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

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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 (E_2) (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, NR3C3), 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).

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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 multiprotein 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).

Post-translational modifications (PTMs) are another regulatory mechanism governing NR function. PTMs represent an important cross-talk mechanism by which other signaling pathways interface with NR activation. In the case of ERα, all domains of the receptor can be phosphorylated in response to ligand and/or growth factor cascades (Ali *et al*. 1993, Le Goff *et al*. 1994, Bunone *et al*. 1996, Weis *et al*. 1996, Chen *et al*. 1999, Yudt *et al*. 1999, Clark *et al*. 2001, Michalides *et al*. 2004, Held *et al*. 2012). Studies in breast cancer cell models have demonstrated that phosphorylation can impact multiple aspects of receptor function, including protein stability, dimerization, DNA binding and co-activator preferences (Arnold *et al*. 1995, Tzeng & Klinge 1996, Chen *et al*. 1999, Henrich *et al*. 2003, Sheeler *et al*. 2003, Calligé *et al*. 2005, Valley *et al*. 2005, Likhite *et al*. 2006, Bhatt *et al*. 2012). ERα is also subject to other modifications, including acetylation (Kim *et al*. 2006), methylation (Le Romancer *et al*. 2008, Subramanian *et al*. 2008), SUMOylation (Sentis *et al*. 2005, Hilmi *et al*. 2012) and palmitoylation (Acconcia *et al*. 2005). Readers are referred to a recent comprehensive review of ERα PTMs (Le Romancer *et al*. 2011). Importantly, ERα and other NRs are targets of ubiquitylation, a PTM that couples receptor protein turnover and transcriptional function at multiple levels of the receptor signaling pathway.

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, ubiquitylation 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*. 1999*a*). 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, Stavreva *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*.

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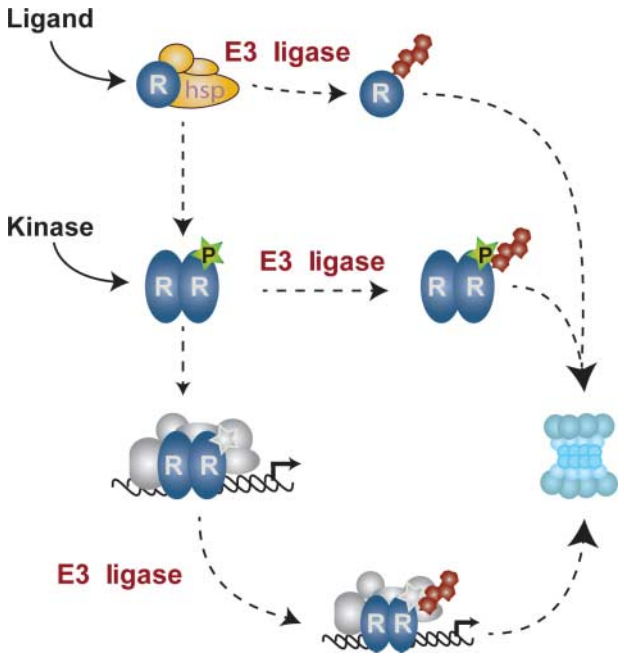

Figure 1
A model of conserved roles of the ubiquitin proteasome pathway in NR signaling. The primary function ascribed to ubiquitin in NR signaling is targeting receptors to the proteasome. Shown here is a simplified NR signaling pathway in which receptor (R) is activated either by ligand or by kinases from growth-factor or membrane-bound NRs. Receptors held bound by heat shock proteins can be targeted for degradation by the 26S proteasome following ubiquitylation by E3 ligases, such as CHIP. Upon binding ligand, receptors undergo dimerization (homo- and heterodimerization are not distinguished here) and can be decorated by multiple post-translational modifications (PTMs) including phosphorylation, shown as a star. Phosphorylated receptors can be directly recognized by E3 ligases, ubiquitylated and directed to the proteasome for degradation. Alternatively, PTMs can be incorporated into active transcriptional complexes of variable co-regulator components, represented by a grey multiprotein complex. The make-up of the co-regulator/receptor complexes can recruit E3 ligases that direct ubiquitylation and degradation of the transcriptional complex. In the degradative pathways, PTMs and co-regulator complexes guide E3 targeting that allows subpopulations of receptors to be selectively degraded in cells.

2014). 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 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.

