

## REVIEW

# Regulation of the androgen receptor by post-translational modifications

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### Abstract

The androgen receptor (AR) is a key molecule in prostate cancer and Kennedy's disease. Understanding the regulatory mechanisms of this steroid receptor is important in the development of potential therapies for these diseases. One layer of AR regulation is provided by post-translational modifications including phosphorylation, acetylation, sumoylation, ubiquitination and methylation. While these

modifications have mostly been studied as individual events, it is becoming clear that these modifications can functionally interact with each other in a signalling pathway. In this review, the effects of all modifications are described with a focus on interplay between them and the functional consequences for the AR.

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### Introduction

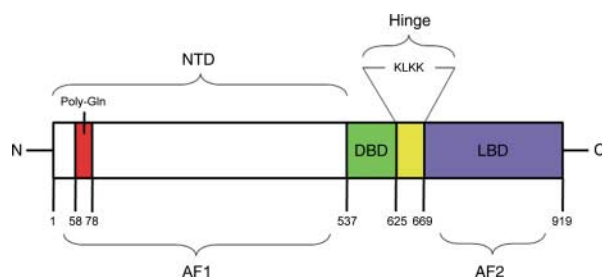
The androgen receptor (AR; Fig. 1) is a steroid hormone receptor that plays a critical role in prostate cancer (PC) progression and development. In addition, it can also cause spinal bulbar muscular dystrophy (commonly referred to as Kennedy's disease). Hence, this 110 kDa protein is the target of many therapeutic strategies. The AR, in the absence of ligand, is sequestered to the cytoplasm by heat-shock proteins, which mask a nuclear localisation sequence (NLS). Upon binding to its androgen ligand, these chaperone proteins dissociate and the AR dimerises, resulting in a conformational change, which exposes the NLS. This results in the AR translocating to the nucleus where it can bind to androgen response elements (AREs) and activate androgen-responsive genes. The regulation of this transcription factor in this process is achieved via a multitude of mechanisms involving interaction with other co-activator and repressor proteins, some of which are responsible for modification of the receptor at the post-translational level to modulate its activity. Specifically, modulation of AR activity by post-translational modifications (PTMs) can be achieved through regulation of protein stability, interaction with other proteins, cellular localisation and structure of the receptor itself. Therefore, discovery and study of these modifications and the enzymes that perform the addition are critical to discover novel therapeutic options.

Many of the enzymes that are responsible for the modification of the receptor are dysregulated in disease, which results in aberrant AR activity. More recently, the interplay between protein PTMs is becoming apparent. Where these modifications were thought to result in a simple signal for the molecule to alter its activity or interaction with other proteins, it is now clear that combinations of modifications act together to provide a more complex message to the cell; a sentence of instructions rather than one simple word.

Numerous PTM sites have been described on the AR. The types of modifications include phosphorylation, acetylation, ubiquitination, sumoylation and more recently methylation (Me).

### Acetylation of the AR

AR acetylation (AR-Ac) was first inferred by studies using the histone deacetylase (HDAC) inhibitor trichostatin A (TSA), which showed that in the presence of dihydrotestosterone (DHT), transcription, chromatin remodelling and AR levels were enhanced (List *et al.* 1999). Direct AR-Ac was first described in 2000 (Fu *et al.* 2000) after other transcription factors, including p53 (Gu & Roeder 1997) and Kruppel-like factor (Song *et al.* 2003), were found to be acetylated. Edman degradation demonstrated that all three lysine residues (K630, K632 and K633) within the KLKK motif can be targeted by



**Figure 1** The structure of the androgen receptor (AR). The AR is composed of a number of domains: the N-terminal domain (NTD) that contains the poly-Gln (CAG) repeat sequence important in Kennedy's disease and the activation function 1 (AF1) domain, the DNA binding domain (DBD), the ligand binding domain (LBD) containing the activation function 2 (AF2) domain and the hinge region where the KLKK motif resides.

the acetylases p300 and p300/CREB binding protein (CBP)-associated factor (PCAF; *Fu et al. 2000*) similar to the KSKK motif in p53 (*Gu & Roeder 1997*). In later studies, Tip60 (*Gaughan et al. 2002*) and *N*-acetyltransferase arrest-defect 1 protein (*Wang et al. 2012*) were also found to directly acetylate the AR.

The role of AR-Ac has been investigated in many studies where acetyl acceptor sites have been mutated to be either non-functional or acetylation mimetic, the findings of which are summarised in *Table 1*. Generally, when the AR cannot be acetylated, TSA can no longer activate the AR, and co-activation by SRC1, p300, PCAF, Ubc9 and Tip60 is abolished resulting in reduced recruitment to AREs. Under these conditions, the AR equilibrium moves towards a repressed form, which has increased binding to co-repressor proteins including NCoR, and subsequent MEKK1 stimulation can no longer induce AR-mediated apoptosis (*Fu et al. 2000, 2003, Xu et al. 2009*). Sumoylation and transrepression functions of the AR remain unaffected by mutation of acetyl acceptor sites (*Fu et al. 2000, 2003, Xu et al. 2009*), suggesting that there is no functional interaction between sumoylation and acetylation. When all acetylation acceptor sites are mutated to glutamine to mimic acetylation, the opposite is observed, with enhanced AR-p300 interaction and reduced interaction with co-repressor proteins such as NCoR, HDACs, and SMAD3 (*Fu et al. 2003*). Under androgen-depleted conditions, AR-Ac mimics exhibit nuclear localisation, while mutants that cannot be acetylated remain in the cytoplasm even with androgen stimulation (*Shiota et al. 2010*). SMAD3/SMAD4 can interact with the AR DNA binding domain (DBD) and ligand binding domain (LBD) to repress AR-Ac and inhibit AR activity (*Kang et al. 2002*). Hence, AR-Ac has been robustly demonstrated to facilitate activation of the AR.

Acetylation is a dynamic process that is reversed by HDAC enzymes. 5'-TG-3' interacting factor recruits HDAC1, mediated by Sin3a (*Sharma & Sun 2001*), to deacetylate the AR and down-regulate AR activity (*Gaughan et al. 2002*).

