Ubiquitylation of nuclear receptors: new linkages and therapeutic implications

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Abstract

The nuclear receptor (NR) superfamily is a group of transcriptional regulators that control multiple aspects of both physiology and pathology and are broadly recognized as viable therapeutic targets. While receptor-modulating drugs have been successful in many cases, the discovery of new drug targets is still an active area of research, because resistance to NR-targeting therapies remains a significant clinical challenge. Many successful targeted therapies have harnessed the control of receptor activity by targeting events within the NR signaling pathway. In this review, we explore the role of NR ubiquitylation and discuss how the expanding roles of ubiquitin could be leveraged to identify additional entry points to control receptor function for future therapeutic development.

Key Words
- E3 ligase
- proteasome
- steroid
- NF-κB

Brief overview of nuclear receptor signaling and drug targets

Nuclear receptors (NRs) comprise a family of transcriptional regulators that control multiple physiological processes including growth, development, reproduction and metabolism through the control of gene expression (Di Croce et al. 1999, Aranda & Pascual 2001). The founding member of the family, estrogen receptor alpha (ERα), was identified via its high affinity binding to radiolabeled estradiol (E2) (Toft & Gorski 1966, Jensen et al. 1967). Following the cloning of the gene encoding the glucocorticoid receptor (GR, NR3C1; Miesfeld et al. 1984, Hollenberg et al. 1985), numerous other NRs were identified and combined into a superfamily composed of a total of 48 receptors in mammals, including ERα (NR3A1) and ERβ (NR3A2), thyroid hormone receptor (TR, NR1A1), progesterone receptor (PR, NR3C1), androgen receptor (AR, NR3C4), retinoic acid receptor (RAR, NR1B1–3), retinoic X receptor (RXR, NR2B1–3), vitamin D receptor (VDR, NR1I1), peroxisome proliferator-activated receptors (PPAR, NR1C1–3) and a number of orphan receptors with no known ligands (Evans & Mangelsdorf 2014). The receptors share a similar architecture consisting of an intrinsically disordered N-terminus, which in some receptors encodes a ligand-independent transactivation domain, a central DNA-binding domain containing two zinc finger motifs, and a C-terminal ligand-binding domain (LBD). The LBD mediates multiple receptor functions, including ligand binding, dimerization, co-regulator interactions and ligand-dependent transcriptional activation function. It is no surprise then that research has focused largely on the LBD and the modulation of receptor actions through both endogenous and synthetic ligands (Gronemeyer et al. 2004, McDonnell & Wardell 2010).
The dissection of the molecular events that regulate receptor function has greatly advanced the NR field and contributed significantly to the drug discovery toolbox. Originally, NRs were thought to participate in a relatively simple signal transduction pathway in which activated receptors directly mediated responses in the nucleus through direct DNA binding and transcriptional activation. Though fundamentally correct, the broadening knowledge of components in the NR-activation mechanism has greatly expanded the model and simultaneously expanded the opportunity to control receptor function. In the contemporary model, ligands bind to receptors in the cytoplasm or nucleus or, in some cases, to plasma-membrane-bound receptors. Ligand binding triggers a series of intracellular events, including the release of inactive receptors from heat shock protein (Hsp) complexes, changes to receptor protein conformation, mobilization, dimerization and recruitment of multi-protein transcriptional complexes. The activated NR transcriptional complexes include co-regulators (activators and repressors), chromatin modifying and remodeling complexes, and components of the basal transcriptional machinery. To date, over 300 NR co-regulators have been identified (Jung et al. 2005, Malovannaya et al. 2011; www.nursa.org). Ligand activation of membrane receptors couples receptor activation to intracellular signaling cascades (Hammes & Levin 2011). Additionally, NRs can be activated indirectly through ligand-independent mechanisms by growth factors. The complexity of NR function and regulation is further expanded by the addition of a temporal component to receptor transcriptional complexes (Métivier et al. 2003, Nagaich et al. 2004). Collectively, the elucidation of this activation cascade forms the basis for the identification of agents targeting receptors at multiple levels, including co-activator interactions (Norris et al. 1999, Parent et al. 2008, Gunther et al. 2009), dimerization, subcellular localization (Tran et al. 2009) and DNA binding (Wang et al. 2006, Mao et al. 2008, Andersen et al. 2010, Caboni & Lloyd 2013).


