticoids on metabolism, development and immune responses throughout the human body (Weikum *et al.* 2017), whereas the AR binds androgens to regulate the development, maintenance and function of the male reproductive organs and the female reproductive physiology (Gao *et al.* 2005, Walters *et al.* 2016). These nuclear receptors (NRs) are also important drug targets: Synthetic glucocorticoid agonists, such as dexamethasone, are widely used pharmaceuticals due to their potent anti-inflammatory and anti-immune effects (Kadmiel & Cidlowski 2013) and due to their cytotoxicity in lymphoid cancer cells (Pui & Evans 2006). Synthetic AR antagonists, antiandrogens, such as enzalutamide (Scher *et al.* 2012, Hussain *et al.* 2018), apalutamide (Clegg *et al.* 2012, Smith *et al.* 2018) and darolutamide (Moilanen *et al.* 2015, Fizazi *et al.* 2019) are used for the treatment of prostate cancer (PC). Recent studies have shown that GR contributes to enzalutamide-resistance of castration-resistant prostate cancer (CRPC), highlighting the importance to study the crosstalk of GR and AR in PC (Kumar 2020).

Upon binding to their cognate agonists, the GR and the AR translocate to the nucleus and bind to enhancers where they modulate the transcriptional state of target genes. Modulation of the transcriptional state is dependent on the recruitment of coregulator proteins. For instance, these proteins bridge the enhancer-bound receptor to the RNA polymerase II (RNA Pol II) machinery, post-translationally modify (PTM) histones and other proteins or remodel the nucleosome composition of chromatin (Millard *et al.* 2013, Meier & Brehm 2014). The interactions of GR and AR with coregulators are influenced by PTMs such as SUMOylation of the receptors. Coregulator dysfunction can lead to severe pathologies, and coregulators are emerging as important drug targets in various diseases (Lonard & O'Malley 2012). However, despite the key role of coregulators in GR and AR function and in disease pathologies, the protein interactomes of these receptors have remained poorly characterized.

The focus of this thesis work was to employ state-of-the-art proteomics methods to elucidate the protein interactomes of the GR and the AR. Furthermore, these methods were utilized to clarify the role of SUMOylation on GR coregulator interactions. The role of BCOR, one of the novel AR-interacting proteins found in this work, in AR signaling in CRPC cells was also characterized. The findings of this thesis contribute towards clarifying the role of coregulators in nuclear receptor function. The interactomes of these receptors can help to elucidate the molecular

mechanisms by which they regulate gene expression and potentially lead to the identification of novel factors involved in GR and AR signaling. These previously uncharacterized coregulators also represent potential drug targets in inflammatory conditions, acute lymphoblastic leukemia and PC.

2 REVIEW OF THE LITERATURE

2.1 TRANSCRIPTION FACTORS

Transcription factors (TFs) constitute a class of proteins that bind to specific DNA sequences to control the transcription of genetic information from DNA to RNA (Vaquerizas *et al.* 2009, Lambert *et al.* 2018). TFs participate in numerous vital cellular processes, such as in the maintenance of cell metabolism, cell differentiation and embryonic development. Indeed, TF mutations are responsible for numerous diseases, such as some forms of cancer and developmental disorders. To date, over 1600 proteins in humans are known to be or predicted as TFs, meaning that TFs represent approximately 8% of all protein-coding human genes (Lambert *et al.* 2018). Whole transcriptome sequencing (RNA-seq) analyses from human adult tissues have revealed that roughly one-third of the currently known TFs are expressed in a tissue-specific manner, while the rest are ubiquitously expressed across diverse tissue types (Lambert *et al.* 2018). Interestingly, in general, TFs are expressed at lower levels than non-TF genes. This has been suggested to be important in maintaining the DNA-binding specificity of TFs by directing them to higher affinity sites, while leaving the lower affinity sites unoccupied. Moreover, the low expression levels may help in triggering regulatory events by altering TF concentrations or activity (Vaquerizas *et al.* 2009).

All TFs contain at least one DNA-binding domain (DBD), which has been used to classify them into different families; the largest TF families in humans being the C2H2-zinc finger, Homeodomain, basic helix-loop-helix, basic leucine zipper, forkhead and nuclear receptor families (Lambert *et al.* 2018). New TFs are identified largely by sequence homology to these characterized DBDs. It is the DBD which is responsible for the binding of the TF to its specific DNA target sequences, "motifs". For these sites, TFs can have even a 1000-fold preference relative to other sequences. TF motifs are usually very short sequences, typically only 4-8 base pairs (bp) long, and one gene may contain multiple binding sites for several different TFs (Reiter *et al.* 2017, Lambert *et al.* 2018). Recent advances in genome-wide methods have revealed that several TFs bind primarily to regulatory elements, enhancers, outside of genes. Most of the functional DNA in the human genome is regulatory in nature (Kellis *et al.* 2014), meaning that sequence-specific TFs are key molecules in decoding the majority of the information in DNA (Lambert *et al.* 2018).

Some TFs recruit the basal transcription machinery directly to gene promoters to initiate transcription. However, most human TFs bind to genomic regulatory elements, enhancers, and activate or repress transcription from target gene core-promoters (short sequence surrounding the transcriptional start site) by recruiting coregulator proteins (Vaquerizas *et al.* 2009, Reiter *et al.* 2017, Lambert *et al.* 2018). TFs can regulate expression of target genes from large distances through chromatin looping, that brings stretches of genomic sequence to closer proximity to each other

than to intervening sequences (Figure 1) (Kim & Shendure 2019). TFs may also cooperate, promoting the chromatin binding of each other (Suter 2020). Chromatin looping, TF cooperativity, and the vast number of different coregulators recruited by TFs have made it challenging to understand how a single TF-binding event on chromatin ultimately regulates the expression of a specific gene.

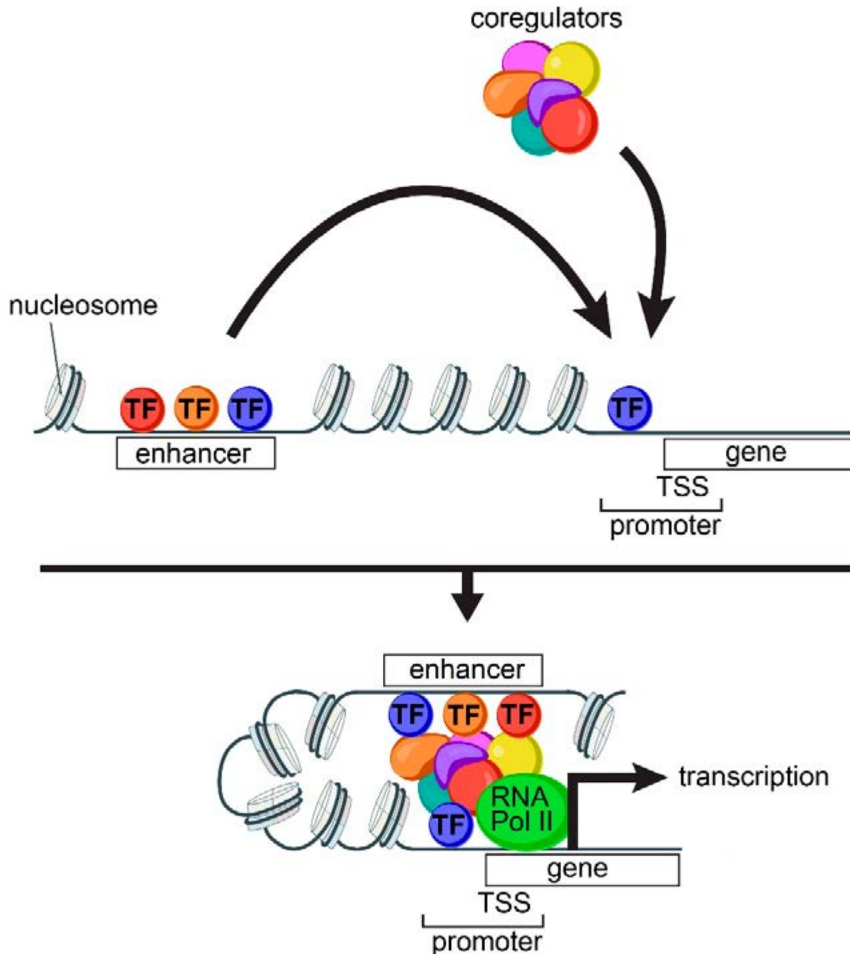

Figure 1. Transcriptional regulation by transcription factors (TFs). Enhancers contain short sequence motifs that are recognized by TFs. Some TFs also bind promoters directly. TFs recruit coregulator proteins that influence transcription through a variety of mechanisms, such as recruitment of RNA polymerase II (RNA Pol II), post-translational modification of histones and chromatin remodeling. TSS, transcription start site.

2.1.1 Nuclear receptors

Nuclear receptors (NRs) form a superfamily of TFs that regulate the transcription of genes in response to a ligand such as steroid and thyroid hormones or other types of lipophilic molecules. In humans, 48 TFs have been categorized as NRs (Zhang *et al.*

2004). Binding to a ligand leads to a conformational change in the receptor, which results in DNA-binding and the regulation of target genes. Some NRs reside in the nucleus continuously, while others remain inactive and bound by chaperone proteins in the cytosol and translocate to the nucleus only after binding their respective ligand, this latter type being exemplified by steroid receptors (SR) (Figure 2A) (Echeverria & Picard 2010). NRs, like all TFs, can bind to DNA cooperatively as homo- or heterodimers, or as higher-order structures (Lambert *et al.* 2018, Paakinaho *et al.* 2019).

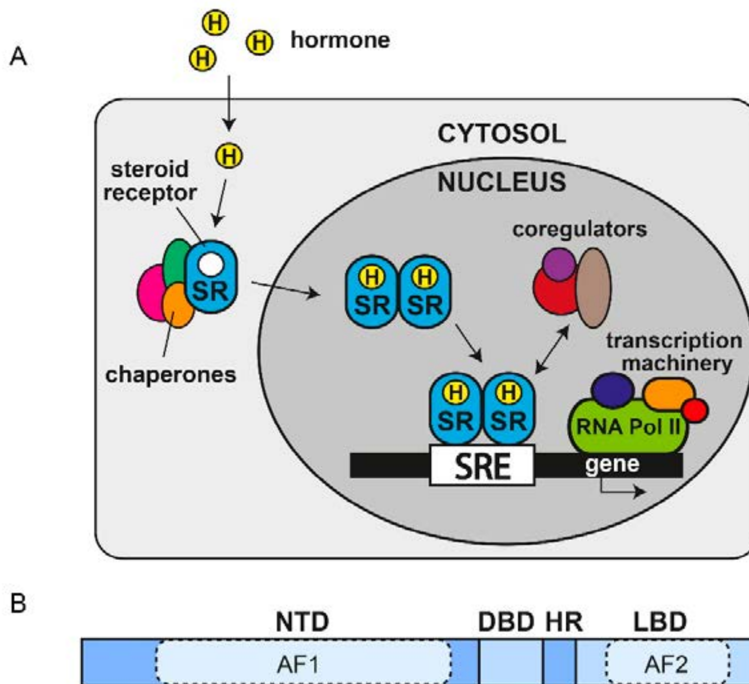

Figure 2. Nuclear receptor (NR) function and domain structure. (A) Diagram shows nuclear translocation of steroid receptors (SRs) upon hormone (H) binding. (B) General domain structure of NRs. SRE, steroid response element; RNA Pol II, RNA polymerase II; NTD, N-terminal domain; AF1, activation function 1; DBD, DNA-binding domain; HR, hinge region; LBD, ligand-binding domain; AF2, activation function 2.

All nuclear receptors possess three main domains: N-terminal transactivation domain (NTD), central DNA-binding domain (DBD), and C-terminal ligand-binding domain (LBD) (Figure 2B). Some NRs also contain a hinge region between the DBD and LBD that includes one or more nuclear localization signals (NLS) that control the nuclear localization of the receptor. The NTD contains a stretch of amino acids that ligand-independently regulates the activity of the receptors, termed activation function (AF1), whereas the LBD contains the second activation function (AF2) that acts in a ligand-dependent manner. The AF1 and AF2 regulate nuclear receptor activity by mediating interactions with transcriptional coregulators (Warnmark *et al.* 2003, Simons *et al.* 2014). The NTD and LBD may also contain transactivation units

(TAU) that influence receptor activity similarly to AF1 and AF2. The DBD is a key characteristic of all NRs; it is composed of two C4 type zinc fingers, where each zinc ion is coordinated to four cysteine residues to stabilize the structure of the domain (Cotnoir-White *et al.* 2011). The C4 zinc finger is distinct from the most common type of mammalian TF DBD, the C2H2 zinc finger, where the zinc ion is coordinated to two cysteine and two histidine residues (Lambert *et al.* 2018).

2.1.1.1 Steroid receptors

Steroid receptors (SRs), a subset of the NR superfamily, are activated by steroid hormones. There are six SRs in vertebrates, and they are further subdivided into two families: 3-keto SRs (NR3C family) and estrogen receptors (NR3A family), according to the type of hormone they recognize (Figure 3A). 3-Keto SRs, the glucocorticoid receptor (GR), the mineralocorticoid receptor (MR), the androgen receptor (AR), and the progesterone receptor (PR) are activated by 3-ketosteroids (GR: cortisol and corticosterone, MR: aldosterone, AR: dihydrotestosterone and testosterone, and PR: progesterone), whereas the estrogen receptor α and β (ERα and ERβ) bind 3-hydroxysteroids (different forms of estrogen: estrone, estradiol, estriol or estetrol) (Busillo *et al.* 2013) (Figure 3B).

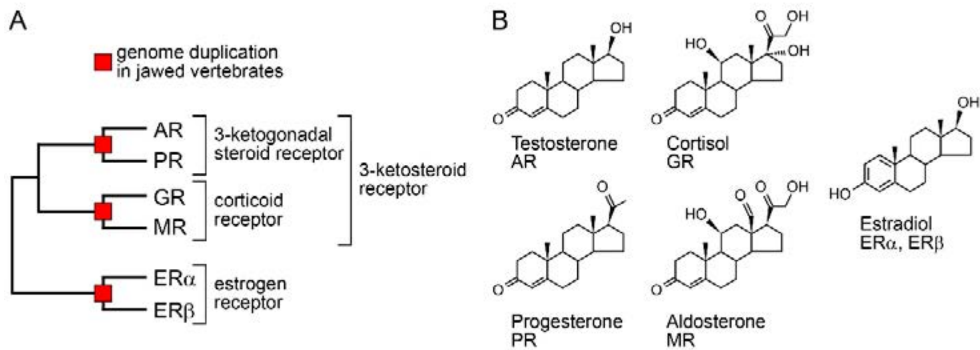

Figure 3. Relatedness of different steroid receptors (SRs) and steroid hormones. (A) Dendogram displays relatedness between different SRs. (B) Structures of different steroid hormones. SR that primarily binds the corresponding hormone is named below. Adapted from (Busillo *et al.* 2013).

These differences can be explained by the evolution of the SRs: Duplications and mutations in the common SR ancestor 600 to 800 million years ago led it to diverge into two main SR types, one of which eventually became the 3-ketosteroid receptors and the other one becoming the ERs. Furthermore, the common ancestor of 3-ketosteroid receptors duplicated, diverging to the ancestor of GR and MR and to the ancestor of AR and PR, explaining why GR and MR are more similar in sequence and function when compared to AR and PR (Eick *et al.* 2012). SRs were originally thought to be evolutionarily relatively new and only exists in vertebrates. However, surprisingly, genes with clear sequence homology to the human ER were discovered

recently in invertebrates proving that SRs are much more ancient in their origin (Thornton *et al.* 2003, Eick & Thornton 2011, Eick *et al.* 2012).

The NTD of SRs is in general longer than in other NRs and it is the most variable domain among SRs in terms of both sequence and size (Simons *et al.* 2014). The DBD is in turn the most conserved domain to the extent that all NR3C family members are capable of binding to the same canonical glucocorticoid/mineralocorticoid/androgen/progesterone response element (GRE/MRE/ARE/PRE), while estrogen receptors bind a different element (Cotnoir-White *et al.* 2011). Likewise, NR3C family members share high sequence similarity between their LBDs, explaining their preference for similar steroids, but they still retain enough differences to distinguish between ligands. In NR3C family members, the AF1 is the more important activation function required for maximal activity of the receptors, while in the estrogen receptors the AF1 and AF2 are more equal in terms of importance (He *et al.* 2004). The AF1 possibly allows specific gene regulation by the NR3C family members when they bind to the same response elements because the NTD is the most variable domain in these different receptors (He *et al.* 2004).

2.1.1.1.1 Glucocorticoid receptor

The GR, a member of the NR3C subfamily of SRs, mediates the effects of glucocorticoid hormones that maintain homeostasis during environmental and physiological stress. The adrenal cortex produces and releases glucocorticoid hormones under the control of the hypothalamic-pituitary-adrenal axis (Revollo & Cidlowski 2009, Weikum *et al.* 2017). The primary function of the GR is to respond to glucocorticoids to increase glucose production when blood glucose levels are low and to suppress the immune system during inflammation (Revollo & Cidlowski 2009). However, GR also regulates other processes, such as bone mineralization, central nervous system function and development (Revollo & Cidlowski 2009, Vandevyver *et al.* 2014, Weikum *et al.* 2017).

Dysregulation of glucocorticoid signaling may lead to pathologies such as Cushing’s or Addison’s disease. In Cushing’s disease, a tumor in the pituitary leads to overproduction of glucocorticoids from the adrenal cortex, resulting in weight gain, hyperglycemia, increased fat mass, immunosuppression, reduced muscle and bone mass and water retention. Opposingly, in Addison’s disease a developmental defect or trauma in the adrenal cortex leads to deficient production and release of glucocorticoids and mineralocorticoids, resulting in weight loss, hypoglycemia and dysregulation of sodium and potassium levels. Cushing’s disease is treated by surgically removing the tumor from the pituitary, whereas Addison’s disease is treated by glucocorticoid- and mineralocorticoid replacement therapy (Revollo & Cidlowski 2009).

Synthetic glucocorticoids, such as dexamethasone, are widely used pharmaceuticals due to their potent immunosuppressant activity. They are used to treat different autoimmune and inflammatory conditions, such as systemic lupus erythematosus, rheumatoid arthritis, asthma, different allergies, and to treat patients

with hematological cancers, such as B cell acute lymphoblastic leukemia (B-ALL) (Pui & Evans 2006, Ramamoorthy & Cidlowski 2016). Dexamethasone was also found to reduce mortality in hospitalized Coronavirus disease 2019 (COVID-19) patients that required respiratory support (RECOVERY Collaborative Group *et al.* 2020). However, long-term use of glucocorticoids leads to adverse effects that resemble the symptoms of Cushing's disease. In addition, some patients with inflammatory conditions or hematological cancers respond poorly to glucocorticoid treatment due to glucocorticoid-resistance (Kadmiel & Cidlowski 2013). Therefore, new GR ligands with increased specificity and efficacy are under constant research and development (De Bosscher *et al.* 2005, Wang *et al.* 2006, Sundahl *et al.* 2015).

NR3C1 that encodes the GR, is located on chromosome 5 (5q21) and is expressed in nearly all vertebrate cells. In mice, whole body deletion of exon 2 (encodes most of the NTD) of the GR gene led to severe developmental abnormalities in the lung causing death within hours from birth (Cole *et al.* 1995). In live humans, GR loss-of-function mutations have been identified in glucocorticoid resistance syndrome that is characterized by partial target-tissue insensitivity to glucocorticoids (Bray & Cotton 2003, Vitellius & Lombes 2020). The insensitivity leads to overactivation of the hypothalamic-pituitary-adrenal axis resulting in increased levels of glucocorticoids, mineralocorticoids and androgens that cause adrenal hyperplasia, hirsutism, high blood pressure and overweight in these patients (Vitellius & Lombes 2020).