Interestingly, HDAC7 has also been found to inhibit AR; however, the mechanism for this is independent of AR acetyl acceptor sites (*Karvonen et al. 2006*). Modulation of AR-Ac is also critical for control of AR activity during the cell cycle. AR-Ac mimics, for example, yield enhanced cell survival, proliferation and AR-regulated gene expression as a consequence of increased levels of the cell cycle control genes, cyclin D and cyclin E (*Fu et al. 2003*). Cyclin D1 can also disrupt the interaction between PCAF and AR, thereby altering the AR-Ac profile and potentially inhibiting AR transcriptional activity at the G1/S transition (*Martinez & Danielsen 2002*).

AR-Ac can also regulate DNA binding and activation of transcription in a promoter-dependent context. For example, upon mutation of all acetyl acceptors, the mouse *Muc1* (*Pem*) promoter is not activated while prostate-specific antigen (*PSA*; *KLK3*) remains active and the *MTTV* promoter is activated to a higher level than with WT AR. This differential transcriptional ability of acetylation site mutants of AR suggests the receptor can recruit different coregulator proteins depending on which sites can be acetylated (*Faus & Haendler 2008*). One study specifically addressed the role of the hinge region, which contains the KLKK motif, in the regulation of AR activity by generating an AR mutant that lacked residues 629–636. As detailed in *Table 1*, this mutant AR displayed a stronger androgen response but it had low affinity for ARE binding sites, which is not surprising as these effects were independent of nuclear localisation. This deletion construct also displayed an enhanced N and C terminal interaction and could be inhibited by anti-androgen treatment (*Haelens et al. 2007*). These findings are contradictory to the published work on AR-Ac mutants, which highlights the role of the intact hinge region in the regulation of AR activity and how PTM can influence its function.

Acetylation of the AR is induced by androgen stimulation (*Fu et al. 2000*); however, there are other cellular stimuli that can influence the levels of AR-Ac. The neuropeptide, bombesin, which has been demonstrated to play a role in PC progression, has a positive impact on p300 activity. This can be further stimulated by Src to enhance AR-Ac levels (*Gong et al. 2006*). Similarly, IL4 has been found to activate the AR in the absence of androgens via elevation of CBP/p300 levels to enhance AR-Ac and stimulate castrate-resistant PC (CRPC; *Lee et al. 2009*). The activity of the AR can also be altered through acetylation of other proteins that can modulate the AR. For example, P300 acetylates  $\beta$ -catenin, regulating its interaction with TCF and the AR to result in enhanced receptor transcriptional activity to regulate a subset of genes in a ligand-dependent manner (*Levy et al. 2004*). Another example is HSP90-Ac, which results in destabilisation of the AR leading to its destruction by the proteasome (*Ai et al. 2009, Gibbs et al. 2009*).

Due to the oncogenic effects of an acetylated AR, many laboratories, including our own, have investigated the potential of histone acetyltransferase (HAT) inhibition as

**Table 1** Androgen receptor acetylation and methylation mutants

| Mutant                           | AR characteristics  | Phenotypic effects   | Reference  |
|----------------------------------|---|--|--|
| AR <sup>(K630A)</sup>            | Decreased activation by DHT<br>No effect on sumoylation<br>Abolished co-activation by SRC1, p300, Ubc9, Tip60<br>No TSA-mediated activation<br>Increased binding to NCoR<br>Transrepression unaffected<br>Impaired activation by PKA inhibition<br>Decreased ligand-induced phosphorylation<br>Decreased N/C interaction            | Resistant to apoptotic stimuli   | Fu <i>et al.</i> (2000)<br>Fu <i>et al.</i> (2002)<br>Fu <i>et al.</i> (2004)<br>Ko <i>et al.</i> (2011)     |
| AR <sup>(K630R)</sup>            | Decreased p300 binding<br>Enhanced co-repressor binding   |  | Fu <i>et al.</i> (2003)<br>Gaughan <i>et al.</i> (2011)  |
| AR <sup>(K630Q)</sup>            | Acetylation mimic<br><br>Enhanced p300 binding<br>Reduced binding of NCoR, HDAC and SMAD3<br>Transrepression unaffected   | Increased cell cycle regulatory genes<br>Decreased apoptosis                     | Fu <i>et al.</i> (2003)  |
| AR <sup>(K630T)</sup>            | Acetylation mimic<br><br>Enhanced p300 binding<br>Reduced binding of NCoR, HDAC and SMAD3<br>Transrepression unaffected   | Increased cell cycle regulatory genes<br>Decreased apoptosis                     | Fu <i>et al.</i> (2003)<br>Fu <i>et al.</i> (2006)   |
| AR <sup>(K632R)</sup>            | Resistant to SIRT1-mediated repression<br>Decreased PCAF interaction<br>Abolished co-activation by Set9<br>No methylation   | Has been found as a somatic mutation   | Shi <i>et al.</i> (2002)<br>Gaughan <i>et al.</i> (2011)   |
| AR <sup>(K632A/K633A)</sup>      | Decreased activation by DHT<br><br>Misfolding and aggregation<br>No effect on sumoylation<br>Abolished co-activation by SRC1, p300, PCAF, Ubc9, Tip60<br>No TSA-mediated activation<br>Increased binding to NCoR<br>Transrepression unaffected<br>Impaired activation by PKA inhibition<br>Decreased ligand-induced phosphorylation | Delayed ligand-dependent nuclear translocation<br>Resistant to apoptotic stimuli | Fu <i>et al.</i> (2000)<br>Thomas <i>et al.</i> (2004)<br>Fu <i>et al.</i> (2002)<br>Fu <i>et al.</i> (2004) |
| AR <sup>(K630/632/633A)</sup>    | Impaired nuclear translocation<br>No alteration in DNA binding  | Effect on gene activation promoter dependent                                     | Faus & Haendler (2008)   |
| AR <sup>(K630R/632R/633R)</sup>  | Remains cytoplasmic despite ligand stimulation  |  | Shiota <i>et al.</i> (2010)  |
| AR <sup>(K630Q/632Q/633Q)</sup>  | Acetylation mimic   |  | Shiota <i>et al.</i> (2010)  |
| AR <sup>(629–636 deletion)</sup> | Translocates to the nucleus even during starvation<br>Enhanced androgen response<br>Reduced DNA binding<br>Can be inhibited by anti-androgens<br>Unaffected nuclear localisation<br>Enhanced N/C interaction  |  | Haelens <i>et al.</i> (2007)   |