NR degradation by the ubiquitin–proteasome pathway

The first studies investigating the mechanisms of NR protein turnover pointed to the role of proteasomes and, subsequently, ubiquitination in targeting receptors to the degradation pathway. Multiple groups demonstrated that proteasome inhibitors disrupted estrogen-induced decreases in ERα protein levels (Alarid et al. 1999, El Khissiin & Leclercq 1999, Nawaz et al. 1999a). Subsequently, it was observed that RARγ2 and RARα were down-regulated in response to their ligand, all-trans retinoic acid, and the down-regulation was blocked by proteasome inhibitors, MG132 and lactacystin (Zhu et al. 1999, Kopf et al. 2000). The TR, GR and mineralocorticoid receptor (MR) were also found to be down-regulated in response to ligand binding via a similar pathway (Dace et al. 2000, Wallace & Cidlowski 2001, Yokota et al. 2004). These studies established the proteasome pathway as a key regulator of NR protein stability (Alarid et al. 2006; Fig. 1). One caveat, however, is that proteasome inhibitor studies also disrupt NR motility and transcription (Lonard et al. 2000, Reid et al. 2003, Elbi et al. 2004, Stavriva et al. 2004). Proteasome inhibitors can also affect NR gene expression and indirectly lead to downstream changes in NR target gene regulation (Powers et al. 2010, Prenzel et al. 2011). Further, the proteasome pathway is not selective for NRs, and inhibition of such a vital cellular function can lead to both inhibition and activation of other signaling pathways, production of reactive oxygen species and induction of apoptosis (Shinohara et al. 1996, Emanuele et al. 2002, Cirit et al. 2012). Recent studies also implicate lysosomes in the degradation of a number of NRs, including ERα, AR and GR (He et al. 2011, Totta et al. 2015).
Owing to the confounding effects of proteasome inhibitors and the complex regulation of NR protein stability, the study of NR proteolysis shifted to better understanding the role and mechanisms of ubiquitylation in targeting NRs to proteasome-mediated degradation.

Ubiquitin is a 76-residue protein that can modify target substrates by covalent attachment of its C-terminal carboxyl group to a lysine residue on the target substrate in a catalytic process involving three classes of enzymes (Hershko & Ciechanover 1998, Pickart 2001, Komander 2009). The first class of enzymes, known as E1 activating enzymes, bind ubiquitin through a catalytic cysteine residue in an ATP-dependent mechanism, creating a high-energy thioester bond. The E1 enzyme is loaded with a second ubiquitin molecule and then recruits the second class of enzymes, E2 conjugating enzymes. The E1-Ub complex then transfers the ubiquitin to a conserved catalytic cysteine residue of the E2 enzyme, forming a thioester-linked E2-Ub complex in a process known as transthioesterification (Lee & Schindelin 2008). Lastly, the third class of enzymes, known as E3 ubiquitin ligases, facilitates the transfer of ubiquitin from the E2 conjugating enzyme, directly or indirectly, to a lysine on the substrate forming an isopeptide bond. To date, two ubiquitin-specific E1 activating enzymes (UBA1 and UBA6), 35 E2 conjugating enzymes, and over 600 E3 ligases have been reported in humans (Bernassola et al. 2008, Deshaies & Joazeiro 2009, Markson et al. 2009, Schulman & Harper 2009, Ye & Rape 2009, van Wijk & Timmers 2010).

Substrate selectivity is primarily guided by E3 ubiquitin ligases, which belong to three subtypes: really interesting new gene (RING), homologous to E6-AP C-terminus (HECT) and RING-between-RING (RBR) (Berndsen & Wolberger 2014). These ligases are classified based on the corresponding motifs (RING, HECT and RBR) required for E3 activity as well as the distinct mechanisms involved. A RING E3 ligase can function as a monomer, dimer (homo or hetero), or multi-protein complex with the RING domain binding to specific E2s and a distinct region of the ligase (or ligase complex) binding to specific substrates. RING ligases promote the transfer of ubiquitin from ubiquitin-charged E2 without itself forming an intermediate thioester with a ubiquitin molecule. In contrast, HECT ligases accept ubiquitin from ubiquitin-charged E2s to its catalytic cysteine, which is then transferred to the substrate. RBR ligases have a combination of RING and HECT mechanisms whereby one of the RING domains binds to specific E2s and a distinct region of the ligase (or ligase complex) binding to specific substrates. RING ligases promote the transfer of ubiquitin from ubiquitin-charged E2 without itself forming an intermediate thioester with a ubiquitin molecule. In contrast, HECT ligases accept ubiquitin from ubiquitin-charged E2s to its catalytic cysteine, which is then transferred to the substrate. RBR ligases have a combination of RING and HECT mechanisms whereby one of the RING domains binds to specific E2s and the other contains a catalytic cysteine that accepts a ubiquitin from ubiquitin-charged E2s and then transfers it to substrates. Table 1 lists the ubiquitin E3 ligases that participate in NR ubiquitylation that are organized by NR types and in a general chronological order of discovery in NR regulation.

In the following sections, we highlight specific ligases that appear to have generalized activities on NRs and that are implicated in distinct aspects of NR signaling pathway with the goal of highlighting processes where interference of NR ubiquitylation may be leveraged as part of on-going drug development targeting the ubiquitin system.
Control of unliganded NR protein stability by carboxyl-terminus of Hsc70-interacting protein

The control of NRs by carboxyl-terminus of Hsc70-interacting protein (CHIP) represents specific ligase activity at early stages in NR signaling, including controlling basal NR expression and receptor availability prior to ligand binding. NRs are held stable in their unliganded state by chaperone complexes, which include Hsps, Hsp70 and Hsp90 (Smith & Toft 1993). The Hsp interaction guides appropriate folding of NR protein and stabilizes the ligand-binding pocket (Bresnick et al. 1989, Smith 1993, Stancato et al. 1996, Pratt 1997). Disruption of the Hsp90–NR complex using a chemical inhibitor, geldanamycin, was shown to cause down-regulation of ERs, PR, GR and AR in a proteasome-dependent manner (Whitesell & Cook 1996, Pratt & Toft 1997, Bagatt et al. 2001, Connell et al. 2001, Lee et al. 2002, Vanaja et al. 2002, Fan et al. 2005). CHIP is a RING E3 ligase that contains a tetratricopeptide repeat, which binds and interacts with numerous ligases, including CHIP, BARD1, BRCA1, and MDM2. It is involved in the regulation of a variety of cellular processes, including cell proliferation, differentiation, and apoptosis.

