REVIEW

Linking the ubiquitin–proteasome pathway to chromatin remodeling/modification by nuclear receptors

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Abstract

Over 25 years ago, eukaryotic cells were shown to contain a highly specific system for the selective degradation of short-lived proteins, this system is known as the ubiquitin–proteasome pathway. In this pathway, proteins are targeted for degradation by covalent modification by a small highly conserved protein named ubiquitin. Ubiquitin-mediated degradation of regulatory proteins plays an important role in numerous cell processes, including cell cycle progression, signal transduction and transcriptional regulation. Recent experiments have shown that the ubiquitin–proteasome pathway is also involved in nuclear hormone receptor (NR)-mediated transcriptional regulation. The idea that the ubiquitin–proteasome pathway is involved in NR-mediated transcription is strengthened by experiments showing that ubiquitin–proteasome components are recruited to NR target gene promoters. However, it is not clear how these components modulate NR-mediated chromatin remodeling and gene expression. In this review, we postulate the role of the ubiquitin–proteasome pathway on NR-mediated chromatin remodeling and gene regulation based on the current knowledge from studies implicating the pathway in chromatin structure modifications that are applicable to NR function. Since evidence from this laboratory, using the glucocorticoid receptor responsive mouse mammary tumor virus (MMTV) promoter organized as chromatin, suggest that the ubiquitin–proteasome system may be involved in the elongation phase of transcription, we particularly concentrate on chromatin modifications associated with the elongation phase.

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Introduction

Overview of the ubiquitin–proteasome pathway

Proteins exist in a dynamic state in the cell, with multiple pathways leading to their degradation (Glickman & Ciechanover 2002). The best-described pathway is the ubiquitin–proteasome system which degrades the bulk of cellular proteins in an energy-dependent manner (Ciechanover *et al.* 2000, Goldberg *et al.* 2001, Glickman & Ciechanover 2002) (Fig. 1). The ubiquitin–proteasome pathway plays a pivotal role in the degradation of short-lived and regulatory proteins important in a variety of cellular processes, including cell cycle progression, modulation of cell surface receptors and ion channels, antigen presentation and more recently nuclear hormone receptors (NRs) (Glickman & Ciechanover 2002, Nawaz & O'Malley 2004). Degradation of cellular proteins via the ubiquitin–proteasome pathway is a highly complex and tightly regulated process. For this reason, dysregulation of the ubiquitin–proteasome system can potentially affect many cellular processes in health and disease (Schwartz & Ciechanover 1999, Ciechanover & Brundin 2003, Ohta & Fukuda 2004). In this review, we postulate

the importance of the ubiquitin–proteasome system in NR-mediated chromatin remodeling and transcriptional regulation.

Ubiquitin activation

Targeting cellular proteins for degradation via the ubiquitin–proteasome pathway involves two discrete steps (Glickman & Ciechanover 2002, Pickart 2004). First, the protein substrate is tagged by covalent attachment of multiple ubiquitin molecules to generate the polyubiquitin degradation signal. Second, the tagged protein is degraded by the 26S proteasomal complex. The process and the biochemical steps involved in ubiquitination and polyubiquitination have been reviewed in detail elsewhere, but we include a brief description of the system in this review (Glickman & Ciechanover 2002, Pickart 2004).

Briefly, conjugation of ubiquitin to the protein substrate involves a three-step enzyme cascade. In the first step, the C-terminus of ubiquitin is activated in an ATP-dependent step and covalently linked by a thioester bond to an active cysteine site on the ubiquitin-activating

Figure 1 An overview of the ubiquitin–proteasome system showing enzymatic cascade involved in activation of ubiquitin and subsequent targeting of the protein substrate to the proteasome. The ubiquitin pathway initiates via an ATP-dependent activation of free ubiquitin (Ub) by a ubiquitin-activating enzyme (E1). The activated ubiquitin is transferred to a ubiquitin-conjugating enzyme (E2) and finally to a ubiquitin ligase (E3) which targets the ubiquitin to a lysine residue of a specific protein substrate. The process is repeated to mark the protein substrate (target) with a polyubiquitin chain, a recognition signal (Ub-S) for the 19S and subsequent degradation of the protein by the 20S proteasome. Listed are key enzymes in the ubiquitin–proteasome pathway involved in NR-mediated transcriptional regulation (Ciechanover & Schwartz 1998, Fan *et al.* 1999, Kang *et al.* 2002, Reid *et al.* 2003, Yan *et al.* 2003, Nawaz & O'Malley 2004, Perissi *et al.* 2004).

enzyme (UAC), E1. In the second step, activated ubiquitin is transferred to one of the many ubiquitin carrier/conjugating enzymes (UBC), E2 enzymes, via another thioester bond. The E2 enzyme then transfers the activated ubiquitin via an isopeptide bond to a lysine residue in the target protein bound to a specific E3 ubiquitin ligase (Fig. 1). Depending on the type of E3 ligase, the transfer of ubiquitin to the substrate is either direct or indirect. In most organisms, including humans and yeast, a single E1 enzyme, activates ubiquitin for an entire array of E2 conjugating enzymes. The human genome project reveals over 40 different potential E2s and over 500 different possible E3s (Wong *et al.* 2003). The E3 ubiquitin ligases exist in multiple families and ultimately it is the E3s alone or in combination with the E2 that determine the specificity of substrate recognition by the 26S proteasome (Glickman & Ciechanover 2002, Ciechanover & Brundin 2003, Pickart 2004).

Recent studies classify several hundred potential E3 ubiquitin ligases in the human genome into two distinct families based on their structural domains and mechanisms of action. These are the Homologous to the

E6-AP C Terminus (HECT) domain ubiquitin ligases and the Really Interesting New Gene (RING) finger E3 ligases (Glickman & Ciechanover 2002, Ciechanover & Brundin 2003). For the HECT domain E3 ligases the E2 first transfers the activated ubiquitin to the E3 to form an E3–ubiquitin bond and subsequently the E3 transfers the ubiquitin to the target substrate. The prototypical HECT domain family member is the E6-AP, a 100 kDa protein required for ubiquitination and rapid degradation of p53 in the presence of the human papilloma virus oncoprotein (Scheffner 1998). E6-AP is also a steroid hormone receptor coactivator, and ablation of the E6-AP gene in mice is associated with hormone resistance (Nawaz *et al.* 1999, Smith *et al.* 2002). Another member of this family is the yeast RSP5 or hRPF1, which was shown to enhance hormone-dependent activation by the progesterone (PR) and glucocorticoid receptors (GR) in *in vitro* experiments (Imhof & McDonnell 1996). RSP5 also conjugates and leads to ubiquitin-dependent degradation of the large subunit of RNA polymerase II (RNA pol II) (Huibregtse *et al.* 1997).