a therapy for PC. AR-Ac has been shown to be inhibited by *Rosa rugosa* Thunb. (Rosaceae) extracts (Lee *et al.* 2008), Resveratrol (a polyphenolic phytoalexin; Harada *et al.* 2011), procyanidin B3 (Choi *et al.* 2011), green tea catechins (Lee *et al.* 2012) and allspice (Lee *et al.* 2007), all of which result in a decrease in cancer cell proliferation but not all by the same mechanism, as detailed in Table 2. Furthermore, many HAT inhibitors have been described recently, which are targeted against specific enzymes such as p300 (curcumin) and Tip60 (NU9056, K Coffey & C N Robson 2012, unpublished data).

## Me of the AR

Regulation of AR activity can be achieved indirectly via Me of histone proteins. Me and demethylation of histone proteins are emerging as a key process in the initiation and regulation of transcription. Furthermore, many lysine demethylases (KDMs) and methyltransferases (KMTs) have been detailed as nuclear receptor coregulator proteins with direct AR-Me being documented in 2011 by Gaughan *et al.* (2011), which was quickly followed by a second report by Ko *et al.* (2011). Both studies described Set9-mediated AR-Me as

**Table 2** Acetylation inhibitors and their effects on AR

| Compound name           | AR-specific effects of reduced AR acetylation  | Functional effects of reduced AR acetylation  | Reference  |
|-------------------------|--|---|--|
| Allspice<br>Rosaceae    | Inhibits p300 and CBP activity<br>Suppressed ligand-mediated activation<br>Enhanced effects of AR antagonist   | Inhibits PC cell growth<br>Inhibits PC cell growth  | Lee <i>et al.</i> (2007)<br>Lee <i>et al.</i> (2008) |
| Procyanidin B3 (Pro-B3) | Inhibition of p300-mediated AR-Ac<br>Inhibits AR-mediated transcription  | Inhibition of proliferation<br>Inhibition of cell cycle gene expression<br>Increased cell death | Choi <i>et al.</i> (2011)                            |
| Resveratrol             | No effect on nuclear translocation at early timepoints<br>Reduced nuclear AR at later timepoints<br>Reduced DNA binding<br>Reduced AR protein levels |   | Harada <i>et al.</i> (2011)                          |
| Green tea catechins     | Inhibited nuclear translocation in response to ligand<br>Suppressed ligand-mediated activation<br>Reduced AR-dependent gene transcription            | PC cell death   | Lee <i>et al.</i> (2012)                             |

a transcriptional activator. In both reports, Set9 was shown to interact with AR and facilitate the interaction between the N and C terminal domains. Set9 was further demonstrated to interact with AREs in response to androgen stimulation. However, there are some discrepancies between the two reports, which need to be addressed. First, the identified sites of Me upon the AR protein are different. Gaughan *et al.* used a combination of mass spectrometry, immunoprecipitation and studies with short mutant AR peptides to demonstrate AR-Me within the KLKK motif of the hinge region at Lys632. Alternatively, Ko *et al.* used immunoprecipitation and mutant AR protein fragments to demonstrate that AR-Me occurs in the same region but at Lys630. While both studies robustly demonstrate their findings, differences in experimentation between the use of short peptides or larger fragments of AR may explain the discrepancy in Me site identification. Alternatively, multiple Me states may exist under different circumstances.

To date, these are the only two studies that describe direct Me of the AR, while demethylation of the AR has not yet been reported. Hence, Set9 may not be the only KMT that can methylate the AR. Indeed, many KMTs and KDMs have been described as AR co-regulator proteins without AR Me/demethylation being investigated.

**Phosphorylation of the AR**

Phosphorylation of the AR has been studied for nearly 40 years and is therefore the most characterised of all PTMs. As this topic has been extensively reviewed in the past (Faus & Haendler 2006, Ward & Weigel 2009, Gioeli & Paschal 2012) and information is comprehensively detailed within phosphosite ([www.phosphosite.org](http://www.phosphosite.org)), a less detailed description will be presented here that highlights the importance of AR phosphorylation (AR-P) in the regulation of its function and how it can interact with other PTMs. The complexity of

AR-P as a component of a signalling pathway is quite staggering and perhaps demonstrates how pathways will evolve for other types of PTMs over the next 40 years.

First reported in 1984 (Goueli *et al.* 1984), AR-P was initially thought to facilitate ligand binding (Golsteyn *et al.* 1989). The first direct evidence of AR-P was reported in 1990, when it was found that stimulation of the LNCaP PC cell line with the androgen DHT resulted in stimulation of AR-P (van Laar *et al.* 1990). As AR-P was only found in nuclear fractions, it was suggested that AR-P takes place after transformation to the active state (Golsteyn *et al.* 1990). In fact, the AR is phosphorylated within 15 min of synthesis to allow ligand binding (Blok *et al.* 1998) but undergoes further phosphorylation events upon androgen stimulation (Kuiper *et al.* 1991, van Laar *et al.* 1991, Kuiper & Brinkmann 1995). Much debate then followed as to whether the AR was phosphorylated in response to androgens or whether this effect was due to total AR levels increasing in response to androgen treatment (Kemppainen *et al.* 1992, Kuiper *et al.* 1993). The AR was later demonstrated to be phosphorylated under conditions of androgen depletion (Beitel *et al.* 1995). Indeed, AR-P can determine whether or not AR ligands perform as agonists or antagonists, and mutations in these sites can convert antagonists into agonists (Wang *et al.* 1999b).

