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Emerging Roles of the 26S Proteasome in Nuclear Hormone Receptor-Regulated Transcription

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Abstract

The mechanisms by which Nuclear Hormone Receptors (NHRs) regulate transcription are highly dynamic and require interplay between a myriad of regulatory protein complexes including the 26S proteasome. Protein degradation is the most well-established role of the proteasome; however, an increasing body of evidence suggests that the 26S proteasome may regulate transcription in proteolytic and non-proteolytic mechanisms. Here we review how these mechanisms may apply to NHR-mediated transcriptional regulation.

BACKGROUND

The 26S proteasome holoenzyme

The 26S proteasome is a large (~2.5 MDa) cytosolic and nuclear protease complex, which functions primarily to degrade ubiquitinated proteins in an ATP-dependent manner. The proteasome regulates the turnover of a diverse array of transcription factors, including NHRs [reviewed in (1–3)]. The proteasome complex possesses multiple enzymatic activities including proteases, ATPases/helicases, and RNAses, which mediate its regulation of various physiological processes.

The 26S proteasome is highly conserved in eukaryotic evolution and exists as two multi-subunit complexes: a core protease, termed 20S catalytic core particle (CP), and a regulatory subunit, referred to as 19S regulatory particle (RP) or the PA700 (4). 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 (5). Some characteristics of the two particles are briefly explained below, but further details can be found in other sections of this review issue and prior reviews (3,4)

The 19S regulatory Particle—The 19S RP has two distinct subcomplexes, the lid and the base. The 19S lid is composed of nine non-ATPase subunits: namely, Rpn3/PSMD3 (S3), Rpn5/PSMD12, Rpn6/PSMD11 (S9), Rpn7/PSMD6 (S10a), Rpn8/PSMD7 (S12), Rpn9/PSMD13 (S11), Rpn11/PSMD14 (S13/ human POH1), Rpn12/PSMD8 (S14) and Rpn15/PSMD9 (S15) (Yeast/human acronyms shown) (2). The 19S lid is required for the degradation of ubiquitinated substrate proteins, although all of the functions and activities of the lid subunits are not fully understood. Of the known lid subunits, five have a PCI (Proteasome, COP9, Initiation factor 3) domain and two have an MPN (Mpr1, Pad1 N-

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terminal) domain. These domains are usually found in subunits of large protein complexes involved in diverse biological functions (6,7). For example, the PCI domain is found in eukaryotic initiation factor 3 (eIF3) protein complexes which are critical for translation of mRNAs, and the presence of these domains on 19S lid subunits may tie the lid to mRNA processing functions (7,8). In addition, lid subunits such as PSMD14 (POH1) also have deubiquitinating activity (9).

The base complex of the 19S RP consists of six distinct AAA-family (ATPases associated with a variety of cellular activities) ATPases designated as Rpt1-6 in yeast and PSMC1-6 in humans, respectively (10). The human 19S base subunits are also referred to as S4/Rpt2, S6/Rpt3, S10b/Rpt4/SUG2, S6/Rpt5/TBP-1 and S8/Rpt6/SUG1/TRIP1 (11). Apart from the ATPases, the base has an additional three non-ATPase subunits Rpn 1, 2 and 10, human PSMD2, 1 and 4 or S2, S1 and S5a (10). Proteasome mutants that affect transcription are typically associated with 19S ATPases (12–14).

The 20S catalytic particle—The 20S CP is a barrel-like structure whose subunits are arranged as a stack of four rings, two α and β rings each with seven distinct subunits α 1–7, β 1–7, β 1–7, α 1–7 (10). Structural features of the 20S core block the random degradation of proteins, providing a high level of substrate specificity. The α -subunits form outer rings with no catalytic activity, but play a role in the interaction of the 20S and 19S regulatory particle (10). In eukaryotes, three of the seven β -subunits (β -1, 2 and 5) have functional proteolytic active sites within the 20S core that differ in their substrate specificities, namely caspase-like (C-L), trypsin-like (T-L) and chymotrypsin-like (ChT-L) cleaving acidic, basic and hydrophobic amino acid residues, respectively (3).

Nuclear hormone receptors as specialized transcriptional regulators

NHRs encompass a large group of ligand-activated, DNA-binding eukaryotic transcription factors that play crucial roles in metazoan life, including embryonic development, growth, cell differentiation, metabolic homeostasis and reproduction (15,16). In humans, there are 48 members of the NHR family of which a number have known ligands, but others are collectively referred to as orphan receptors and have no known ligand (15,16). The various NHRs share several structural features: a highly conserved zinc-finger DNA-binding domain (DBD), a C-terminal ligand-binding domain (LBD) and two transcriptional activation function domains, namely AF-1, which encompasses the variable and plastic N-terminus, and the C-terminal AF-2, which harbors the ligand-binding domain. AF-1 is thus constitutively active whereas AF-2 activity depends on ligand binding. The DBD binds highly specialized DNA sequences known as response elements that determine the affinity and specific binding for each receptor. The LBD confers ligand specificity of the specific NHR, whereas AF-1 and AF-2 domains mediate receptor interaction with specific co-regulators (17,18).