The second group is the RING finger E3 ligases, including the U-box and the plant homeo-domain (PHD) proteins that catalyze a direct transfer of the ubiquitin moiety from E2 to the E3 bound substrate (Aravind & Koonin 2000, Joazeiro & Weissman 2000). The RING finger E3 ligases are classified into two distinct groups: single- or multi-subunit complexes. Single-subunit E3s such as murine double minute 2 (Mdm2) and Parkin contain the substrate recognition element and the RING finger on the same polypeptide (Fang *et al.* 2000). Among the multi-subunit RING finger complexes are the anaphase promoting complex (APC), the von-Hippel Lindau-Elongins B and C (VBC)-Cul2 complex and the Skp1-Cullin/cdc53-F-box (SCF) complex (Peters 1998, Jackson *et al.* 2000, Tyers & Jorgensen 2000, Blagosklonny 2001).

Other RING finger proteins shown to mediate ubiquitination in *in vitro* assays include the product of the breast and ovarian cancer susceptibility gene 1 (BRCA1) (Brzovic *et al.* 2001). BRCA1 decreases estrogen receptor (ER) and increases androgen receptor (AR) transcriptional activity (Fan *et al.* 1999, Park *et al.* 2000). The U-box derivatives of the RING finger E3s such as C-terminus of Hsp interacting protein (CHIP), may act as E4 ligases to facilitate polyubiquitination of specific substrates, including NRs (Patterson 2002). Thus, as we shall see, the HECT and RING finger domain family of E3 ligases is critically involved in NR function (Nawaz & O'Malley 2004).

The 26S proteasome holoenzyme

The proteasome holoenzyme, also known as the 26S proteasome, is the major cytosolic and nuclear protease complex that is responsible for the ATP-dependent proteolytic degradation of cellular proteins (Bochtler *et al.* 1999, Glickman & Ciechanover 2002). The proteasome is highly conserved in eukaryotic evolution and exists as two major subcomplexes: the 19S regulatory particle and the 20S catalytic core particle (CP). The 20S core particle is sandwiched between two 19S regulatory particles (RP), also called PA700 (Fig. 1). Mammalian cells also contain a second regulatory complex, the 11S regulator or PA28, which does not catalyze degradation of proteins but is involved in antigen presentation (Rechsteiner *et al.* 2000). The 19S particle has two structural subunits: the 'lid' and the 'base'. Initially the proteasome was regarded primarily as a 'cycler' of misfolded proteins, but over the last decade, the function of this multi-enzyme complex has proven to be multifaceted. Individual components of the complete 26S proteasome are involved in different tasks as discussed below.

The 19S regulatory particle The 19S regulator has ubiquitin hydrolysis activity and serves multiple roles for

regulating the activity of the proteasome, including selecting substrates, preparing them for degradation, translocating them into the core particle and dictating the end products generated by the core particle after digestion. Two nomenclatures are used to designate subunits for the lid and the base of the 19S complex. The S (subunit) or RP (regulatory particle) designate the mammalian and yeast *Saccharomyces cerevisiae* 19S subcomplex respectively (Dubiel *et al.* 1995, Finley *et al.* 1998). Each designation orders the subunits by their molecular mass, but the RP nomenclature also differentiates between Rpt ATPase (Rpt) and non-ATPase (Rpn) subunits. The lid has eight non-ATPase subunits – namely S3/Rpn3, Rpn5, S9/Rpn6, S10a/Rpn7, S11/Rpn9, S12/Rpn8, S13/Rpn11 and S14/Rpn12 – and a unique subunit Rpn4/S5b. Degradation of the ubiquitinated substrate requires the lid, suggesting an interaction between the lid and the polyubiquitin chain on the target protein.

The base complex contains the six ATPases – S7/Rpt1, S4/Rpt2, S6/Rpt3, S10b/Rpt4, S6/Rpt5/TBP-1 and S8/Rpt6/SUG1/Trip1 – and three non-ATPase subunits S1/Rpn2, S2/Rpn1 and S5a/Rpn10 (Voges *et al.* 1999). Each of the six ATPases belongs to the family of ATPases associated with a variety of cellular activities (AAA). The base components of the 19S are critical for recognition of the ubiquitinated substrate by the proteasome.

Several of the 19S subunits are known for their role in processes other than protein degradation. For example, S6/Rpt5/TBP-1, the HIV Tat-binding protein 1, was first defined as a modulator of HIV Tat transactivation (Nelbock *et al.* 1990, Apcher *et al.* 2003). The S8/Rpt6/SUG1/Trip1 subunit has been described as a mediator of the TATA-binding protein and a DNA helicase that interacts specifically with transcription factor TFIIF, a component of the RNA polymerase holoenzyme (Weeda *et al.* 1997). The subunit S5a/Rpn10 is also similar to the p44 subunit of the basal transcription factor TFIIF (Aravind & Ponting 1998). Consequently, some of these components are thought to directly regulate RNA pol II activity and enhance NR function (Ferdous *et al.* 2001, Ishizuka 2001, Gonzalez *et al.* 2002).

The 20S catalytic core The 20S proteasome of eukaryotes contains fourteen subunits. Eukaryotic proteasomes may be thought of as a stack of four rings, two α and β rings each with seven distinct subunits arranged in a general barrel structure $\alpha 1-7$, $\beta 1-7$, $\beta 1-7$, $\alpha 1-7$. Structural features of the 20S core block the random degradation of proteins providing a high level of substrate specificity. The α -subunits forming outer rings have no catalytic activity, but play a role in the interaction of the 20S and 19S regulatory particle. In eukaryotes, three of the seven β -subunits have functional proteolytic sites that differ in

their specificities. These include two sites of each of the following types: chymotrypsin-like activity that cut hydrophobic residues, trypsin-like sites that cleave basic amino acids and caspase-like sites also known as post-glutamyl peptide hydrolase-like (PGPH) sites, that cleave acidic residues. Recently, the development of specific proteasome inhibitors that target specific catalytic sites has proved valuable for research probing the function of the ubiquitin–proteasome pathway in cellular processes (Kisselev & Goldberg 2001). Proteasome inhibitors, such as the *Streptomyces* metabolite lactacystin and synthetic compounds such as MG132, have been used extensively to study the effects of the ubiquitin–proteasome pathway on NR function (Lonard *et al.* 2000, Wallace & Cidlowski 2001, Deroo *et al.* 2002).

COP9 signalosome

Recently a novel protein complex called the COP9 signalosome (CSN) has been identified in eukaryotes (Deng *et al.* 2000). The CSN complex is highly conserved from plants to mammals (Wei *et al.* 1994). The eight subunits (CSN1–8) of the CSN share sequence homologies with eight subunits of the lid of the 19S regulatory particle of the 26S proteasome (Glickman *et al.* 1998). The CSN is thought to cooperate with the 26S proteasome in regulating protein stability (Schwechheimer & Deng 2001). Due to its control over the ubiquitin–proteasome system, CSN is implicated in a variety of biological processes also regulated by the 26S proteasome. These include, but are not limited to, *Drosophila* and mammalian development, transcriptional regulation, cell cycle control and cell signaling (Bech-Otschir *et al.* 2002, Harari-Steinberg & Chamovitz 2004). Biochemically, the CSN has been implicated in two distinct processes: regulation of protein degradation through deneddylation of the cullin subunit of the RING finger complex, SCF (Skp1/cullin/F-box) E3 ubiquitin ligases; and modulation of kinase signaling pathways (reviewed in Bech-Otschir *et al.* 2002, Wei & Deng 2003). In all eukaryotes tested, the CSN acts to scaffold the NEDD8, a small ubiquitin-like protein, from the cullin subunit of the SCF–E3 ubiquitin ligase complex, thereby inactivating the complex (von Arnim 2003, Chiba & Tanaka 2004). The most conserved of the CSN subunits are CNS2/Alien and CSN5/Jab1. CSN2/Alien, also known as thyroid hormone receptor (TR) interacting protein (Trip15) is a co-repressor of NR, TR and vitamin D receptor (VDR) (Lee *et al.* 1995, Dressel *et al.* 1999, Altincicek *et al.* 2000). CSN5/Jab1 is a c-Jun activating binding protein that specifically stabilizes c-Jun DNA complexes to activate c-Jun-mediated transcription (Chamovitz & Segal 2001). Using yeast and mammalian two-hybrid system, CSN5 was shown to interact with both progesterone receptor (PR) and the steroid receptor co-activator 1 (SRC-1) to potentiate PR