Table 1  E3 ligases involved in NR ubiquitylation

<table>
<thead>
<tr>
<th>Nuclear receptor</th>
<th>E3 ligase</th>
<th>Class of ligase</th>
<th>Type of Ub</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogen receptor alpha</td>
<td>CHIP</td>
<td>RING (U-box)</td>
<td>Poly</td>
<td>Nawaz et al. (1999b), Li et al. (2006), Sun et al. (2012) and Rajbhandari et al. (2014)</td>
</tr>
<tr>
<td></td>
<td>Mdm2</td>
<td>RING</td>
<td>Poly</td>
<td>Duong et al. (2007)</td>
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<tr>
<td></td>
<td>BRCA1/BARD1</td>
<td>RING</td>
<td>Mono</td>
<td>Eakin et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>EFP (TRIM25)</td>
<td>RING (Cullin)</td>
<td>Poly K48</td>
<td>Nakajima et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>SPOP</td>
<td>RING</td>
<td>Poly</td>
<td>Byun &amp; Jung (2008)</td>
</tr>
<tr>
<td></td>
<td>RBCK1</td>
<td>RING (RBR)</td>
<td>?</td>
<td>Gustafsson et al. (2010)</td>
</tr>
<tr>
<td></td>
<td>CUEDC2</td>
<td>?*</td>
<td>?</td>
<td>Pan et al. (2011)</td>
</tr>
<tr>
<td></td>
<td>Skp2</td>
<td>RING (F-box)</td>
<td>Poly</td>
<td>Bhatt et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>VHL</td>
<td>RING</td>
<td>Poly</td>
<td>Jung et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>NEDD4</td>
<td>HECT</td>
<td>Poly</td>
<td>Zhu et al. (2014)</td>
</tr>
<tr>
<td></td>
<td>RNF6</td>
<td>RING</td>
<td>Pol K48</td>
<td>Tateishi et al. (2006)</td>
</tr>
<tr>
<td></td>
<td>Siah2</td>
<td>RING</td>
<td>Poly</td>
<td>Picard et al. (2008)</td>
</tr>
<tr>
<td></td>
<td>UBR1</td>
<td>RING</td>
<td>Poly</td>
<td>Sanchez et al. (2013)</td>
</tr>
<tr>
<td>Glucocorticoid receptor</td>
<td>CHIP</td>
<td>RING (U-box)</td>
<td>Pol K6 or K27</td>
<td>Xu et al. (2009b)</td>
</tr>
<tr>
<td></td>
<td>Mdm2</td>
<td>RING</td>
<td>Pol K48</td>
<td>Qi et al. (2013)</td>
</tr>
<tr>
<td></td>
<td>NEDD4</td>
<td>HECT</td>
<td>Poly</td>
<td>Sultana et al. (2013)</td>
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<tr>
<td></td>
<td>RNF6</td>
<td>RING</td>
<td>Poly</td>
<td>Li et al. (2008)</td>
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<tr>
<td></td>
<td>Siah2</td>
<td>RING</td>
<td>Pol K48</td>
<td>Xie et al. (2013)</td>
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<tr>
<td></td>
<td>UBR1</td>
<td>RING</td>
<td>Poly</td>
<td>Li et al. (2014)</td>
</tr>
<tr>
<td>Progesterone receptor</td>
<td>CUEDC2</td>
<td>?*</td>
<td>?</td>
<td>Zhang et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>BRCA1/BARD1</td>
<td>RING</td>
<td>Poly</td>
<td>Calvo &amp; Beato (2011)</td>
</tr>
<tr>
<td>Retinoic acid receptor alpha</td>
<td>FLRF (Rnf41)</td>
<td>RING</td>
<td>?</td>
<td>Jing et al. (2008)</td>
</tr>
<tr>
<td>Retinoic X receptor</td>
<td>RNF8</td>
<td>RING</td>
<td>?</td>
<td>Takano et al. (2004)</td>
</tr>
<tr>
<td>Mineralocorticoid receptor</td>
<td>CHIP</td>
<td>RING (U-box)</td>
<td>Poly</td>
<td>Faresse et al. (2010)</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Siah2</td>
<td>RING</td>
<td>Poly</td>
<td>Kilroy et al. (2012)</td>
</tr>
<tr>
<td></td>
<td>MKRN1</td>
<td>RING</td>
<td>Poly</td>
<td>Kim et al. (2014)</td>
</tr>
<tr>
<td>Estrogen-related receptors</td>
<td>Parkin</td>
<td>RING (RBR)</td>
<td>Poly</td>
<td>Ren et al. (2011)</td>
</tr>
</tbody>
</table>