Members of this structurally related NHR superfamily can be subdivided into at least three classes. Class 1 includes the classical receptors for steroid hormones, such as the glucocorticoid receptor (GR), progesterone receptor (PR), estrogen receptor (ER), androgen receptor (AR) and mineralocorticoid receptor (MR). Class 1 receptors are bound to heat shock proteins and do not interact with promoter DNA independent of ligand. Upon ligand binding, this class of receptors binds directly and with high affinity to inverted repeats of DNA hormone response elements (HREs) as homodimers (19). The second class of NHRs bind non-steroid hormones, which include retinoic acid receptor (RAR), thyroid hormone receptor (TR), vitamin D receptor (VDR) and receptors that bind diverse lipophilic products such as fatty acids and prostaglandins [peroxisome proliferator-activated receptor (PPAR) and liver X receptor (LXR)]. These receptors bind to direct repeats as heterodimers with

another nuclear receptor, retinoid X receptor (RXR). This class of receptors is in the nucleus and bound to DNA in the absence of ligand (19). The other class of NHRs are the orphan receptors which include ER-related receptors, steroidogenic factor 1 (SF1), nerve growth factor-induced receptor (NGFI-B), X-linked orphan receptor-1 (DAX-1) and testis receptors (TR2 and R4) (20). These third classes of NHRs are transcription factors that have similar structure to other NHRs, but no known associated ligand. It is of interest to note that although classical canonical DNA sequences have been described for each class of NHRs, recent data from DNA microarray technology combined with chromatin immunoprecipitation (ChIP) assays have led to discovery of new NHR-regulated genes and global identification of DNA HREs divergent from the canonical HREs with respect to GR, ER and AR (21–24). Furthermore, studies with these receptors have highlighted binding sites at distances significantly greater than 10 kb downstream or upstream of transcription start sites, an observation that could change our view on how NHRs interact with chromatin to modulate different steps of transcription (25,26).

Nuclear receptors recruit both noncovalent and covalent chromatin modifying complexes to alter chromatin architecture and assemble basal transcriptional machinery at promoter regions of target genes (27,28). Among prominent NHR coregulators are the ATPase SWI/SNF family of chromatin remodeling complexes and histone-modifying complexes (27,28). Nuclear receptors work with diverse histone-modifying complexes as both coactivators and corepressors. The coactivator complexes are represented by histone acetyltransferases (HATs) and histone methyltransferases (HMTs), whereas corepressors include complexes such as NCoR/SMRT and Mi-2 NURD which contain histone deacetylases (HDACs) or CoREST which contains histone demethylase (HDM) activity (29–31).

A key characteristic of nuclear receptors is their ability to directly interact with DNA sequences within chromatin. Within eukaryotic nuclei, DNA is complexed with histones to form a dynamic polymer known as chromatin (32). The fundamental structural unit of chromatin is the nucleosome, which consists of approximately 146 bp of DNA wrapped around an octamer of histones containing two copies each of four core histone proteins: H2A, H2B, H3, and H4 (33). A fifth class of histone, the linker histone H1, associates with DNA between nucleosomes and may facilitate the formation of higher order chromatin organization such as chromatin fibers (34). The organization of DNA into chromatin *in vivo* generates regulatory constraints that have central roles in many cellular processes including transcription (35). Chromatin presents a significant physical barrier for regulatory proteins such as transcription factors to access DNA target sites (36). Unlike many other transcription factors, some NHRs are capable of binding to their HRE embedded in chromatin and to recruit multi-subunit enzyme complexes exhibiting various activities that enable them to modify chromatin via at least four mechanisms: 1. post-translational modifications such as acetylation, methylation and ubiquitylation of the amino terminal tails of histone (37–39); 2. changes in plasticity and fluidity of chromatin facilitated by ATP-dependent chromatin remodeling complexes which disrupt nucleosome structure (28); 3. reorganization of heterochromatin by enzymes that directly alter DNA such as DNA methyltransferases (40); 4. the ability to dynamically modulate hormone mediated transcriptional response by tight regulation of the transcriptional complexes and coordination of chromatin dynamics by the 26S proteasome (26,41). Together these activities enable efficient NHR-mediated gene transcription. In this review we will focus on the role of the 26S proteasome in nuclear hormone receptor-mediated gene transcription and discuss its dynamic function as a master regulator of transcriptional complexes and chromatin dynamics.

26S Proteasome and Transcription

Although the proteasome was initially perceived as a graveyard for proteins, recent advances in molecular biology have redefined its role as a regulatory system that influences the fate of many cellular processes including transcription (42,43). A view of the 26S proteasome as a “static garbage disposal unit for cellular waste” presents a tremendous problem to understanding how this complex regulates transcription. To understand the role of the proteasome in NHR-regulated transcription, we first examine the historical connection between the proteasome and transcription.