Alternative splicing generates GRα, GRβ, GRγ, GR-A and GR-P isoforms of the human GR. GRα is 777-amino-acid long and has been the most extensively investigated (Revollo & Cidlowski 2009, Kadmiel & Cidlowski 2013). All splice variants are expressed throughout the body, but in general, expression levels of GRα are much higher than that of the other variants and the physiological role of the other variants has remained largely unclear (Revollo & Cidlowski 2009). GRα and GRβ are identical through amino acid 727 but differ in their LBD (Lewis-Tuffin & Cidlowski 2006, Kino *et al.* 2009). In the absence of ligand, GRα resides in the cytosol and translocates to the nucleus only after binding glucocorticoids. GRβ, in contrast, does not bind glucocorticoids, is constitutively active in the nucleus and acts as a dominant negative inhibitor of GRα (Oakley & Cidlowski 2011). Increased expression of GRβ has been shown to contribute to glucocorticoid resistance, for instance, in lymphoblastic leukemia (Longui *et al.* 2000), systemic lupus erythematosus (Piotrowski *et al.* 2007), rheumatoid arthritis (Goecke & Guerrero 2006) and steroid resistant asthma (Goleva *et al.* 2006).

GRγ, in contrast, includes an arginine residue insertion between the two zinc fingers in the DBD (Ray *et al.* 1996). It is widely expressed and binds glucocorticoids and DNA but differs in its gene regulation pattern from that of GRα (Ray *et al.* 1996, Meijsing *et al.* 2009). GRγ expression is also associated with glucocorticoid resistance (Ray *et al.* 1996). GR-A and GR-P are splice variants that lack large regions from the LBD. They were originally found in glucocorticoid-resistant multiple myeloma and are thought to contribute to glucocorticoid-insensitivity in small lung cancers and

hematological malignancies (Moalli *et al.* 1993, Gaitan *et al.* 1995, Krett *et al.* 1995, de Lange *et al.* 2001). In addition, alternative initiation of translation gives rise to eight additional GRα isoforms with progressively shorter NTDs (GRα-A, GRα-B, GRα-C1, GRα-C2, GRα-C3, GRα-D1, GRα-D2, GRα-D3) that exhibit different tissue-expression patterns and regulate unique sets of genes (Lu & Cidlowski 2005, Lu & Cidlowski 2006). Similar set of translational isoforms is expected to exist for other GR splice variants (Oakley & Cidlowski 2011).

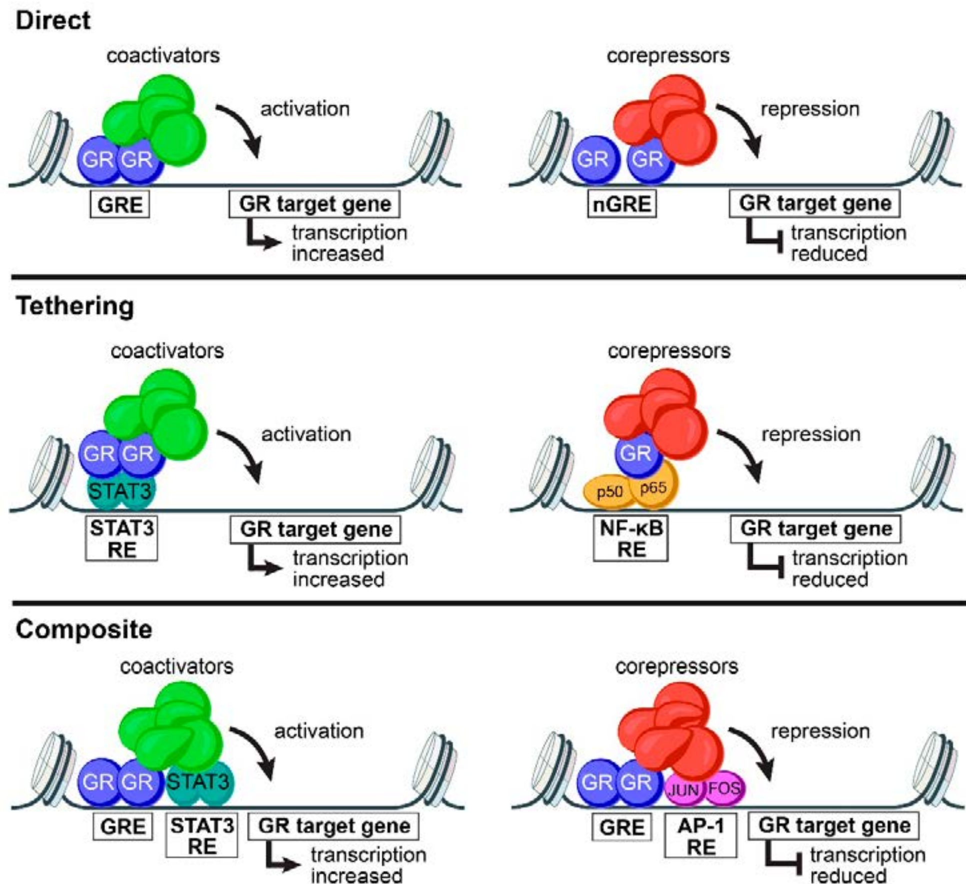

Figure 4. Modes of GR chromatin-binding in the regulation of transcription. Adapted from (Oakley & Cidlowski 2011). RE, response element.

In the absence of glucocorticoids, the non-liganded GRα (apo-GRα) associates as monomers with heat-shock proteins (HSPs) in the cytosol. After binding glucocorticoids, the liganded GRα (holo-GRα) dissociates from HSPs, translocates to the nucleus and binds to enhancers to regulate transcription together with coregulators (Figure 2A) (Echeverria & Picard 2010, Weikum *et al.* 2017). Classically, the genomic GR-binding events have been categorized into three main types: direct, tethering and composite (Figure 4). In direct binding, GR binds directly to

glucocorticoid response elements (GREs) as a homodimer, whereas in tethering it binds to another TF instead of binding to DNA directly. In composite binding, the GR binds next to other TFs in order to conjointly regulate the expression of target genes. STAT3, NFκB (e.g. consisting of p50 and p65 dimer) and AP-1 (e.g. consisting of JUN and FOS dimer) are among the TFs that are known to regulate the target genes of the GR (Oakley & Cidlowski 2011, Kadmiel & Cidlowski 2013, Weikum *et al.* 2017).

These different binding modes explain why the GRE is not the only GR motif that is enriched, when GR-binding sites are examined in genome-wide ChIP-seq experiments (Sacta *et al.* 2018) (in addition to the relatively low ~200 bp resolution of ChIP-seq as compared to the 15 bp sized GRE). Regardless of the mode of chromatin-binding, the GR is known to recruit coregulator proteins that possess the necessary enzymatic and protein-binding capabilities to enhance or attenuate transcription (Weikum *et al.* 2017). Even though the GR is expressed ubiquitously throughout the human body, it can have very diverse functions in many different cell types. This has been suggested to originate from the context-dependent function of the GR that is influenced by nearby DNA sequences and therefore on the binding of other TFs, the presence of a certain set of coregulators, post-translational modifications and the type of ligand (in the case of synthetic ligands for instance) (Weikum *et al.* 2017). Moreover, chromatin structure (i.e. topologically associated domains) is likely to play a role in the cell-type specific effects of glucocorticoids by influencing the access of the GR to specific enhancers and by influencing enhancer-promoter interactions.

2.1.1.1.2 Androgen receptor

The AR is also a member of the NR3C subfamily of SRs, but it mediates the effects of androgens, specifically testosterone and 5α-dihydrotestosterone (5α-DHT), to regulate the development, maintenance and function of the male reproductive organs and sexually dimorphic characteristics (Gao *et al.* 2005, Banerjee *et al.* 2018). AR also regulates normal ovarian, uterine and mammary gland function in females (Walters *et al.* 2016). Androgen production and release is tightly regulated by the hypothalamic-pituitary-gonadal axis. Testosterone is primarily synthesized by Leydig cells of the testis, but also in the adrenal cortex, liver, and ovary in women (Gao *et al.* 2005). Testosterone is reduced to 5α-DHT in the prostate gland, liver and skin by 5α-reductase (Thigpen *et al.* 1993, Russell *et al.* 1994). 5α-DHT is the primary androgen found in the prostate and approximately 10-times more potent in activating the AR than testosterone (Deslypere *et al.* 1992, Dai *et al.* 2017).

Classically, testosterone is used to reverse symptoms caused by low testosterone levels in conditions such as male hypogonadism (defects in the testes, hypothalamus or pituitary) and Klinefelter syndrome (one or two extra X chromosomes leading to underdeveloped testes), whereas antiandrogens are primarily used to treat prostate cancer (PC) (Gao *et al.* 2005). In females, increased androgen production may lead to the development of multifollicular ovaries (Lucis *et al.* 1966, Chang 2007, Becerra-Fernandez *et al.* 2014), and AR knockout female mice exhibit underdevelopment of follicles (Walters *et al.* 2009, Cheng *et al.* 2013). Increased AR expression has been

associated with poor survival in a subgroup of breast cancer patients (Lehmann *et al.* 2011, Caiazza *et al.* 2016), and some patients with breast cancer have been shown to benefit from antiandrogen therapy (Gucalp *et al.* 2013, Zhu *et al.* 2016).

The AR gene, *AR* (alias *NR3C4*), is located in the X-chromosome (Xq11-12) (Gao *et al.* 2005). In male mice, AR was shown to be most active in the testes, prostate, seminal vesicles and bone marrow, whereas in females the AR was most active in the ovaries, uterus, omentum tissue and mammary glands (Dart *et al.* 2013). In both sexes, AR was also expressed in the skeletal muscle, salivary glands, spleen, adipose tissue, the eyes and regions of the brain, highlighting the importance of AR signaling also outside the reproductive organs (Dart *et al.* 2013). The mouse AR expression and activity also correlated well with AR expression in humans (Dart *et al.* 2013).

The canonical and longest naturally occurring AR/AR-B isoform is 919 amino acids long. At least six other AR splice variants have been reported: AR45/AR-A, AR3/AR-V7, AR4/AR-V1, AR5/AR-V4, AR6/AR-V3 and AR-V567es (Guo & Qiu 2011). Most of these isoforms are truncated from the LBD and have been only found in PC cells (Guo & Qiu 2011). However, AR45 is a naturally occurring variant and is truncated from the NTD (Ahrens-Fath *et al.* 2005). It is especially expressed in the heart and skeletal muscle, and to a lesser extent in the prostate, lung, uterus and breast. AR45 has been shown to either repress or enhance AR transcriptional activity depending on the context (Ahrens-Fath *et al.* 2005).

Mutations in the AR gene are associated with androgen insensitivity syndrome and PC (Gao *et al.* 2005). Symptoms of androgen insensitivity syndrome depend on the severity in AR disruption: In complete androgen insensitivity syndrome, males are characterized with a female phenotype, whereas in partial insensitivity some male characteristics remain (Hughes & Deeb 2006). In PC, AR mutations may contribute, for instance, to antiandrogen resistance (Fenton *et al.* 1997, Balbas *et al.* 2013).

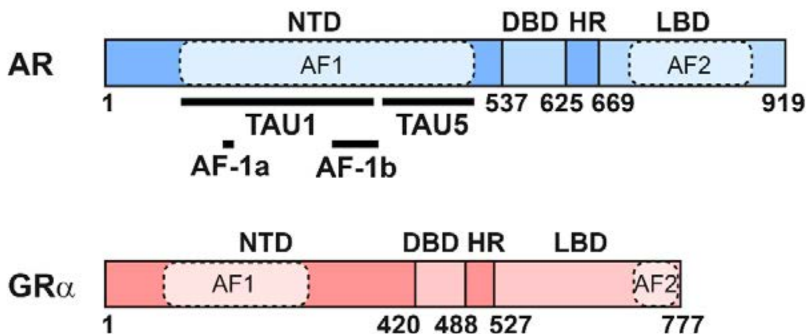

Figure 5. Domain structure of the androgen receptor (AR) and glucocorticoid receptor isoform α (GRα). ; NTD, N-terminal domain; AF1, activation function 1; DBD, DNA-binding domain; HR, hinge region; LBD, ligand-binding domain; AF2, activation function 2; TAU, transactivation unit.

The model for AR subcellular localisation dynamics was built largely on initial studies with the GR: The unliganded AR is bound by HSPs in the cytosol, and after

ligand binding translocates to the nucleus to bind target response elements as homodimers (Dehm & Tindall 2007, Echeverria & Picard 2010). Recent genome-wide ChIP-seq experiments have revealed that most of the AR-binding sites are located on enhancers rather than on gene promoters, suggesting that the AR primarily regulates target genes through chromatin looping (Massie *et al.* 2011, Toropainen *et al.* 2015, Toropainen *et al.* 2016).

The AR NTD is more than 100 amino acids longer than that of the GR and contains polyglutamine and polyglycine sequences that vary in length between individuals (Ferro *et al.* 2002, Ding *et al.* 2004, Ding *et al.* 2005) (Figure 5). In addition, as opposed to the other SRs, an interaction between the AR N-terminal AF-1 and C-terminal AF-2 is needed for full activity of the receptor (Ikonen *et al.* 1997, He *et al.* 2002). Deletion experiments led to the identification of two additional functional regions within the AR AF1 that are important for full AR activity; the transactivation unit (TAU) 1 and TAU5 (Jenster *et al.* 1995). TAU1 can be further divided into two regulatory domains; AF-1a and AF-1b (Chamberlain *et al.* 1996). Interestingly, it was reported that deleting regions from the NTD impaired AR activity only in some of the tested cell lines, suggesting that the NTD AFs and TAUs have context-specific roles in regulating AR activity (Dehm & Tindall 2007).

2.1.1.1.3 AR and GR in prostate cancer

Prostate cancer (PC) is among the most common cancers diagnosed in men in Western nations (Siegel *et al.* 2020). PC is usually diagnosed by elevated prostate-specific antigen (PSA) levels in the blood. PSA is a serine protease that is included in normal prostate secretions but is released to the blood when prostate morphology is disrupted (Lilja *et al.* 2008). PC is classified according to differentiation status (Gleason score 1-5), invasiveness of the primary cancer (T1-4), lymph node metastasis (N0 or 1) or presence of distant metastases (M0 and 1a-c) (Shen & Abate-Shen 2010). Role of androgen signaling in PC was established already in 1941, when Huggins and Hodges showed that orchiectomy (removal of the testicles) induces considerable regression in PC tumors (Huggins & Hodges 1941). The treatment for local primary PC is radical prostatectomy (removal of the prostate and the tissue surrounding it), which can be curative. Androgen deprivation therapy (ADT) is typically applied before and after prostatectomy. ADT uses surgery or chemical castration to lower the androgen levels produced by the testicles. ADT is also an adjuvant treatment for metastasized primary PC in combination with radiation therapy. If the disease recurs, as shown by increasing PSA levels in the blood, targeted therapy is typically applied (Dai *et al.* 2017).

Despite initial successful treatment, in many patients PC eventually evolves to castration-resistant PC (CRPC), that progresses even during ADT, when androgen levels are extremely low (Banerjee *et al.* 2018). CRPC is essentially untreatable, with standard chemotherapy increasing the survival time on average by 2 months (Petrylak *et al.* 2004, Tannock *et al.* 2004). Progression to CRPC is usually

characterized by increasing expression levels of PSA, that is also a direct AR target gene. Increasing expression levels of PSA indicate that PC cells, typically expressing PSA, are proliferating, for instance, at metastatic sites. It also indicates that AR signaling is restored in CRPC (Scher & Sawyers 2005, Ryan & Tindall 2011). Amplification of the AR gene was found in androgen-independent tumors, suggesting that AR amplification sensitizes the cancer cells to very low androgen concentrations (Visakorpi *et al.* 1995). Later, other mechanisms have been shown to contribute to development of CRPC, such as AR gain-of-function by splice variants, mutations, aberrant AR coregulation and dysregulation of epigenetics downstream of AR signaling (Dai *et al.* 2017, Baumgart & Haendler 2017). However, progression of PC to CRPC still remains incompletely understood (Banerjee *et al.* 2018).

In the recent years, the struggle against CRPC has led to the development of various second-generation antiandrogens (AR antagonists), such as enzalutamide (Scher *et al.* 2012, Hussain *et al.* 2018), apalutamide (ARN-509) (Clegg *et al.* 2012, Smith *et al.* 2018) and darolutamide (ODM-201) (Moilanen *et al.* 2015, Fizazi *et al.* 2019) that have passed clinical trials and are now used in treatments against CRPC. These novel drugs, such as enzalutamide and apalutamide, have been shown to prolong patient survival but they are not curative due to eventual resistance developed by the cancer (Watson *et al.* 2015, Banerjee *et al.* 2018). Some of the antiandrogen-resistant PC tumors have been classified as neuroendocrine PC (NEPC) because they express markers normally found only in neuroendocrine cells (Vlachostergios *et al.* 2017). Other CRPC variants include small cell carcinoma, "AR indifferent" castration-resistant adenocarcinoma, intermediate atypical, aggressive variant and ductal (Vlachostergios *et al.* 2017).

One mechanism of antiandrogen resistance is thought to be mediated by constitutively active AR splice variants that lack the LBD, such as AR-V7, that regulate the expression of AR target genes even in the absence of ligands (Ciccarese *et al.* 2016, Cao *et al.* 2016). Knockdown of AR-V7 was shown to sensitize CRPC cells to growth inhibition by enzalutamide (Li *et al.* 2013). AR mutations that contribute to PC are often located at the AR LBD and increase its activity, for instance, by extending ligand-binding capacity (Veldscholte *et al.* 1990). AR mutations may also alter the conformation of the receptor to enable coactivator interactions when it is bound by antiandrogens, and thus lead to an agonist-like response by antiandrogens (Joseph *et al.* 2013).

Interestingly, in androgen-dependent PC glucocorticoids have been shown to slow the proliferation of tumor cells, whereas in CRPC glucocorticoids promote tumor growth (Montgomery *et al.* 2014, Huang *et al.* 2018). Antiandrogen therapy was shown to increase GR expression levels and GR upregulation to bypass AR signaling and contribute to antiandrogen resistance (Arora *et al.* 2013, Rodriguez-Vida *et al.* 2015, Hirayama & r 2018). The ability of the GR to partially substitute the AR in CRPC is thought to originate from the similarities of these two receptors (Claessens *et al.* 2017). In PC cells, several genes that are under androgen regulation have been shown to respond to glucocorticoids (Sahu et al. 2012), and GR can still

upregulate anti-apoptotic genes when the AR is inhibited with antiandrogens (Jaaskelainen *et al.* 2011, Isikbay *et al.* 2014). Therefore, clinical trials are underway to test whether simultaneous inhibition of AR and GR signaling using enzalutamide and mifepristone (RU486) is beneficial in patients with CRPC (Kumar 2020). However, more specific GR modulators for CRPC treatments are under development because mifepristone also weakly modulates the activity of AR (Taplin *et al.* 2008, Clark *et al.* 2008, Kach *et al.* 2017).

2.2 CHROMATIN STRUCTURE

Genomic DNA in eukaryotes is packaged into a nuclear structure called chromatin, which also contains proteins and RNA. Chromatin consists of basic repeating units, nucleosomes, in which 145-147 bp of double-stranded DNA is wrapped around histone proteins. Each nucleosome has eight histone proteins, canonically, two H3/H4 heterodimers that form the central tetramer, which is capped on each end by a H2A/H2B heterodimer (Luger *et al.* 1997, Zhou *et al.* 2019). Each histone in the nucleosome contains a central globular histone fold region which extends to the more flexible and unstructured histone tail at the N-terminus that protrudes out from the globular nucleosome core particle. Histone H2A and H2B also contain a second tail at the C-terminus (Iwasaki *et al.* 2013). Nucleosomes are further organized into 10-nm chromatin fibres where the nucleosomes are regularly spaced with ~200 bp distance from each other (Li *et al.* 2010, Maeshima *et al.* 2019). The fibres form larger functional compartments, such as the topologically associated domains (TADs) (Dixon *et al.* 2012) and A- and B-compartments (Lieberman-Aiden *et al.* 2009).

The wrapping of DNA into nucleosomes allows very tight packaging of the genome to the small volume of the nucleus but also functions as an inactivating mechanism for transcription by sterically occluding TFs and the basal transcription machinery from binding to DNA. Multiple studies have shown that accessible chromatin, euchromatin, associates with active transcription whereas inaccessible tightly packed chromatin, heterochromatin, is transcriptionally inactive (Johnson & Dent 2013, Voss & Hager 2014). However, the classification of chromatin into these two states is not clear-cut, and different states of transcriptional activity in chromatin regions can be identified, for instance, by characterizing post-translational modifications (PTMs) of histones (Ernst & Kellis 2010).