*Serine phosphorylation*

There are multiple serine residues within the AR that can be phosphorylated (Table 3). Some are better characterised than others, although overall the function and validity of some of the sites is still unclear due to conflicting findings. For example, Ser81 was described as phosphorylated by protein kinase C (PKC); however, this was not supported by inhibitor studies (Gioeli *et al.* 2002). Caution should also be taken when investigating AR-P in the literature as some of the phosphorylation sites are given different numbers, which can confuse the reader. For example, Ser210 is described as

**Table 3** Phosphorylation sites within the AR

| Residue         | Stimulator                        | Enzymes  | Crosstalk | Functional outcomes  | Cellular localisation | Inhibitors   | Disease                   |
|-----------------|-----------------------------------|--|-----------|--|-----------------------|--------------|---------------------------|
| Ser16           |                                   | PKA <sup>a</sup> , calcium calmodulin 2 <sup>a</sup> |           |  |                       |              |                           |
| Ser81           | Androgen<br>Sapostin <sup>b</sup> | PKC, CDK9, CDK5, CDK1, Src, PP2A                     |           | Cell growth<br>Chromatin binding<br>Transcriptional activity<br>AR stabilisation<br>Promoter specificity<br>Constitutive P<br>P300-mediated activation | Nuclear               | Camptothecin | CRPC                      |
| Ser94           |                                   | PP2A   |           |  | Cytoplasm             |              |                           |
| Ser118          |                                   | Casein kinase 2 <sup>a</sup>                         |           |  |                       |              |                           |
| Ser213 (Ser210) | R1881, DHT, IGF1                  | Akt  |           | Nuclear translocation<br>Inhibition/activation <sup>c</sup><br>Turnover  | Cytoplasm             |              |                           |
| Ser256          |                                   | PP2A   |           |  | Nuclear               |              | PC                        |
| Ser293          |                                   | Aurora A   |           | Cell survival<br>Transcriptional activity<br>Inhibition of AR  | Nuclear               |              |                           |
| Ser308          |                                   | Cyclin D3/CDK1 p58                                   |           |  |                       |              |                           |
| Ser424          |                                   | PP2A   |           |  |                       |              |                           |
| Ser515 (Ser514) | EGF                               | MAPK, CDK7   | Ser650-P  | Caspase 3-mediated AR cleavage<br>Transcription  |                       |              | CRPC<br>Kennedy's disease |
| Ser578          | IGF1                              |  | Ser515-P  | Interaction with Ku70/80<br>Localisation<br>Constitutive P   |                       | Forskolin    |                           |
| Ser650 (Ser641) | EGF, Forskolin, SNARE1            | CK2 <sup>a</sup> , MKK4, p38, Jnk, ERK1              | AR-Ub     | Decreased AR activity<br>Nuclear export<br>CHIP binding  |                       |              |                           |
| Ser662          |                                   | PP1  | Ser515-P  |  |                       |              |                           |
| Ser791          |                                   | Akt  |           | Cell survival  |                       | Forskolin    |                           |
| Tyr267          | EGF, Gas6, HRG                    | Ack1   |           |  |                       | Dasatinib    |                           |
| Tyr363          | HRG                               | Ack1   |           |  |                       | Dasatinib    |                           |
| Tyr534          | EGF, HRG, IL6, FBS, Etk, Sapostin |  |           | Conformation change <sup>a</sup><br>Decreased CHIP binding   |                       |              | CRPC                      |

<sup>a</sup>Predicted.<sup>b</sup>Cell line specific.<sup>c</sup>Dependent on cell line passage number.



phosphorylated (Lin *et al.* 2001b, 2003, McCall *et al.* 2008, Shank *et al.* 2008, Bryant *et al.* 2011); however, when referring to the standard AR protein sequence (ANDR HUMAN, P10275), an arginine is found at this site, while in other studies, the same serine in question is referred to as Ser213 (Wen *et al.* 2000, Nan *et al.* 2003, Burgdorf *et al.* 2004, Taneja *et al.* 2005, Kim & Lee 2009, Lee 2009). In this review, the nomenclature used will correspond to the standard sequence to avoid such confusion.

Constitutive phosphorylation of the AR is seen at Ser94 (Jenster *et al.* 1994) and Ser650 (Wong *et al.* 2004). Similar to Ser81, p-Ser94 is required to generate the 112 kDa form of the AR (Jenster *et al.* 1994). However, in contrast to Ser81, which is a nuclear event, p-Ser94 occurs in the cytoplasm (Kesler *et al.* 2007). Interestingly, mutation of Ser94 and Ser81 has little effect on transcription (Zhou *et al.* 1995). In response to androgens, p-Ser81 is the most abundant (Lee *et al.* 2007); however, it can occur at low levels of androgen, for example, in LNCaP cells Ser81 is phosphorylated in response to as little as 1 nM DHT, while in C4-2 cells concentrations of between 10 and 100 pM is sufficient (Chen *et al.* 2006). Ser308 was found to be phosphorylated in the presence of androgens using MALDI/TOF-MS (Zhu *et al.* 2001), with mutation of this site to alanine inconsistently increasing transcriptional activity (Gioeli *et al.* 2002), yet cyclin D3/CDK11p58 can phosphorylate this site both *in vitro* and *in vivo* to result in inhibition of AR activity (Zong *et al.* 2007), suggesting that this residue may be phosphorylated as a mechanism for receptor down-regulation in the nucleus.

#### *Protein kinases involved in AR-P*

Initially, protein kinase A (PKA) and PKC were investigated as AR kinases as their activation resulted in enhanced AR activity in the presence of ligand (Ikonen *et al.* 1994). PKA-mediated AR activation is not a result of increased AR levels or increased DNA binding capacity; furthermore, AR does not contain PKA recognition sites, which led the investigators to conclude that the effects of PKA on AR are indirect (Ikonen *et al.* 1994). Similarly, while PKC activation stimulates AR activity, no additional phosphorylation was observed, suggesting an indirect mode of action (de Ruiter *et al.* 1995). A later study revealed that PKA activation with forskolin resulted in dephosphorylation of the AR at certain serine residues. In contrast with previous studies, dephosphorylation stimulated by forskolin treatment resulted in decreased AR transcriptional activity and reduced ligand binding (Blok *et al.* 1998). An *in vivo* study of AR-P revealed that in the absence of androgens, both isoforms (AR and AR-P) were present, but upon stimulation with androgens, the intensity of the phosphorylated form was increased whereas upon treatment with forskolin the AR-P isoform was depleted. When both androgens and forskolin were applied together, androgen-induced phosphorylation of the AR was inhibited (Wang & Fondell 2001). In the absence of androgens, PKA activates the AR (Gioeli *et al.* 2002), an effect

that can be inhibited by bicalutamide and flutamide and is believed to be a consequence of increased nuclear AR levels (Nazareth & Weigel 1996).