Historical ties between proteasome and transcription—The 26S proteasome and transcription are intimately linked historically. Critical subunits of the 26S proteasome were identified as transcriptional regulators before being classified as proteasomal. In the early 1990s, Johnston and colleagues using yeast genetics found that the 19S base ATPase subunit Rpt6/SUG1 (PSMC6) was required for GAL4-mediated transcriptional activation (44). Following these experiments, SUG1 was identified as a part of a mediator complex that was able to activate transcription, increase basal transcription and facilitate efficient phosphorylation of RNA Pol II by TFIIF-associated C-terminal domain kinase (45). Using the yeast two-hybrid system, additional experiments within this period identified a human transcriptional mediator that interacted with both the thyroid hormone receptor and RXR (46). The authors showed that this mediator TRIP1 (Thyroid Hormone Receptor Interacting Protein 1) could substitute for SUG1 to activate GAL4 transcription in yeast. Additionally, human HIV-TAT binding protein and MSS1 (mammalian suppressor of CDC28/G cyclins), proteins that closely resemble SUG1 and were later identified as proteasome subunits PSMC3/Rpt5 and PSMC2/Rpt1 respectively, were shown to modulate HIV-Tat mediated transcription (47,48). These studies implied that the 19S ATPases could form a complex with RNA Pol II holoenzyme and act as transcriptional co-regulators, facilitating GAL4 and THR activator-mediated transcription independent of the 26S proteasome multi-enzyme complex. However, in the mid 1990s other studies identified SUG1 as a component of the 26S proteasome, a protease involved in ATP-dependent degradation of ubiquitinated proteins, disputing the classification of SUG1 as a component of the RNA Pol II holoenzyme (49). These data suggest that SUG1 function in transcription is indirect and is dependent on proteolysis by the 26S proteasome holoenzyme, a theme supported by others (49,50). The controversy was fueled by another study arguing that SUG1 was not a component of the proteasome, but a component of the RNA Pol II machinery, since there was a direct interaction shown between SUG1 and the TATA binding protein, a key component of the TFIID basal transcriptional machinery. This suggests that SUG1 ATPase activity could mediate its effects on transcription (51). To date, involvement of the 26S proteasome in transcription by proteolytic and non-proteolytic mechanisms remains controversial and has recently gained more interest (43,52–54). Two models can be postulated for delineating the role of the proteasome in nuclear hormone receptor-mediated gene transcription.

Proteolytic activities of the proteasome are important in the control of transcription factors, their coregulators and RNA Pol II transcriptional machinery (53,55). The dynamic exchange of these factors on chromatin maintains multiple rounds of transcription (56). **Non-proteolytic activities** of the proteasome are important for co-activator recruitment and assembly of the pre-initiation complex (PIC) to initiate transcription, stimulate transcriptional elongation and termination and mediate chromatin modifications that modulate RNA Pol II transcription efficiency (42,57,58). To date most studies linking the proteasome to transcription have been done in yeast, with less known about 26S proteasome and transcription in mammalian cells. A major caveat of mammalian studies is the lack of discrete model systems as available by yeast genetics for particular proteasome subunits (52,53). Using the NHRs as prototypical transcriptional factors we will review what is

currently known about the proteasome and receptor-mediated transcriptional regulation (26,42,43,54). To do this most effectively, we will present two models based on recent published and unpublished information. Consequently they are necessarily incomplete, and represent a “*working hypothesis*” of the mechanisms that underlie this remarkable collaboration of what were once thought to be quite disparate cellular systems.

Model 1: Proteolytic activity of the proteasome is required for hormone receptor-mediated transcription

The first model argues that proteolytic activity of the 26S proteasome is required for NHR-mediated transcription. This model posits that the proteolytic activities of the 26S proteasome are required for limiting hormone response via control of the levels of NHRs and their coregulators and disassembling RNA Pol II PICs to tightly regulate hormone response (59,60). Indeed most hormone receptors including GR, ER, PR, TR, RAR and VDR undergo ligand-dependent proteasome-mediated proteolysis providing a direct link between NHR-mediated gene transcription and proteolytic activities of the 26S proteasome (55,61). Proteasomal degradation is required for the dynamic exchange of receptors, coregulators and transcriptional complexes for transcription to proceed efficiently (31,56). In line with this model, several years ago, it was shown that the estrogen receptor (ER α) is degraded via the proteasome and that blocking proteolysis with the synthetic proteasome inhibitor MG132 attenuates ligand-dependent transactivation despite an increase in ER α protein levels (62,63). A combination of fluorescence recovery after photobleaching (FRAP) and ChIP assays demonstrated that proteolytic activity of the proteasome controls the dynamic exchange between receptor, receptor coregulators, RNA Pol II complexes and DNA template to regulate ER-mediated transcriptional activity (64). Similar to ER, inhibiting proteasome activity suppresses AR- and PR-mediated expression of specific genes (65,66). However, in contrast, to ER, work from our laboratory and other laboratories showed that blocking proteasomal activity did not inhibit GR-mediated gene activation (67,68). Furthermore, inhibiting proteolysis blocked GR mobility and immobilized GR within the nuclear matrix, but this was correlated with increased transcriptional output, suggesting a multilayered interaction of proteasome with the GR and its subsequent transcriptional regulation (69,70).

Indeed the differential mechanisms by which proteasome activity modulates transcription by ER and GR suggest a surprisingly sophisticated relationship between the 26S proteasome and the NHR superfamily. Since previous experiments only examined a limited number of gene targets, we used a genomic approach to examine the impact of proteasome activity on GR- and ER-mediated gene expression in MCF-7 breast cancer cells treated with dexamethasone or 17 β -estradiol or in combination with MG132. Transcript profiling revealed that in fact the two receptors are not different in their requirement for proteolytic activity of the proteasome. Similar to ER, several endogenous GR target genes are downregulated after proteasome inhibition, whereas many ER endogenous targets are upregulated after proteasome inhibition, suggesting the requirement for proteolytic activity is gene specific (71). Many questions remain to be answered on the mechanisms that render proteolysis to be gene specific (Figures 1 and 2).