BARD1, BRCA1-associated RING domain protein 1; BRCA1, breast cancer type 1 susceptibility protein; CHIP, carboxyl terminus of Hsc70-interacting protein; CUEDC2, CUE domain containing 2; E6AP, E6-associated protein; EFP, estrogen-responsive finger protein; FBXW7, F-box/WD repeat-containing protein 7; FLRF, fetal liver ring finger; Hdm2, human double minute 2; HECT, homologous to the E6-AP C-terminus; Mdm2, mouse double minute 2; MKRN1, makorin ring finger protein 1; NEDD4, neural precursor cell expressed, developmentally down-regulated 4; PPARγ, peroxisome proliferator-activated receptor gamma; RBCK1, RanBP-type and C3HC4-type zinc finger containing 1; RING, really interesting new gene; RNF6, ring finger protein 6; RNF8, ring finger protein 8; RNF31, ring finger protein 31; Rnf41, ring finger protein 41; Siah2, seven in absentia homolog 2; Skp2, 5-phase kinase-associated protein 2; SPOP, speckle-type POZ protein; Ub, ubiquitin; UBR1, ubiquitin protein ligase E3 component N-recognition 1; VHL, Von Hippel-Lindau tumor suppressor.

*CUEDC2 is a CUE domain-containing gene that promotes the degradation of PR and ERs through ubiquitylation, see references.
Regulated NR ubiquitylation by phosphorylation and coactivator complexes

Beyond control of basal NR protein levels, ligand-induced turnover of NRs revealed additional layers of complexity in E3 ligase action in NR signaling. As described above, ligand binding to NRs triggers a series of events associated with the transcriptional activation mechanism, including phosphorylation and the recruitment of multi-protein transcriptional complexes. Ubiquitylation is integrated within this activation mechanism through both phosphorylation and the protein complexes recruited to NRs (Fig. 1). For example, the RING E3 ligase mouse double minute 2 (Mdm2) is implicated in the turnover of many NRs, including AR, ERα, ERβ and GR (Table 1, references therein). The targeting of NRs for ubiquitylation by Mdm2 is triggered by at least two levels of regulation, NR phosphorylation and the composition of the NR transcriptional complex. In the case of AR, phosphorylation of AR on Ser515 by cyclin-dependent kinase 7 (Cdk7), a component of the basal transcriptional machinery, is critical for recruitment of Mdm2 and the subsequent ubiquitylation and degradation of AR by the proteasome (Chymkowitch et al. 2011). Ubiquitylation of AR by Mdm2 can also be signaled following phosphorylation of AR by Akt on Ser210 and Ser790 (Lin et al. 2002). Mutation of Ser210 and Ser790, or Ser515, to alanine prevents recruitment of Mdm2 and ubiquitylation of AR. Like AR, Mdm2 is also recruited to ERα and ERβ complexes when the corresponding ER is phosphorylated (Valley et al. 2005, Picard et al. 2008, Sanchez et al. 2013). However, degradation of ERα upon Mdm2 over-expression provides an example in which specific NR protein complexes are also a requirement for this response – in this case, a complex with p53 (Duong et al. 2007). Similarly, GR degradation following dexamethasone treatment involves the formation of a GR complex containing p53 and Hdm2 (Sengupta & Wasylyk 2001). In the case of ERβ, Mdm2 works in concert with a different coregulator, CREB-binding protein (CBP), to form a complex that results in ubiquitylation and ultimate degradation of ERβ (Sanchez et al. 2013). Interestingly, unlike ERβ, the Mdm2–CBP complex was unable to target ERα for degradation. These observations suggest that Mdm2 is recruited to NRs as part of larger multi-protein complexes that impart specificity of Mdm2 action in controlling ubiquitylation and degradation. Given that receptor complexes are dynamic (Métivier et al. 2003), this protein complex specificity, in addition to phosphorylation events, could impart temporal and context-specific regulation on the NR ubiquitylation, stability and associated functions.