and other NR-mediated transcriptional activation (Chauchereau *et al.* 2000). The functional significance and the molecular mechanisms of these interactions are not characterized. Interestingly, CSN subunits are homologous to the 19S proteasome and CSN5/Jab1 corresponds to the Rpn11 subunit of the 19S regulatory particle. Whether COP9 signalosome is involved in NR stability/degradation is unknown. However, given the function of COP9 in deneddylation of the SCF–E3 ligase complex, we can speculate a role of CSN5 on NR protein stability. Modification by NEDD8 (neddylation) is required for proteasome-mediated degradation of estrogen receptor alpha (ER α) (Fan *et al.* 2003). It would be interesting to explore whether the CSN5/Jab1 subunit is involved in deneddylation and neddylation of ER α as observed for the SCF complex. Other evidence for a role of CSN in NR receptor function, comes from experiments showing that Jab1/CSN5 colocalizes with NEDD8 in murine embryogenesis, a process controlled by a number of NRs, including the estrogen receptor (Carrabino *et al.* 2004). In the future, it will be critical to determine whether any of the enzymatic activities associated with the signalosome are involved in the control of NR protein stability and transcriptional regulation.

Role of the ubiquitin–proteasome pathway in chromatin structure and gene expression

Ubiquitin and chromatin structure

While the function of the ubiquitin system in protein degradation is well established, it is becoming clear that modification by ubiquitin may have additional and distinct roles in biology (Conaway *et al.* 2000, Ulrich 2002, Lipford & Deshaies 2003, Muratani & Tansey 2003). Indeed, there is a growing body of evidence indicating that ubiquitin and proteasomes are intimately involved in chromatin structure modifications and gene control (Zhang 2003, Osley 2004). Within the eukaryotic nuclei, DNA associates with nuclear proteins to form chromatin. The fundamental structural unit of chromatin, the nucleosome, is composed of 146 base pairs of DNA wrapped around an octamer of highly conserved histone proteins H2A, H2B, H3 and H4 (Luger *et al.* 1997). Organization of the genome into chromatin impedes a number of biological processes, including transcription. As a result, chromatin has to undergo a number of structural modifications for transcription to occur (Wolffe & Hayes 1999). Recent studies indicate that covalent modifications of histone tails – including acetylation, methylation, phosphorylation, ubiquitination, sumoylation, and poly-ADP-ribosylation – play a vital role in regulating chromatin structure dynamics

and gene expression (Fischle *et al.* 2003b, Nathan *et al.* 2003). Modification of histones by ubiquitin has recently come to the forefront as a previously unrecognized level of transcriptional control (Jason *et al.* 2002, Ulrich 2002, Muratani & Tansey 2003, Zhang 2003). The involvement of ubiquitin in chromatin structure was realized over 25 years ago, when histone H2A was identified as the first protein to be ubiquitinated (Goldknopf *et al.* 1975, Zhang 2003, Pickart 2004). To date the precise role of this modification in regulating chromatin structure and/or function is unknown, although a role of ubiquitinated H2A in higher order chromatin folding has been proposed (Jason *et al.* 2002, Zhang 2003).

In addition to histone H2A, histone H2B is also ubiquitinated in both yeast and humans (Thorne *et al.* 1987). A number of yeast studies have explored the impact of ubiquitinated histone H2B (uH2B) on chromatin structure and transcriptional regulation (reviewed in Osley 2004). Together with histone H2A and H2B, histone H3 and H1 are also ubiquitinated (Chen *et al.* 1998, Pham & Sauer 2000). Specifically, TAFII250, a component of general transcription factor TFIID, exhibits ubiquitin-activating/conjugating (E1/E2) activity leading to the ubiquitination of the linker histone H1 (Pham & Sauer 2000). Currently, it is not clear how ubiquitinated histones regulate chromatin structure and function. In the sections that follow, we review the role of these modifications within chromatin and their potential contributions to NR-mediated gene regulation.

Ubiquitin and chromatin structure: implications for NR-mediated gene transcription

Transcription occurs within the context of chromatin. In higher eukaryotes, histone ubiquitination is a prominent modification that is associated with alteration of nucleosome structure (Fischle *et al.* 2003b, Nathan *et al.* 2003). While the role of ubiquitinated histones has not been extensively explored with respect to NR-mediated transcriptional regulation, it is well known that NRs interact with chromatin to regulate gene expression. A number of earlier studies suggest that histone ubiquitination is involved in gene transcription both in a positive and negative manner. For example, ubiquitinated H2A is abundant in actively transcribing regions of the mouse dihydrofolate reductase (DHFR) and hsp70 genes (Barsoum *et al.* 1982, Levinger & Varshavsky 1982). In other reports, ubiquitinated histones colocalize with inactive transcriptional regions. Even more interesting with respect to NR function is the fact that ubiquitinated H2A is found in transcriptionally inactive compartments such as the sex body of mouse spermatids (Baarends *et al.* 1999). Other data suggest that ubiquitinated H2A is involved in chromatin rearrangement during spermatogenesis (Baarends *et al.* 1999).

Rad6/Ubc2 ubiquitinates histone H2A *in vitro* and is implicated in the ubiquitination of H2A *in vivo* (Baarends *et al.* 1999, Haas *et al.* 1991). The mammalian homologs of yeast Rad6/Ubc2, HR6A and HR6B function in mouse spermatogenesis. HR6A expression is decreased during spermatogenesis, but the HR6B is enriched in the testis at the time of histone–protamine transition (Roest *et al.* 1996). Deletion of HR6B in mice results in male sterility and defects in spermatogenesis (Roest *et al.* 1996). In addition, extensive ubiquitination of histone H2A is observed in pachytene stage of prophase I and in elongating spermatids, while ubiquitination by HR6B is proposed to trigger removal of histones to allow chromatin condensation and packaging in spermatogenesis (Baarends *et al.* 1999). Intriguingly, ubiquitinated histone H3 is also found in elongating spermatids (Chen *et al.* 1998). Since spermatogenesis is a process regulated by androgen and estrogen receptors (members of the NR super family), these observations may imply a role for ubiquitinated histone H2A and H3 in NR function.