Model 2: Direct involvement of specific proteasome subunits in transcription: Non-proteolytic effects of the proteasome in transcription

Our genome wide experiments suggest a second model for mechanisms that point towards how 26S proteasome regulates gene transcription. There is an increasing body of evidence suggesting that the 26S proteasome can modulate transcription via non-proteolytic mechanisms. In eukaryotes, transcription is mechanistically divided into distinct steps; PIC

formation and initiation, elongation and termination (72,73). In yeast, proteasome subunits have been implicated in each step of transcription (74).

ChIP analysis: Specific proteasome subunits independently associate with DNA sequences: Proteasome subunits interact with chromatin

In yeast, numerous ChIP experiments have demonstrated that 19S ATPases are recruited to transcriptionally active genes both at promoters and transcribed regions suggesting that these ATPases function in transcriptional initiation and elongation (75–77). Furthermore, genome-wide ChIP analyses have revealed that several yeast genes associate with either the 19S RP or the 20S CP subunits, but not both, suggesting the subunits may function independent of one another and of the intact 26S proteasome catalytic complex (77,78). Indeed, whereas the 19S ATPases are localized throughout the gene and are involved in transcriptional initiation and elongation, the 20S associates mainly with the 3' end of genes and is implicated in transcriptional termination (74,79). It is not entirely clear whether differential distribution of proteasome subunits on genes denotes a non-proteolytic function of the proteasome, although some experiments have attempted to answer this question. For example, the 19S lid and base subunits as well as the 20S subunits have been detected on the CDC20 gene in budding yeast (13). However, mutants of the 20S subunit exhibit defects in proteolysis, but do not affect CDC20 gene transcription, whereas mutations in the 19S subunits have severe defects in CDC20 expression, arguing that the 19S proteasome acts in a proteolytic-independent manner to modulate CDC20 expression (13).

Consistent with the observations in yeast, proteasome components associate with NHR-regulated genes in human cells. At the pS2 promoter, ChIP and re-ChIP experiments show that TRIP1/SUG1 is in the same complex with ER, RNA Pol II and the basal transcription factors TBP and TFIID, supporting a role of the 19S RP in transcriptional initiation (64,75). The 19S ATPase subunit TRIP1/SUG1, but not the 20S PSMA2/ α 2 subunit, is recruited to the pS2 promoter in a ligand-dependent manner, further supporting a role for SUG1 in transcriptional initiation (64). Similar experiments have shown that the 19S ATPase proteins independent of 20S (APIS) complex is recruited to the AR-responsive prostate antigen promoter (66). Since the temporal recruitment of the 19S RP and receptor correlated with transcription, whereas inhibiting the proteolytic activity retains the 19S RP at the promoter and inhibits transcription of the target gene, the authors proposed that the 19S RP is required as a chaperone to mediate the turnover of the receptor/transcriptional complex by the 26S proteasome to allow multiple rounds of transcription (64,66).

The main dilemma presented by current studies is the inability to distinguish between proteolytic and non-proteolytic mechanisms of the proteasome in NHR-regulated gene transcription. Based on ChIP assays it has been proposed that the differential interaction of the 19S and 20S proteasome subunits with NHR target genes implies mechanisms of regulating transcription that are independent of proteolysis referred to as non-proteolytic. For example, although degradation of receptor complexes is attributed to the presence of the 19S RP on the pS2 promoter, there is no 20S on the promoter, suggesting an independent role of the 19S RP. It seems most likely that the 19S RP may function in transcription at least in two distinct ways: one as a chaperone complex directly interacting with transcriptional activators and the other as an adaptor dedicated to feeding the 20S proteasome.

While there is tremendous evidence supporting a role for the 19S RP in transcription, much less is known about the interaction of the 20S CP with DNA, primarily due to a lack of good antibodies for many 20S subunits. Genome wide ChIP experiments in yeast have shown differential interactions of the 19S and 20S subunits with yeast chromatin. This interaction is gene specific in that some genes exhibit the 19S exclusively at the promoter and the 20S at

the 3' end, whereas other genes have the two subunits throughout the gene (77,78). Perhaps because of the issues of antibodies there is very little known of the role of the 20S CP in NHR-mediated transcription. A recent study has shown that the 20S subunit low molecular mass polypeptide LMP2/PSMD9 directly interacts with the steroid hormone receptor coactivator SRC-1 to modulate ER-mediated transcription (80). ChIP analysis showed that LMP2 is recruited to the entire pS2 sequence, indicating a function at all steps of transcription. Importantly, this study showed that the 20S subunit is required for the elongation step of transcription, since re-ChIP experiments show that LMP2 is within the same complex with elongin A, an elongation factor. The authors also show that over expression of LMP2 did not have an effect on the stability of SRC-1 or other coactivators. Despite this observation, the authors concluded that the effects of LMP2 on ER-mediated transactivation were dependent on proteolytic activity since inhibiting the proteasome with MG132 blocked LMP2 from activating transcription from an ERE-Luc reporter gene (80). Alternatively, LMP2 might have non-proteolytic function in this context, since others have demonstrated that MG132 inhibits translation of the Luc gene reporter and the authors did not monitor endogenous pS2 expression after treatment with proteasome inhibitor (69,81). Interestingly, LMP2 is an immunoproteasome subunit which replaces the constitutive proteasome to generate antigenic peptides presented to MHC class I molecules. It is not entirely clear how LMP2 would regulate ER degradation if in fact this is the mechanism of LMP2-mediated regulation of ER response. The correlation between ER and the LMP2 20S subunit is also demonstrated by other experiments that show PA28 γ (REG γ or PSME3), an 11S complex that replaces the 19S regulator and activates the 20S CP during immune-presentation, interacts with and actively degrades the ER coactivator SRC3 (82).