Some E3 ligases control NR function through both ligase activity-dependent and -independent mechanisms. A primary example of dual-action E3 ligases is E6-associated protein (E6AP). The first studies to demonstrate ubiquitylation of endogenous NRs were done on ERα (Wijayaratne & McDonnell 2001). Subsequently, E6AP was found to be recruited to ERα in a calmodulin-dependent manner, leading to ubiquitylation and degradation of ERα (Li et al. 2006). In addition to calmodulin-dependent ubiquitylation, recruitment of E6AP to ERα as well as ERβ requires phosphorylation of the receptor (Picard et al. 2008, Rajbhandari et al. 2014). Consistent with the ligase function of E6AP, mammary and prostate glands of E6AP-null mice show elevated levels of ERα (Gao et al. 2005). However, E6AP also functions as a coactivator for ERα, as well as other NRs such as PR, AR and GR (Nawaz et al. 1999b, Ramamoorthy & Nawaz 2008). In these cases, the disruption of ubiquitin ligase activity as well as the HECT domain of E6AP by mutagenesis had no effect on NR coactivating activity (Nawaz et al. 1999b). Similar ligase-independent coregulator function has been noted in NR regulation by other HECT ligases, including NEDD4-1, Rsp5 and HACE1. For example, HACE1 was identified as an NR-interacting partner in a yeast two-hybrid screen, and shown to interact with RARx, RARy, ERα and TRα. Mutation of critical cysteine residues in the HECT ligase domain had no effect on its transcriptional repressor activity toward RARs (Zhao et al. 2009). Likewise, the ligase activity of Rsp5 is not essential for Rsp5...
ubiquitylation of Skp2, which is implicated in cancer progression through loss of cell-cycle control, transcriptional regulation, apoptosis and genomic stability (Deng 2006, Roy 2000). For example, attachment of a single ubiquitin molecule (monoubiquitylation) on NRs by BRCA1 has been described. BRCA1, along with its partner BARD1, form a heterodimeric RING E3 ligase implicated in numerous cellular processes including DNA repair, cell cycle control, transcriptional regulation, apoptosis and genomic stability (Deng 2006, Roy et al. 2012). BRCA1 ubiquitylation of both ERα and PR contributes to their transcriptional function (Eakin et al. 2007, Calvo & Beato 2011). BRCA1/BARD1 monoubiquitylates ERα in vitro and in vivo (Eakin et al. 2007, Dizin & Irminger-Finger 2010, Ma et al. 2010, Zhu et al. 2014). This monoubiquitylation is dependent on BRCA1/BARD1 ligase activity as cancer predisposing BRCA1 mutations (C61G and C64G) affecting the ligase activity abolish the ability of BRCA1 to monoubiquitylate ERα. The site of monoubiquitylation on ERα was identified through mass spectrometry to be K302; however, the K302A ERα mutant was still monoubiquitylated in vitro. The adjacent lysine residue, K303, can be targeted for monoubiquitylation in lieu of K302 (Eakin et al. 2007). The precise function of ERα monoubiquitylation by BRCA1/BARD1 in vivo is still unclear, although it is hypothesized to play a role in inhibition of cell-cycle control and transcription (Bloom & Pagano 2003, Kamata et al. 2005, Davidovich et al. 2008). Skp2 ligase activity was shown to be dependent on E2-induced phosphorylation, leading to ubiquitylation of p27kip1 (Lecanda et al. 2007, Huang et al. 2012). The loss of nuclear p27kip1 has been shown to occur in E2-induced type 1 endometrial carcinogenesis (Lecanda et al. 2007). Using a small molecule screen, specific agents have been identified that block Skp2-dependent ubiquitylation of p27kip1, thus preventing its degradation. Treatment of E2-induced endometrial carcinoma cell lines with these small molecules resulted in increased levels of p27kip1 along with decreased proliferation (Pavlides et al. 2013). These experiments demonstrate that alterations of E3 ligase activity using small molecule inhibitors could be a viable strategy for future therapeutic development. This possibility is further supported by a recent report of peptide and small molecule inhibitors of HECT ligases (Mund et al. 2014).

To date, three E3-targeting drugs have been approved by the FDA, and all three target the same enzyme, cereblon (CRBN). CRBN is a part of the Cul4-Rbx1-DDB1-CRBN RING ubiquitin E3 ligase complex, and the three drugs that target CRBN – thalidomide, lenalidomide and pomalidomide, commonly referred to as immunomodulatory drugs – bind to CRBN and promote the recruitment of substrates, including Ikaros (IKZF1) and Aiolos (IKZF3), which are subsequently ubiquitylated and degraded (Ito et al. 2010, Chamberlain et al. 2014, Fischer et al. 2014, Lu et al. 2014). Currently these drugs are approved for multiple myeloma therapy (Martiniani et al. 2012, Terpos et al. 2013).

Current ubiquitin-targeting therapeutics

The examples provided above indicate the potential for targeting multiple steps (protein folding, coactivator interactions and transcriptional function) in the NR signaling pathway via control of ubiquitylation. Clinical approaches in cancer therapy have thus far focused on inhibiting the 26S proteasome (Teicher et al. 1999). Bortezomib (Velcade, PS-341) is a general proteasome inhibitor that is FDA approved for the treatment of multiple myeloma and mantle cell lymphoma. Second-generation proteasome inhibitors have also been developed, including carfilzomib, which was approved in 2012 for multiple myeloma patients that are refractory to bortezomib therapy (Mitsiades et al. 2011). While the preclinical data supports the efficacy of proteasome inhibitors in other cancer types, the results outside of hematological malignancies have been disappointing (Yang et al. 2006). Hence, efforts are underway to more specifically target the ubiquitylation machinery and their substrates.

A glimpse into the relevance to the NR field is provided by studies of Skp1-Cullin1-F-box (SCF)–Skp2 and p27kip1. Skp2 is an F-box protein and component of the SCF RING ubiquitin E3 ligase complex. Skp2 ligase ubiquitylates and degrades ERα and a high Skp2 expression in human tumors correlates with an ERα-negative status (Bhatt et al. 2012). Skp2 is overexpressed in human cancers, and deregulation of Skp2 is implicated in cancer progression through loss of cell-cycle control and transcription (Bloom & Pagano 2003, Kamata et al. 2005, Davidovich et al. 2008). Skp2 ligase activity was shown to be dependent on E2-induced phosphorylation, leading to ubiquitylation of p27kip1 (Lecanda et al. 2007, Huang et al. 2012). The loss of nuclear p27kip1 has been shown to occur in E2-induced type 1 endometrial carcinogenesis (Lecanda et al. 2007). Using a small molecule screen, specific agents have been identified that block Skp2-dependent ubiquitylation of p27kip1, thus preventing its degradation. Treatment of E2-induced endometrial carcinoma cell lines with these small molecules resulted in increased levels of p27kip1 along with decreased proliferation (Pavlides et al. 2013). These experiments demonstrate that alterations of E3 ligase activity using small molecule inhibitors could be a viable strategy for future therapeutic development. This possibility is further supported by a recent report of peptide and small molecule inhibitors of HECT ligases (Mund et al. 2014).