To allow access for DNA-binding proteins, nucleosomes undergo dynamic changes; unwrapping, rewrapping, sliding, assembly and disassembly (Bowman & Poirier 2015). Nucleosomes may undergo spontaneous unwrapping and wrapping of DNA, so-called “DNA breathing”, that may momentarily expose DNA segments and lead to further unwrapping, if a protein binds at that time (Li & Widom 2004, Li *et al.* 2005). In addition, there are four mechanisms by which nucleosome dynamics are modulated with the help of assisting proteins: (1) PTMs of histones, (2) ATP-dependent chromatin remodelers, (3) variant histones, and (4) histone chaperones (Zhou *et al.* 2019). These regulatory mechanisms enable a delicate control of

transcription by influencing chromatin-binding by TFs, such as the general TFs of the basal transcription initiation complex (Collingwood *et al*. 1999). Transcription is not initiated when the DNA-binding sequence recognized by the general TFs (TATA-box) is assembled into nucleosomes (Figure 6).

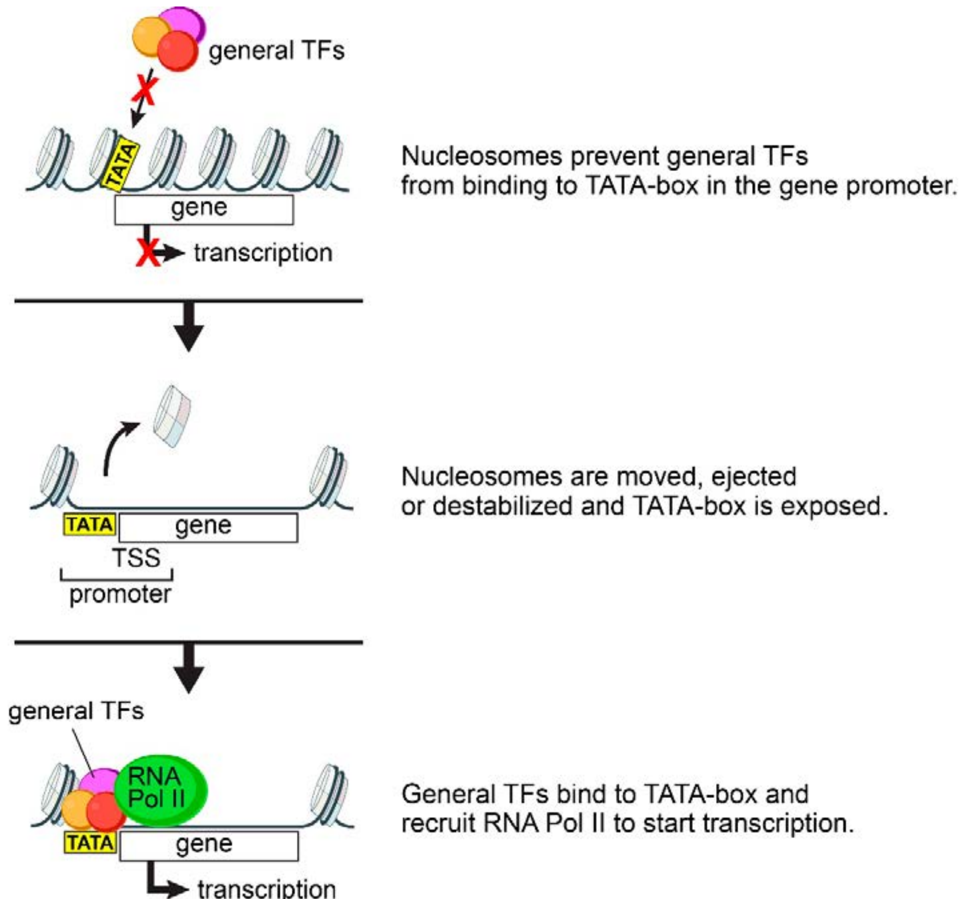

Figure 6. Role of nucleosomes in the regulation of transcription. Some TFs, such as the general TFs in the basal transcription initiation complex, are unable to bind to motifs (TATA-box) that are assembled into nucleosomes. Destabilization of nucleosomes by histone modifications and/or chromatin remodelers exposes the motifs for TF binding. TSS, transcription start site.

2.2.1 Post-translational modification of histones

Post-translational modifications (PTMs), i.e. the covalent attachment of chemical moieties or small proteins, of histones regulate multiple cellular processes, including DNA replication, repair and transcription (Zentner & Henikoff 2013). Histone PTMs exert these functions by altering intrinsic histone-DNA and histone-histone interactions to influence nucleosome dynamics (unwrapping, rewrapping, sliding, assembly and disassembly) (Bowman & Poirier 2015). They also mediate the recruitment of hundreds of different chromatin-binding proteins as individuals or as

multi-protein complexes that further influence chromatin accessibility (Iwasaki *et al.* 2013, Bowman & Poirier 2015, Zhao & Garcia 2015).

Histone PTMs are located on the folds and tails of the histones. PTMs on histone tails are especially important in protein recruitment because the histone tails protrude out of the nucleosome and are more readily accessible for interactions than the histone fold (Iwasaki *et al.* 2013). However, crystal structures have revealed that protein interactions with nucleosomes are mediated by a combination of different surfaces on the nucleosome, such as segments of DNA, the acidic patch on the H2A/H2B dimer surface, and other surfaces on histone folds and tails (Zhou *et al.* 2019).

Acetylation, methylation, phosphorylation and ubiquitination are among the best-studied histone PTMs (Zhao & Garcia 2015). Specific histone marks have been shown to associate with certain DNA regulatory elements: Promoters usually associate with high levels of H3K4me3, whereas enhancers are generally marked with H3K4me1 and H3K27ac or H3K27me3. At active genes enhancers tend to be marked with H3K27ac and at inactive genes with H3K27me3 (Zentner *et al.* 2011, Rada-Iglesias *et al.* 2011).

Some of these modifications are highly dynamic. For example, in the case of histone acetylation, enzymes catalysing and removing these moieties work in seamless collaboration (Waterborg 2002, Zentner & Henikoff 2013). The rapid and reversible nature of epigenetic modifications is thought to be important in the adaptation of organisms to environmental changes (Zentner & Henikoff 2013). In addition, histone PTMs can function in an ordered fashion where one type of modification leads to the formation of another, as exemplified by the repressive H3K27me3 mark that recruits canonical polycomb group protein complexes that further generate H2AK119ub1 to repress gene expression. Furthermore, the enzymes catalysing these histone modifications are not specific for histones alone – they also modify and regulate the activity other chromatin-associated proteins such as TFs (Gaughan *et al.* 2002, Ito *et al.* 2006).

2.2.1.1 Acetylation

Hyperacetylation of histone lysine residues has been shown to be associated with active transcription of genes. Histone acetylation neutralizes the positive charge of lysine groups, thus weakening histone-DNA and histone-histone interactions leading to a destabilization of the nucleosome and exposure of DNA to the transcription machinery (Zentner & Henikoff 2013). Histone acetyltransferases (HATs) and histone deacetylases (HDACs) regulate the acetylation status of histone lysine residues and are both associated with sites of active transcription (Wang *et al.* 2009). The enzymes are thought to work in rapid cycles where histone acetylation facilitates RNA polymerase transit and deacetylation promotes the reassembly of chromatin after transcription (Waterborg 2002).

Histone acetylation was also shown to unwrap nucleosomes to provide access for the DNA replication and repair machinery (Unnikrishnan *et al.* 2010, Xu & Price

2011). Bromodomains (BRDs) on chromatin-binding proteins recognize acetylated lysine residues. BRDs especially on chromatin remodelers such as the bromodomain and extraterminal (BET) family members (BRD2, BRD3, BRD4 and BRDT), direct these remodelers to acetylated histones to facilitate further unwrapping of the nucleosomes (Zeng & Zhou 2002, Zentner & Henikoff 2013). Proteins with BRDs also contain other interaction domains which facilitate the formation of large protein complexes (Fujisawa & Filippakopoulos 2017, Lambert *et al.* 2019).

2.2.1.2 Methylation

Histones can be mono-, di- or trimethylated on lysines and mono- or dimethylated on arginines. Histone lysine methylations have been more extensively studied than arginine methylations (Shi & Whetstine 2007, Zentner & Henikoff 2013). In contrast to acetylation, methylation of the lysine residues on histones does not influence their positive charge and thus does not affect nucleosome dynamics as directly as acetylation. Instead, histone methylations are thought to exert their functions mainly by recruitment of proteins with methylation-recognizing domains, such as the chromodomain (CHD) and plant homeodomain (PHD) (Zentner & Henikoff 2013).

In general, high levels of mono-, di- or tri- H3K4 methylations, di- and trimethylations of H3K36, and monomethylations of H2BK5, H3K9, H3K27, H3K79 and H4K20 are associated with actively transcribed genes, whereas trimethylations of H3K9, H3K27 and H3K79 are linked to repression (Barski *et al.* 2007, Huang & Zhu 2018). Moreover, H3K4 monomethylations (H3K4me1) are primarily associated with enhancers, dimethylations (H3K4me2) with both promoters and enhancers, and trimethylations (H3K4me3) with promoters (Barski *et al.* 2007, Heintzman *et al.* 2007, Heintzman *et al.* 2009, Zentner & Henikoff 2013).

Histone methyltransferases (HMTs) and demethylases (HDMs) regulate the methylation status of histones (Shi & Whetstine 2007, Nicholson & Chen 2009). For instance, the activating H3K4 methylations are generated by mixed-lineage leukemia (MLL) complexes (Li *et al.* 2016), whereas the repressive H3K27 trimethylations are catalysed by type 2 polycomb repressive complexes (PRC2) (Schuettengruber *et al.* 2017). These histone methylations then function as recruitment sites for other protein complexes. For example, H3K4me3 are recognized by the ISWI chromatin remodeling complex by a subunit that contains a PHD finger, and the ISWI complex then promotes the expression of developmentally important HOX genes (Wysocka *et al.* 2006). Similarly, CHD-domain containing Polycomb proteins direct type 1 polycomb complexes (PRC1) to H3K27me3 sites, but in this instance, they induce the formation of heterochromatin to repress genes (Fischle *et al.* 2003).

2.2.1.3 Phosphorylation

Histone phosphorylation is thought to destabilize nucleosomes by altering the charge between the histone-DNA interfaces, similarly to acetylation. However, the charge introduced by phosphorylation is negative, suggesting that it is the charge

repulsion between the negatively charged DNA backbone and the phosphorylated histone residues that leads to nucleosome destabilization (Banerjee & Chakravarti 2011, North *et al.* 2011). Currently, the best-characterized role of histone phosphorylation is in DNA repair. Kinases are recruited to DNA damage sites to phosphorylate the variant histone H2AX at serine 139 to decondense chromatin and recruit DNA repair machinery proteins (Rossetto *et al.* 2012, Gil & Vagnarelli 2019). The phosphorylated H2AX is then either removed or dephosphorylated by dephosphatases to enable DNA damage checkpoint recovery (Gil & Vagnarelli 2019). For instance, in the regulation of transcription, histone phosphorylations are induced at expressed genes after treatment with growth hormone or androgens (Gil & Vagnarelli 2019).

2.2.1.4 Ubiquitylation

Monoubiquitylation of histone lysine residues also regulates histone dynamics, but compared to histone acetylation, methylation and phosphorylation, its mechanism of function is not as well understood (Zentner & Henikoff 2013). Ubiquitin is a small (76 amino-acids) globular protein but still far larger than the other histone PTMs that consist of small chemical moieties. In contrast to polyubiquitination that directs target proteins to proteasomal degradation, monoubiquitination of histones regulates DNA damage responses and transcription. The modification sites are thought to function as molecular docking platforms for the proteins involved in DNA repair and transcription. Histone monoubiquitinations have been associated with both gene activation and repression and therefore their function is thought to be largely context-dependent (Fleming *et al.* 2008, Zhou *et al.* 2008, Minsky *et al.* 2008, Lee *et al.* 2012, Zentner & Henikoff 2013). Histone monoubiquitylations are generated by E3 ubiquitin ligases and removed by deubiquitinases (DUBs) (Schuettengruber *et al.* 2017). Monoubiquitination of histone H2B at lysine 120 (H2BK120ub1) and of histone H2A at lysine 119 (H2AK119ub1) are the best-characterized histone monoubiquitinations.

H2BK120ub1 promotes transcriptional elongation by disrupting chromatin compaction (Sun & Allis 2002, Minsky *et al.* 2008, Fierz *et al.* 2011, Fierz *et al.* 2012). H2BK120ub1 is catalysed by the E3 ubiquitin ligase complex that consists of RNF20 and RNF40 (Kim *et al.* 2005). RNF20 and RNF40 have been shown to coregulate the AR (Jaaskelainen *et al.* 2012) and the ER (Prenzel *et al.* 2011, Nagarajan *et al.* 2014). The mechanism of H2BK120ub1 in transcription regulation remained unknown until very recently: Using cryo-electron microscopy, Huang and colleagues showed that H2BK120ub1 recruits MLL histone methylase complexes via an interaction with the RBBP5 subunit in these complexes (Xue *et al.* 2019). These MLL complexes then catalyse H3K4 methylations that can further recruit chromatin remodelers to induce chromatin accessibility and activate transcription (Wysocka *et al.* 2006, Li *et al.* 2016).

In contrast, H2AK119ub1 induces chromatin silencing and is important in regulating development and cell differentiation through polycomb-mediated silencing (Wang *et al.* 2004, Zhou *et al.* 2008, Wang *et al.* 2018). H2AK119ub1 is

catalysed by type 1 polycomb repressor complexes (PRC1) that contain either the RING1 (RING1A) or RNF2 (RING1B) ubiquitin ligase. The PRC1 complexes are further divided into canonical (cPRC1) and non-canonical (ncPRC1) complexes depending on the subunit composition (Gao *et al.* 2012, Schuettengruber *et al.* 2017). Remodeling and spacing factor 1 (RSF1) was shown to bind to H2AK119ub1 and to be required for H2AK119ub1 -mediated gene repression in coordination with linker histone H1 (Zhang *et al.* 2017). However, a more detailed mechanism as to how RSF1 remodels H2AK119ub1 -modified chromatin to repress genes and the possible involvement of other factors remains to be determined.

2.2.2 Classification of chromatin remodelers

Chromatin remodelers are classified into four main families: imitation switch (ISWI), chromodomain helicase DNA-binding (CHD), switch/sucrose non-fermentable (SWI/SNF) and INO80 (Clapier *et al.* 2017). In addition, various BRD-containing chromatin remodelers have been identified, such as the BET-family members (Fujisawa & Filippakopoulos 2017). Regardless of the family, every remodeler contains an ATPase-translocase domain that carries out ATP-dependent sliding of DNA around a nucleosome. ATP is used as the energy source to break histone-DNA contacts where one round of ATP hydrolysis leads to 1-2 bp translocation of DNA around the nucleosome (Harada *et al.* 2016). The activity and specificity of the ATPase-translocase domain in each remodeler type depends on adjacent domains and/or proteins that are part of the remodeler complex (Mashtalir *et al.* 2018, Pan *et al.* 2019). These adjacent domains, such as chromodomains in CHD remodelers (Tran *et al.* 2000) and BRDs in SWI/SNF remodelers (Hassan *et al.* 2002) or in BET family members (Fujisawa & Filippakopoulos 2017), recognize histone PTMs that influence remodeler binding and activity. Different domains or protein subunits may also facilitate interactions with proteins, such as TFs, that direct chromatin remodelers to specific sites on chromatin (Trotter & Archer 2008, Murawska & Brehm 2011).

Remodeler activity can lead to three main outcomes: (1) nucleosome assembly and even spacing, (2) induction of chromatin access, and (3) nucleosome editing (Clapier *et al.* 2017). ISWI and CHD remodelers function in nucleosome assembly and spacing, where they help in nucleosome maturation and spacing of freshly replicated chromatin (Xiao *et al.* 2001, Lusser *et al.* 2005), but also after transcription where nucleosomes have been moved or ejected (Lusser *et al.* 2005, Torigoe *et al.* 2011). The mechanism for regular spacing of nucleosomes by the *D. melanogaster* ISWI remodeler has been resolved in quite a detailed manner: Autoinhibitory domains in the remodeler are inactivated once a DNA-binding domain binds to the linker DNA between nucleosomes. However, when the nearby nucleosome is reeled closer as a result of remodeler activity, the DNA-binding domain dissociates, releasing the autoinhibitory domain that subsequently renders the remodeler inactive. This action leads to regular spacing of nucleosomes along chromatin (Clapier & Cairns 2012).

A similar mechanism has been proposed for the CHD chromatin remodelers that also contain autoinhibitory and DNA-binding domains (Lusser *et al.* 2005). As also

indicated by their nomenclature, CHD remodelers contain chromodomains that recognize methylated lysines on histones (Sims *et al.* 2005, Watson *et al.* 2012a). The tighter nucleosome spacing that results from ISWI and CHD activity can also contribute to gene repression by preventing access for DNA-binding proteins (Murawska & Brehm 2011, Clapier *et al.* 2017).

SWI/SNF are more potent in translocating DNA on nucleosomes than for example the ISWI remodelers (Clapier *et al.* 2016), possibly explaining why the function of the SWI/SNF remodeler often leads to nucleosome ejection and subsequently to more accessible chromatin (Clapier *et al.* 2017). However, specialized remodelers in the other remodeler families are also known to induce chromatin accessibility (Clapier *et al.* 2017). As for the other remodelers, SWI/SNF complexes contain subunits that recognize histone modifications to direct remodeler binding and activity (Asturias *et al.* 2002, Leschziner *et al.* 2005, Skiniotis *et al.* 2007). DNA translocation by SWI/SNF complexes can lead to nucleosome repositioning or ejection. Nucleosome ejection may be a consequence of too high DNA tension on the nucleosome, or a nearby nucleosome may become destabilized and ejected due to simply being reeled too close to another nucleosome by the remodeler (Clapier *et al.* 2017).

Finally, chromatin remodelers can edit nucleosomes by exchanging histone proteins for variant forms. The INO80 family of remodelers are the main remodelers responsible of histone variant exchange, but specialized members in other remodeler families are also capable of altering nucleosome composition (Clapier *et al.* 2017). Similar to the nucleosome ejection induced by SWI/SNF remodelers, the INO80 remodeler activity leads to high DNA tension in the nucleosome and histone ejection, but only a histone dimer is ejected instead of the whole octamer (Ranjan *et al.* 2015, Clapier *et al.* 2017). The ejected histones, often a H2A-H2B heterodimer, can then be replaced with a variant form, such as H2A.Z-H2B, with the assistance of histone chaperones (Mizuguchi *et al.* 2004, Ranjan *et al.* 2015).

2.3 TRANSCRIPTION FACTOR COREGULATORS AND COOPERATING FACTORS

Most human TFs are thought to regulate transcription by interacting with coregulatory proteins at enhancers in the regulatory regions of the genome instead of recruiting RNA Pol II directly to gene promoters. By definition, coregulators are proteins that regulate the rate of transcription after being recruited to chromatin by sequence-specific TFs. Coregulators themselves are unable to bind to DNA in a sequence-specific manner (Millard *et al.* 2013). Histone PTM modifiers and chromatin remodelers can be classified as coregulators when they are recruited to chromatin by TFs: For instance, TF-mediated recruitment of the histone acetyltransferase p300 leads to the acetylation of nearby histones. The acetylation destabilizes nucleosomes or recruits BRD-domain containing SWI/SNF complexes for further remodeling. p300 may also acetylate other chromatin-associated proteins, such as TFs, or it may directly modify the activity of RNA Pol II (Reiter *et al.* 2017) (Figure 7).