Ubiquitinated histone H2B is also associated with gene silencing in yeast *Saccharomyces cerevisiae*. For example, a recent study shows that Rad6 ubiquitinates histone H2B and leads to silencing of the ARG1 gene, which encodes argininosuccinate synthetase (Turner *et al.* 2002). Additional evidence for a role of uH2B in gene regulation is provided by studies showing that ubiquitination of histone H2B is associated with methylation of histone H3 at specific lysine residues, an activity that regulates transcription both in a positive and negative manner (Briggs *et al.* 2002, Dover *et al.* 2002, Sun & Allis 2002, Henry *et al.* 2003, Hwang *et al.* 2003, Wood *et al.* 2003). The regulated interplay between ubiquitination of histone H2B and methylation of histone H3 indicates that histone ubiquitination is an integral part of the ‘histone code’ used by cells to distinguish between transcriptionally active and inactive chromatin (Fischle *et al.* 2003a). Such studies can be relevant to NR, as interaction between various histone modifications has been demonstrated to play a role in NR function (Ma *et al.* 2001, Li *et al.* 2002, 2003).

The role of 26S proteasome holoenzyme components in chromatin modification and transcription

While there is ample evidence that ubiquitination and ubiquitin enzymes can influence some aspects of chromatin structure and transcription, there remains a question as to whether the 26S holoenzyme components are involved in these processes (Conaway *et al.* 2000, Muratani & Tansey 2003). Recent evidence suggests that the 19S components are directly involved in

transcription in yeast systems. Analysis of DNA using chromatin immunoprecipitation assays shows that 19S proteasome components occupy the GAL1–10 promoter (Gonzalez *et al.* 2002). RNA pol II transcription may be viewed as involving four steps: the formation of the pre-initiation complex (PIC), followed by initiation, elongation and termination of transcription (Roeder 1996). Genetic experiments in which yeast strains lacking alleles of SUG1/Rpt6 and SUG2/Rpt4 exhibit elongation defects suggest that SUG1/Rpt6 is directly involved in transcriptional elongation in yeast (Ferdous *et al.* 2001, 2002, Sun *et al.* 2002). In these experiments the role of 19S complex in transcriptional elongation is further supported by the observation that SUG1/Rpt6 physically interacts with the elongation factor Cdc68, a component of the facilitates chromatin transcription (FACT) complex (LeRoy *et al.* 2000, Ferdous *et al.* 2001).

Further support for a role of the 26S proteasome in transcription comes from a recent report demonstrating that the 20S proteasome physically interacts with RNA pol II and is recruited to the sites of active transcription on the GAL1 and HSP82 promoters of yeast (Gillette *et al.* 2004). This supports the idea that the 19S AAA proteins independent of 20S (APIS) complex is required early in the transcription process and at the start of elongation while the 20S is exclusively recruited to the coding region of the gene and is involved in clearing the stalled RNA pol II and regulating the termination step (Gillette *et al.* 2004).

Phosphorylation of the carboxy-terminal domain (CTD) of the largest subunit of pol II is known to switch the RNA pol II from the initiation to the elongation phase of transcription (Komarnitsky *et al.* 2000, Ahn *et al.* 2004). Phosphorylation of the CTD then signals ubiquitination of the large subunit of RNA pol II, allowing the elongating polymerase to recruit a diverse collection of elongation factors (Mitsui & Sharp 1999, Shilatifard *et al.* 2003). Studies in yeast *Saccharomyces cerevisiae* and *Drosophila melanogaster* have demonstrated that recruitment of elongation factors by the elongating RNA pol II is linked to specific chromatin modifications (Gerber & Shilatifard 2003, Hampsey & Reinberg 2003). In *Drosophila melanogaster* the transcriptionally active/elongating form of RNA pol II phosphorylated at serine 2 colocalizes with histone H3 methylated at lysine 4 and 79, suggesting that these modifications are associated with transcriptionally active chromatin (Gerber & Shilatifard 2003). Additionally, in yeast, ubiquitination of histone H2B directs methylation of histone H3 at lysine 4 and 79, modifications associated with active chromatin (reviewed in Osley 2004). More precisely, recent evidence links 19S proteasomal ATPase components to ubiquitination of histone H2B and methylation of histone H3 lysine 4 and 79, and indeed directly ties the 26S proteasome components to specific

chromatin modification and transcription (Ezhkova & Tansey 2004).

The link between the ubiquitin–proteasome pathway and NR-mediated transcriptional regulation

Thus far, the body of evidence suggests that post-translational modification by ubiquitin is directly tied to specific chromatin structure modifications independent of proteolysis. The question remains whether these chromatin modifications or proteolysis via the ubiquitin–proteasome pathway is involved in transcriptional regulation with respect to NRs. Eukaryotic transcription is a complex process tightly regulated by the activity of the RNA pol II enzyme (Roeder 1996). The activity of the large subunit of RNA pol II is, at least in part, regulated by the ubiquitin–proteasome pathway, demonstrating a direct link between ubiquitin-mediated proteolysis and transcription (Bregman *et al.* 1996, Huibregtse *et al.* 1997). The HECT domain E3 ubiquitin ligase Rsp5, which is also a steroid hormone receptor coactivator, has been shown to ubiquitinate and target RNA pol II to the proteasome during DNA damage (Imhof & McDonnell 1996, Beaudenon *et al.* 1999).

The levels of transcription factors that act in conjunction with RNA pol II to effectively synthesize mRNA are critical to transcriptional regulation. Consequently, the levels and activities of transcriptional activators and repressors are tightly controlled. Accumulating evidence indicates that ubiquitin plays an important role in transcriptional regulation through proteasome-dependent and -independent degradation of transcriptional activators (Conaway *et al.* 2000, Muratani & Tansey 2003). The ubiquitin–proteasome pathway tightly regulates a large number of key transcriptional regulators including p53, c-Fos, c-Jun and more recently NRs and their cofactors (Salvat *et al.* 1999, Nawaz & O'Malley 2004).

NRs are one of the largest groups of transcription factors, with more than 49 distinct members now identified in the human genome (Maglich *et al.* 2001, Robinson-Rechavi *et al.* 2001, Evans & O'Malley 2004 (www.nursa.org)). NR-mediated transcriptional regulation is controlled by multiple factors. The classical mode of action of NRs involves binding of a small lipophilic ligand to the receptor and the interaction of the receptor–ligand complex with hormone response elements embedded within chromatin in target gene promoters, followed by recruitment of basal transcription machinery and activation/repression of transcription (Mangelsdorf *et al.* 1995). This cascade of events is facilitated by numerous factors that include chromatin remodeling factors, coactivators and corepressors (Collingwood *et al.* 1999, Kinyamu & Archer 2004, Privalsky 2004). Consequently, NR-mediated transcriptional regulation is subject to multiple levels of control, including changes in

chromatin structure within gene promoters and regulation of receptor and cofactor levels by the ubiquitin–proteasome pathway (Kinyamu & Archer 2004, Nawaz & O'Malley 2004). The transcriptional signal induced by an activated NR is highly dependent on receptor and cofactor protein levels. Subsequently, any changes in receptor levels would affect gene expression. Studies showing that several members of the NR super family – including the glucocorticoid (GR), estrogen (ER), progesterone (PR), androgen (AR), thyroid (TR), retinoic acid (RAR) and vitamin D (VDR) receptors – undergo ligand-dependent proteasome-mediated proteolysis provide a direct link between NR-mediated gene transcription and the ubiquitin–proteasome pathway (reviewed in Nawaz & O'Malley 2004).