Recent studies have shown that proteasome subunits are differentially distributed on a GR-activated gene (83). Chemically inhibiting proteolytic activity of the proteasome leads to an increase in GR-mediated MMTV transcription (67,83). Concomitant to this increase, the 19S APIS complex is depleted from the promoter and the coding region of the gene, while the 20S complex is increased particularly at the coding region of the gene (83). Furthermore, association of the 20S with MMTV DNA at the coding region correlates with both hormone-dependent and -independent transcription (83). Additionally, depleting SUG1 augments hormone-activated transcription with no effect on basal transcription, but depleting the 20S subunit PSMA3 enhances both basal and hormone activated transcription (83). Consistent with this observation, depleting either the 19S (SUG1) or the 20S (PSMA3) fails to block ligand-dependent degradation of GR (83). Taken together, these experiments reveal differential function of specific subunits of the 26S proteasome in GR-mediated transcription. Further studies examining the differential association of the proteasome subunits with the GR-regulated chromatin loci may offer mechanistic distinctions between proteolytic or non-proteolytic mechanisms employed in proteasome regulated hormone responses. The non-proteolytic mechanism, implied by our recent data, is supported by the *in vitro* transcription assays using HeLa cell nuclear extracts that show that depleting the 19S ATPases significantly alters activated, but not basal transcription and that this effect is not dependent on proteolysis (12).

A chaperone activity of the 19S ATPases has been suggested as a non-proteolytic mechanism in transcription. For the purpose of this discussion, a chaperone is a molecule able to change the transcriptional dynamics of an activator or coregulator (84,85). In yeast the 19S RP stabilizes the GAL4 activator and stimulates SAGA/GCN5 activity in an ATPase-dependent manner at the GAL1-GAL10 promoters. Earlier pioneer studies established that mouse and human 19S ATPases could interact with and act as transcriptional activators for a number of nuclear receptors including TR, RAR/RXR and VDR, suggesting a role of the ATPases in NHR-mediated transcriptional initiation (46,86). Recent experiments provide additional support for a chaperone role for the 19S RP in NHR-

mediated gene transcription. Specifically, TAT-Binding Protein (TBP-1/PSMC3), an ATPase subunit of the 19S was shown to enhance AR-dependent transcription in conjunction with TBP-1 interacting protein (TBPIP (87). ChIP experiments showed both TBP-1 and AR associate with the PSA promoter and correlate with transcription, but over expression of TBP-1 did not change AR protein levels (87). In similar experiments, subunits of the 19S RP, TBP-1, TRIP1/SUG1/PSMC5 and RPN10, increase SRC-1 driven transcription of TR-responsive promoters without changes in TR protein levels (88). At RAR target genes, SUG1 interacts with SRC3 coactivator, acting as a chaperone to assemble transcriptional complexes and to increase RAR-mediated gene transcription, similar to what was shown for GAL4/SAGA/GCN5 activity (58,89). The authors also demonstrate the proteolytic role of this complex, since over expressing SUG1 promotes SRC3 degradation and represses RAR-mediated gene transcription implying a proteolytic and non-proteolytic function for SUG1 in RAR-mediated gene transcription (89).

Proteasome modulates transcriptional elongation and termination: Regulation of RNA Pol II phosphorylation by the proteasome

While much attention has focused on effects of the 26S proteasome on transcriptional activation and activators, the 26S proteasome is implicated in multiple steps of transcription. In yeast, the 19S ATPases stimulate elongation and SUG1 mutants have defects in elongation *in vivo*, whereas in *in vitro* transcription assays, immunodepletion of the 19S complex leads to aberrant synthesis of long transcripts, which is a hall mark of transcriptional elongation defects (12). At nuclear receptor regulated genes, 19S ATPases associate with gene promoters, but currently there is no clear evidence how they participate in NHR-mediated transcriptional elongation (64,66,83).

In yeast, the 20S proteasome components associate with 3' regions of genes and the appearance of 20S on genes correlates with accumulation of transcripts (74). Furthermore, the 20S proteasome interacts directly with RNA Pol II and inhibiting proteolytic activity increases read through of a transcription termination site (74). Analogous to the studies in yeast, the 20S components are implicated in the elongation and termination steps of transcription in NHR-mediated transcription in human cells. On the ER-responsive pS2 gene, the 20S subunit LMP2 associates with the coding region and is shown to associate with the termination site (80). At these sites LMP2 is within the same complex with elongin A and RNA Pol II, and depleting LMP2 with siRNA disrupts these associations suggesting that these factors cooperate to enhance pS2 elongation.

In the case of GR, the association of the 20S subunit with the coding region at GR-regulated genes correlates with increased transcription (83). In addition, inhibiting proteolytic activity further increases the association of 20S concomitantly with phosphorylated RNA Pol II, further establishing a role for this subunit in transcriptional elongation (83). Phosphorylation of the carboxy terminus 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 (90). 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 which leads to differential gene expression (91). Mechanistic details on how the 20S coordinates and regulates RNA Pol II phosphorylation to facilitate elongation and other processes that determine the final gene transcript are not clear, but should be a priority in the future.