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Table 1 E3 ligases involved in NR ubiquitylation

Nuclear receptor	E3 ligase	Class of ligase	Type of Ub	References
Estrogen receptor alpha	E6AP	HECT	Poly	Nawaz *et al.* (1999*b*), Li *et al.* (2006), Sun *et al.* (2012) and Rajbhandari *et al.* (2014)
	CHIP	RING (U-box)	Poly	Fan *et al.* (2005)
	Mdm2	RING	Poly	Duong *et al.* (2007)
	BRCA1/BARD1	RING	Mono	Eakin *et al.* (2007)
	EFP (TRIM25)	RING	Poly K48	Nakajima *et al.* (2007)
	SPOP	RING (Cullin)	Poly	Byun & Jung (2008)
	RBCK1	RING (RBR)	?	Gustafsson *et al.* (2010)
	CUEDC2	?[a]	?	Pan *et al.* (2011)
	Skp2	RING (F-box)	Poly	Bhatt *et al.* (2012)
	VHL	RING	Poly	Jung *et al.* (2012)
	RNF31	RING (RBR)	Mono	Zhu *et al.* (2014)
Estrogen receptor beta	CHIP	RING (U-box)	Poly K48	Tateishi *et al.* (2006)
	E6AP	HECT	Poly	Picard *et al.* (2008)
	Mdm2	RING	Poly	Sanchez *et al.* (2013)
Androgen receptor	Mdm2	RING	Poly	Lin *et al.* (2002)
	CHIP	RING (U-box)	Poly	Cardozo *et al.* (2003)
	NEDD4	HECT		Li *et al.* (2008)
	RNF6	RING	Poly K6 or K27	Xu *et al.* (2009*b*)
	Siah2	RING	Poly K48	Qi *et al.* (2013)
	UBR1	RING	?	Sultana *et al.* (2013)
	Skp2	RING	Poly	Li *et al.* (2014)
Glucocorticoid receptor	Hdm2	RING	Poly	Sengupta & Wasylyk (2001)
	CHIP	RING (U-box)	Poly	Connell *et al.* (2001)
	FBXW7	RING (F-box)	Poly	Malyukova *et al.* (2013)
	UBR1	RING	?	Sultana *et al.* (2013)
Progesterone receptor	CUEDC2	?[a]	?	Zhang *et al.* (2007)
	BRCA1/BARD1	RING	Poly	Calvo & Beato (2011)
Retinoic acid receptor alpha	FLRF (Rnf41)	RING	?	Jing *et al.* (2008)
Retinoic X receptor	RNF8	RING	?	Takano *et al.* (2004)
Mineralocorticoid receptor	CHIP	RING (U-box)	Poly	Faresse *et al.* (2010)
PPARγ	Siah2	RING	Poly	Kilroy *et al.* (2012)
	MKRN1	RING	Poly	Kim *et al.* (2014)
Estrogen-related receptors	Parkin	RING (RBR)	Poly	Ren *et al.* (2011)

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, S-phase kinase-associated protein 2; SPOP speckle-type POZ protein; Ub, ubiquitin; UBR1, ubiquitin protein ligase E3 component N-recognin 1; VHL, Von Hipple–Lindau tumor suppressor.
[a]CUEDC2 is a CUE domain-containing gene that promotes the degradation of PR and ERα through ubiquitylation, see references.

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 ERα, PR, GR and AR in a proteasome-dependent manner (Whitesell & Cook 1996, Pratt & Toft 1997, Bagatell *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 decreases the ATPase activity of Hsps. This association ultimately decreases the efficiency of the chaperone and impairs its function, leading to misfolding and subsequent degradation of its substrate by proteasomes (Ballinger *et al.*

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1999, Connell *et al*. 2001). CHIP interacts with Hsp90 and incorporates itself into the NR–Hsp90 heterocomplex, causing remodeling that favors degradation of the NR. In the case of GR, CHIP can ubiquitylate GR both *in vitro* and *in vivo* and directly target it for degradation by interacting with the S5a subunit of the 26S proteasome (Connell *et al*. 2001). CHIP was also found to preferentially associate with unliganded ERα, increasing ubiquitylation and degradation of the receptor. Other NRs also appear to be ubiquitylated by CHIP, including ERβ, AR, GR and MR, suggesting that ubiquitylation of NRs by CHIP is a conserved mechanism (Cardozo *et al*. 2003, Wang & DeFranco 2005, Tateishi *et al*. 2006, Faresse *et al*. 2010). Hence, CHIP ligase is a major regulator of unliganded NR protein expression (Fig. 1), which has relevance in scenarios in breast and prostate cancer where therapies such as aromatase inhibitors and abiraterone decrease hormone production yet maintain the unliganded receptor.