Rapid degradation of a number of key transcriptional regulators, such as p53, by the ubiquitin–proteasome is correlated to their phosphorylation state (Meek 1999). Phosphorylation is thought to signal substrate recognition by the enzymes in the ubiquitination pathway. Similarly, the phosphorylation state of NRs plays a pivotal role in the ligand-mediated stability of these receptors. The GR upon ligand binding is hyperphosphorylated and degraded via the ubiquitin–proteasome pathway (Webster *et al.* 1997, Wallace & Cidlowski 2001, Ismaili & Garabedian 2004). Phosphorylation of the PR by mitogen-activated protein kinase (MAPK) leads to ligand-dependent degradation of the receptor (Lange *et al.* 2000). Phosphorylation of the retinoic acid receptor $\gamma 2$, (RAR $\gamma 2$) by p38 MAPK signals its ligand-dependent degradation (Gianni *et al.* 2002). Furthermore, the phosphorylation state of RARs dictates differential regulation between RAR $\gamma 2$ and RAR α by the ubiquitin–proteasome pathway (Kopf *et al.* 2000). Specifically, upon phosphorylation, RAR $\gamma 2$ undergoes RA-mediated degradation, while the phosphorylated RAR α is stabilized. Finally, the peroxisome proliferator-activated receptor alpha (PPAR α) is also stabilized by phosphorylation in the presence and absence of its ligand (Blanquart *et al.* 2004).

A direct role of the ubiquitin-dependent proteolysis in transcriptional regulation is suggested by recent reports showing an overlap between transcription activation domains and sequences within transcriptional activators that specify protein turnover (Salghetti *et al.* 2001, Muratani & Tansey 2003). Interestingly, the PEST (Pro (P), Glu (E), Ser (S) and Thr (T)) motifs within transcriptional activators that contain recognition sites for ubiquitination, referred to as 'degrons' are also sequences targeted for phosphorylation (Salghetti *et al.* 2000). Intriguingly, receptor phosphorylation and degradation is coupled to receptor-mediated transactivation. Indeed, for the GR, mutation of the PEST sequence within the site of phosphorylation inhibits GR degradation and consequently GR-mediated transcriptional activity (Wallace & Cidlowski 2001). Phos-

phorylation of the PR by MAPK couples PR-mediated transcriptional activity to ligand-dependent degradation (Shen *et al.* 2001). Likewise phosphorylation of the AF-1 domain of the retinoic acid receptor, RAR $\gamma 2$ by p38 MAPK and recruitment of SUG1 a 19S regulatory subunit to AF-2 domain signals RA-dependent degradation and transactivation of RAR $\gamma 2$ receptor (Gianni *et al.* 2002). Conversely, phosphorylation of PPAR α stabilizes the receptor and induces PPAR α transcriptional activity (Blanquart 2004). Thus, the transcriptional potency NRs is correlated with their protein degradation as shown for other transcriptional activators (Molinari *et al.* 1999, Salghetti *et al.* 2001).

Additional factors can influence ligand-dependent degradation of NRs. A recent study provides evidence that specific cofactors can facilitate ligand-dependent degradation of the ER. The p160 coactivator AIB1 uniquely mediates agonist-dependent, but not antagonist-dependent, ER α protein degradation via the ubiquitin–proteasome pathway (Shao *et al.* 2004). While the effect of AIB1 on ER α stability is independent of the E3 ligase E6-AP, AIB1 may modulate ER α degradation via post-translational modifications including, but not limited to, phosphorylation of the receptor (Shao *et al.* 2004). Of note, is that AIB1-mediated ligand-dependent degradation of ER α is correlated with recruitment of transcriptional machinery, including RNA pol II, to ER-target promoter, again tying ubiquitin–proteasome proteolysis of the receptor to transcriptional regulation.

Recent reports suggest cross-signaling between receptors as another means of modulating ligand-dependent degradation of NR via the ubiquitin–proteasome pathway. For example, ER mediates a decrease in GR levels in the presence of estradiol, while hypoxic conditions and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-activated aryl hydrocarbon receptor target the ER for degradation (Stoner *et al.* 2002, Kinyamu & Archer 2003, Wormke *et al.* 2003). Such negative crosstalk is sometimes mediated by the receptor interacting with ubiquitin–proteasome enzymes. In the case of the GR, estradiol increases protein expression of Mdm2, which physically interacts and targets the GR to the proteasome (Sengupta & Wasylyk 2001, Kinyamu & Archer 2003).

NR-mediated transcriptional regulation is a highly complex process involving multiple coregulatory factors. NR coregulators were first discovered as proteins that enhance NR-mediated transcriptional activation (McKenna & O'Malley 2002). Subsequently, coactivators were shown to contain enzymatic activities such as histone acetyltransferase (HAT), histone methyltransferase (HMT) and ATPase activities (Collingwood *et al.* 1999, Kinyamu & Archer 2004, McKenna & O'Malley 2002).

Similar to the receptors, the turnover of NR cofactors is tightly regulated by the proteasome (Zhang *et al.* 1998,

Lonard *et al.* 2000, 2004, Yan *et al.* 2003). Degradation of specific coregulators by the proteasome is ligand specific. For example, 17 β -estradiol, but not 4-hydroxytamoxifen or raloxifene decrease SRC-1 and SRC-3 protein stability (Lonard *et al.* 2004). Cofactor stability can be modulated by post-translational modifications. Phosphorylation of glucocorticoid receptor interacting protein 1 (GRIP1) by cAMP-dependent protein kinase (PKA) induces its degradation via the ubiquitin–proteasome pathway (Hoang *et al.* 2004).

The recognition that a number of enzymes in the ubiquitin–proteasome pathway are also NR cofactors provides another link between the ubiquitin–proteasome pathway and NR function. These include RSP5/RPF1, E6-AP, UBC9 and more recently Mdm2 (Imhof & McDonnell 1996, Nawaz *et al.* 1999, Poukka *et al.* 1999, Saji *et al.* 2001). The E3 ligase RSP5/RPF1 and E6-AP belong to the HECT domain family and the MDM2 E3 ligase belongs to RING domain family (Glickman & Ciechanover 2002). A mutation of the HECT domain does not affect the coactivator function of E6-AP, suggesting that the coactivator function is independent of the ligase activity (Nawaz *et al.* 1999). Additionally, components of the 19S regulatory particle SUG1/Trip1/Rpt6 and TBP-1/Rpt5 regulate NR transcriptional activity (reviewed in (Nawaz & O'Malley 2004). Other evidence shows that ubiquitin-like molecules, including small ubiquitin-like modifier 1 (SUMO-1) and NEDD8 may also regulate NR function (Poukka *et al.* 2000, Fan *et al.* 2003). In this regard, the ubiquitin–proteasome components act as cofactors and function to activate or repress NR transcription.