The 26S proteasome and chromatin plasticity

Within the eukaryotic cell, the transcription template is organized into chromatin. The fundamental unit of chromatin, the nucleosome, is a DNA/histone complex that acts as a barrier to transcriptional processes, generally occluding transcription factors from their

binding sites (35,92). Nuclear receptors are one class of transcriptional factors that recruit various chromatin remodeling activities to access DNA sites within chromatin in order to regulate transcription (56,93,94). Chromatin remodeling is a critical step for nuclear receptor-mediated transcriptional activation from chromatin templates (reviewed in (28)). Transcriptional activation by NHRs involves alteration of chromatin structure by ATP-dependent chromatin remodeling enzymes in conjunction with histone-modifying enzymes (28,95). The effect of NHR-mediated transcription on chromatin structure is determined by changes in accessibility of nucleosomal DNA within target promoters, which is facilitated by the SWI/SNF complex, an ATP-dependent chromatin remodeling complex, and post-translational modifications of histones such as acetylation, phosphorylation, methylation and ubiquitination (28,31,39,95). Changes in chromatin architecture driven by different enzymatic activities present a highly dynamic epigenetic landscape that allows complex regulation of NHR-mediated gene transcription (26,28,41).

Proteasome and histone modifications—An important question is whether the 26S proteasome is involved in establishing this dynamic chromatin state. Proteasome components have been tied to histone modifications (26,52). In yeast the 19S ATPases are necessary for methylation of histone H3 lysine 4 and 79 although such correlation has not been shown in mammalian cells (57). Furthermore, SUG1 has been shown to enhance SAGA/GCN5 dependent acetylation on GAL4 driven promoters (58). There is not a known connection between the 26S proteasome and histone modifications in NHR-mediated transcription. However, a recent study has shown that SUG1 interacts with SRC3 physically and on DNA as shown by reciprocal chromatin immunoprecipitation experiments to enhance RAR-mediated transcription (89). The authors did not measure histone acetylation, although SRC3 in conjunction with other coregulators such p300/CBP and GCN5 possess histone acetyltransferase activity specific for H3 and H4 (96). A similar study has shown that the 20S subunit LMP2 interacts with SRC-1 to mediate ER-mediated transcription (80).

Although the mechanisms are not well understood, recent studies imply a connection between histone modifications and the 26S proteasome (83). Inhibiting proteolytic activity of the proteasome results in a global increase of tri-methyl H3-K4 levels and an increase of this mark on GR-regulated promoters. There is an increase in Mixed Lineage Leukemia (MLL) protein, concomitant with the increase in H3-K4 tri-methylation levels, suggesting that perhaps H3-K4 methylation increases due to stabilization of the respective histone methyltransferase. However, knockdown of either 19S (SUG1) or 20S (PSMA3) resulted in a hormone-dependent increase in MLL mRNA expression, but not SMYD3, another putative HMT for H3-K4 (83). Additionally, global transcript profiling indicates that many mRNA encoding histone methyltransferases and demethylases change due to proteasome inhibition (71). More work is needed to determine whether the proteasome can alter the chromatin architecture by modulating histone marks.

The Proteasome and Chromatin Accessibility

The proteasome regulates the SWI/SNF complex—Chromatin remodelling is a critical step in hormone receptor-mediated transcriptional regulation (28,56,92,94). We have demonstrated that the SWI/SNF complex plays a vital role in NHR-mediated transcriptional regulation. Brg-1 and a number of Brg-1-Associated Factors (BAFs) have been shown to be required for this tightly regulated process, and have also been shown to be regulated by the proteasome. This in turn attributes a role in NHR-mediated transcriptional regulation to the proteasome.

The link between the proteasome and SWI/SNF regulation was first made by Chen and Archer as they discovered that SWI/SNF subunit stoichiometry is regulated by the

proteasome (1). By generating a stable cell line expressing a mutant form of exogenous BAF57, they found that endogenous levels of BAF57 were diminished in a proteasome-dependent manner. Additionally, they found that BAF155 interacts with and stabilizes BAF57, thereby protecting it from proteasomal degradation. The stoichiometry of additional SWI/SNF subunits were later also found to be regulated by SRG3, the murine homolog of BAF155 (97). BAF47, BAF60a and Brg-1 were each found to be stabilized by SRG3 and thereby rescued from proteasomal degradation. As mentioned above, BAF57 directly interacts with ER and enhances transcriptional activation at the pS2 promoter suggesting that BAF57 may recruit the SWI/SNF complex to the promoter (98,99). If the proteasome did not regulate the levels of BAF57 within the cell, then the free, uncomplexed protein could behave as a dominant negative and prevent the recruitment of a fully complemented SWI/SNF complex to the promoter. Similarly, were it not for its regulation by the proteasome, the altered stoichiometry of BAF60a, which has been shown to interact with GR, might disrupt GR-mediated transcription (100). Additionally, similar dominant negative or inhibitory mechanisms may arise from the altered stoichiometry of BAF47, Brg-1 or other SWI/SNF complex subunits if their protein levels were not closely regulated by the proteasome. The proteasome therefore plays an important role in maintaining SWI/SNF subunit stoichiometry, which in turn is crucial for the proper regulation of NHR-mediated transcription.