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*. 1999*b*, 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 coactivation activity (Nawaz *et al*. 1999*b*). 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 RARα, RARγ, 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

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coactivation of PR and GR transactivation (Imhof & McDonnell 1996). Among the HECT ligases, E6AP alone thus far has been directly shown to function as a key NR regulator via ubiquitin ligase-dependent and -independent mechanisms.

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 $p27^{kip1}$. 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 E_2-induced phosphorylation, leading to ubiquitylation of $p27^{kip1}$ (Lecanda *et al.* 2007, Huang *et al.* 2012). The loss of nuclear $p27^{kip1}$ has been shown to occur in E_2-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 $p27^{kip1}$, thus preventing its degradation. Treatment of E_2-induced endometrial carcinoma cell lines with these small molecules resulted in increased levels of $p27^{kip1}$ 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).

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 the 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

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ERα transcriptional activity as well as E_2-induced cell proliferation (Ma *et al.* 2010, La Rosa *et al.* 2011*a*). It should be noted, however, that BRCA1 mutant breast cancers are almost always ERα-negative and thus potential therapies targeting the interaction between ERα 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 poly-ubiquitylation (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 ERα (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 ERα levels. Further, manipulation of RNF31 by knockdown or overexpression decreases and increases ERα-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 ERα can regulate receptor transcriptional activation. The control of ERα 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 poly-ubiquitin 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.* 2009*a*). 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, ERα 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.* (2009*b*) 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.* 2009*b*).

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

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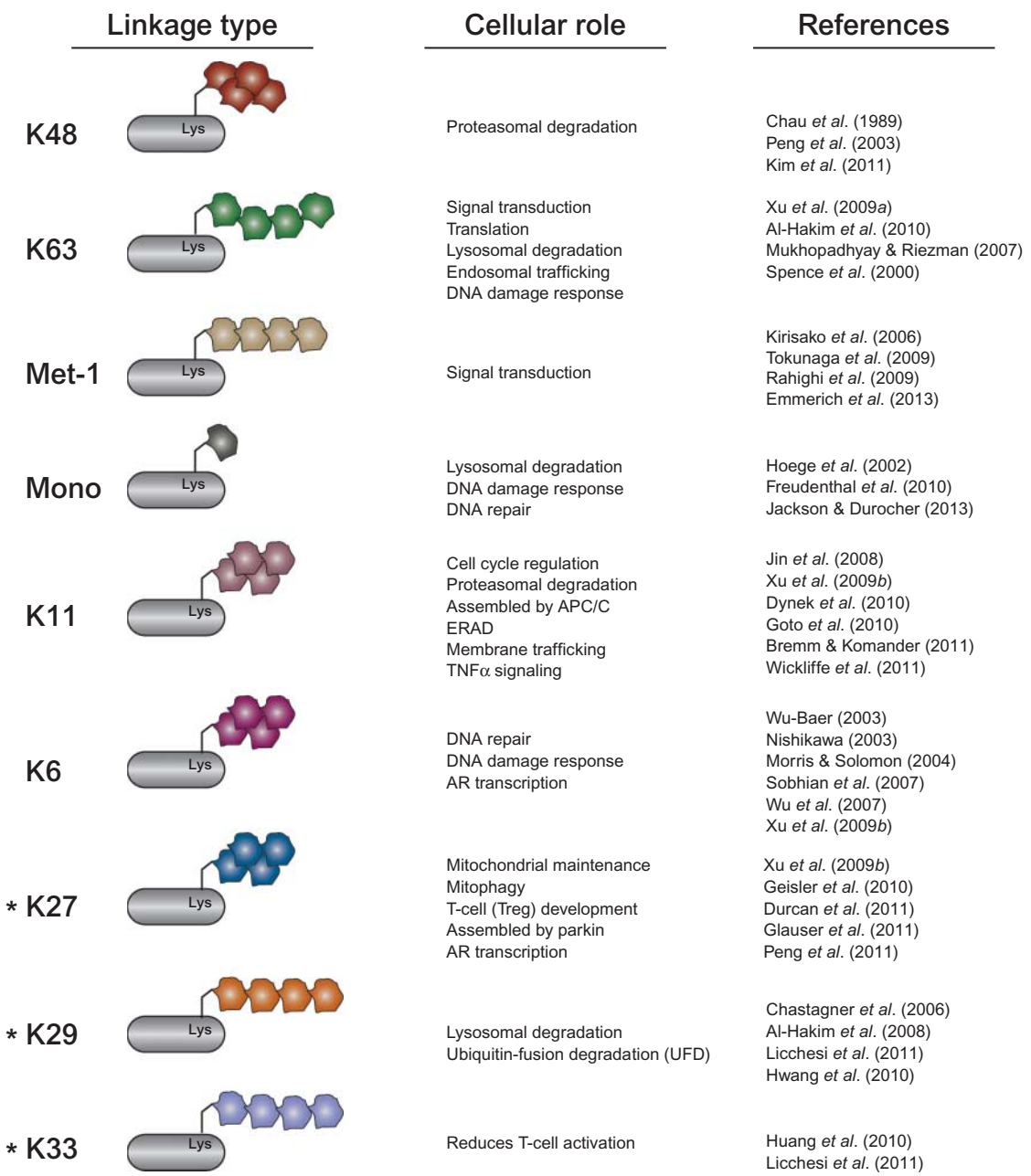