DNA-PK has also been suggested to phosphorylate the AR hinge region (Konno *et al.* 1996) within a consensus site at Ser656 (Shank *et al.* 2008). It is suggested that DNA-PK might play a role in the nuclear export of the AR by the double-stranded DNA-dependent pathway. Furthermore, mutation of Ser578 inhibits the interaction with Ku 70/80 and nuclear retention is promoted with hypophosphorylation at Ser515 (Ponguta *et al.* 2008). Indeed, AR-P was found to be the rate-limiting step in nuclear export (Shank *et al.* 2008).

#### *AR-P under androgen-depleted conditions*

Akt binds and phosphorylates AR at Ser213 (Wen *et al.* 2000) and Ser790 (Wen *et al.* 2000) under androgen-independent conditions. Upon mutation of these sites, PTEN, which inhibits Akt activity, can still inhibit AR, suggesting that neither p-Ser213 nor p-Ser790 is solely responsible for PTEN-mediated AR inhibition (Nan *et al.* 2003). Mutation of Ser213 also prevents Akt-mediated degradation of the AR, suggesting that this residue plays a role in AR turnover (Shank *et al.* 2008). Ser213 can also be phosphorylated by Akt in response to IGF1 (Lin *et al.* 2003) to result in receptor inhibition, probably by inhibiting coregulator interactions (Lin *et al.* 2001a). Similarly, IGF1 stimulation in skeletal muscle cells results in increased AR protein and transcript levels and enhanced p-Ser213 (Kim & Lee 2009) via Akt (Lee 2009) to promote AR nuclear translocation in the absence of androgens (Kim & Lee 2009). Interestingly, overexpression of caveolin 1, a co-activator of AR, is associated with increased phosphorylation of Ser213 (Bryant *et al.* 2011). A phospho-specific antibody has now been developed against p-Ser213, which revealed that the appearance of this modification in response to androgens depends on exactly which androgen is used. R1881 (a synthetic androgen) and DHT can promote p-Ser213 but testosterone has a very weak ability to do this. It is also suggested that a conformational change is required to expose this residue and enable phosphorylation (Taneja *et al.* 2005). The effects of Akt on AR do appear to depend on the number of times cell lines have been passaged; for example, in low passage LNCaP cells Akt inhibits AR, while in high passage cells Akt can activate the AR (Lin *et al.* 2003), which might explain why different functional outcomes are being observed. Upon comparing matched hormone-sensitive and refractory clinical samples of PC in terms of AR-P (Ser213) and the Akt signalling pathway, positive associations between expression and decreased time to death, time to relapse and survival were found (McCall *et al.* 2008). GSK-3 $\beta$ , which is inhibited by Akt-mediated phosphorylation, also mediates AR-P but inhibits transcriptional activity by impeding the interaction between the N and C terminal regions, without affecting localisation or stability (Salas *et al.* 2004, Wang *et al.* 2004). Furthermore, PKA, Akt and MAPK inhibit GSK-3 $\beta$ , which may explain some of the proposed indirect effects of

these kinases on AR-P. Akt-mediated phosphorylation of the AR is not responsible for the synergy observed for AR and Akt in the progression of tumours to relapse following androgen ablation therapy (Xin *et al.* 2006).

At reduced androgen levels, EGF can activate the AR by phosphorylating Ser515 (Ponguta *et al.* 2008), which can influence phosphorylation of Ser650; however, this residue does not play a role in regulating the function of the AR (Wong *et al.* 2004). In conjunction with p-Ser515, p-Ser578 is also required for IGF1-induced AR activation at reduced androgen levels (Ponguta *et al.* 2008). Upon mutation of Ser578, EGF can no longer result in an AR-mediated transcriptional response. EGF, and more noticeably forskolin treatment, can stimulate p-Ser650 at suboptimal concentrations of androgens (Gioeli *et al.* 2002). Similar to Ser256 and Ser118 (Kemppainen *et al.* 1992), Ser650 is a candidate for casein kinase 2 (CK2)-mediated phosphorylation, with stress signalling, MKK4, JNK, ERK1 and p38 being able to enhance p-Ser650 (Gao *et al.* 2004). One study found that phosphorylation of Ser650 has no effect on AR function (Wong *et al.* 2004), but other studies found that mutation of Ser650 decreased transcription by about 30% (Zhou *et al.* 1994) and reduced nuclear export (Gioeli *et al.* 2006). Consistent with this, SNARE1, a protein that translocates AR from the nucleus to the cytoplasm and promotes p-Ser650, is involved in C-terminal HSP-interacting protein (CHIP) binding (E3 ubiquitin (Ub) ligase; Rees *et al.* 2006) and AR-Ub (Narayanan *et al.* 2010), suggesting that p-Ser650 down-regulates AR activity.

IKK and IKK2 have recently been shown to phosphorylate the AR *in vitro*, consistent with the IKK inhibitor BMS345541 being able to reduce AR-P *in vivo* (Jain *et al.* 2012), indicating crosstalk with yet another pathway and further demonstrating the increasing complexity of AR signalling.