The ubiquitin–proteasome components involved in NR-mediated transactivation represent different steps of the ubiquitin–proteasome pathway and different enzymatic activities, including ubiquitin conjugation, ubiquitin ligase and ATPase activities (Fig. 1) (Imhof & McDonnell 1996, Fraser *et al.* 1997, Nawaz *et al.* 1999, Poukka *et al.* 1999, Dennis *et al.* 2001). The complexity of the system is increased by the finding that specific ubiquitin–proteasome enzymes target specific receptors and coregulators (Yan *et al.* 2003, Perissi *et al.* 2004). The E3 ligase Mdm2 targets the GR and AR to the proteasome and is implicated in ER-dependent degradation of the GR (Sengupta & Wasylyk 2001, Lin *et al.* 2002, Kinyamu & Archer 2003). The C-terminal Hsp-interacting protein (CHIP) an E3/E4 ubiquitin ligase stimulates GR and AR degradation (Connell *et al.* 2001, Cardozo *et al.* 2003). Of particular significance is the recent report showing that transducin- β -like (TBL1) and TBL1-related protein (TBLR1), two subunits of the nuclear receptor-corepressor (NCoR) and silencing mediator for retinoic acid receptor and thyroid hormone receptor (SMRT) co-repressor complexes, contain an F-box motif similar to that of the SCF–E3 ligase complex that mediates degradation of the NCoR on NR

gene promoters allowing transcription to proceed (Perissi *et al.* 2004). The study also demonstrates a distinct requirement of ubiquitin enzymes in NR function *in vivo*. Disruption of the TBL1 gene in embryonic stem cells decreases PPAR γ -induced adipogenic differentiation, indicating a biological function for TBL1 in NR-mediated gene activation events (Perissi *et al.* 2004).

The requirement for the ubiquitin–proteasome components in NR-mediated transcription was first suggested by studies using proteasome inhibitors (Nawaz *et al.* 1999, Lonard *et al.* 2000). Treatment of mammalian cells with 26S proteasome inhibitors inhibits ER-, AR- and PR-mediated gene activation, but increases GR levels and facilitates GR-mediated transactivation (Nawaz *et al.* 1999, Wallace & Cidlowski 2001, Deroo *et al.* 2002). This suggests a differential effect of proteasome inhibitors among receptors, which is quite surprising since the effect of proteasome inhibitors on the mobility of the ER and GR within the nuclear matrix is similar (Stenoien *et al.* 2001, Deroo *et al.* 2002, Reid *et al.* 2003, Schaaf & Cidlowski 2003). This may be explained by the fact that ubiquitin–proteasome components show some specificity with regard to NR-mediated transcription. The E2 enzyme Ubc2 and E3 ligase E6-AP are necessary for TR transcriptional activation (Perissi *et al.* 2004). SUG1/Trip1 an ATPase component of the 19S regulatory particle promotes ubiquitination and degradation of ER α and ER β , thereby suppressing their transcriptional activity (Masuyama & Hiramatsu 2003). In contrast to the effect on ER α and ER β , SUG1 has a stimulatory effect on TR transcriptional activity (Fraser *et al.* 1997). These studies implicate different components of the ubiquitin–proteasome system in NR protein stability and transcriptional activation and suggest that specificity of various components involved permit different receptors to adopt varying functional regulatory mechanisms within the cell. For example, Mdm2 increases ER transcriptional activity, but is also associated with a decrease in GR-mediated transactivation (Saji *et al.* 2001, Sengupta & Wasylyk 2001, Kinyamu & Archer 2003, Reid *et al.* 2003).

Transcriptional activation/repression involves alteration of chromatin structure within promoters of target genes. Together with the activated receptors, coregulators and RNA pol II, proteasome components are also recruited to NR target gene promoters, suggesting their involvement with chromatin. The SUG1/Rpt6/hTrip1 ATPase, a component of the 19S base particle, is required for efficient transcription by human RNA pol II and binds directly to activation domains of Gal4 and the viral activator VP16 (Swaffield *et al.* 1995, Chang *et al.* 2001, Ferdous *et al.* 2002). Similarly, ubiquitin–proteasome components are recruited to natural NR promoters, as exemplified by the occupancy of the E3 ubiquitin ligases (Mdm2, E6-AP) together with the 19S

base component SUG1/Trip1/Rpt6 to the ER responsive pS2 promoter using chromatin immunoprecipitation assays (Reid *et al.* 2003, Perissi *et al.* 2004). The S1/Rpn2/p112, a component of the 19S, occupies the AR responsive prostate specific antigen (PSA) promoter (Kang *et al.* 2002). TAFII250, a component of the basal transcriptional machinery and a ubiquitin activating/conjugating enzyme, is part of the initiation complex on NR target promoters (Collingwood *et al.* 1999).

Although ubiquitin–proteasome components are well represented on NR-regulated target gene promoters, their function in NR-dependent transcription is not well understood. One hypothesis is that the ubiquitin–proteasome system directs the turnover of chromatin bound receptors and corresponding cofactors, allowing for attenuation of the transcriptional signal and the promoter to continuously reload transcription factors to ensure further cycles of transcription (Kang *et al.* 2002, Metivier *et al.* 2003, Stavreva *et al.* 2004). It is important to note that alternative mechanisms could explain the effects of proteasome-mediated proteolysis on NR-mediated transcription.

Ubiquitin–proteasome and NR-dependent chromatin remodeling: MMTV as a classic system

The organization of chromatin is of fundamental significance in transcriptional control by NRs (reviewed in Collingwood *et al.* 1999, McKenna & O'Malley 2002, Kinyamu & Archer 2004, Privalsky 2004). Transcriptional activation by NRs involves alteration of chromatin structure by ATP-dependent chromatin remodeling enzymes in conjunction with histone-modifying machines (McKenna & O'Malley 2002, Narlikar *et al.* 2002, Kinyamu & Archer 2004). The effect of NR-mediated transcription on chromatin structure is determined by changes in accessibility of nucleosomal DNA within target promoters, facilitated by the SWI/SNF, an ATP-dependent chromatin remodelling complex and post-translational modifications of histones such as acetylation, phosphorylation, methylation and ubiquitination (Collingwood *et al.* 1999, Fischle *et al.* 2003b, Nathan *et al.* 2003). In as much as the ubiquitin–proteasome components can modify chromatin directly, they represent a critical regulatory consideration in NR-mediated transactivation. For example, ER recruits the E3 ligases, E6-AP and Mdm2, and the 19S proteasome component to the pS2 promoter concomitantly with other factors that have both HAT and HMT activity and have the ability to modify chromatin via histone modifications (Reid *et al.* 2003, Perissi *et al.* 2004).