Figure 2

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. Polyubiquitin 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.

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).

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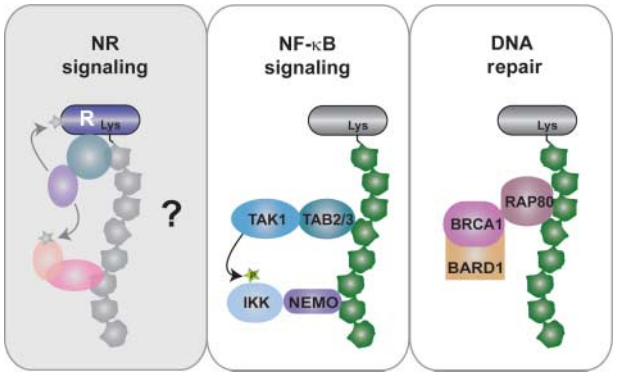

Figure 3
A hypothetical model of non-degradative ubiquitin in NR signaling. Non-degradative polyubiquitin chains can serve as protein assembly scaffolds. Examples shown in the middle and right panels are assemblages described in NF-κB signaling and DNA damage repair. Based on these models, we speculate the potential for non-degradative ubiquitin chains providing similar scaffolding in NR signaling, bringing together tertiary complexes with enzymatic activity to post-translationally modified receptor or other coregulator proteins to affect transcription. Such scaffolds could also bring other ubiquitin-binding domain (UBD) proteins into the NR complex without directly binding to NR. TAK1, TGF-beta-activated kinase; TAB2/3, TGF-beta activated kinase 1/MAP3K7 binding proteins 2 & 3; IKK, I-kappa-B kinase complex; NEMO, NF-kappa-B essential modulator; BRCA1, Breast cancer type 1 susceptibility protein; BARD1, BRCA1 associated RING domain 1; Rap80, Receptor-associated protein 80. Stars represent post-translational modifications such as phosphorylation, denoted as 'P'.

Thus, NF-κB 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*. 2009*b*). 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 TFIIH 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-κB 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 E_2 (La Rosa *et al*. 2011*b*). 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 2 NR coregulators that contain RING finger domains

Coregulator	Ring finger designation	Receptor	References
ARNIP	RNF199	AR	Beitel *et al*. (2002)
BRCA1	RNF53	ERα	Fan *et al*. (1999), Zheng *et al*. (2001), Kawai *et al*. (2002) and Calvo & Beato (2011)
		PR	
EFP	RNF147	ERα	Inoue *et al*. (1993) and Nakajima *et al*. (2007)
MAT1	RNF66	ERα, PPARγ	Talukder *et al*. (2003) and Helenius *et al*. (2009)
RNF8	RNF8	RXR	Takano *et al*. (2004)
RLIM	RNF12	ERα	Johnsen *et al*. (2009)
TIF1	RNF82 and RNF96	RXR, RAR	Vom Baur *et al*. (1996), Thénot *et al*. (1997) and Teyssier *et al*. (2006)
		ERα, VDR, AR and TR	
SNURF	RNF4	AR, GR	Moilanen *et al*. (1998), Poukka *et al*. (2000) and Saville *et al*. (2002)
		PR and ERα	

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; RLIM, ring finger protein, LIM domain interacting; TIF1, transcriptional intermediary factor; SNURF, SNRPN upstream reading frame.

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mutants (K48R, K63R, etc.), as well as replacement of endogenous ubiquitin with ubiquitin mutants (Xu *et al.* 2009*c*), has been applied to interrogate the role for specific ubiquitin chains. Finally, mass spectrometry techniques have also be 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 polyubiquitylation 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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