Additional evidence that the proteasome regulates the SWI/SNF complex, particularly at the level of Brg-1, has been shown in embryonic stem (ES) cells (101). At the pluripotent stage, the proteasome prevents binding of transcription factors and RNA Polymerase II and proteasome inhibition has been shown to increase levels of intergenic transcription in ES cells (101). This data suggests that the proteasome may play a role in suppressing the formation of PICs. One subunit that is known to be crucial in PIC assembly is Brg-1. In fact, in ES cells and mature B cells, binding of Brg-1 to specific gene loci increased upon proteasome inhibition (101). Therefore, the role of Brg-1 in PIC formation and thus transcriptional initiation in ES cells is dependent on proteasome activity. Interestingly, proteasomal inhibition experiments revealed that the increased binding of Brg-1 was not due to an increase in Brg-1 protein levels (101). Nonetheless, the fact that Brg-1 binding is limited by proteasomal activity suggests that the proteasome may play a role in controlling the levels of active chromatin in ES cells (101).

The catalytic activity of the proteasome has also recently been found to govern the actions of Brg-1 and SWI/SNF chromatin remodelling in the regulation of inflammatory genes (102). It was shown that the proteasome plays a crucial role in controlling both the extent and duration of inflammatory responses by controlling the proteasomal degradation of Brg-1 and thus regulating chromatin accessibility at specific promoters. For example, proteasome activity influences the removal of Brg-1 from the IL-6 promoter, thereby restricting nucleosome remodelling, so as to prevent premature gene induction, limit persistent gene transcription and induce timely gene shut-off (102). It comes as no surprise, therefore, that inhibiting the proteasome resulted in unabated Brg-1 recruitment to the IL-6 promoter. This, in turn, led to uninterrupted IL-6 transcription as the chromatin remained in a remodelled and active state. These results define a mechanism, at least within immunoresponsive cells, by which the proteasome antagonizes Brg-1 and SWI/SNF function in order to “fine-tune” the kinetics of inflammatory responses (102).

Brg-1 is a critical regulator of GR-mediated transcriptional regulation (103,104). It would seem from above studies that the proteolytic activity of the proteasome controls SWI/SNF levels and thus this could influence nucleosomal accessibility. Our recent studies show that inhibiting proteolytic activity of the proteasome increases GR-mediated transcription, but chromatin accessibility does not change on the MMTV promoter (83). Compared with GR,

Brg-1 protein levels did not change significantly and proteasome inhibition did not increase hormone-dependent interaction between Brg-1 and MMTV DNA, although MMTV RNA increases under these conditions (83). From these experiments we conclude that the proteasome acts at a step downstream of promoter activation and different mechanisms function to alter chromatin dynamics and to regulate transcription independent of the proteolytic activity of the proteasome. In the case of GR, an increase in tri-methyl H3-K4 at the gene correlates with hormone-dependent transcription when the proteasomal proteolysis is inhibited. The question remains whether the changes in chromatin architecture under these conditions can be attributed to non-proteolytic mechanisms of the proteasome. Whereas the SWI/SNF complex is a bonafide chromatin remodeler, the proteasome ATPases possess ATPase and DNA-helicase activity which can break interactions between histones and DNA to enable subsequent modifications to chromatin (105). The 19S ATPases facilitate RNA Pol II elongation by interacting with elongation factors such as FACT (FACilitates Chromatin Transcription) (79). However, a recent study has shown that the 19S regulator (PA700) cannot remodel mononucleosomes assembled *in vitro* (106). More work is necessary to unravel the role of the proteasome in modulating chromatin architecture and the impact of such changes on NHR-mediated transcriptional regulation.

CONCLUSIONS and Future Questions

It is clear that the 26S proteasome regulates NHR-mediated transcription at multiple levels, but the mechanisms of this regulation remain less than fully realized. Consequently, while the link between proteolytic activities of the 26S proteasome in NHR-mediated transactivation is well established, important questions remain unanswered. Although earlier studies showed that some NHRs require the proteolytic activity of the proteasome for transactivation, this dogma is not all encompassing. For example, although proteasomal degradation is required for dis-assembly of receptor/RNA Pol II complexes, some genes do not require proteolytic activity of the proteasome (71,81). Determining the architectural differences in the promoter and enhancer regions of genes that require proteolysis, relative to those that do not, will be valuable in understanding why some, but not all, genes require the proteolytic activity of the proteasome for enhanced transcription. This could be especially important since promoter/enhancer architecture in most cases determines activator/cofactor dynamics and subsequent formation of different PIC complexes, which may be subject to degradation in a gene specific manner (64,70,71). Apart from transcription initiation, knowing how proteolytic activity contributes to NHR-mediated transcriptional elongation, termination and RNA processing remain intriguing areas of investigation. For example, how does RNA Pol II transition from the initiation to elongation competent forms when the proteasome is inhibited?