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Regulation of NR function by E3 ligase-mediated monoubiquitylation

While the focus in the NR field and therapeutic approaches has largely been directed to the role of ubiquitin in degradation and stability of receptors, the scope of the ubiquitin field extends well beyond degradative mechanisms associated with the proteasome pathway that requires the attachment of a ubiquitin polymer (or chain) with four or more ubiquitin moieties (Thrower et al. 2000). For example, attachment of a single ubiquitin molecule (monoubiquitylation) on NRs by BRCA1 has been described. BRCA1, along with its partner BARD1, form a heterodimeric RING E3 ligase implicated in numerous cellular processes including DNA repair, cell cycle control, transcriptional regulation, apoptosis and genomic stability (Deng 2006, Roy et al. 2012). BRCA1 ubiquitylation of both ERα and PR contributes to their transcriptional function (Eakin et al. 2007, Calvo & Beato 2011). BRCA1/BARD1 monoubiquitylates ERα in vitro and in vivo (Eakin et al. 2007, Dizin & Irminger-Finger 2010, Ma et al. 2010, Zhu et al. 2014). This monoubiquitylation is dependent on BRCA1/BARD1 ligase activity as cancer predisposing BRCA1 mutations (C61G and C64G) affecting the ligase activity abolish the ability of BRCA1 to monoubiquitylate ERα. The site of monoubiquitylation on ERα was identified through mass spectrometry to be K302; however, the K302A ERα mutant was still mono-
ERβ transcriptional activity as well as E2-induced cell proliferation (Ma et al. 2010, La Rosa et al. 2011a). It should be noted, however, that BRCA1 mutant breast cancers are almost always ERβ-negative and thus potential therapies targeting the interaction between ERβ and BRCA1 would not be suitable in these cases (Karp et al. 1997, Loman et al. 1998). In the case of PR, BRCA1 induces ubiquitylation, but whether PR ubiquitylation is polyubiquitylation (attachment of a ubiquitin chain on a lysine) or multi-monoubiquitylation (multiple single ubiquitin attachments on different lysine sites on the substrate) is unresolved (Calvo & Beato 2011).

In addition to BRCA1, RNF31 – also known as HOIP or ZIBRA – can also monoubiquitylate ERβ (Zhu et al. 2014). RNF31 is a RBR E3 ligase, and a component of the linear ubiquitin assembly complex (LUBAC). Studies by Zhu et al. (2014) show a positive correlation between RNF31 and ERβ levels. Further, manipulation of RNF31 by knockdown or overexpression decreases and increases ERβ-mediated transcriptional activity, respectively. Importantly, the effects on receptor transcriptional function were shown to be dependent on RNF31 E3 ligase activity, supporting the idea that monoubiquitylation of ERβ can regulate receptor transcriptional activation. The control of ERβ by RNF31 and BRCA1/BARD1 suggests that there may be other NRs regulated in the same manner via monoubiquitylation. Moreover, given that RNF31 can act in the context of the LUBAC E3 ligase complex (see below), additional forms of ubiquitylation may also play regulatory roles in NR function.

Non-degradative ubiquitin code

Advances in the ubiquitin field have led to an emerging concept of the ‘ubiquitin code’ (Kulathu & Komander 2012). Ubiquitin itself has seven lysine residues – K6, K11, K27, K29, K33, K48 and K63 – each of which can serve as ubiquitylation sites to assemble ubiquitin chains connected via distinct internal lysine residues (Fig. 2). The attachment of ubiquitin chains linked via lysine K48 marks the substrate for degradation by the 26S proteasome and, as mentioned above, a minimum of four ubiquitin molecules is needed for efficient recognition and degradation of substrates tagged with K48-linked polyubiquitin chains (Thrower et al. 2000). The aforementioned NR associated ligases form this type of ubiquitin chains. However, polyubiquitin chains linked by each of the other ubiquitin lysines has been shown to be present in vivo through analysis by mass spectrometry (Xu et al. 2009a). Moreover, the amino group of the N-terminal methionine of ubiquitin can serve to assemble ‘M1-linked’ polyubiquitin chains. Many substrates can also be multi-monoubiquitylated at multiple lysine sites as described above. Finally, different types of ubiquitin configurations could occur in a single substrate (i.e., ‘mixed’ ubiquitin linkages). These varying ubiquitin chains significantly expanded the roles that ubiquitin plays in multitudes of molecular, cellular, physiological and pathological processes. The primary decoding of the information built into distinct types of ubiquitin chains is mediated by an array of ubiquitin-binding domains (UBDs) or ubiquitin receptors (Dikic et al. 2009). For a more comprehensive overview of UBDs, we direct the reader to recently published reviews on UBDs (Dikic et al. 2009, Husnjak & Dikic 2012, Searle et al. 2012). The role for these less commonly studied forms of polyubiquitin linkages in NR regulation is a relatively untapped and emerging area of research. For example, ERβ has recently been shown to contain a UBD in its AF-2 domain (Pesiri et al. 2013).