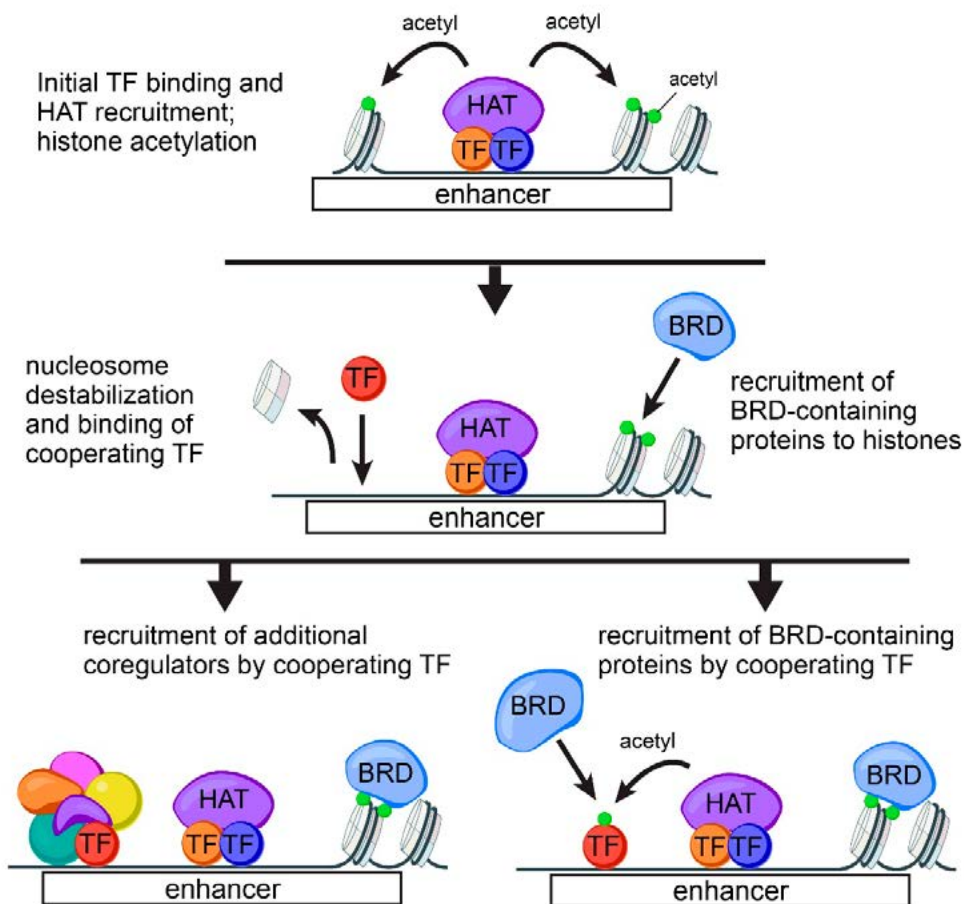

Figure 7. Hypothetical example of cooperativity between TFs, coregulators and post-translational modifications (PTMs) at an enhancer. TFs recruit a histone acetyltransferase (HAT) that acetylates histones. Histone acetylation in turn destabilizes nucleosomes, enabling the binding of a cooperating TF, and the recruitment of BRD-containing proteins (BRD). The cooperating TF may recruit coregulators or BRD-containing proteins. Adapted from (Reiter *et al.* 2017).

The coregulators of NRs have been the most widely studied (Millard *et al.* 2013) (Table 1). Classically, based on their effect in simple reporter gene assays, NR coregulators have been divided into coactivators and corepressors, where coactivators potentiate and corepressors suppress the expression of reporter genes. NR corepressors have been thought to be recruited by unliganded receptors and coactivators by agonist-bound NRs (Perissi & Rosenfeld 2005). To date, over 200 interacting proteins have been identified for both GR and AR in human cells (Stark *et al.* 2006, Heemers & Tindall 2007, DePriest *et al.* 2016, Chatr-Aryamontri *et al.* 2017). Some of the long-established GR and AR -interacting coactivators, corepressor and chromatin remodelers are described below.

Table 1. Some of the classical NR coregulators. Adapted from (McKenna & O'Malley 2010, Ruepp *et al.* 2010, Mashtalir *et al.* 2018).

Coregulator/Family	Symbols	Functions	Complex
Steroid receptor coactivators	NCOA1/SRC-1	Recruits acetyltransferases, weak intrinsic acetyltransferase activity	SRC-1 complex: NCOA1, NCOA2, EP300, CREBBP, TROVE2 SRC-3 complex: NCOA3, NCOA2, EP300, CREBBP, TROVE2
	NCOA2/SRC-2/GRIP1/TIF2		
	NCOA3/SRC-3/AIB1/ACTR/ TRAM-1/RAC3		
Adenovirus E1A-associated 300 kDa protein	EP300/p300	Acetyltransferase	SRC-1 and SRC-3 complex
cAMP response element-binding protein (CREB) binding protein	CREBBP/CBP		
Nuclear receptor corepressor	NCOR1/N-CoR	Recruits histone deacetylases	N-CoR complex: NCOR1, NCOR2, HDAC3, TBL1XR1, CORO2A, GPS2 Other interactions: HDAC1, HDAC2
Silencing mediator of retinoid and thyroid receptors	NCOR2/SMRT		
Histone deacetylase 1	HDAC1	Histone deacetylase	Several complexes: Sin3a, BHC, NuRD
Histone deacetylase 2	HDAC2		
SMRT/HDAC1-associated repressor protein	SPEN/SHARP/ MINT	Steroid-inducible corepressor, recruits histone deacetylases	
Paired amphipathic helix protein Sin3a	SIN3A/Sin3a	Recruits histone deacetylases	Sin3 complex: SIN3A, HDAC1, HDAC2, SAP18, SAP30, RBBP4, RBBP7
Protein CoREST	RCOR1/RCOR	Recruits histone deacetylases and demethylases	BHC complex: RCOR1, HDAC1, HDAC2, KDM1A, GSE1, HMG20B, PHF21A
Thyroid receptor-associated protein 220	MED1/TRAP220/PPARBP/ DRIP205/CRSP200	Coactivator for NR superfamily, bridges TFs to RNA pol II	Mediator complex
Activating signal cointegrator-2	NCOA6/ASC-2/RAP250/TRBP/AIB3	Coactivator for NR superfamily and other TFs	
Metastasis-associated 1	MTA1	Recruits histone deacetylases	NuRD complex: MTA1, MTA3, HDAC1, HDAC2, CHD4, CHD3, MBD3, RBBP4, RBBP7
Transcription intermediary factor-1α	TRIM24/TIF1α/RNF82	Associates with chromatin, has intrinsic E3 ubiquitin ligase activity	
Transcription intermediary factor-1β	TRIM28/TIF1β/RNF96/KAP-1/KRIP-1	Associates with chromatin, has intrinsic E3 SUMO ligase activity	
Transcription activator BRG1	SMARCA4/BRG1/BAF190A	Remodels chromatin, ATPase subunit of the BAF (SWI/SNF) chromatin remodeling complex	BAF (SWI/SNF) complex: SMARCA4, SMARCA2, ARID1A, ARID1B, SMARCC1, SMARCC2, SMARCB1, SMARCD1, SMARCD2, SMARCD3, SMARCE1, DPF1, DPF2, DPF3, BCL7A, BCL7B, BCL7C, SS18, SS18L1, ACTL6A, ACTB

2.3.1 GR and AR coregulators

2.3.1.1 Classical coactivators

The first NR coregulators were identified in yeast two-hybrid screens using NR LBDs as baits. The very first of these authentic NR coregulators was steroid receptor coactivator 1 (SRC-1, also known as NCOA1) found in 1995 by O'Malley and colleagues as an interactor of the PR LBD (Onate *et al.* 1995). It was also found to coactivate several other NRs including GR (Onate *et al.* 1995, Leo & Chen 2000) and later AR (Alen *et al.* 1999, Bevan *et al.* 1999, Ma *et al.* 1999). NCOA1 belongs to the p160 protein family that includes two other homologous proteins, SRC-2 (NCOA2/GRIP1/TIF2) and SRC-3 (NCOA3/ACTR/pCIP/AIB1/TRAM-1) (Dasgupta & O'Malley 2014) that are also GR and AR coactivators (Hong *et al.* 1996, Voegel *et al.* 1996, Chen *et al.* 1997, Ma *et al.* 1999, Tan *et al.* 2000).

All SRCs contain an acetyltransferase domain and an interaction domain for acetyltransferases p300 and cyclic AMP response-element binding protein (CREB)-binding protein (CBP, CREBBP). However, the intrinsic acetyltransferase activity of SRCs is thought to be non-essential for their coactivatory function and their main mode of coactivation to rely on the recruitment of other coactivators (Collingwood *et al.* 1999, Dasgupta & O'Malley 2014). Recently, O'Malley and colleagues used cryoelectron microscopy to describe the spatial organization and stoichiometry of NCOA3 when bound to ER: two NCOA3 proteins are recruited to a DNA-bound ER dimer, and the two NCOA3 proteins in turn bind together to different regions of one p300 protein (Yi *et al.* 2015) (Figure 8).

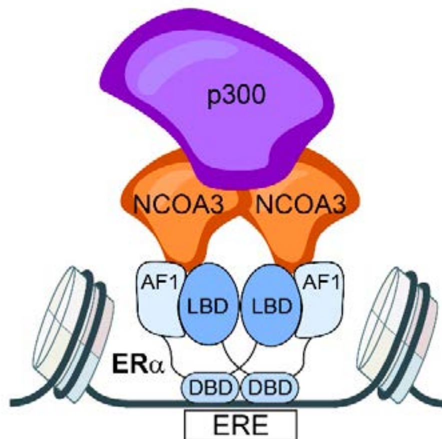

Figure 8. Spatial organization and stoichiometry of NCOA3 (SRC3) and p300 proteins bound to the estrogen receptor α (ERα) as resolved by Yi and colleagues. SRC complexes may interact in a similar manner with the GR and the AR. Adapted from (Yi *et al.* 2015). ERE, estrogen response element.

The interaction motif LxxLL (NR box) on SRCs mediates the interaction to the AF-2 in the NR LBD. SRC interactions with GR are mainly dependent on these domains. However, with the AR, the NR box -mediated interaction with AF-2 is much weaker and less important than the interaction with AF-1. The interaction with AF-1 is mediated by another interaction domain in the SRCs that is distinct from the NR box (Ding *et al.* 1998, Bevan *et al.* 1999). Intriguingly, the AF-1 on SRs is especially long in comparison with the other NRs and it influences the activity of SRs more than AF-2 (Simons *et al.* 2014). The AF-1 is also structurally less conserved than AF-2 and the AR AF-1 is significantly longer than that of the GR (Warnmark *et al.* 2003).

The NTD where the AF-1 resides is intrinsically disordered and lacks a stable secondary and tertiary structure, but has large surface areas to allow coregulator interactions and can momentarily form an ordered structure after interacting with coregulators (Simons *et al.* 2014). The disordered nature of the NTD has made it difficult to crystallize in laboratory conditions and thus the conformation of AF-1 and how it regulates NR activity has remained poorly characterized as compared to AF-2 (Warnmark *et al.* 2003, Simons *et al.* 2014). Interestingly, recent studies have shown that the disordered NTD of NRs may promote liquid-liquid phase separation that compartmentalizes and concentrates coactivators to specific regions of the genome to drive robust expression of genes (Sabari *et al.* 2018, Soltys & Ozyhar 2020).

2.3.1.2 Classical corepressors

At the same time as NCOA1 was identified in 1995, various laboratories searched for the proteins that were responsible for gene repression by unliganded thyroid hormone and retinoic acid receptors that in the absence of ligand were known to remain chromatin bound but in a repressive state. This search led to the identification of two corepressor proteins; the nuclear receptor corepressor (NCoR, NCOR1) and silencing mediator of retinoic acid and thyroid hormone receptors (SMRT, NCOR2) (Horlein *et al.* 1995, Kurokawa *et al.* 1995, Chen & Li 1998). These corepressors are thought to function mainly by recruiting histone deacetylases (HDACs) and HDAC-containing protein complexes, such as the SIN3 protein complex (Perissi *et al.* 2010, Watson *et al.* 2012b).

The interaction of GR with NCOR1 and NCOR2 was originally shown with RU486 (mifepristone) -bound GR and this was suggested to be responsible for the reduced activity of GR when bound to antagonists (Schulz *et al.* 2002). Similarly, NCOR2 was shown to interact with AR in the presence of the antiandrogen cyproterone acetate and shown to mediate the antihormone-induced inactivity of AR (Dotzlaw *et al.* 2002). In a separate study, NCOR1 was shown to attenuate the activity of the agonist-bound AR (Cheng *et al.* 2002).

According to the classical definition, coactivators contain the NR box, while corepressors use the LxxH/IIxxxI/L motif (CoRNR box) to bind to the same hydrophobic groove on the NR AF-2 (Millard *et al.* 2013, Simons *et al.* 2014). The binding of a coactivator or corepressor to the AF-2 is thought to be mutually exclusive and regulated by ligand binding to the LBD (Millard *et al.* 2013).

2.3.1.3 Chromatin remodelers and pioneering factors

Other classical coregulators of GR and AR include SWI/SNF chromatin remodelers. In 1992, Yoshinaga and colleagues showed that the yeast SWI/SNF complex components SWI1, SWI2 and SWI3 interact with rat GR and are necessary for GR activity (Yoshinaga *et al.* 1992). Later, the BRG1- or BRM-associated factor (BAF) complex (human homolog of SWI/SNF) was shown to be ligand-dependently recruited to the GR where it enhances GR activity (Muchardt & Yaniv 1993, Fryer & Archer 1998). Human SWI/SNF components were also shown to enhance the activity of the AR (Inoue *et al.* 2002).

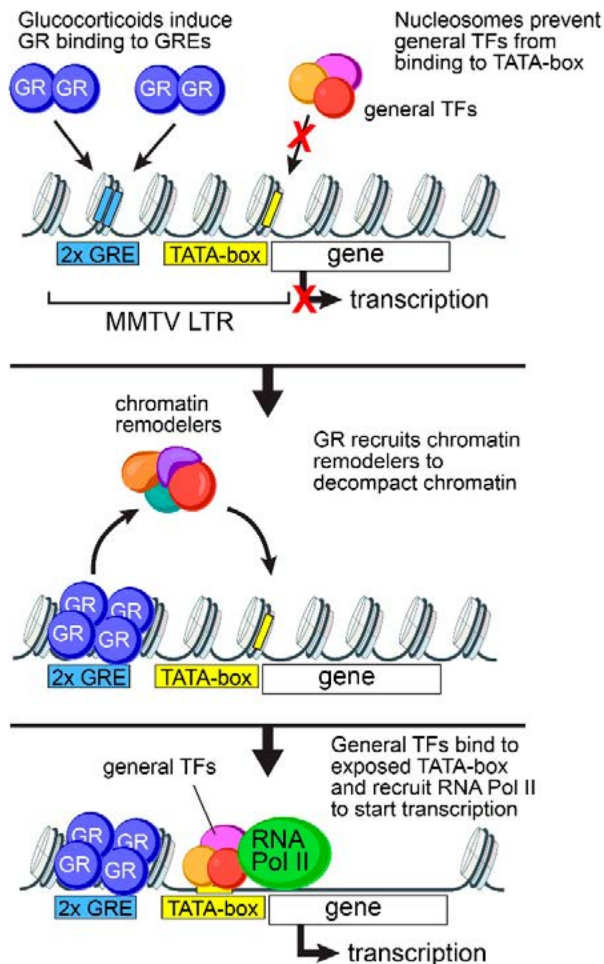

Figure 9. Model depicting the role of GR in regulating the accessibility of the mouse mammary tumor virus (MMTV) long terminal repeat (LTR) promoter. Activated GR binds to two GREs on the surface of a nucleosome. GR then recruits chromatin remodelers that expose the TATA-box for general TFs. Subsequently, the general TFs recruit RNA polymerase II (RNA Pol II) to begin transcription. Adapted from (Collingwood *et al.* 1999).

Role of GR in chromatin remodeling was established already in the early 90s by Hager and colleagues (Archer *et al.* 1991, Archer *et al.* 1992). GR was shown to activate transcription from a mouse mammary tumor virus (MMTV) promoter even when the GREs recognized by GR were wrapped into nucleosomes (Figure 9). GR recruitment to nucleosomal DNA led to chromatin remodeling that exposed DNA-binding sequences to the transcription initiation complex (TATA-box) (Archer *et al.* 1991, Archer *et al.* 1992, Collingwood *et al.* 1999). The ATPase subunit BRG1 from the BAF chromatin remodeling complex was shown to be the key molecular machine recruited to MMTV by GR (Fryer & Archer 1998). Later, regulation of MMTV activity by AR was also shown to be dependent on BRG1 machinery (Huang *et al.* 2003).

TATA-boxes need to be nucleosome-free because binding of the general TFs to these elements causes a severe distortion in DNA that only nucleosome-free DNA can undergo. Thus, assembly of a TATA-box into nucleosomes is a very powerful mechanism of transcriptional repression, that specialized TFs, such as the GR, can regulate (Collingwood *et al.* 1999). The role of GR as a pioneering factor (i.e. TF that can directly bind condensed chromatin) has later been verified with genome-wide methods, that show glucocorticoid-induced chromatin opening at GR-binding sites (Johnson *et al.* 2018, Hoffman *et al.* 2018, Paakinaho *et al.* 2019). However, the role of AR in opening inaccessible chromatin has not been as extensively studied.

GR was shown to induce chromatin openness at some genes also in a BRG1-independent manner, suggesting that other chromatin remodelers are involved (John *et al.* 2008). Indeed, various chromatin remodelers, such as CHD- and BRD-containing proteins, have been identified as GR and AR interactors (Stark *et al.* 2006, Chatr-Aryamontri *et al.* 2017). However, it remains to be investigated whether they mediate pioneering-type functions of these receptors. Especially in AR signaling in PC, the CHD- and BRD-containing remodelers have of wide interest recently (Urbanucci & Mills 2018, Augello *et al.* 2019). For instance, deletion of the CHD1 chromatin remodeler is among the most common alterations found in PC (Liu *et al.* 2012, Wedge *et al.* 2018). CHD1 loss led to AR redistribution on chromatin and increased tumor growth, indicating that CHD1 functions as a tumor suppressor by limiting AR binding to chromatin (Augello *et al.* 2019). In contrast, the BRD-containing BET-family of chromatin remodelers seem to be oncogenic in nature, since their inhibition attenuates the growth of CRPC cells (Asangani *et al.* 2014, Asangani *et al.* 2016, Urbanucci & Mills 2018, Faivre *et al.* 2020).

Induction of chromatin accessibility is possibly a shared mechanism of many different NRs since other NRs in addition to GR and AR have been found to interact with chromatin remodelers (Trotter & Archer 2007). In addition, recent studies have shown that other pioneer TFs, such as FOXA1, can induce chromatin openness and promote the activity of GR, AR and ER (Holmqvist *et al.* 2005, Carroll *et al.* 2005, Jozwik & Carroll 2012, Hankey *et al.* 2020). However, these effects seem to be largely context-dependent, at least in the case of FOXA1: In breast cancer cells, depletion of FOXA1 restricts ER binding to chromatin (Hurtado *et al.* 2011), whereas in PC cells depletion of FOXA1 mostly enhances AR and GR chromatin binding (Sahu *et al.* 2011,

Wang *et al.* 2011). In breast cancer cells, the FOXA1-dependent ER-binding sites were mostly located at compacted chromatin, suggesting that FOXA1 functions as a pioneering factor at these sites to allow ER binding (Hurtado *et al.* 2011). In contrast, in PC cells, FOXA1 may compete with AR and GR for chromatin binding sites or maintain inaccessible chromatin (Sahu *et al.* 2011). However, in PC cells, FOXA1 was also shown to promote GR and AR binding at a subset of binding-sites that are not shared between the receptors, further indicating that the function of FOXA1 is largely context-specific (Sahu *et al.* 2013). Indeed, different pioneering factors, FoxA1, Hnf4α (hepatocyte nuclear factor 4α) and AP-2α (activating enhancer binding protein 2α), were shown to guide AR to specific genomic loci in murine prostate, kidney and epididymis, respectively (Pihlajamaa *et al.* 2014)(Sahu et al. 2014).

The role of SR crosstalk in the normal function of male and female reproductive physiology and development, and in cancers such as PC and breast cancer, remains underinvestigated. If SRs are expressed at sufficient levels in the presence of their cognate hormones, they may influence each other's binding to chromatin. In PC cells, overexpressed GR and endogenous AR were shown to enhance each other's binding to chromatin at specific binding sites (Sahu *et al.* 2013). Since pioneering-type function of the GR has already been reported (Johnson *et al.* 2018, Hoffman *et al.* 2018, Paakinaho *et al.* 2019), recruitment of chromatin remodelers could be one mechanism of cooperativity between SRs.

2.3.1.4 Dilemma of coregulator classification

Various reports have shown that the activating or repressing effect of NR coregulators is largely context-dependent (Perissi & Rosenfeld 2005, Wu *et al.* 2014, Lempiainen *et al.* 2017). For instance, the lysine demethylase KDM1A (LSD1) represses gene expression by demethylating H3K4, but it can enhance gene expression, if it demethylates H3K9. It also functions as a component of the LSD1/CoREST repressive complex that contains HDACs (Perissi & Rosenfeld 2005). However, it coactivates AR in PC cells by demethylating H3K9 (Shi *et al.* 2004, Metzger *et al.* 2005). In addition, we showed that the depletion of KDM1A attenuates GR activity, suggesting that KDM1A also coactivates GR (Lempiainen *et al.* 2017).