Despite the presence of the ubiquitin–proteasome components on NR target gene promoters, work from our laboratory excludes nucleosomal DNA accessibility as a means of altering chromatin structure by these

molecules (Deroo *et al.* 2002). Transcriptional regulation by the GR is concomitant with chromatin remodeling exemplified by restriction enzyme hypersensitivity on the MMTV promoter (reviewed in Deroo & Archer 2001, Hsiao *et al.* 2002, Hebbar & Archer 2003). Proteasome inhibitors increase GR transactivation of the MMTV promoter in the absence of increased chromatin remodeling as measured by restriction enzyme hypersensitivity (Deroo *et al.* 2002). This observation suggests that the increased transcriptional ability of the GR in the presence of proteasome inhibitors is downstream of chromatin remodeling normally associated with GR recruiting the SWI/SNF complex to the MMTV promoter (Fryer & Archer 1998, Fletcher *et al.* 2002). This idea is supported by the finding that proteasome inhibitors potentiate GR transcriptional activity of transient templates not organized as chromatin (Wallace & Cidlowski 2001, Deroo *et al.* 2002). This suggests that the ubiquitin–proteasome system components are involved in a step downstream of transcriptional initiation including, but not limited to, the elongation step of transcription. Support for this concept comes from experiments showing that ensuing rounds of transcription and recycling of the ER are dependent on the release of the receptor from the promoter, which is dependent on a functional proteasome and requires phosphorylation of the C-terminal domain of RNA pol II, a requirement for transcriptional elongation (Shang *et al.* 2000, Reid *et al.* 2003). Studies in *Saccharomyces cerevisiae* and *Drosophila melanogaster* suggest that the ubiquitin–proteasome pathway is directly tied to transcription such that direct and specific chromatin modification by ubiquitin may regulate transcriptional elongation (reviewed in Conaway *et al.* 2000, Muratani & Tansey 2003, Zhang 2003). If the ubiquitin–proteasome system is linked to the elongation step of transcription, then it may explain how proteasome inhibitors would increase GR transcriptional activity. In the section that follows we will examine how the events orchestrated by the ubiquitin–proteasome system may target the elongation step of transcription within NR gene regulation (Deroo *et al.* 2002, Reid *et al.* 2003, Archer & Kinyamu 2004).

A role of chromatin structure modifications associated with the elongating RNA pol II in NR-mediated gene transcription: what can we explore in the future?

Phosphorylation of the CTD of the largest subunit of pol II is known to switch the RNA pol II from the initiation to the elongation phase of transcription (Komarnitsky *et al.* 2000, Ahn *et al.* 2004). Phosphorylation of CTD

then signals ubiquitination of the large subunit of RNA pol II, allowing the elongating polymerase to recruit a diverse collection of elongation factors (Mitsui & Sharp 1999, Shilatfard *et al.* 2003). Studies in yeast *Saccharomyces cerevisiae* and *Drosophila melanogaster* have demonstrated that recruitment of elongation factors by the elongating RNA pol II is associated with specific chromatin modifications (Gerber & Shilatfard 2003, Hampsey & Reinberg 2003). In *Drosophila melanogaster* the transcriptionally active/elongating form of RNA pol II phosphorylated at serine 2 colocalizes with histone H3 methylated at lysine 4 and 79, suggesting that these modifications are associated with transcriptionally active chromatin (Gerber & Shilatfard 2003). Additionally, in *Saccharomyces cerevisiae* ubiquitination of histone H2B directs methylation of histone H3 at K4 and K79, modifications associated with active chromatin (reviewed in Osley 2004). Recent evidence links 19S proteasomal ATPase components to ubiquitination of histone H2B and methylation of histone H3-K4 and K79, and indeed directly ties the 26S proteasome components to specific chromatin modification and transcription (Ezhkova & Tansey 2004).

To date no published studies have addressed whether the ubiquitin–proteasome system is associated with the elongating RNA pol II and histone H3 lysine modification with respect to NRs. Most studies have shown that arginine methyltransferases are NR coactivators, but a role in lysine methylation is not well documented (Kouzarides 2002, Stallcup *et al.* 2003). Methylation of histone H3-K9 is associated with transcriptional repression by steroid hormone receptors (Ma *et al.* 2001, Li *et al.* 2002, 2003). Two recent studies provide evidence that methylation of histone H3 lysine 4 plays a role in transcriptional regulation by the androgen and ecdysone receptors, members of the NR super family (Kim *et al.* 2003, Sedkov *et al.* 2003). Interestingly, on the AR-regulated PSA promoter, the tri-methylated histone H3-K4 is decreased in the promoter region and increased in the coding region in the presence of AR agonist (Kim *et al.* 2003). If methylation of H3-K4 is linked to the ubiquitin–proteasome system, this finding supports the observation that proteasome inhibitors silence the PSA promoter (Kang *et al.* 2002). On the other hand, the direct relationship of histone modification on transcription in higher eukaryotes relative to what is known in yeast is controversial. For example, one study shows histone H3 lysine methylation patterns in higher eukaryotes similar to those in yeast, while another study does not (Liang *et al.* 2004, Schneider *et al.* 2004).

What other potential chromatin histone modifications can be orchestrated by the ubiquitin–proteasome components on the NR-regulated genome? The presence of ubiquitinated histones on NR target gene promoters has not been demonstrated to date. However, histone H2A ubiquitination is implicated in sper-

matogenesis, a process driven by steroid hormone receptors (Roest *et al.* 1996, 2004). The E3 ubiquitin ligases, such as Mdm2 and E6-AP, are recruited to NR target gene promoters (Reid *et al.* 2003). There is also evidence suggesting that Mdm2 can ubiquitinate histone H2B *in vitro* and *in vivo* (Minsky & Oren 2003). On the occupied promoter, Mdm2 may function like yeast Bre1, providing a means to alter chromatin structure within the occupied promoter (Wood *et al.* 2003). Future studies can evaluate whether histone ubiquitination plays a role in NR-mediated changes in chromatin structure.

Evidence from this and other laboratories suggests that the ubiquitin–proteasome system is involved in GR-mediated transcriptional elongation (Deroo *et al.* 2002, Reid *et al.* 2003). There is a potential to utilize the MMTV promoter, organized as chromatin, to explore chromatin modifications associated with the transition from the initiation to the elongation phase of transcription. On the MMTV promoter the elongating polymerase was shown to have a capacity to decondense chromatin, an aspect attributed both to the mechanical effect of the RNA polymerase on the DNA or the ability of the RNA polymerase to associate with a factor or factors that decondense chromatin (Muller *et al.* 2001).

Changes in histone modifications may provide the platform for association with other factors that can alter chromatin structure (Krogan *et al.* 2003, Ng *et al.* 2003, Xiao *et al.* 2003). In yeast the ATPase of the chromatin remodeling complex ISWI/hSNF2H was shown to recognize chromatin marked by di- and trimethyl histone H3-K4 and to associate with the elongating RNA pol II (Morillon *et al.* 2003, Santos-Rosa *et al.* 2003). On the pS2 promoter, the phosphorylated RNA pol II occupies the promoter concomitantly with the elongation factors (Elp1 and 3) and the SWI/SNF complex, although it is not clear whether this association correlates with nucleosomal DNA accessibility (Metivier *et al.* 2003). Other evidence supporting a role of chromatin remodeling and modifying machines in the elongation step comes from the observation that the SWI/SNF complex is recruited to the coding region of hsp70 promoter (Corey *et al.* 2003). The elongating polymerase/phosphorylated RNA pol II can recruit putative elongation factors with chromatin remodeling activities such as FACT, although FACT complex occupancy on NR target promoters has not been demonstrated (Orphanides *et al.* 1999, Mason & Struhl 2003, Saunders *et al.* 2003). Conversely, it is known that the FACT complex associates with chromatin remodeling complexes found in the VDR complex WINAC, which also contains SWI/SNF and is required for NR function (Kitagawa *et al.* 2003).