#### AR-P and disease

Suggesting a role in a more aggressive disease state, non-liganded activation of the AR can feature enhanced AR-P. For example, overexpression of Her2/Neu results in elevated AR-P levels in AR-transfected DU145 cells (Sugita *et al.* 2004), IL6 activates AR via MAPK and Src (Ueda *et al.* 2002) and the activation of the CXCL12/CXCR4 axis can increase PI3K-dependent AR-P, all in the absence of androgens (Kasina & Macoska 2012). Furthermore, neuropeptides can influence AR-P, which is likely important in PC due to the occurrence of a neuroendocrine phenotype within some cells. This is further emphasised by observations that AR-P and increased AR activity are only seen in combination with very low androgen levels. This process appears to be dependent on PKC, PKA and MAPK, as revealed by use of specific inhibitor molecules (Dai *et al.* 2002). There is also a precedent that mutations within the AR can result in novel phosphorylation sites with functional outcomes, for example a missense mutation within exon 1 at Arg405 to a serine residue. However, in this case, the AR is less active than the

WT molecule as the PTM interferes with the binding of p300 (Lagarde *et al.* 2012).

Dysregulated AR-P at numerous sites has been implicated in PC development and progression. For example, Ser292 can be phosphorylated by Aurora A kinase, which is frequently overexpressed in numerous cancers, including PC, to promote cell survival, phosphorylation-dependent trans-activation and PSA induction in the presence or absence of androgens (Shu *et al.* 2010). One of the best-characterised sites is Ser81, where p-Ser81 is increased in carcinoma compared with normal tissue (Li *et al.* 2006). P-Ser81 is inhibited by the topoisomerase inhibitor camptothecin, which results in decreased AR activity as a result of reduced androgen binding and nuclear translocation (Liu *et al.* 2010a). Androgen-independent PC cell line models have reported increased levels of p-Ser81 (Hsu *et al.* 2011), with AR stabilisation (Golsteyn *et al.* 1989, Faus & Haendler 2008) and regulation of promoter specificity (Lee *et al.* 2007) being described as functional outcomes. Ser81 phosphorylation occurs in the nucleus, similar to Ser256 and Ser308 (Kesler *et al.* 2007), ~4 h post-androgen stimulation, suggesting that this residue is not responsible for enhancing nuclear translocation or DNA binding; hence, rapidly induced genes do not require this phosphorylation event (Chen *et al.* 2006). P-Ser81 can also be induced by activation of cells with conditioned media from stromal cells, indicating that this modification can play a role in stromal-epithelial interaction (Shigemura *et al.* 2009). Enzymes responsible for p-Ser81 include CDK9 (Gordon *et al.* 2010), CDK5 (Hsu *et al.* 2011) and CDK1 (Chen *et al.* 2012). Furthermore, Src can enhance p-Ser81 in a ligand-independent manner upon exposure to hepatitis B virus (HBV) viral factors (Chiu *et al.* 2007). P-Ser81 increases during mitosis when CDK1 is active, and inhibition of CDK1 and CDK9 results in decreased p-Ser81 while inhibiting AR chromatin binding and transcriptional activity (Chen *et al.* 2012). Mutation of two phenylalanine residues (Phe582 and Phe583) within the DNA recognition helix, which is seen in androgen insensitivity syndrome, results in hypophosphorylation at Ser81. This mutant AR localises in sub-nuclear foci with both CBP and glutamate receptor interacting protein 1 in the presence of androgen, suggesting that p-Ser81 occurs after nuclear localisation, ligand binding and interaction with CBP and other co-activators (Black *et al.* 2004).

#### Tyrosine phosphorylation

For many years, tyrosine phosphorylation of the AR remained undiscovered partly due to the fact that DHT cannot stimulate this PTM but EGF, Heregulin (HRG), IL6, FBS (Guo *et al.* 2006) and Etk can (Dai *et al.* 2010). Using mass spectrometry, p-Tyr534 was identified (Guo *et al.* 2006) and subsequently verified (Kraus *et al.* 2006). Interestingly, AR mutations at this site cannot be activated by EGF but they can still be activated by DHT, although are unresponsive to lower DHT concentrations. Codon switch experiments

demonstrated that this phosphorylation event is required for PSA induction at low levels of DHT or in response to growth factor stimulation. Furthermore, Tyr534-mutant AR is not stimulated to translocate to the nucleus by Src activation (Guo *et al.* 2006). The scaffold protein GNB2L1 (RACK1), which interacts with the AR to facilitate ligand-independent AR nuclear translocation (Rigas *et al.* 2003), can also modulate AR tyrosine phosphorylation and its interaction with Src (Kraus *et al.* 2006). Tyr534 phosphorylation is increased in refractory xenograft models and is present in refractory human samples of PC. The mechanism by which this modification results in the observed functional outcomes is unclear, but a conformational change, which leads to improved ligand binding capabilities, has been suggested (Guo *et al.* 2006). A further two p-Tyr sites have since been documented: Tyr267 and Tyr363. Both sites are phosphorylated in response to HRG via Ack1 (cdc42-associated tyrosine kinase). Tyr267 appears to be the dominant functional site as mutation studies demonstrated that, whereas mutation of Tyr363 can inhibit AR activity, mutation of Tyr267 completely abolished HRG-stimulated AR activation (Mahajan *et al.* 2007). EGF and Gas6 can also result in Tyr267 phosphorylation (Liu *et al.* 2010b). Anti-androgens have no effect on tyrosine phosphorylation at 267 (Mahajan *et al.* 2010); however, inhibition can be achieved using Dasatinib, a Src and Ack1 inhibitor (Liu *et al.* 2010b). The AR isoform, AR8, has recently been identified. This isoform is membrane bound via two palmitoylation sites. Upon over-expression, increased Tyr-P of AR is observed alongside enhanced interactions between Src and EGFR and AR and EGFR (Yang *et al.* 2011a).

The glycoprotein precursor, prosaposin, is amplified in PC and is expressed at high levels in metastatic androgen-insensitive cancers where it stimulates growth, migration and invasion. Treatment of LNCaP cells with prosaposin resulted in increased Ser81 phosphorylation but had no effect on serine phosphorylation in LNCaP-AI or prostate stromal cells. However, when stromal cells were treated with either DHT or saposin C, an increased AR tyrosine phosphorylation was observed (Koochekpour *et al.* 2008).