The non-proteolytic function of the proteasome in NHR-mediated transcriptional regulation remains similarly incompletely characterized, with many questions to be asked. With current genome wide techniques, a global localization of proteasome subunits in the mammalian genome is both necessary and urgently needed. As with above the questions are numerous: If proteasomes act as chaperones, how do they get to the gene loci with respect to NHR-regulated genes? Do proteasome subunits modulate chromatin architecture of NHR target genes? Do the 19S ATPases possess enzymatic activities that facilitate or modulate other enzymatic activities such as SWI/SNF chromatin remodeling activities at NHR target genes? Is there a connection between the proteasome and histone modifications in mammalian cells? How do the proteasome subunits and concomitant chromatin modifications facilitate RNA Pol II phosphorylation to coordinate the escape from initiation to productive elongation? Can the proteolytic and non-proteolytic activities of the proteasome be separated in mammalian cells? Given that proteasome subunits have several functional domains, do mutations in domains that affect degradation still have an effect on transcription? At present

a lack of genetic models make these questions difficult to answer. The net result is that the function and role(s) of the proteasome in NHR-mediated transcriptional regulation remains an important, interesting and provocative area of research.

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Figure 1. General mechanisms linking proteasome-mediated degradation and NHR-regulated transcription

Ligand bound nuclear hormone receptors (NHRs) bind directly to inactive chromatin (green nucleosomes) and **(1)** recruit chromatin remodeling complexes (SWI/SNF), histone acetyltransferases (HAT), histone deacetylases (HDAC), histone methyltransferases (HMTs), histone demethylases (HDM), the 26S proteasome (26S), specific proteasome subunits (19S) and ubiquitination machinery (UM). **(2)** Some of these cofactors modify chromatin by altering the accessibility of nucleosomal DNA (SWI/SNF) or by covalently modifying histone tails [HMT, arginine (R) methylation; HAT, acetylation (Ac)] to generate active chromatin (red nucleosomes) **(3)**. This facilitates the recruitment of RNA Pol II and general transcription factors/mediator (GTFs/MED) in the assembly of the pre-initiation complex (PIC) to activate transcription. **(4)** Subsequent to transcriptional initiation, NHRs and PIC complexes are rapidly degraded by the proteasome following promoter escape. This presumably allows the procession of elongation and re-assembly of transcriptional complexes, thus ensuring multiple rounds of transcription.

Figure 2. Proposed mechanisms for gene specific requirement for proteolytic activity of the 26S proteasome

(A) Gene is repressed after proteasome inhibition. Inhibiting proteolytic activity represses gene activation. Examples of genes that may represent this mechanism are , GR-regulated Galanin or ER-regulated SDF-1 (71). Ligand bound nuclear hormone receptors (NHRs) bind directly to chromatin and recruit various enzymatic complexes, represented as nuclear receptor cofactors [NRC, see Figure 1, for the different enzymatic activities]. There are two possibilities to explain gene repression when proteasome activity is inhibited: **(i)** gene activation requires proteolytic removal of a repressor complex. In this case, ubiquitin machinery (UM) targets a repressor protein for ubiquitination. The 19S as a part of general NHR coregulators recognizes the ubiquitinated repressor protein (19S is an ubiquitin receptor) and targets the repressor to 26S proteasome. The 19S ATPase acts as part of the 26S proteasome holoenzyme and chemical inhibition of the proteasome with MG132 blocks degradation of the repressor and the gene is repressed due to accumulation of the repressor at the promoter. **(ii)** Alternatively a productive PIC requires proteolytic degradation of activator/PIC complexes to release RNA Pol II from promoter (promoter escape) and to switch to productive elongation. Proteolytic activity is required to limit DNA/NHR/GTF contacts to inhibit unproductive PIC and nonproductive transcripts as well as reassembly of the transcriptional complexes to ensure multiple rounds of transcription. The 19S acts in concert with the 26S proteasome to recognize ubiquitinated RNA Pol II and RNA Pol II machinery including Mediator (MED) and GTFs as shown in Figure 1. All the events occur post-initiation and inhibiting proteasome activity represses transcription. Transcriptional activation or initiation is the rate-limiting step.

(B) Gene is activated after proteasome inhibition. Inhibiting proteolytic activity leads to gene activation, for example GR-regulated S100P or ER-regulated DDX10 (71). Activated receptor recruits a milieu of coregulators as in **(A)** including the 19S which acts as a chaperone for chromatin-modifying enzymes (HMTs/HATs) or ATP-dependent chromatin factors which modify chromatin (red nucleosomes) via specific histone modifications (ubiquitination, Ac-acetylation, K4/R-CH3- methylation) the 19S also acts to bridge the MED, GTF and RNA Pol II to activate transcription. The changes in chromatin structure, notably specific histone modifications mediated by specific 19S subunits, permit rapid assembly of a stable, high fidelity productive PIC.

Formation of a productive PIC promotes promoter escape and early elongation through phosphorylation of the RNA Pol II carboxy terminus domain (Ser5). The 19S ATPases may act as chaperones for the phosphorylated RNA Pol II (Ser 5) to help maintain chromatin architecture in a state that is conducive to continued elongation by recruiting HMTs, capping factors (CF) and elongation factors (ELF). As the elongating RNA Pol II (Ser 2) moves along more factors (splicing factors, SF) are recruited. The 20S may have similar chaperone activity for termination factors to move along with the phosphorylated RNA Pol II and promote increased read-through of termination sites to ensure increased gene transcription. Notably, a gene that does not require proteolytic activity for activation must rapidly assemble a productive PIC. Promoter architecture is the key to the rapid assembly of a productive PIC.