The study of RNF6 in AR biology provides a proof of principle that non-degradative ubiquitin chains can contribute to NR transcriptional function. Xu et al. (2009b) discovered that AR was polyubiquitylated by the E3 ubiquitin ligase, RNF6, in prostate cancer cells. Following identification as an interacting partner of AR in GST-pull down assays, the authors showed that RNF6 overexpression leads to an increase in polyubiquitylated AR without changes in AR protein levels. In vitro ubiquitylation assays revealed that RNF6 added K6- or K27-linked polyubiquitin chains to the AR. Overexpression of RNF6 increased the recruitment of co-factors, specifically ARA54, to androgen response elements, which suggests the possibility that specific ubiquitin linkages may contribute to recruitment and specificity of coregulator complexes on DNA. This may have implications in hormone-refractory prostate cancer where RNF6 has been shown to be overexpressed (Xu et al. 2009b).

To begin to elucidate the potential involvement of the ubiquitin code in NR regulation, we briefly provide an example (NF-κB) where the roles of distinct types of ubiquitylation are better understood (Chen & Chen 2013). NF-κB is a family of related dimeric transcription factors that are held inactive in the cytoplasm by a class of inhibitor proteins, inhibitor of κB (IκB). Cell signaling leads to the degradation of IκB, liberating NF-κB to the nucleus to initiate transcription. IκB degradation is mediated by phosphorylation of IκB, which creates a docking site for the β-transducing repeat containing protein (β-TrCP) RING ubiquitin E3 ligase complex that induces K48-linked polyubiquitylation of IκB and its degradation by the 26S proteasome. Degradation of
IκB is analogous to the polyubiquitylation and degradation of NRs by the proteasome. Interestingly, the mechanism of activation of IκB kinase (IKK) complex that phosphorylates IκB involves non-degradative polyubiquitin chains, such as K63 and M1 chains, assembled by combinations of multiple E2s and E3s. In a simplified model, these polyubiquitin chains are recognized by UBD proteins TAK1 binding proteins 2 and 3 and NF-κB essential modulator to induce TAK1-dependent IKK activation (Yamaoka et al. 1998; Fig. 3, middle panel).

### Figure 2
A schematic of the cellular processes in which different polyubiquitin chain species have been implicated based on linkage type. The different types of ubiquitylated species are represented as cartoons in the left-hand column. Polyubiquitinated chains can be organized into a ‘closed’ or ‘open’ conformation based solely on the type of linkage that connects them. A (*) symbolizes that structural data is currently unavailable for these linkages; however, modeling of these structures predicts the conformation of each chain type. Cellular roles are determined based on the identification of each chain type in a specific cellular process. Currently the function of many of these chains is still unknown. This list is not meant to be comprehensive but rather to highlight the many diverse roles of ubiquitin.

<table>
<thead>
<tr>
<th>Linkage type</th>
<th>Cellular role</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>* K33</td>
<td>Reduces T-cell activation</td>
<td>Huang et al. (2010) Licchesi et al. (2011)</td>
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</tbody>
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Complexes involved in DNA double-strand break repair include DNA repair and transcriptional activation. For instance, DNA repair components such as the NHEJ complex (Brown & Jackson 2015; Fig. 3, right panel). It is conceivable that non-degradative ubiquitin chains could also be used to assist in assembly of NR transcriptional complexes (Fig. 3, left panel).

Thus, NF-kB signaling highlights how non-degradative ubiquitin chains can serve as a novel scaffold to assemble multi-protein complexes. Similarly, non-degradative ubiquitin chains are used to assist in the assembly of protein complexes involved in DNA double-strand break repair (Brown & Jackson 2015; Fig. 3, right panel). It is conceivable that non-degradative ubiquitin chains could also be used to assist in assembly of NR transcriptional complexes (Fig. 3, left panel).

A new frontier in ubiquitin regulation of NR

The RNF6 study in AR mentioned above suggests that non-degradative polyubiquitin chains may interface with NR activation mechanisms at the level of co-activators (Xu et al. 2009b). Indeed, an inspection of NR co-activators can identify several of them to contain RING finger domains (Table 2). Whether the RING domains in these coactivators contribute to their activities in NR signaling awaits further investigation. However, intriguingly, TIF1 assembles ternary coactivator complexes as part of AR transcriptional activation (Teyssier et al. 2006), and MAT1 is part of the assembly of TFIH and Cdk-activating kinase complex involved in receptor phosphorylation and transcriptional synergy (Rochette-Egly et al. 1997, Bastien et al. 2000, Chen et al. 2000, Chymkowitch et al. 2011). It is tempting to speculate that the RING domains of these factors could produce ubiquitin-based assembly scaffolds similar to what is observed in NF-kB signaling. The limited studies of different ubiquitin linkages in biological contexts, including the NR field, could be due in part to the difficulty in detecting and quantifying endogenous proteins modified by specific ubiquitin linkages. Linkage-specific antibodies are commercially available for the detection of K48, K63 or M1 linkages. These antibodies can be used in immunoprecipitation–western analyses (Haglund & Dikic 2005, Emmerich et al. 2013, Jackson & Durocher 2013). This approach has been used to investigate the types of ubiquitin chains formed on ERα in response to E2 (La Rosa et al. 2011b). Alternatively, overexpression and knockdown or knockout of specific E2s and E3s (e.g., Ubc13 for K63 chains or LUBAC subunits for M1 chains; Kirisako et al. 2006, Tokunaga et al. 2009, 2011, Ikeda et al. 2011), or overexpression of ubiquitin