It has been proposed that coregulators should be defined by their mechanism of action rather than by subdivision into coactivators and corepressors according to the transcriptional outcome (Millard *et al.* 2013). Moreover, NR coregulators do not function in isolation, but as components of large coregulator complexes that may consists of tens of different proteins (Millard *et al.* 2013). According to this mechanism-centered classification, coregulators may function in four main ways: (1) by directly interacting with TFs to recruit coregulator complexes to specific genomic loci, (2) by altering ("writing") the PTM status of histones or other chromatin-bound proteins or functioning as chromatin remodelers, (3) by recognizing ("reading") specific PTMs on histones or other chromatin-bound proteins or functioning as a substrate for PTMs, or (4) by functioning as a scaffold that holds the coregulator complex subunits together (Millard *et al.* 2013).

2.3.1.5 GR and AR coregulators as drug targets

NR coregulators are known to be dysregulated in various human diseases, such as metabolic disorders, heritable syndromes and cancer (Lonard *et al.* 2007, Lonard & O'Malley 2012, Dasgupta & O'Malley 2014). Coregulators have been considered difficult molecules to target, because they may lack a defined enzyme catalytic surface or a high-affinity ligand-binding pocket (Lonard & O'Malley 2012). However, even in these cases small molecule inhibitors can be developed that target protein-protein interaction surfaces or cause protein degradation. Examples of those include molecules that have been designed against SRCs (Lonard & O'Malley 2012, Dasgupta & O'Malley 2014). Targeting NR coregulators that are expressed in a tissue-specific manner could potentially aid in developing more tissue-specific therapies with less adverse effects (Chen 2008).

GR coregulators as drug targets are especially of interest because prolonged glucocorticoid therapy often leads to severe side effects and/or glucocorticoid resistance (Oakley & Cidlowski 2011). Synthetic GR ligands have been developed in an attempt to improve the specificity and efficacy of glucocorticoid therapies (De Bosscher *et al.* 2005, Wang *et al.* 2006, Sundahl *et al.* 2015), but these approaches have led to the discovery of disappointingly few ligands (De Bosscher *et al.* 2016). However, especially in leukemia, GR coregulators have been suggested as potential targets to improve the sensitivity to glucocorticoids (Clarisse *et al.* 2017, Poulard *et al.* 2019). Small molecule inhibitors and activators against SRCs could be potentially used in treatments against hematological malignancies (Clarisse *et al.* 2017). For instance, the small molecule inhibitor verrucarin A causes the degradation of NCOA3 and the inhibitor bufalin evokes the degradation of both NCOA1 and NCOA3 (Yan *et al.* 2014, Wang *et al.* 2014, Lonard & O'Malley 2016). Both of these inhibitors attenuate cancer cell proliferation (Yan *et al.* 2014, Wang *et al.* 2014, Lonard & O'Malley 2016). On the other hand, the small molecule stimulator MCB-613 hyperstimulates the transcriptional activity of SRCs, ultimately killing cancer cells (Wang *et al.* 2015b).

AR coregulators provide valuable alternative drug targets especially in the lethal CRPC (Mostaghel *et al.* 2014, Cato *et al.* 2017). Inhibitors against different AR coregulators, such as those for the BET-family members (Urbanucci & Mills 2018) and KDM1A (Etani *et al.* 2015) are in preclinical or clinical evaluation (Urbanucci & Mills 2018). Interestingly, the turmeric spice isoflavone curcumin was shown to attenuate proliferation of CRPC cells and reduce tumor growth *in vivo* (Shah *et al.* 2012). Curcumin inhibited AR signaling by suppressing occupancy of pioneering factors at AR-bound enhancers (Shah *et al.* 2012). In particular, coregulators interacting with the AR N-terminal AF-1 are thought to be relevant drug targets because AF-1 controls AR activity more than AF-2, and the multiple AR variants that are expressed in CRPC lack a LBD (Claessens *et al.* 2008, Guo *et al.* 2009, Cato *et al.* 2017).

2.3.2 Post-translational modifications in coregulator interactions

In addition to ligands, NR-coregulator interactions are regulated by PTMs both on coregulators and on NRs. For instance, PTMs such as phosphorylation, acetylation, ubiquitination, methylation and SUMOylation of SRCs fine-tune their activity when they bind to NRs (Dasgupta & O'Malley 2014). On the other hand, the highly variable NTD on SRs, such as that of the GR and AR, is especially rich in sites for PTMs (Faus & Haendler 2006) (Figure 10).

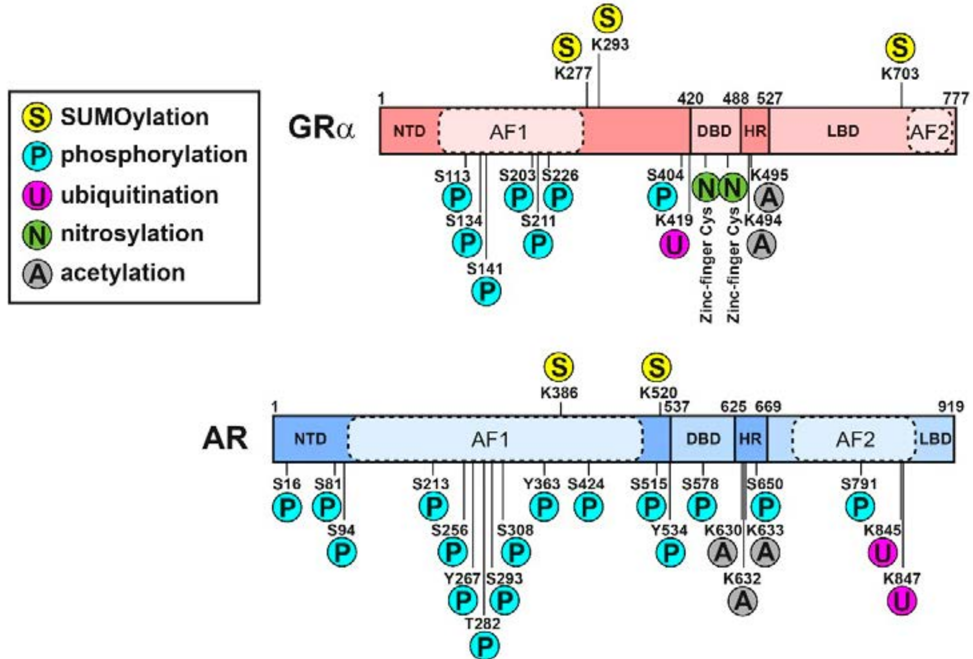

Figure 10. Sites of major GR and AR post-translational modifications (PTMs) mapped onto the GR and AR domain schematics. Adapted from (Gioeli & Paschal 2012, Weikum *et al.* 2017).

2.3.2.1 SUMOylation and GR

SUMO (small ubiquitin-like modifier) is a ~100-amino acid long protein which is attached as a covalent modification primarily onto nuclear proteins. In mammalian cells, it is expressed as three isoforms (SUMO1, -2 and -3) from which SUMO2 and SUMO3 are nearly (97%) identical (herein collectively referred to as SUMO2/3), whereas SUMO1 is only about ~50% identical with SUMO2/3 (Tatham *et al.* 2001, Gill 2004). The SUMOylation process starts from the SAE1/2 heterodimer that activates SUMOs so that they can be conjugated to their target lysines by UBC9. Additional SUMO ligases, such as PIAS proteins, can guide the target lysine to UBC9 to assist in the SUMOylation process (van Wijk & Timmers 2010). Finally, SUMO-specific proteases (SENPs) release free SUMO for a new cycle of conjugation by cleaving SUMOs from target proteins; this makes the process reversible (Mukhopadhyay & Dasso 2007).

Different mutation experiments have shed some light on the function of NR NTDs by identifying important regulatory subregions, such as the so-called synergy control motifs of the GR (Iniguez-Lluhi & Pearce 2000, Holmstrom *et al.* 2008). Surprisingly, and unlike many other NTD subdomains identified for the GR or other NRs, the synergy control motifs repress GR activity (Iniguez-Lluhi & Pearce 2000, Holmstrom *et al.* 2008). The GR synergy control motifs were later shown to contain sites for SUMOylation, and mutating these SUMO target lysines (K277 and K293) to arginines significantly increased GR activity in a reporter assay (Tian *et al.* 2002), suggesting that it is the SUMOylation at these control motifs that represses GR activity. On the other hand, a slight decrease in GR activity was observed when an additional SUMO target lysine (K703R) at the LBD was mutated (Tian *et al.* 2002), indicating that the SUMOylation of the LBD is less important for GR activity and is functionally distinct from the SUMOylation of the NTD.

The importance of SUMOylation on GR function was further demonstrated with genome-wide ChIP-seq which revealed that the SUMOylation mutant GR (GR3KR, all three aforementioned SUMO target sites mutated to arginines) binds to chromatin more avidly than its wild-type counterpart (Paakinaho *et al.* 2014). Moreover, the mutations influenced the expression of GR target genes that regulate cell growth, enhancing the proliferation of GR3KR -expressing cells (Paakinaho *et al.* 2014). These findings indicate that GR SUMOylation has physiologically important functions.

2.4 METHODS TO STUDY PROTEIN-PROTEIN INTERACTIONS OF TRANSCRIPTION FACTORS

Protein-protein interactions were originally studied by biochemical methods, such as co-immunoprecipitation (Co-IP) and chromatographic co-fractionation. Co-IP relies on a specific antibody that binds the protein of interest to an affinity matrix that captures interacting proteins. The interactors from eluates are then detected by western blotting, which also requires the availability of specific antibodies. In co-fractionation, a mixture of proteins is passed through a chromatographic column that separates the proteins into different fractions according to their specific qualities, e.g. the molecular mass of the complex. In the original co-fractionation studies, proteins in the same fraction were identified by western blotting with specific antibodies. Co-IP is still considered as one of the standard methods for studying protein-protein interactions and is used in parallel with other methods. Improved versions of co-fractionation can be beneficial, for instance in studying protein-protein interactions of nuclear proteins (Zhuang *et al.* 2014).

However, the invention of cloning and protein tags, such as the FLAG and hemagglutinin (HA), also allowed co-IP of proteins for which antibodies were not available. They also enabled the development of efficient generic affinity purification (AP) reagents (Agbo & Lambert 2019). Cloning and protein tags also enabled the development of genetic methods such as the yeast two-hybrid (Y2H) screen (Fields & Song 1989); this was especially important in the NR coregulator field, because it

allowed the identification of the first NR coregulators (Onate *et al.* 1995). In Y2H, the N-terminal DNA-binding domain and C-terminal transcriptional activation domain of the yeast GAL4 TF are fused to two proteins. If these two proteins interact, the GAL4 TF reconstitutes and activates the expression of a reporter gene downstream of a GAL4-binding sequence (Fields & Song 1989). Various laboratories have later improved the method and applied it in an automated manner to screen massive numbers of protein pairs, as was done for NRs (Albers *et al.* 2005). Y2H is still considered an extremely valuable method to study direct protein-protein interactions (Gingras & Raught 2012).

The improvements in mass spectrometers (MS) have enabled the sensitive identification of protein-protein interactors in various types of experiments. In addition, the development of fluorescent tags, such as green fluorescent protein (GFP), enabled the emergence of various microscopy-based methods to study protein-protein interactions. Some of the current MS and microscopy -based methods that have been used to study TF protein-protein interactions, especially those of the GR and the AR, are described below.

2.4.1 Mass-spectrometric methods

After the development of generic AP reagents, it was quickly realized that MS could achieve sensitive and unbiased detection of proteins in pulldown eluates. In a typical MS-based proteomic experiment, the protein eluates are first digested with a sequence-specific protease (e.g. trypsin) after which the peptides are separated with chromatography, electrosprayed and introduced into the vacuum of the MS (Aebersold & Mann 2016). The MS data can then be acquired in three main ways: data-dependent acquisition (DDA), data-independent acquisition (DIA) or by targeted acquisition. In DDA, the MS instrument first acquires the spectra of all the peptides in the sample (MS1), after which selected peptides are further fragmented and the spectra of these fragments acquired (MS2, tandem MS, MS/MS) (Aebersold & Mann 2016, Agbo & Lambert 2019). DDA is the preferred method for proteome discovery and used in bottom-up analysis where peptides are fragmented before analysis (Aebersold & Mann 2016, Agbo & Lambert 2019). In DIA, peptides belonging to a given range of mass-to-charge (m/z) ratios are selected for fragmentation and analysis, whereas in targeted acquisition, a specific peptide of known m/z is selected. DIA and targeted acquisition are more sensitive and reproducible than DDA, but they are not suitable for identification of the whole spectrum of peptides in the sample (Aebersold & Mann 2016, Agbo & Lambert 2019).

After MS data acquisition, the identified spectra are inferred to determine the amino acid composition of the peptides. The peptide sequences are then compared to protein databases to identify the proteins from which the peptides originated. The sensitivity of MS has been improved to such a level that in a single analysis up to ~90% of the proteome of a human cancer cell line can be detected (Meier *et al.* 2018, Agbo & Lambert 2019). The high sensitivity of the equipment compensates for the

fact that protein samples cannot be amplified, and protocols have also been improved to minimize sample loss during preparation (Agbo & Lambert 2019). Furthermore, applications, such as stable isotope labeling by amino acids in cell culture (SILAC), can further increase the sensitivity of MS methods (Ong *et al.* 2002). In SILAC, mammalian cell lines are grown in the presence of non-radioactive isotopically labeled amino acids that are incorporated into proteins. In MS runs, the isotopes can be identified and used for the relative quantitation of changes in protein abundance between samples. The benefit of this method is that samples can also be combined in the preparation steps which reduces the technical error (Ong *et al.* 2002).

However, identification of protein-protein interactions of chromatin-associated proteins, such as TFs results in a unique set of problems due to the low solubility of these proteins when they are chromatin-bound (Lambert *et al.* 2012, Agbo & Lambert 2019). In recent years, various improved MS-based methods have been employed for the identification of protein-protein interactions on chromatin, especially in the SR field (Agbo & Lambert 2019). In principle, protein interactors for the identification with MS can be retrieved in three main ways: affinity purification, proximity biotinylation or co-fractionation (Gingras *et al.* 2019). Some of the current MS-based methods belonging to these categories are described below.

2.4.1.1 Affinity purification mass spectrometry

Affinity purification mass spectrometry (AP-MS) is the most widely used method for mapping protein-protein interactions (Liu *et al.* 2018). It involves a pulldown of a protein of interest (bait) with its associating proteins (preys) that are subsequently identified with MS. The AP phase is performed using an antibody against an endogenous bait protein or by the expression of an epitope-tagged bait (Dunham *et al.* 2012). Strep-tag is considered as the gold standard of epitope tags in AP-MS protocols due to the high protein purity it achieves in pulldowns and the gentle elution it allows in physiological conditions with biotin (Liu *et al.* 2018).

In 2003, Ishitani and colleagues published the first AP-MS study of the AR by using the AR AF-1 as a bait after which eluates were analyzed with MS (Ishitani *et al.* 2003). Later, Jasavala and colleagues identified 421 putative AR-binding proteins using full-length AR (Jasavala *et al.* 2007). In these experiments, an affinity-tagged AR was expressed in HEK293 cells, and an antibody against the tag was used to purify AR and its interactors in a single purification step from the cytosolic and nuclear compartments (Jasavala *et al.* 2007). In 2006, Hedman and colleagues conducted the first AP-MS interactome study of the GR, identifying 27 putative GR-binding proteins in rat liver (Hedman *et al.* 2006). Purifications with an anti-GR antibody were used to extract cytosolic GR interactors that were subsequently identified by MS (Hedman *et al.* 2006). Since then, various AP-MS studies have characterized GR and AR interactors in different cell lines and models (Cao *et al.* 2014, Li *et al.* 2015, Wang *et al.* 2015a, Hsiao *et al.* 2016, Ptushkina *et al.* 2017, Luo *et al.* 2017, Zhu *et al.* 2018, Stelloo *et al.* 2018).

Several large-scale AP-MS studies have been conducted with human and yeast coregulator protein complexes (Gavin *et al.* 2006, Malovannaya *et al.* 2010, Malovannaya *et al.* 2011, Li *et al.* 2015) . For instance, Malovannaya and colleagues characterized more than 1000 endogenous NR coregulators in HeLa cells by using antibody-based pulldowns with minimal washes (Malovannaya *et al.* 2010). In a follow-up study, 1796 distinct antibodies in 3290 AP-MS experiments were used to determine an even larger number of coregulators (Malovannaya *et al.* 2011). Later, Liu et al. used a modified tandem affinity purification (TAP) to elucidate the protein-protein interactomes of 56 TFs in 214 AP-MS experiments (Li *et al.* 2015). Triple-tagged TFs were expressed in HEK293T cells with AP being performed in two steps leading to the identification of 2156 high-confident interactions (Li *et al.* 2015).

Traditional AP-MS methods are not always optimal for the identification of protein-protein interactions on chromatin, because chromatin-associated proteins are often highly insoluble and cannot be retrieved under mild lysis conditions. Harsher conditions, on the other hand, can easily lead to the loss of interactions (Gingras *et al.* 2019). For instance, an AP-MS protocol that successfully identifies protein-protein interactions of cytosolic kinases (Varjosalo *et al.* 2013) did not retrieve any commonly known GR interactors of the agonist-activated GR in HEK293 cells (Lempiäinen *et al.* unpublished observations). Most likely, the GR interactors were not properly solubilized or they were lost during the washing steps. In general, protocols with multiple purification steps and high salt concentrations are more specific, but can result in the loss of chromatin-dependent and transient interactions (Lambert *et al.* 2012). Efficient chromatin solubilization can be achieved by sonication and/or with nucleases that break down chromatin. However, chromatin solubilization often leads to higher background contamination and appropriate controls are needed (Lambert *et al.* 2012).

Especially in recent years, modified AP-MS protocols that aim to identify TF interactors on chromatin have been developed (Mohammed *et al.* 2013, D'Santos *et al.* 2015, Mohammed *et al.* 2016, Rafiee *et al.* 2016). Rapid immunoprecipitation mass spectrometry of endogenous proteins (RIME) combines formaldehyde crosslinking, antibody pulldown of an endogenous bait protein with an on-bead digestion in the purification of endogenous interactors (Mohammed *et al.* 2013). Formaldehyde crosslinking has been routinely used in mainstream techniques including chromatin immunoprecipitation (ChIP) of DNA-protein complexes and immunohistological analyses, and when coupled with MS, it can improve the detection of novel protein-protein interactions (Sutherland *et al.* 2008). In formaldehyde crosslinking, covalent bonds are formed between proteins, DNA, and other reactive molecules within 2.3-2.7 Å (0.23-0.27 nm) distance (Sutherland *et al.* 2008). RIME has been successfully applied in the identification of protein interactors of at least ER, PR and NCOA1 in breast cancer cells (Mohammed *et al.* 2013, D'Santos *et al.* 2015, Mohammed *et al.* 2016, Browne *et al.* 2018) and those of AR (Barfeld *et al.* 2017, Stelloo *et al.* 2018) and MYC (Barfeld *et al.* 2017) in PC cells.

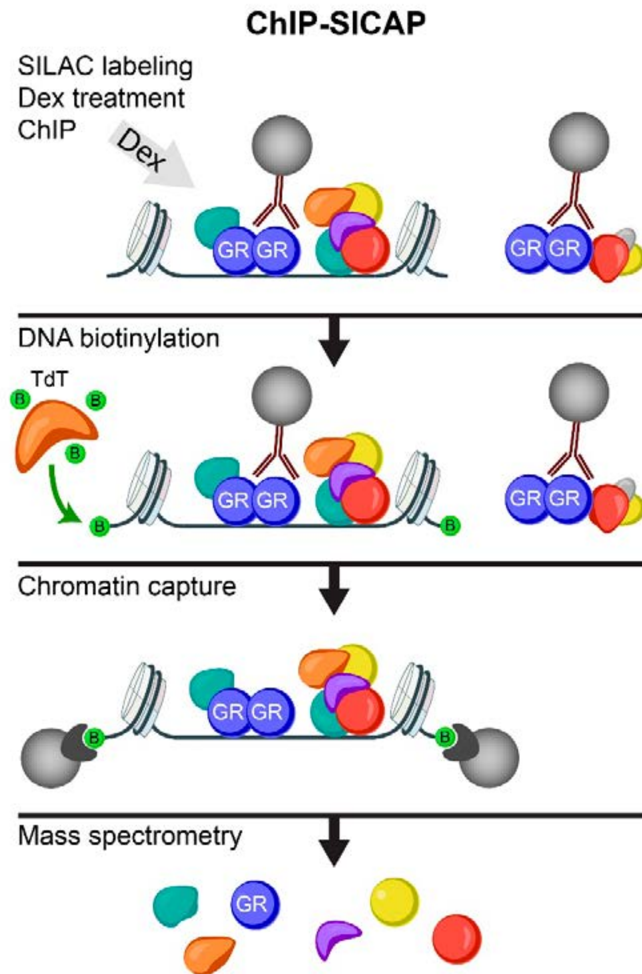

Figure 11. Schematic representation of the ChIP-SICAP protocol. Dex, dexamethasone.