Additional chromatin modifications that can arise from components of the ubiquitin–proteasome system

Initiation

Elongation

Figure 2 Model for the role of the ubiquitin–proteasome system in NR-mediated chromatin structure modification and transcriptional regulation. In the presence of hormone, NRs bind to hormone response elements embedded within chromatin. The activated NR recruits various groups of chromatin-modifying machines (SWI/SNF, HATs, p160 and HMTs) to remodel chromatin followed by recruitment of the mediator complex (MED) and the general transcriptional machinery (TFIID) allowing transcriptional initiation by RNA pol II. There is a distinct requirement for the ubiquitin enzymes (UBE) and the 26S proteasome (26S) in NR-mediated transcriptional regulation at least via the traditional role in proteolysis of NR and NR co-activators (p160) and co-repressor complexes (REP). (The arrows indicate dynamic interactions between various factors as opposed to a static model (Hager *et al.* 2004, Stavreva *et al.* 2004). Based on studies in yeast *Saccharomyces cerevisiae*, the ubiquitin–proteasome system (UBE, 26S) appears to function at the elongation step of transcription. In NR-mediated transcriptional regulation, we propose that ubiquitin–proteasome components might play a part in switching the RNA pol II from a hypo-phosphorylated to a hyper-phosphorylated state. The elongation phase of transcription is regulated by phosphorylation of the C-terminal domain (CTD) of RNA pol II. The phosphorylated RNA pol II may then recruit various factors that modify chromatin (SWI/SNF, ISWI, FACT and HMT) and gene-specific elongation factors (EL(X) or EL(Y) allowing differential gene regulation by NRs. Kme, methylated histone lysine residue; Rme, methylated histone arginine residue; AC, acetylated histone lysine residue.

may involve modifications in higher-order chromatin structure and/or NR function. TAFII 250, which is a component of the basal transcriptional machinery and a coactivator of NR, is a ubiquitin-activating/-conjugating enzyme that ubiquitinates the linker histone H1. Histone H1 has clear effects on higher-order chromatin structure and phosphorylation of histone H1 is a critical component of GR-mediated MMTV activation (Bhattacharjee *et al.* 2001, Georgel *et al.* 2003). During GR-mediated chromatin remodeling, histone H1 is lost from the MMTV promoter (Bresnick *et al.* 1992; reviewed in Deroo & Archer 2001, Hebbar & Archer 2003, Kinyamu & Archer 2004). Upon prolonged glucocorticoid exposure, the following prolonged exposure to hormone is accompanied by dephosphorylation

of the global and promoter-associated histone H1 that is mimicked by kinase inhibitors (Lee & Archer 1998, Bhattacharjee *et al.* 2001). Glucocorticoid withdrawal results in a competent promoter and rephosphorylation of histone H1. In addition, recent studies using electrospray mass spectrometry demonstrate that prolonged glucocorticoid treatment leads to dephosphorylation of specific histone H1 isoforms in mouse cells (Banks *et al.* 2001). While *in vivo* data as to the effects of linker histone H1 in NR-mediated gene regulation are still lacking, linker histones are essential for mouse development and play a role in spermatogenesis, biological processes known to be regulated by NRs (Fan & Skoultschi 2003, Lin Q. *et al.* 2004). In particular, inactivation of three mouse histone H1 isoforms (H1-2,

H1.3 and H1.4) leads to embryonic death at midgestation (Fan & Skoultschi 2003).

Recent studies show that histone-modifying machines involved in NR function have ubiquitin ligase activity targeted at other proteins. The histone acetyltransferase p300 is a ubiquitin ligase for p53 (Grossman *et al.* 2003). Histone deacetylase 6 (HDAC6) was shown to interact directly with ubiquitin (Hook *et al.* 2002, Kawaguchi *et al.* 2003). The ubiquitin binding of HDAC6 has recently been linked to chromatin compaction in mouse oocytes and fertilized egg (Verdel 2003). Thus, proteins normally recruited to NR target promoters may function as ubiquitin–proteasome components apart from their normal function as a HAT or HDAC, respectively.

The above studies provide a rationale for further exploring chromatin modifications associated with the ubiquitin–proteasome pathway with regard to NR regulation of the elongation phase of transcription.

Conclusion and future prospects: what lays ahead?

Substantial and elegant observations over the past 5 years support the notion that the ubiquitin–proteasome system plays a role in NR-mediated transcriptional regulation. Most of the evidence suggests that the system contributes to transcriptional regulation via receptor/cofactor turnover thereby enabling recycling and reutilization of receptor/cofactor complexes to increase transcriptional efficiency (reviewed in Nawaz & O'Malley 2004). However, proteasome-mediated receptor/cofactor turnover may not be the only mechanism by which the proteasome modulates NR-mediated transcription. For example, as discussed earlier, inhibiting the proteasome increases GR-mediated transcriptional activity from the MMTV promoter, an effect presumed to be due to an increase in GR levels in the cell (Wallace & Cidlowski 2001, Deroo *et al.* 2002). However, inhibiting the proteasome increases MMTV transcription independent of an increase in GR protein levels (Stavreva *et al.* 2004). Conversely, for the ER, partial agonists such as trans-hydroxytamoxifen – which stabilize ERs and do not lead to ER degradation – support ER-mediated gene expression independent of proteolysis of the receptor (Wijayarathne 2001, Frasor 2004). These findings support the idea that the ubiquitin–proteasome system is involved in NR gene transcription by other mechanisms apart from receptor turnover. In pursuance of this idea, it will be exciting to explore the possibility that the effects of the ubiquitin–proteasome pathway on NR-mediated gene regulation may be gene specific and dependent on chromatin structure/architecture of the target promoters rather than being receptor specific.

Ligand-activated NRs bind to hormone response elements embedded within chromatin and recruit various chromatin-modifying machines – including SWI/SNF, HATs and HMTs – which remodel chromatin to initiate transcription (Fig. 2). There is evidence supporting a distinct requirement for the ubiquitin enzymes and the 26S proteasome in NR-mediated transcriptional regulation at least via the traditional role in proteolysis of NR and NR co-factor complexes. However, more studies are required to elucidate exactly how ubiquitin–proteasome components function in each step of the NR-mediated transcription process, particularly the elongation step where specific chromatin modifications are associated with gene activity (Fig. 2).

In summary, regulation of NR-mediated gene transcription by the ubiquitin–proteasome system is complex and may include multiple levels of control involving chromatin modification(s) and the possibility of dynamic interplay between histone modifications that facilitate binding of existing or novel chromatin modifying factors.

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