<table>
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<tr>
<th>Coregulator</th>
<th>Ring finger designation</th>
<th>Receptor</th>
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<tbody>
<tr>
<td>ARNIP</td>
<td>RNF199</td>
<td>AR</td>
</tr>
<tr>
<td></td>
<td>RNF53</td>
<td>ERα</td>
</tr>
<tr>
<td>EFP</td>
<td>RNF147</td>
<td>PR</td>
</tr>
<tr>
<td>MAT1</td>
<td>RNF66</td>
<td>ERα</td>
</tr>
<tr>
<td>RNF8</td>
<td>RNF8</td>
<td>ERα, PPARγ</td>
</tr>
<tr>
<td>RLM</td>
<td>RNF12</td>
<td>RXR</td>
</tr>
<tr>
<td>TIF1</td>
<td>RNF82 and RNF96</td>
<td>RXR, RAR</td>
</tr>
<tr>
<td>SNURF</td>
<td>RNF4</td>
<td>ERα, VDR, AR and TR</td>
</tr>
</tbody>
</table>

RNF, ring finger protein; ARNIP, androgen receptor N-terminal interacting protein; BRCA1, breast cancer type 1 susceptibility protein; EFP, estrogen-responsive finger protein; MAT1, menage-a-trois homologue 1; RLM, ring finger protein, LIM domain interacting; TIF1, transcriptional intermediary factor; SNURF, SNRPN upstream reading frame.
mutants (K48R, K63R, etc.), as well as replacement of endogenous ubiquitin with ubiquitin mutants (Xu et al. 2009c), has been applied to interrogate the role for specific ubiquitin chains. Finally, mass spectrometry techniques have also been used to map the ubiquitylation sites that are aided by the development of anti-di-Gly antibodies to enrich ubiquitylated peptide species following trypsin digestion (Kirkpatrick et al. 2005, Kim et al. 2011). Each of these approaches has limitations in cell systems (e.g., over-expression/knockdown can have effects on multiple substrates, background of endogenous ubiquitin in mM concentrations, multiple genes encoding ubiquitin, and effects on cell viability). Despite these challenges, in combination these techniques have been instrumental in providing insight into the roles of alternative forms of ubiquitin linkages in cell signaling and regulation.

The role of non-degradative ubiquitin and the ubiquitin code in regulation of NR function is in its infancy and despite some of the current technical challenges, understanding how this protein modification regulates NR function may open new avenues of research and therapeutic design. There are many critical reagents being generated (e.g., antibodies that specifically detect different ubiquitin linkages (Newton et al. 2008, Matsumoto et al. 2012), new techniques being developed (e.g., advanced, sensitive and quantitative MS analyses (Peng et al. 2003, Xu & Peng 2006, Phu et al. 2011)) and specific ubiquitin E2s, E3s and deubiquitinases that act on specific ubiquitin linkages are being identified (Komander et al. 2009, Ye & Rape 2009, Kar et al. 2012). These advances may accelerate the elucidation of the roles for non-degradative poly-ubiquitylation in regulation of the NR family of proteins. While capitalizing on receptor ubiquitylation has yet to be tapped for clinical application, there is much to be gained by better understanding of the expanding role of ubiquitin in NR signaling. Just as the increased complexity of receptor genomic and non-genomic activities is providing new avenues of rationale design of therapeutics for NR-associated disease, the growing roles of ubiquitin in receptor protein control and transactivation provide an alternative to existing ligand-based therapies. The marriage of NR and ubiquitin fields presents an opportunity for both fields to explore fundamental biology of these important systems with high translational potential.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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References


Bastien J, Adam-Stittah S, Riedl T, Egly JM, Chambon P & Rochette-Egly C 2000 TRF1HH interacts with the retinoic acid receptor γ and


Brenschel EH, Dalman FC, Sanchez ER & Pratt WB 1989 Evidence that the 90-kDa heat shock protein is necessary for the steroid binding conformation of the L cell glucocorticoid receptor. Journal of Biological Chemistry 264 4992–4997.


Chymkytowich P, May NL, Chaneau P, Compe E & Egly JM 2011 The phosphorylation of the androgen receptor by TFIIH directs the ubiquitin/proteasome process. EMBO Journal 30 468–479. (doi:10.1038/emboj.2010.337)


Pesiri V, Rosa PL, Stano P & Acconcia F 2013 Identification of an estrogen receptor α non covalent ubiquitin-binding surface: role in 17β-


Ramamoorthy S & Nawaz Z 2008 E6-associated protein (E6-AP) is a dual function coactivator of steroid hormone receptors. *Nuclear Receptor Signaling* **6** 6006. (doi:10.1621/nrs.06006)


Smith DF & Toft DO 1993 Steroid receptors and their associated proteins. *Molecular Endocrinology* **7** 4–11. (doi:10.1210/mend.7.1.8441607)


Sun J, Zhou W, Kalaiappan K, Nawaz Z & Slingerland JM 2012 E6α phosphorylation at Y537 by Src triggers E6-AP–ERα binding, ERα
ubiquitination, promoter occupancy, and target gene expression. Molecular Endocrinology 26 1567–1577. (doi:10.1210/me.2012-1140)


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