ChIP combined with selective isolation of chromatin associated proteins (SICAP) and SILAC metabolic labeling was successfully used to identify the protein networks of Oct4, Sox2 and Nanog TFs in mouse embryonic stem cells (Rafiee *et al.* 2016). As in RIME, the protocol also involves formaldehyde crosslinking, but in the subsequent steps, DNA fragments are purified, resulting in the identification of exclusively chromatin-bound proteins or complexes (Figure 11). In the ChIP-SICAP protocol, sonication is used to break the crosslinked chromatin into 200-300 bp fragments, after which an antibody against the bait protein is used to immunoprecipitate both soluble and chromatin-bound bait-prey complexes. The ends of DNA fragments are then labeled with biotin by terminal deoxynucleotidyl transferase (TdT) in the presence of biotinylated nucleotides. TdT is a DNA polymerase that extends DNA from the 3′ end template independently and without the need for a complementary strand (Jones

& Dive 1999, Rafiee *et al.* 2016). The non-crosslinked interactions are then released and the antibody is denatured in the presence of ionic detergents and a reducing agent. Streptavidin beads are used to capture the biotinylated DNA-protein complexes and stringent washing applied to remove contaminating proteins and the IP antibody. Finally, heating is used to reverse the protein-DNA crosslinks, proteins digested and identified with MS.

DNA can also be utilized to capture interacting proteins that then can be identified with MS. For instance, DNA containing EREs (estrogen-response elements) was used to pull down ER interactors in nuclear extracts, identifying all three classical SRCs (NCOA1-3 or SRC1-3), NCOA6, MED1, EP300, CREBBP and NRIP1 as ER interactors (Foulds *et al.* 2013). Similarly, the *Abca1* gene promoter was exploited to identify interactors of the liver X receptor (Gillespie *et al.* 2015).

2.4.1.2 Proximity labeling

Proximity-dependent biotinylation coupled with MS (PDB-MS) has been utilized in the study of protein-protein interactions more recently than AP-MS (Roux *et al.* 2012, Gingras *et al.* 2019). In PDB-MS, the bait protein is fused with an enzyme that catalyzes the activation of biotin or a phenolic biotin derivative. The reactive biotin derivatives then diffuse from the active site of the enzyme and covalently attach to nearby proteins. The proteins can then be purified with streptavidin and identified by MS (Gingras *et al.* 2019). PDB-MS methods are well suited for the identification of transient interactions of chromatin-bound proteins, because the complexes do not have to remain intact during purification and harsh conditions can be used to efficiently solubilize proteins. Moreover, interactions are not susceptible to post-lysis artifacts (i.e. false positives resulting from mixing proteins from different intracellular locations) because proteins are labeled in living cells before cell lysis (Gingras *et al.* 2019). Biotin ligases (BirA*, BioID2, BASU, TurboID, miniTurbo) and peroxidases (APEX, APEX2, HRP) are the two main classes of enzymes that have been applied in PDB-MS methods (Gingras *et al.* 2019).

The original proximity-dependent biotin identification (BioID) utilizes a mutated form (R118G) of the *Escherichia coli* biotin ligase (BirA*) that releases activated biotin which readily reacts with primary amines in nearby polypeptides within the ~10 nm range (Roux *et al.* 2012, Gingras *et al.* 2019). In the identification of chromatin-bound interactions, the BirA-fused bait protein is expressed, after which excess biotin is added to induce the ligation of biotin. Cells are lysed, chromatin sonicated and treated with nucleases to efficiently solubilize chromatin-associated proteins. The biotinylated proteins are then purified with streptavidin, washed, eluted with biotin and finally identified with MS (Figure 12).

BioID has been applied in various interactomics studies of proteins after its initial application in 2012 (Roux *et al.* 2012, Sears *et al.* 2019, Gingras *et al.* 2019). It has been successfully used especially for insoluble bait proteins, such as chromatin bound TFs, histones, nuclear pore complexes, nuclear lamin and membrane proteins (Sears *et al.* 2019, Gingras *et al.* 2019). BioID was used to explore the effect of TNFα on GR

interactions (Dendoncker *et al.* 2019) and to identify interactors of other TFs, such as the ER (Zhu *et al.* 2019) and the Hox TF Ultrabithorax (Carnesecchi *et al.* 2020). In addition, BioID and AP-MS have been used in parallel to complement each other (Couzens *et al.* 2013, Hardt *et al.* 2018). For instance, BioID with SILAC metabolic labeling and in parallel with AP-MS were used to identify interactions of the fatty acid 2-hydroxylase in HEK293 cells (Hardt *et al.* 2018). Even combination tags suitable for both BioID and AP-MS experiments as a single expression construct have been developed recently (Liu *et al.* 2018).

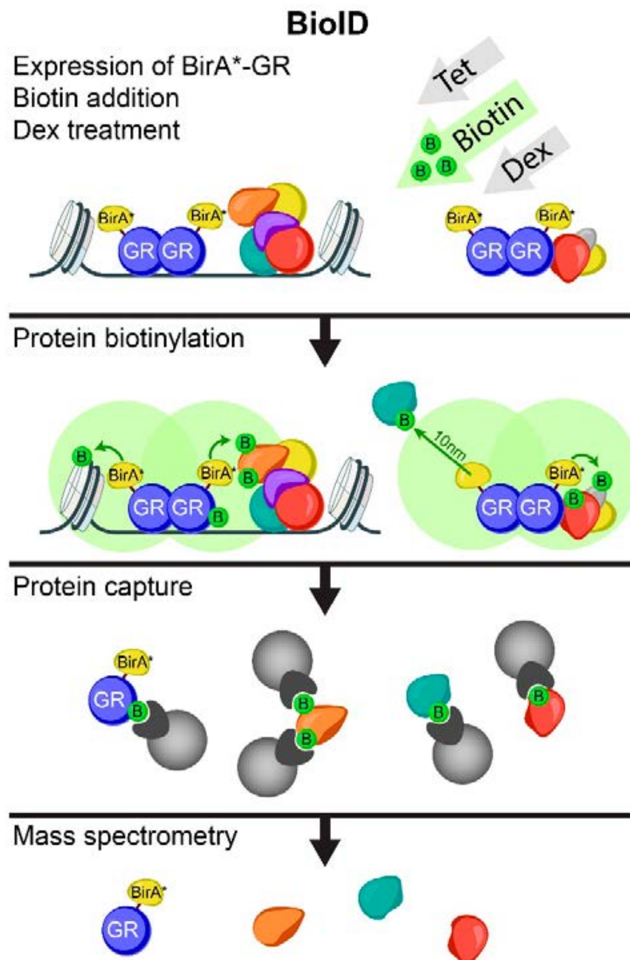

Figure 12. Schematic representation of the BioID protocol. Tet, tetracycline; Dex, dexamethasone.

Peroxidases, such as the engineered ascorbate peroxidase (APEX), and the more catalytically active APEX2, catalyze the oxidation of biotin-phenol to a reactive biotin-phenoxyl that covalently attaches to tyrosines (and possibly also to

tryptophan, cysteine, and histidine) on target polypeptides within ~20 nm distance (Gingras *et al.* 2019, Trinkle-Mulcahy 2019). Peroxidases can generate sufficient levels of biotinylated proteins for MS analysis within minutes, whereas the traditional BirA* requires several hours if it is to achieve the same level of biotinylation (Gingras *et al.* 2019). In addition, APEX is smaller than the original BirA* (27 versus 35 kDa) and functions better at temperatures below 37 °C that are used to grow model organisms, such as *Drosophila* and yeast (Chen & Perrimon 2017, Trinkle-Mulcahy 2019). Peroxidases have been successfully used in various protein interactomics studies (Lobingier *et al.* 2017, Paek *et al.* 2017, Trinkle-Mulcahy 2019). However, in order to generate the activated biotin-phenoxyl compound, peroxidases also require H_2O_2, which is toxic for living samples (Branon *et al.* 2018). Improved versions of BirA* have been developed, such as miniTurbo and TurboID, that have reduced the size of the ligase and increased its activity. These improvements potentially enable the use of biotin ligases also in shorter protocols with the benefit of less toxicity than peroxidase-based methods (Branon *et al.* 2018, Gingras *et al.* 2019, Samavarchi-Tehrani *et al.* 2020). Nonetheless, TurboID is also toxic when utilized for elongated durations (Branon *et al.* 2018).

Intriguingly, the development of the clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) gene editing methodology has allowed the identification of proteins at specific gene loci (Trinkle-Mulcahy 2019). CRISPR/Cas9 employs a guide RNA (gRNA) that targets a Cas9 nuclease to a specific location on the chromatin to edit target sequences. For instance, it allows the addition of complete tag sequences, such as those coding for fluorophores, to specific locations on the genome (Adli 2018). In MS applications, a catalytically dead Cas9 mutant (dCas9) can be fused to an affinity tag, a biotin ligase or a peroxidase. The fusion protein can then be targeted to a specific gene locus when co-expressed with gRNA (Trinkle-Mulcahy 2019). For example, in CasID, dCas9 is fused to BirA* that biotinylates nearby proteins at a specific target locus (Schmidtmann *et al.* 2016). Similarly, dCas9 has been fused to APEX2 and targeted to telomeric and centromeric regions (Gao *et al.* 2018) and to hTERT and MYC promoter regions (Myers *et al.* 2018). Different AP-MS protocols have also utilized CRISPR/Cas9 by fusing Cas9 to affinity tags, but they are considered less effective and less sensitive than methods employing PDB-MS (Trinkle-Mulcahy 2019).

2.4.1.3 Co-fractionation

Biochemical fractionation techniques can be combined with MS to characterize protein complexes or organelle composition. For instance, size or charge-based separation techniques can be used to purify biochemically stable complexes with the fractions being analyzed with MS (Yates *et al.* 2005). Kristensen et al, combined size-exclusion chromatography with quantitative proteomics to map 291 coeluting complexes with the same depth and accuracy as AP-MS but with less work and without overexpression or tagging (Kristensen *et al.* 2012). The identified interactors also included GR interactors, such as the histone deacetylase 1 (HDAC1) and

metastasis-associated protein 2 (MTA2) (Kristensen *et al.* 2012). Similarly, Havugimana and colleagues used co-fractionation coupled with MS to identify 622 putative protein complexes (Havugimana *et al.* 2012).

2.4.2 Microscopic methods

Various microscopic assays have been developed to monitor protein-protein interactions. The bimolecular fluorescence complementation assay (BiFC) employs an N-terminal and a C-terminal fragment of a fluorophore, such as the green fluorescent protein (GFP), that are fused to the proteins of interest. The interaction between the two proteins reconstitutes the fluorophore, which can then be detected with microscopy (Cabantous *et al.* 2005). Localization of the interaction (i.e. cytosolic or nuclear location) can also be determined with BiFC (Cabantous *et al.* 2005). BiFC was applied to confirm protein interaction pairs and interaction localizations, for instance, of different kinases (Varjosalo *et al.* 2013), TFs (Yazaki *et al.* 2016) and fatty acid 2-hydroxylase (Hardt *et al.* 2018).

Quantitative live-cell microscopy such as fluorescence resonance energy transfer (FRET) can also be used to study direct protein-protein interactions (Royen *et al.* 2009, Lam *et al.* 2012). This approach is based on a physical phenomenon where the excitation energy of a donor fluorophore is transferred to a second nearby fluorophore that subsequently emits the energy at a lower energy wavelength (Forster 2012). The emitted fluorescence can then be detected with a microscope. For instance, FRET has been used to study the effect of DNA-binding to the interaction between the AR N- and C-termini (N/C interaction) and on AR coregulator interactions (Royen *et al.* 2007). More recently, FRET was used to study the effect of different ligands on AR N/C interactions (Roell *et al.* 2019) as well as the effect of TNFα on the homodimerization and p300 interactions of the GR (Dendoncker *et al.* 2019).

Fluorescence recovery after photobleaching (FRAP), fluorescence correlation spectroscopy (FCS) and single-molecule tracking (SMT) are also quantitative live-cell microscopic methods that have been applied to study the effects of different ligands, mutations and coregulators on the nuclear dynamics of GR and AR (McNally *et al.* 2000, Schaaf & Cidlowski 2003, Royen *et al.* 2007, Groeneweg *et al.* 2014, Royen *et al.* 2014, Paakinaho *et al.* 2017, Keizer *et al.* 2019). In FRAP, a brief high intensity laser pulse is used to bleach an area of fluorophores after which the recovery of fluorescence to the bleached area is measured (Axelrod *et al.* 1976). In a study of TF nuclear dynamics, the FRAP area is typically a strip spanning the nucleus (Royen *et al.* 2014). In FCS, fluctuations in fluorescence intensity are analyzed within a much smaller volume (~1 μm^3) which allows an assessment of both diffusion and binding of molecules (Magde *et al.* 1974, Stasevich *et al.* 2010). In SMT, diffusion and binding of molecules are also measured, but at the single-molecule level (Ober *et al.* 2015). SMT was developed much later, because it needed more powerful instrumentation and advanced signal and image processing techniques (Ober *et al.* 2015).

FRET, FRAP, FCS and SMT have often been used in parallel to complement or cross-validate each other (Royen *et al.* 2007, Stasevich *et al.* 2010, Royen *et al.* 2014, Keizer *et al.* 2019). Early FRAP experiments showed that increased residence times of GR are associated with greater transcriptional output (Stavreva *et al.* 2004). This finding has been confirmed in various studies that show increased residence times of GR, AR and other SRs in the presence of ligand (Royen *et al.* 2014, Groeneweg *et al.* 2014, Paakinaho *et al.* 2017).

FRAP, FCS and SMT can be used in the study of TF-coregulator interactions in an indirect manner when the effect of overexpression, depletion or mutation of coregulators on the nuclear mobility of TFs are measured. For instance, transfecting fragments of the AR coregulator ARA54 (RNF14) was shown to slow down the nuclear mobility of the AR in FRAP experiments (Royen *et al.* 2007). Similarly, FRAP was used to show that overexpression of the AR interactor FOXA1 decreases the nuclear mobility of the AR and that this effect is enhanced with SUMOylation-deficient FOXA1 (Sutinen *et al.* 2014a). More recently, SMT experiments revealed that the nuclear mobility of GR increases when the GR-interacting AP-1 TF complex is disrupted by a mutation of its FOS subunit (Paakinaho *et al.* 2017). On the other hand, the effect of TF activation to coregulator mobility can be measured as was shown in recent SMT experiments with GR coregulators (Paakinaho *et al.* 2017).

3 AIMS OF THE STUDY

GR has been shown to contribute to enzalutamide-resistance of castration-resistant prostate cancer (CRPC), highlighting the importance to study the crosstalk of GR and AR in PC. Recent advances in proteomic methods have enabled unbiased identification of protein-protein interactions of insoluble chromatin-associated proteins. In this thesis work, state-of-the-art proteomics methods were utilized to uncover novel protein-protein interactions of the GR and the AR on chromatin. Moreover, these methods were used to elucidate the effect of the post-translational modification, SUMO, on the protein interactome of the GR, exemplifying how a post-translational modification may fine-tune the response of a signal-activated TF. In addition, this study aimed to identify the role of BCOR, one of the novel AR-interacting proteins found in this work, in AR signaling in PC cells.

The specific aims of the study were:

- To uncover novel protein-protein interactions of the GR and the AR by utilizing state-of-the-art proteomics methods.
- To elucidate the effect of SUMOylation on the protein interactome of the GR and on the ability of the receptor to promote accessible chromatin.
- To determine the role of BCOR in H2AK119 monoubiquitination and in the expression of AR target genes in PC cells.

4 MATERIALS AND METHODS

A wide range of cellular and molecular biology methods were used in this thesis (Table 2). Experimental procedures are described in detail in the original publications (I-III).

Table 2. Summary of the methods used in this thesis.

Method	Original publication
Cell apoptosis assay	III
Cell culture	I, II, III
Generation of cell lines expressing BirA*-fused bait	I, II
Cell proliferation assay	III
Co-immunoprecipitation	III
Fluorescence recovery after photobleaching (FRAP)	I, II
Genome-wide sequencing methods	
Assay for transposase-accessible chromatin sequencing (ATAC-seq)	II
Chromatin immunoprecipitation sequencing (ChIP-seq)	II, III
RNA sequencing (RNA-seq)	III
Immunoblotting	I, II, III
Immunofluorescence and confocal microscopy	I
Isolation of RNA	I, II, III
Mass-spectrometric methods	
Affinity-purification coupled with MS (AP-MS)	II
ChIP coupled with selective isolation of chromatin associated proteins (ChIP-SICAP)	II
Proximity-dependent biotinylation (BioID)	I, II
Molecular cloning	I, II
Quantitative RT-PCR	I, II, III
Reporter gene assay	I
RNA interference	I, III
Transient transfection assays	I, II

5 RESULTS AND DISCUSSION

5.1 PROTEIN INTERACTOME OF THE GR

Initial NR coregulators were identified in yeast-two hybrid screens that utilized NR LBD regions as a bait protein (Nolte *et al.* 1998). However, interactomes of full-length NRs, such as those of the GR, have remained poorly defined (Khorasanizadeh & Rastinejad 2016). AP-MS has been used to identify protein interactomes of full-length NRs, but these interactomes have usually included no, or very few, well-established coregulators, or they have only focused on cytosolic interactions (Hedman *et al.* 2006, Gillespie *et al.* 2015). For instance, an AP-MS protocol that successfully identified protein-protein interactions of cytosolic kinases (Varjosalo *et al.* 2013) did not retrieve any commonly known GR interactors of the dexamethasone-activated GR in HEK293 cells (Lempiäinen *et al*. unpublished observations).

The lack of *bona fide* NR interactions in early AP-MS experiments could be explained by inefficient solubilization of chromatin-associated proteins or the loss of transient interactions in the purification steps (Lambert *et al.* 2012). Especially in recent years, modified versions of AP-MS protocols have been developed in an attempt to improve the purification of chromatin-associated proteins (Lambert *et al.* 2012, Mohammed *et al.* 2013, Lambert *et al.* 2014, Rafiee *et al.* 2016, Mohammed *et al.* 2016). For instance, different ChIP -based interactomics protocols, such as ChIP-SICAP, include a formaldehyde crosslinking step to form protein-protein and protein-DNA crosslinks to help preserve interactions during purification. ChIP-SICAP also includes a chromatin isolation step, meaning that it exclusively identifies protein-interactions of chromatin-associated proteins (Rafiee *et al.* 2016). In addition, orthogonal methods that rely on covalent biotinylation of proximal proteins, such as BioID, have been successfully utilized in the NR field (Dendoncker *et al.* 2019, Zhu *et al.* 2019). These biotinylation methods are also capable of identifying transient interactions, making them a very attractive alternative to AP-MS protocols (Gingras *et al.* 2019).

BioID and ChIP-SICAP successfully identified both well-established and putatively novel protein interactions of the agonist-activated GR (Figure 2 in I and Figure 1B in II). For instance, BioID identified all three members of the steroid receptor coactivator (SRC) family (NCOA1-3 or SRC1-3) and the classical corepressor NCOR1 (NCoR) as GR interactors (Figure 2 in I), whereas ChIP-SICAP identified NCOA1 (SRC1), NCOA2 (SRC2) and NCOR1 (Figure 1B in II). The SRCs were the first coactivators and NCOR1 the first corepressor identified for NRs (Chen & Evans 1995, Onate *et al.* 1995) and genome-wide studies have shown that they associate with chromatin (Percharde *et al.* 2012, Qin *et al.* 2014, Browne *et al.* 2018) (Figure 4A in II). BioID and ChIP-SICAP also identified multiple subunits of the BAF chromatin remodeling complex (Figure 2 in I and Figure 1B in II), that is a well-established interactor of the GR (Fryer & Archer 1998). These findings support the concept that

BioID and ChIP-SICAP are powerful unbiased methods for identification of protein interactions of the GR on chromatin.