### AR phosphatases

Phosphorylation of the AR is a reversible process. Currently, only two protein phosphatases have been described to interact with and dephosphorylate the AR. Protein phosphatase 2A (PP2A) was the first enzyme to be identified to bind to ligand-activated AR and result in dephosphorylation at multiple sites (Table 3; Lin *et al.* 2002, Yang *et al.* 2005, 2007). Based on the sites, which are dephosphorylated, PP2A can result in enhanced AR stability and transcriptional activity. In addition, inhibition of PP2A maintains ligand-independent phosphorylation (Bhardwaj *et al.* 2011), which could be explained by dephosphorylation of Ser94, which is constitutively phosphorylated. A second phosphatase, PP1, has been shown to dephosphorylate Ser650 to result in

enhanced AR levels, transcriptional activity and nuclear localisation (Chen *et al.* 2009). As mentioned previously, Ser650 and Ser662 can be dephosphorylated in response to PKA stimulation by forskolin, resulting in decreased AR transcriptional activity and reduced ligand binding (Blok *et al.* 1998). The proteoglycans, Decorin (Hu *et al.* 2009), bicalutamide and estramustine can also reduce the levels of AR-P (Wang *et al.* 1999a,b); however, some anti-androgens can induce AR-P levels and enhance AR nuclear levels, although whether the increased AR-P is either cause or consequence is not apparent. In comparison to phosphorylation, dephosphorylation is understudied. With the commercialisation of kinase and phosphatase siRNA screens, it will only be a matter of time before a plethora of novel AR phosphatases are identified with characterisation of their cellular effects. However, due to the complexity of phosphorylation sites and stimuli, this will not be an easy task. Furthermore, while there is evidence that a number of phosphorylation sites can influence each other, it will be interesting to see how this idea develops and whether another layer of complexity can be added to this signalling pathway.

### Ubiquitination of the AR

Ub, a 76 amino acid protein with a molecular mass of ~8.5 kDa, is highly conserved and expressed in all tissues. Ubiquitination is the enzymatic process by which one or more Ub proteins becomes attached to another protein via the carboxylic acid group of the C-terminal glycine residue within activated forms of the protein. This process is catalysed by a series of enzymes, which function in an ordered sequence of events. First, an E1 enzyme functions to prepare the Ub moiety for protein attachment. An E2 enzyme then functions to conjugate the Ub to lysine residues within the target protein and thirdly an E3 Ub ligase provides specificity for the protein being targeted (Hicke 2001, Kerscher *et al.* 2006, Ande *et al.* 2009). Ubiquitination is an important mechanism by which the cell regulates the turnover of proteins; however, it can also function as a signalling moiety to affect functional outcomes of signalling pathways.

The downstream consequences of protein ubiquitination depend on the type of modification. For example, mono-ubiquitination vs poly-ubiquitination or variant chains formed through one of seven lysine residues in Ub, e.g. Lys48 vs Lys63 chains. Generally, poly-ubiquitination linked to Lys48 signals for the protein to be degraded by the proteasome, but poly-ubiquitination at Lys63 can indicate other processes including DNA damage sensing and repair, receptor internalisation, vesicular transport and signal transduction. The function of chains derived from other lysine sites in Ub (K6, K11, K27, K29 and K33) are less well characterised.

MDM2 was the first E3 Ub ligase identified for the AR (Lin *et al.* 2002). More recently, other E3 ligases have been identified including CHIP (Chymkowitch *et al.* 2011) and



RNF6 (Xu *et al.* 2009). The RNF6 E3 ligase can promote poly-Ub of AR similar to MDM2, but it can also promote AR mono-Ub (Chymkowitch *et al.* 2011). AR-Ub on the promoters of androgen-responsive genes is thought to function to clear the promoter ready for a subsequent round of transcription.

A number of proteins have been described to regulate this PTM. The chaperone protein, HSP27, can prevent MDM2-mediated AR-Ub by associating with the AR in response to androgen stimulation (Zoubeidi *et al.* 2007). Tumour susceptibility gene 101 (*TSG101*) can increase AR-mediated transcription, which coincides with increased mono-ubiquitination of ligand-bound AR while having no significant impact on the stability of the AR itself. It is thought that *TSG101* functions by preventing poly-ubiquitination rather than stimulating mono-ubiquitination (Burgdorf *et al.* 2004).

Interestingly, AR-P is a requisite for MDM2-mediated ubiquitination, and AR-P mutants are more stable than WT AR. A mechanism was proposed whereby Akt is activated by ligands including IGF1 or IL6 to result in the phosphorylation and subsequent activation of MDM2 concurrently with AR-P. This in turn permits MDM2-mediated AR-Ub to target AR to the proteasome for degradation (Lin *et al.* 2002). Phosphorylation of AR at Ser515 is required for AR-MDM2 association and subsequent ubiquitination. However, when this phosphorylation cannot occur and MDM2 fails to interact, CHIP is recruited to facilitate interaction with the proteasomal machinery (Chymkowitch *et al.* 2011). A later study demonstrated that HDAC1 was also present in a complex with AR and MDM2 at androgen-regulated promoters where it can attenuate AR transcriptional activity. Furthermore, AR poly-Ub is androgen dependent and can be attenuated by the lysine acetyltransferase and AR co-activator Tip60 (*KAT5*; Gaughan *et al.* 2005).

Proposed sites of ubiquitination are within the KLKK motif due to the dependency on deacetylation. However, poly-Ub at Lys845 and Lys847 incorporating Lys6 and/or Lys27 chains catalysed by the E3 Ub ligase, RNF6, has been reported. This modification functions as a binding site for other co-activator proteins, e.g. ARA54 (Xu *et al.* 2009). Interestingly, this is the first reported incidence of AR poly-Ub to enhance transcriptional activity rather than result in proteasomal degradation. RNF6 can facilitate AR-mediated transcription at a subset of genes that does not include PSA and transmembrane protease, serine 2 (*TMPRSS2*), but it can still modulate AR function under androgen-deprived conditions (Xu *et al.* 2009).