Intriguingly, both methods also identified GR interactors that were not known before to contribute to SR signaling. For instance, both BioID and ChIP-SICAP identified interferon regulatory factor 2 -binding protein 2 (IRF2BP2) (Figure 2 in I and Supplementary Table 1 in II), that corepresses the TF interferon regulatory factor 2 (IRF2) but had not been recognized as coregulating SRs. By using ChIP-seq and RNA-seq, we characterized further the role of IRF2BP2 in glucocorticoid signaling (Manjur *et al.* 2019). Another novel SR coregulator that was identified as a GR interactor is BCL6 co-repressor (BCOR) (Figure 2 in I); we are also characterizing it in more detail in the context of GR signaling (Manjur *et al.* manuscript in preparation). Other putatively novel GR interactors include proteins that have been reported to coregulate other SRs. For example, both BioID and ChIP-SICAP identified transducing-like enhancer protein 3 (TLE3) (Figure 2 in I and Supplementary Table 1 in II), that was shown to corepress ER target genes (Jangal *et al.* 2014), but is not known to coregulate GR.

Importantly, many of the Dex-induced GR ChIP-SICAP interactions were among the genes that influence the growth and Dex-sensitivity of NALM-6 cells that model B cell acute lymphoblastic leukemia (B-ALL) (Poulard *et al.* 2019) (Supplementary Figure 2 in II). These findings indicate that many of the GR chromatin partners in HEK293 cells have physiologically relevant functions also in B-ALL cells. Novel GR coregulators could have implications in treatments against B-ALL.

5.2 COMPARISON BETWEEN BIOID- AND CHIP-SICAP - DERIVED INTERACTOMES

The number of agonist-dependent GR interactors identified with ChIP-SICAP (314 proteins) is roughly three times greater than with BioID (108 proteins). In addition, the overlap of proteins identified with these two methods is surprisingly small, with only about 30 proteins detected with both methods (Figure 13A). The different nature of the methods possibly explains these differences: ChIP-SICAP identifies chromatin-bound proteins in chromatin fragments sized roughly 200-300 bp, whereas BioID tags proteins within a 10 nm distance. The 200-300 bp fragment size equals about a 60-90 nm distance (if one bp on linear DNA is 0.3 nm), meaning that ChIP-SICAP can potentially detect chromatin interactors from a far greater distance than BioID. This may account for to the greater number of interactors identified with ChIP-SICAP. For instance, ChIP-SICAP detected more histone proteins than BioID (Figure 13B).

On the other hand, unlike ChIP-SICAP, BioID is not designed to exclusively identify chromatin-associated proteins. Therefore, the unique interactions that are identified only with BioID could potentially include interactions that are off chromatin. However, these unique interactions are most likely still nuclear, because in these experiments, the agonist was added at the same time with biotin, forcing the nuclear translocation of the fusion protein at the same time as the initiation of

biotinylation. Moreover, almost all high confidence GR interactions identified with BioID were compromised when the GR DBD was mutated (Figure 4B in I), implying that these interactions are primarily taking place on chromatin.

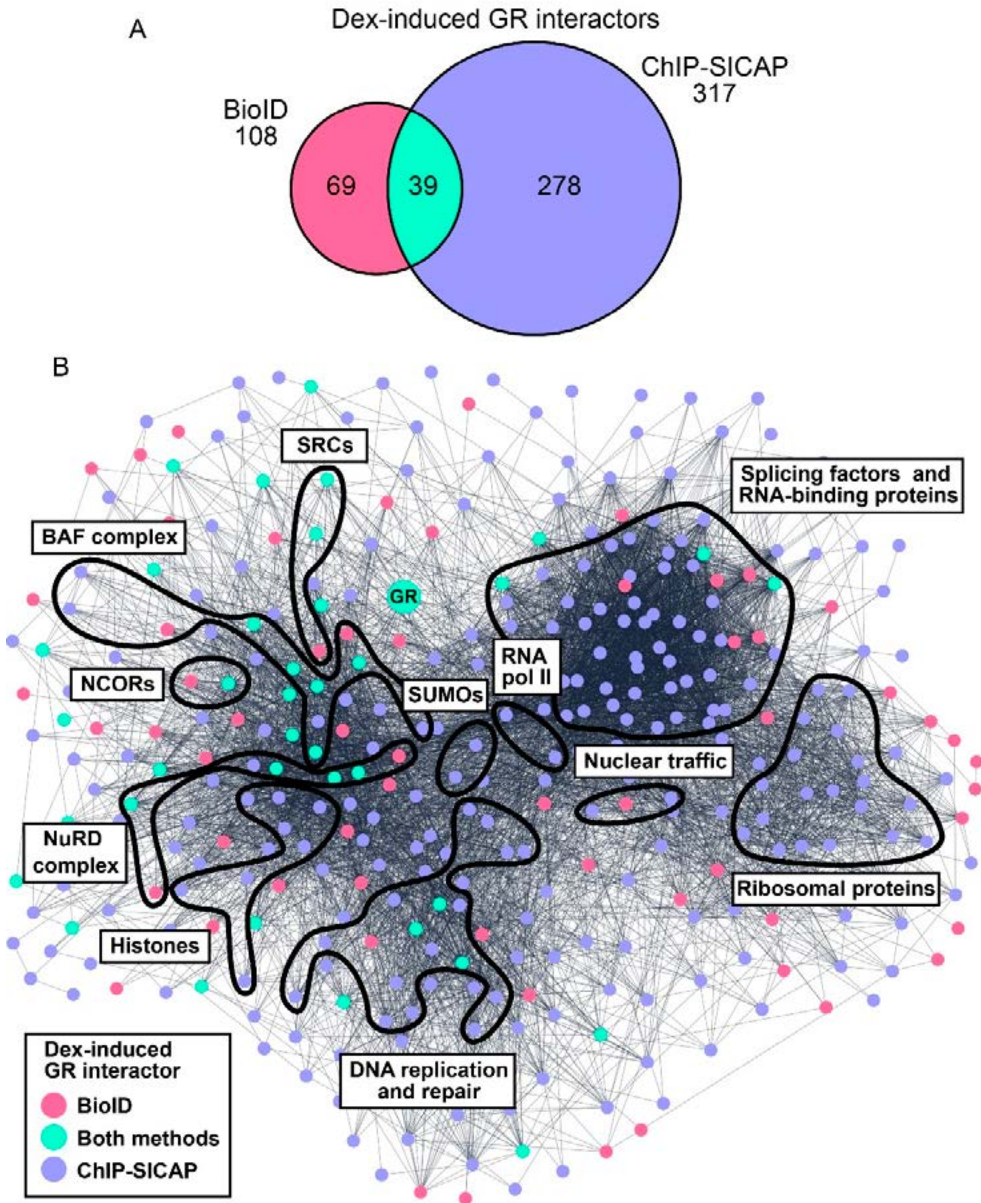

Figure 13. Comparison of statistically significant Dex-dependent GR interactomes identified with BioID and ChIP-SICAP. (A) Venn-diagram shows the overlap of Dex-dependent GR interactions identified with both methods. (B) Combined GR interactome identified with both methods. Members belonging to different protein complexes or functional groups are highlighted. Interactions between proteins were acquired from the STRING-database

(Szklarczyk *et al.* 2019). Proteins are clustered using Perfuse Force Directed Layout in Cytoscape software (Shannon *et al.* 2003). Proteins with no interactions in STRING-database were omitted.

5.3 SELECTION OF CONTROLS FOR BIOID

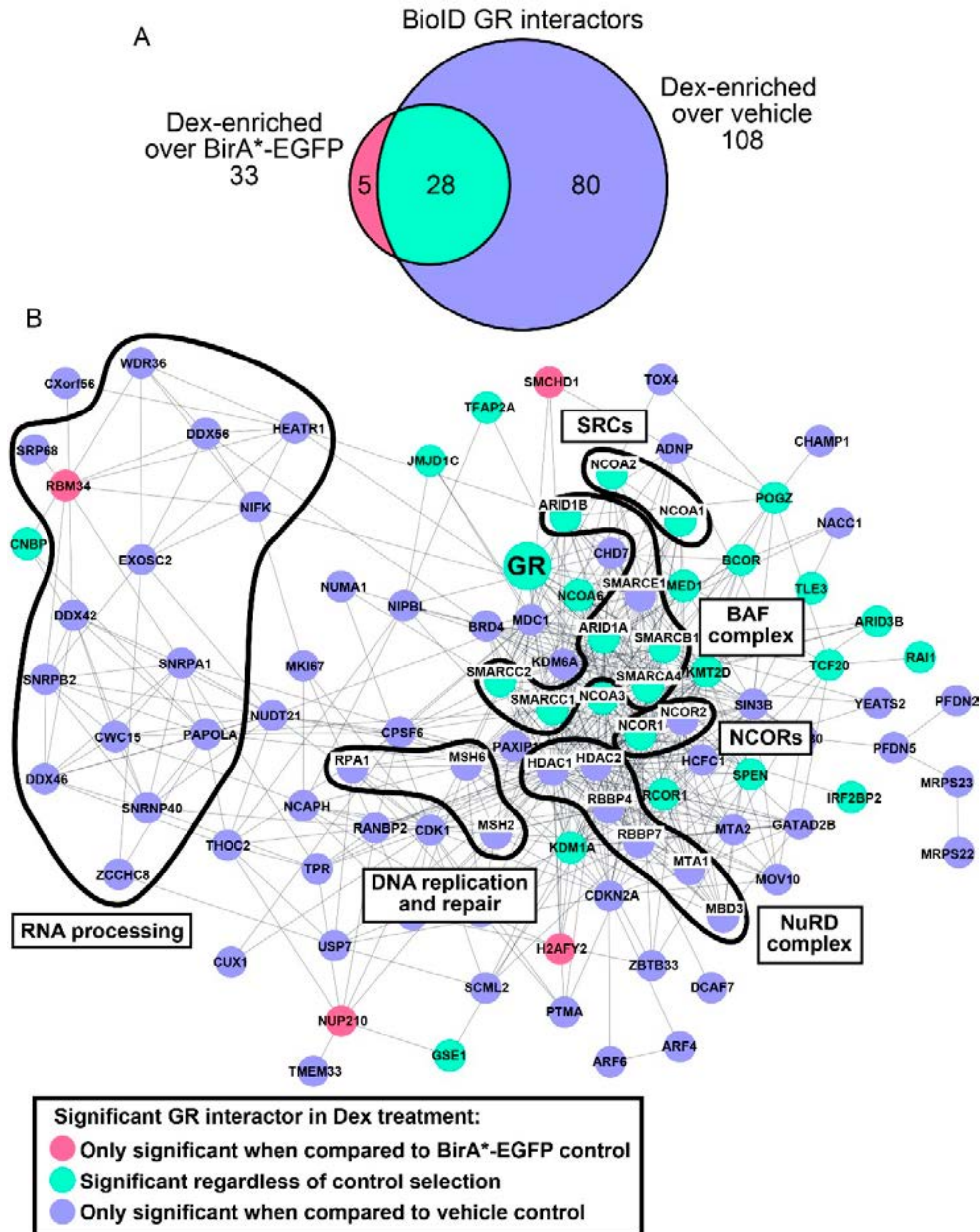

Figure 14. Effect of control selection on the GR interactome identified with BioID. (A) Venn-diagram shows the overlap of statistically significant (SAINT FDR < 0.05) GR interactions

identified by using BirA*-EGFP or vehicle treatment as the control. (B) Comparison of the GR BioID interactome using either BirA*-EGFP or vehicle control. Members belonging to different protein complexes or functional groups are highlighted. Interactions between proteins were acquired from the STRING-database (Szklarczyk *et al.* 2019). Proteins are clustered using Perfuse Force Directed Layout in Cytoscape software (Shannon *et al.* 2003). Proteins with no interactions in STRING-database were omitted.

In the BioID experiments, BirA* fused to enhanced green fluorescent protein (EGFP) was used as the control to distinguish between specific interactors. BirA*-EGFP labels proteins in both nucleus and the cytosol, potentially providing background information about both compartments (Supplementary Figure 2 in I). These controls help in the removal of highly abundant proteins, such as actins, tubulins and ribosomal proteins that may remain in the pulldown resin even after the washing steps. They also help in the removal of other proteins that may unspecifically bind to the pulldown resin and contaminants that may be introduced in sample preparation steps, such as skin keratins.

Mammalian cells contain low amounts of endogenous biotin and some proteins are endogenously biotinylated, namely carboxylases. Controls also help in distinguishing these endogenously biotinylated proteins, that will always be present in BioID eluates. However, this also means that protein interactions of endogenously biotinylated proteins cannot be studied using BioID. It is also worth noting that EGFP may interact with ubiquitination pathway components, subsequently leading to their removal from BioID hits, even if they were *bona fide* interactors. The latter problem could be fixed by using different controls, such as BirA* fused to a nuclear localization signal. However, in the case of SRs, control purifications in the absence of ligand can also be performed and used as a control to define specific interactions. Unfortunately, this approach does not define the background of nuclear proteins since the fusion protein is not localized to the nucleus. In the case of the GR, using the unliganded samples as the control in the statistical analysis with significance analysis of interactome (SAINT) (Choi *et al.* 2011) yields 108 statistically significant (false discovery rate < 0.05) agonist-specific interactions. However, with BirA*-EGFP the number of statistically significant interactors is merely 33 (Figure 14A). The interactors that are statistically significant with both controls include SRCs and BAF complex subunits that are among the best-established GR interactors (Figure 14A). However, selecting vehicle treatment as the control expands the interactome to span components from the NuRD complex, DNA replication and repair machinery, histones and proteins involved in RNA processing (Figure 14A).

5.4 SUMOYLATION OF THE GR MODULATES THE PROTEIN INTERACTOME OF THE RECEPTOR

Human cells express three SUMO isoforms: SUMO1 and the nearly identical SUMO2 and -3 (collectively referred to as SUMO2/3), that are covalently conjugated to specific lysine residues on target proteins, most often nuclear proteins (Tatham *et al.* 2001, Gill 2004). AP-MS experiments with SUMO2/3 antibody show that GR and

many of its interactors are SUMOylated in HEK293 cells (Figure 2A in II). After GR activation with an agonist, SUMO2/3 is recruited to GR-binding sites as assessed with ChIP-seq (Figure 2B in II). This can be explained by the recruitment of SUMOylated GR and its interactors to these binding sites and also by SUMOylation of the receptor itself.

The most prominent SUMOylation sites on the GR are at three lysine residues that are located at ΨKxE (where Ψ is a hydrophobic residue and x is any amino acid) SUMO consensus motifs (Figure 10A). Two of these sites (K277 and K293) are in the GR N-terminal domain, whereas the third (K703) and weakest one is in the LBD (Hendriks et al. 2018). Mutating the lysine residues at these three SUMO consensus motifs to arginines produces a SUMOylation-defective GR (GR3KR) that in reporter gene assays shows enhanced activity (Tian *et al.* 2002) and in genome-wide ChIP-seq studies displays a distinct chromatin-binding pattern different from its wild-type counterpart (GRwt) (Paakinaho *et al.* 2014). In addition, the GR3KR-expressing cells exhibited enhanced proliferation compared to GRwt-expressing cells (Paakinaho *et al.* 2014).

Interestingly, both BioID and ChIP-SICAP identified different subunits of the BAF chromatin remodeling complex as enriched interactors for the GR3KR when compared to GRwt (Figure 3 in II). Several CHD chromatin remodelers also prefer GR3KR over GRwt, in addition to various components of the AP-1 TF complex and the classical coactivator NCOA1 (SRC1). The enhanced interaction of GR3KR with NCOA1 is reflected on the genome-wide chromatin-binding: NCOA1 recruitment to GR-binding sites is pronounced in HEK293 cells expressing GR3KR compared to cells expressing GRwt (Figure 4A-C in II). However, the corepressor NCOR1, which does not show any preference for GR3KR or GRwt in the proteomics analysis, does not reveal a difference in chromatin-binding between the two cell lines (Figure 4A-C in II). The enhanced interaction between GR3KR and NCOA1 is also mirrored in FRAP experiments: transfection of NCOA1 slows down the nuclear mobility of GR3KR more than GRwt (Figure 4D-E in II).

Moreover, GR3KR is more potent than GRwt at decompacting chromatin especially at *de novo* sites, that are closed before GR activation, as determined with ATAC-seq (Figure 6A-B in II). The enhanced interactions with chromatin remodelers by the GR3KR may explain why it is also more potent at increasing chromatin accessibility. These findings are in line with previous observations of the enhanced activity of GR3KR in reporter gene assays (Tian *et al.* 2002) and increased genome-wide binding of GR3KR at chromatin (Paakinaho *et al.* 2014). SUMOylation-deficient forms of other TFs, MITF (microphthalmia-associated TF), AR, and a yeast TF Sko1, have also exhibited enhanced chromatin binding (Bertolotto *et al.* 2011, Sutinen *et al.* 2014b, Sri Theivakadadcham *et al.* 2019, Rosonina 2019), suggesting that SUMOylation has a conserved role in restricting TF binding to chromatin.

5.5 PROTEIN INTERACTOMES OF THE GR AND THE AR ARE SIMILAR BUT NOT IDENTICAL

Interestingly, BioID experiments with GR and AR revealed that these receptors share more than half of their interactions in HEK293 cells (Figure 5 in I). The GR and the AR are structurally and functionally similar and exhibit a similar chromatin-binding pattern (Sahu *et al.* 2013, Pihlajamaa *et al.* 2015), possibly explaining the similarities in their interactomes. Moreover, they share more than 90% of their binding sites in HEK293 cells that were used in the BioID experiments (Paakinaho *et al.* 2014, Sutinen *et al.* 2014a). Binding to the same chromatin sites may expose the receptors to a similar set of coregulators. These findings are in line with the previous knowledge that SRs share several coregulators (McKenna & O'Malley 2010).

Overlapping protein interactomes of the GR and the AR may also contribute to their crosstalk in PC. In PC cells, GR and AR have significantly overlapping cistromes and transcriptomes (Sahu *et al.* 2013), that allow GR to drive enzalutamide-resistant growth by regulating expression of AR target genes (Arora *et al.* 2013, Shah *et al.* 2017). Efforts have been made to inhibit GR signaling in CRPC (Taplin *et al.* 2008, Kach *et al.* 2017), and clinical trials are underway to test simultaneous inhibition of GR with mifepristone (RU486) and AR with enzalutamide in CRPC (Kumar 2020). TFs may also compete for common coactivators, if the coactivators are expressed in limiting numbers. For instance, the inflammatory TF NF-κB was shown to compete with GR for the coactivator p300 (Dendoncker *et al.* 2019). This competition leads to reduced activity of agonist-bound GR when cells are co-treated with tumor necrosis factor α (TNFα). TNFα is a cytokine that acts through a signaling cascade to activate NF-κB. Similarly, simultaneous activation of GR and AR could lead them to modulate each other's functions through competition for the same coactivators. This concept is supported by the recent study by Gillespie and colleagues, who showed that at the protein level, coactivators are very rare (in hundreds of molecules per nucleus) compared to TFs (in tens of thousands) and corepressors (in hundreds of thousands) (Gillespie *et al.* 2020).

However, the BioID experiments also revealed protein complexes that differ in their preference for GR or AR (Figure 5 in I). For instance, SRC, MLL4 and mediator complexes seem to prefer GR, whereas BAF, neural BAF, N-CoR, BHC and PRC1.1 complexes favored AR. Some of these differences can possibly be explained by differences in the N-terminal domain that is the most variable domain between the receptors. For instance, ZMIZ2 was previously shown to coactivate AR by binding to the AF-1 in the N-terminal domain of the receptor (Huang *et al.* 2005) and it is also an AR-exclusive interactor in BioID (Supplementary Figure 8 in I). Interestingly, ZMIM2 may have promoted the preferential interaction of the AR with the BAF complex (Figure 5 in I), because it also interacts with components of the BAF complex (Huang *et al.* 2005).

5.6 INTERACTORS COREGULATE GR AND AR IN A TARGET-GENE SELECTIVE FASHION

In early NR coregulator studies, agonist-bound SRs were thought to interact with coactivators and the antagonist-bound SRs associate with corepressors (Collingwood *et al.* 1999, Perissi & Rosenfeld 2005, Dasgupta & O'Malley 2014). However, the agonist-dependent protein interactomes of GR and AR contain both coactivator and corepressor proteins (Figure 5 in I and Figure 1B in II), and antagonist-treatment leads to an overall reduction in interactions, rather than to an increase in corepressor interactions (Figure 4A and D in I). Moreover, the relative amount of interactions is reflected in the activity of the receptors in the presence of antagonist: the RU486 (mifepristone, partial antagonist) -bound GR is still able to undergo some interactions (Figure 4A in I) and has some activity (Supplementary Figure 4 in I), while enzalutamide (potent antiandrogen) -bound AR undergoes nearly no interactions (Figure 4D) and is transcriptionally almost completely inactive (Tran *et al.* 2009). Binding of the antagonist most likely leads to a sub-optimal conformation of the receptors, thus decreasing the number of interactions.

RNA-seq experiments demonstrate that GR and AR agonists lead to the repression of hundreds of genes in addition to inducing gene expression (Manjur *et al.* 2019) (Figure 3C in III). These findings suggest that agonist-bound GR and AR could directly recruit corepressors to repress expression of target genes. The local chromatin environment (e.g. DNA sequence, histone modifications or other chromatin-bound TFs) could determine if coactivators or corepressors are being recruited. For instance, agonist-bound GR was shown to recruit corepressor proteins to repress target genes when it directly binds to the evolutionarily conserved negative response elements (nGRE) that are distinct GR-binding sequences from GREs (Surjit *et al.* 2011). GR is also known to repress genes via tethered indirect transrepression by binding to other TFs such as AP-1 and NF-κB (Langlais *et al.* 2012, Ratman *et al.* 2013). Genome-wide ChIP-seq did not detect direct GR binding at glucocorticoid-repressed enhancers, but these sites contained motifs for AP-1 and NF-κB TFs (Johnson *et al.* 2018). Similarly, global run-on sequencing (GRO-seq) experiments in VCaP cells revealed that treatment with AR agonist leads to both activation and repression of enhancers, but AR binding is only detected at activated enhancers (Toropainen *et al.* 2016). The presence of motifs for other TFs at these sites suggests that they may be repressed via tethered repression.

However, the absence of GR and AR binding at repressed sites also indicates that competition for coactivators could function as a repressive mechanism. Recent findings by Gillespie et al. that showed the relative rareness of coactivators compared to repressors suggests that the nucleus is a highly repressive environment that passively represses genes not only by forcing corepressor binding but also by limiting coactivator binding (Gillespie *et al.* 2020). Interestingly, TF protein levels were shown to be between that of corepressors and coactivators (Gillespie *et al.* 2020). In the case of the GR and the AR, their activation may redirect the limited number of

coactivators to genes that are induced, leaving the remaining genes without coactivators and thus repressed. The highly abundant corepressors may then further repress genes in the absence of coactivators. Agonist-bound GR and AR may transiently interact with corepressor proteins even when bound to ligand-induced enhancers, if these enhancers are constantly scanned by highly abundant corepressor proteins. For instance, histone acetylation and deacetylation have been shown to function in rapid repetitive cycles with an acetylation half-life of only a few minutes (Waterborg 2002).

It is also possible that the GR- and AR-interacting proteins are not functioning as corepressors when they interact with the agonist-bound receptors. Several studies have shown that coactivators and corepressors may behave in opposing manners depending on the cell line and target gene, as was shown for coregulators, such as KDM1A, NCOA1, NCOR1 and NCOR2 (Jeyakumar *et al.* 1997, Tagami *et al.* 1997, Weiss *et al.* 1999, Berghagen *et al.* 2002), and for other GR coregulators (Wu *et al.* 2014). In line with these studies, depletion of putative GR corepressors RCOR1, IRF2BP2 and BCOR enhanced or attenuated dex-induced gene expression in HEK293 and A549 cells depending on the selection of the target gene (Figure 3 in I), suggesting that despite their previously reported role in repression (Huynh *et al.* 2000, You *et al.* 2001, Childs & Goodbourn 2003) they may also function as GR coactivators at specific genes. Moreover, genome-wide RNA-seq from HEK293 cells after IRF2BP2 depletion shows that a reduction of IRF2BP2 protein levels not only enhances but also represses expression of a subset of GR target genes (Manjur *et al.* 2019), suggesting that it can function as a coactivator and as a corepressor for the GR in a target-gene dependent manner. Similar results are seen in VCaP cells at AR target genes after depletion of BCOR; the effects of depletion are largely gene-dependent (Figure 3 in III).

However, it is challenging to determine the activating or repressing function of a coregulator if the analysis is based on a relatively long-lasting depletion of the coregulator protein, because secondary effects may take place (i.e. depleted protein regulates expression of another coregulator or a TF whose target genes are also influenced). The secondary effects may lead to incorrect assumptions of the activating or repressing function of a coregulator. In addition, ligand-activated TFs, such as the GR and the AR, require additional incubation periods in the presence of hormone to induce measurable mRNA expression changes. These limitations could be circumvented for instance by using fast-acting small molecule inhibitors against the coregulator together with rapid genome-wide assays, such as GRO-seq that assesses changes in transcription in a shorter time window. However, only a limited number of small-molecule inhibitors exist for currently known coregulators and they are difficult to design against proteins that do not contain any intrinsic enzymatic activity (Lonard & O'Malley 2012).

In VCaP cells, more than half of the AR-binding sites are occupied by BCOR (Figure 1A and Supplementary Figure 1C in III), suggesting that BCOR has a significant role in regulating the function of the AR. Roughly half of the total number of BCOR-binding sites are responsive to androgens (androgen-enriched, A-enriched

or vehicle-enriched, V-enriched), while the other half remains unresponsive (V-and-A-shared) (Figure 1A and Supplementary Figure 1D in III). At the androgen-responsive sites, BCOR-binding is either increased (A-enriched) or reduced (V-enriched) upon activation of AR. Most of these androgen-responsive sites are A-enriched (Figure 1A-D in III), suggesting that the main mode of androgen-dependent BCOR coregulatory function is mediated by direct recruitment to activated AR. Similar hormone-dependent recruitment of IRF2BP2 at GR-binding sites was observed in HEK293 cells (Manjur *et al.* 2019).

The majority of the BCOR-binding sites in VCaP cells are located at intergenic and intronic regions (Figure 15A) and in these regions, A-enriched and V-and-A-shared sites are found in roughly equal numbers, while the V-enriched sites form only a small fraction of the binding sites (Figure 15B). Interestingly, some BCOR binding is also seen at promoters, where 90% of the BCOR-binding sites are V-and-A-shared (Figure 15A and B). In HEK293 cells, binding of IRF2BP2 follows a similar pattern at these genomic regions (Manjur *et al.* 2019).

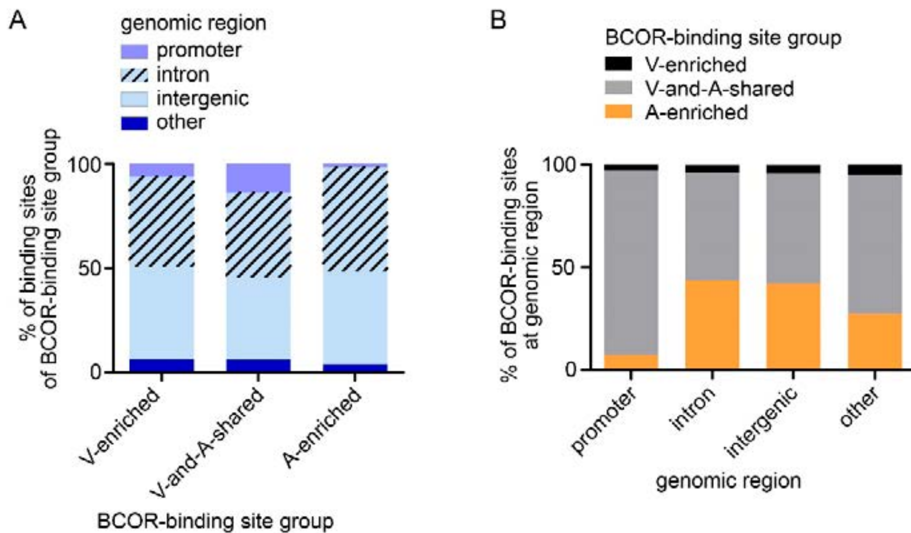

Figure 15. Distribution of BCOR-binding sites at annotated genomic regions. (A) Columns show how BCOR-binding sites in each group (V-enriched, V-and-A-shared and A-enriched) are distributed to different genomic regions. (B) Columns show what type of BCOR-binding sites (V-enriched, V-and-A-shared or A-enriched) each type of genomic region contains. BCOR-binding site groups are the same as clusters 1-3 in Figure 1A in III. A, androgen; V, vehicle.

RNA-seq analysis reveals that BCOR depletion both up- and down-regulates the expression of hundreds of AR target genes (Figure 3D-E in III), further supporting the concept that the BCOR is a significant coregulator for AR in VCaP cells. A comparison of the RNA-seq data to BCOR ChIP-seq data indicates that more than half of the A-enriched BCOR-binding sites are near androgen up-regulated genes rather than repressed genes (Figure 16A and B). In an opposing manner, the V-

enriched BCOR-binding sites associate with androgen down-regulated genes rather than up-regulated genes (Figure 16A and B). Recruitment of BCOR to up-regulated genes and its dissociation from the down-regulated counterparts may represent one function of BCOR as a coactivator. The limited abundance of coactivator proteins in the nucleus (Gillespie *et al.* 2020) supports a model in which coactivator complexes dissociate from repressed genes and are recruited to the induced genes. However, all three types of BCOR-binding sites are also seen in different environments (A-enriched near down-regulated genes and V-enriched and V-and-A-shared near up-regulated genes) (Figure 16A and B). These findings indicate that BCOR coactivator or corepressor function cannot be explained simply by androgen-dependent changes in its chromatin-binding pattern and that BCOR may associate with coactivator and corepressor complexes in a context-dependent manner (i.e. depending on the local chromatin environment).

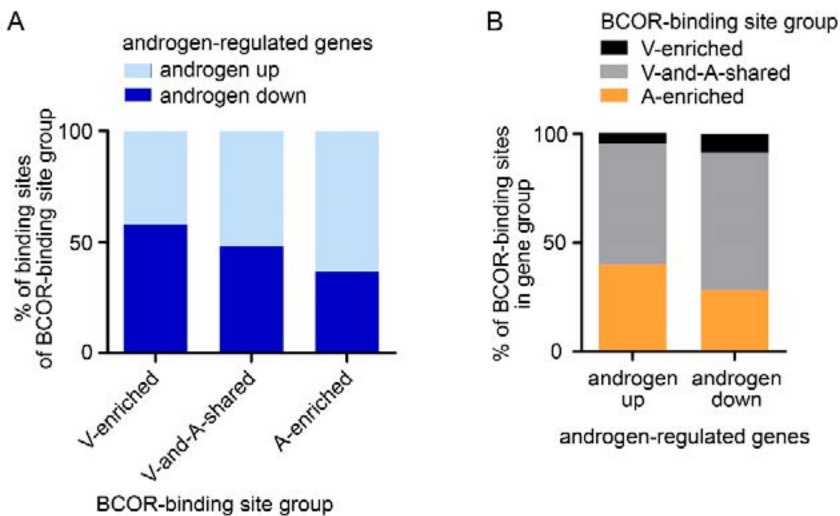

Figure 16. Distribution of BCOR-binding sites at androgen-regulated genes. (A) Columns show how BCOR-binding sites in each group (V-enriched, V-and-A-shared and A-enriched) are distributed near androgen up- and down-regulated genes. (B) Columns show what type of BCOR-binding sites (V-enriched, V-and-A-shared or A-enriched) androgen up- and down-regulated genes contain. BCOR-binding site groups are the same as clusters 1-3 in Figure 1A in III and androgen up- and down-regulated genes the same as in Figure 3D in III. A, androgen; V, vehicle.

Nevertheless, the largest number of BCOR-influenced genes are androgen-repressed genes that are de-repressed (i.e. mRNA expression upregulated) upon BCOR depletion (Figure 3D-E in III), indicating that BCOR is mainly a corepressor of the AR. These findings are in line with the previously characterized role of BCOR in repression (Huynh *et al.* 2000, Hatzi *et al.* 2013, Granadino-Roldan *et al.* 2014). A comparison of the RNA-seq data to H2AK119ub1 ChIP-seq data shows that BCOR depletion decreases H2AK119ub1, especially at genes that BCOR corepresses (Figure

4C in III), suggesting that BCOR represses these genes by maintaining H2AK119ub1. This concept is supported by previous studies showing that BCOR participates in H2AK119ub1 regulation as a component of the non-canonical PRC1.1 complex (Kelly *et al.* 2019). These BCOR repressed genes included several homeobox (HOX) genes that have been associated with the development of PC (Javed & Langley 2014, Yao *et al.* 2019). Surprisingly, depletion of BCOR de-repressed almost all HOX genes that are expressed in VCaP cells (Supplementary Figure 6A in III). Moreover, in primary PC tumors, BCOR expression negatively correlates with expression of several HOX genes (Supplementary Figure 8D in III), suggesting that BCOR represses these genes also in PC tumors.

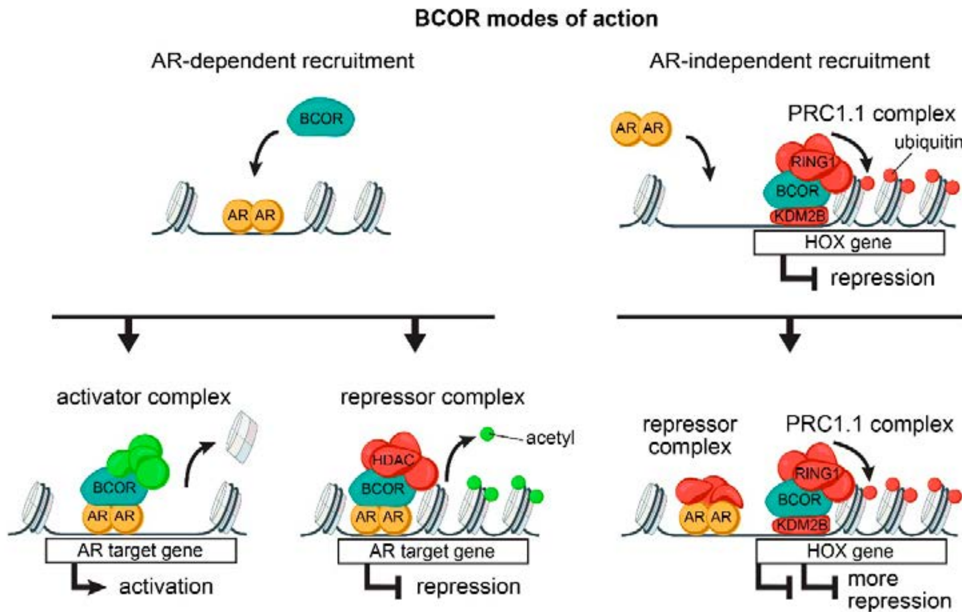

Figure 17. Suggested model for BCOR modes of action in the regulation of AR target gene expression. Left side of the figure shows BCOR recruitment to chromatin by AR and subsequent association with coactivator or corepressor complexes. The right side of the figure shows BCOR-mediated repression of HOX-genes via H2A K119 monoubiquitination by the PRC1.1 complex. Many of the HOX-genes in VCaP cells are also repressed by the AR, leading to further repression.

Interestingly, depletion of BCOR de-represses HOX genes prior to androgen-treatment, suggesting that BCOR contributes to repression of these genes upstream of the AR. In addition to HOX genes, many of the BCOR co-repressed genes are characterized by the androgen-unresponsive type of BCOR-binding at promoter regions (Supplementary Figure 6B and Figure 4F in III). The presence of an excess of corepressors in the nucleus (Gillespie *et al.* 2020), may drive more stable binding by corepressor complexes. Interestingly, especially at promoter regions, BCOR binding was mainly androgen-unresponsive (Figure 15B). Taken together, these results

suggest a model where BCOR associates in a context-dependent manner with coactivator or corepressor complexes to regulate AR target gene expression (Figure 17).

Importantly, BCOR depletion attenuates the proliferation and induces apoptosis of VCaP cells (Figure 6 in III). Interestingly, BCOR is overexpressed in metastatic (Grasso *et al.* 2012, Robinson *et al.* 2015, Su *et al.* 2019) and primary PC (Cancer Genome Atlas Research Network 2015) (Supplementary Figure 8C in III), and its expression negatively correlates with disease-free survival in PC patients (Supplementary Figure 11 in III). These findings suggest that BCOR has an important role in regulating the proliferation and viability of CRPC cells and it potentially contributes to the growth of PC.

6 CONCLUSIONS AND FUTURE PROSPECTS

Dysregulation of SR signaling drives various clinically relevant pathological states, such as cancer. SRs recruit coregulator proteins to specific target sequences at enhancers to regulate the expression of target genes. Coregulators are emerging as important drug targets in pathologies to complement direct targeting of SRs, including the AR in CRPC. However, protein interactomes of full-length SRs have remained surprisingly poorly defined. This is partially due to the technical challenges in assessing the very transient interactions of chromatin-bound proteins, which are highly insoluble. Improvements in the sensitivity of instrumentation and the introduction of novel proteomic methods now enable the unbiased characterization of protein interactomes of chromatin-bound SRs.

In this thesis, unbiased state-of-the-art proteomic methods, BioID and ChIP-SICAP, were utilized to elucidate the protein interactomes of the GR and the AR. Furthermore, these methods were employed in parallel with ChIP-seq and ATAC-seq to clarify the role of GR SUMOylation on the coregulator interactions, chromatin binding and transcriptional regulation of the receptor. In addition, ChIP-seq and RNA-seq were utilized to define the role of BCOR, one of the novel AR-interacting proteins found in this work, in AR signaling in CRPC cells.

The main findings of this thesis are:

- Agonist-bound GR and AR interact with both coactivators and corepressors and many of these interactions are shared between the receptors.
- Antagonist-bound GR and AR do not interact efficiently with coactivators or corepressors.
- DNA-binding -deficient GR shows an impaired ability to interact with coregulators.
- SUMOylation of the GR restricts the ability of the receptor to interact with chromatin remodelers and thus it inhibits opening of chromatin at GR-binding sites.
- In CRPC cells, BCOR is recruited to AR chromatin-binding sites, where it regulates AR target gene expression, in part by contributing to the monoubiquitination of H2AK119.
- Proliferation and apoptosis of CRPC cells are sensitive to the protein levels of BCOR.

The findings of this thesis contribute towards a better understanding of the role of coregulators in the function of SRs. The findings show that SUMO regulates GR activity by influencing the protein interactome of the receptor, introducing a mechanism to explain how SR PTMs may alter the transcriptional activity of SRs through coregulator interactions. Moreover, the protein interactomes of GR and AR discovered in this thesis represent a valuable resource in the NR field. Novel interactors may have previously unrecognized roles in SR function, as was shown for the BCOR in AR's function in CRPC cells. These previously uncharacterized coregulators also provide potential drug targets in inflammatory conditions, acute lymphoblastic leukemia and PC.

Recent studies have shown that gene transcript levels are poorly reflected in terms of protein abundance, underlining the importance of examining transcriptional regulation at the protein level (Latonen *et al.* 2018, Sinha *et al.* 2019, Gillespie *et al.* 2020). For instance, the fundamental conclusion that the nucleus is a highly repressive environment at least in erythroid cells, could not be drawn from estimating solely mRNA transcript levels (Gillespie *et al.* 2020). Characterizing in a similar manner in different disease models, the TF, coactivator and corepressor protein stoichiometry, may provide valuable insights into transcriptional dysregulation in disease states such as cancer. In combination with interactome studies, the most relevant coregulators can possibly be identified and the interactions then targeted. Taken together, modern MS-based protein interactomics methods are powerful tools that, in parallel with genome-wide methods, can provide valuable insights into TF and coregulator biology.

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The glucocorticoid and androgen receptors (GR and AR) regulate the expression of genes by interacting with DNA and other chromatin-bound proteins. The GR is targeted in inflammatory diseases and leukemia, and the AR is a key drug target in prostate cancer. In this thesis, state-of-the-art proteomic and genomic methods were utilized to identify novel protein interactions of the GR and the AR. The findings are valuable for future drug design in inflammatory conditions, leukemia and prostate cancer.

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