Deubiquitinase (DUB) enzymes are responsible for the removal of Ub moieties from proteins. They function by cleaving the Ub modification at Gly76. This process can have multiple outcomes, for example generating free Ub protein that can be reattached to another protein, or reversing proteasomal targeting. In 2005, USP10 was identified as the first DUB to co-activate the AR, although direct AR deubiquitination was not investigated in this study (Faus *et al.*

2005). USP26 was also identified by siRNA screening, as a DUB that can interact with AR in the nucleus and modulate AR-Ub in a cell line-specific and context-dependent manner. Interestingly, this study did not identify USP10 as an AR regulator (Dirac & Bernards 2010). More recently, USP10 was found to deubiquitinate chromatin proteins at AR-regulated genes in response to androgens to enable transcriptional activation. Moreover, when USP10 levels are reduced, the levels of AR remain unchanged suggesting that USP10 functions as an AR co-activator but does not focus its enzymatic activity towards the AR itself (Draker *et al.* 2011). Similarly, USP22 co-activates the AR without altering AR levels; however, AR-USP22 interaction has only been described in the absence of androgens (Zhao *et al.* 2008).

## AR-sumoylation

Sumoylation is the process by which small Ub-like modifier (SUMO) is added to a protein (Gareau & Lima 2010, Wilkinson & Henley 2010). There are three members of the SUMO protein family: SUMO1, SUMO2 and SUMO3. All these proteins are strongly conserved through evolution, indicating their importance in cellular mechanisms. The process by which the SUMO moiety is added to the target protein is very similar to that of the Ub system. The process requires an E1 activating enzyme, an E2 conjugating enzyme and an E3 ligating enzyme. The E1 activating enzyme consists of two subunits, Aos1 and Uba2, that show homology with the yeast activating enzyme Uba1, for Ub substrate (Johnson *et al.* 1997). In this first step in the pathway, the Gly-Gly residues are exposed on the C-terminus of SUMO, then a thiol ester adduct is formed between Uba2 and SUMO in an ATP-dependent process. The only E2 conjugating enzyme discovered to date is Ubc9 (Poukka *et al.* 2000). Finally, the E3 ligase enzyme attaches the SUMO to the protein of interest. Currently, there have been three types of E3 discovered. The first is the protein inhibitor of activated STAT (PIAS) family of proteins, the second is RanBP2 (localised to the nuclear pore complex) and the third is Pc2 (component of the polycomb protein complexes). The function of sumoylation is thought to determine cellular localisation, for example RanGAP1 is located in the cytoplasm but upon sumoylation is relocated to the nuclear envelope and, in some cases, can regulate transcriptional activity. Furthermore, sumoylation has been suggested as a protective mechanism to prevent structural alterations to proteins in response to cellular stress (Mukherjee *et al.* 2009). It has also been suggested that sumoylation may compete with the ubiquitination process as quite often the same residues within proteins are targeted by both pathways.

AR-sumoylation (AR-Su) was first described in 2000 after the SUMO conjugating enzyme, Ubc9, was found to interact with the AR to result in increased transcriptional activity (Poukka *et al.* 2000). The consensus sequence for protein sumoylation, (I/L/V)-K-X-D/E, was detected twice within

the AR protein. AR-Su occurs within the N-terminal domain of the protein at Lys386 predominantly and at Lys520 to a reduced extent. This process is enhanced in the presence of ligand, which was thought to be a result of the increased levels of AR within the nucleus where Ubc9 resides (Poukka *et al.* 2000). However, it has since been noted that constitutively nuclear apo-AR (unliganded AR) or antagonist-bound AR are only weakly sumoylated compared with agonist-bound AR (Kaikkonen *et al.* 2009). Sumoylation of AR at these highly conserved SUMO-acceptor sites results in negative regulation of the AR. It has been demonstrated that sumoylation within the N terminal domain of the AR impairs the intramolecular interaction with the ligand-binding domain, which is required for efficient transcriptional activation (Kaikkonen *et al.* 2009).

Interestingly, the increased transcriptional activity detected following overexpression of Ubc9 is not dependent on its enzymatic activity, suggesting that a more complicated chain of events is involved (Kaikkonen *et al.* 2009). Furthermore, upon mutation of the SUMO-accepting sites within the AR, greater transcriptional activity is observed, suggesting that AR-Su is in fact an inhibitory process, similar to sumoylation of c-Jun. This may be expected as sumoylation sites are within the N terminal region, which is involved in the intramolecular interaction with the LBD upon hormone binding. Therefore, the addition of any large moieties to this region may interfere with this critical process. Furthermore, sumoylation is thought to result in the recruitment of co-repressors. For example, sumoylated p300 recruits HDAC6 while sumoylated ELK1 recruits HDAC2. This is indeed the case for AR where Daxx has recently been described as a SUMO-reader protein, which interacts with the AR in a sumoylation-dependent manner to inhibit DNA binding (Shih *et al.* 2007).

Early studies of AR-Su focused on the addition of SUMO1 to the AR, but later studies also demonstrated that SUMO3 and SUMO2 can function as AR regulators. However, in contrast to SUMO1, which inhibits AR activity, SUMO2 and -3 strongly enhance AR transcriptional activity in LNCaP cells. This differs from their effect on AR activity in primary prostate epithelial cells, where SUMO3 has a weak negative effect on AR activity, suggesting that effects are dependent on cell type and the cellular background. Interestingly, the sites of AR-Su and the ligase activity were found to be independent for the effects (Zheng *et al.* 2006).

HDAC4 can promote AR-Su, suggesting that it may function as an E3 ligase, similar to activity of other HDACs for other proteins. As HDAC4 is itself a target of sumoylation, it has been suggested that a sumoylation cascade may take place, yet a SUMO-deficient HDAC4 can still promote AR-Su (Yang *et al.* 2011b).

The E2 enzyme, Ubc9, provides substrate specificity and no other factors are required for it to function. Ubc9 has been found to interact with at least five nuclear receptors including the AR, where it can stimulate transcriptional activity. Interestingly, upon mutation of the cysteine residue to

which SUMO1 binds, Ubc9 can still enhance AR transcriptional activity. It is thought that Ubc9 interaction with other proteins via its C terminus, specifically residues 151–158, is important for AR activation (Chang *et al.* 2007).

The inhibitors of activated STAT (PIAS) family of proteins are important E3 ligase enzymes in the sumoylation pathway. There are five members of the family, - $\alpha$ , - $\beta$ , - $\gamma$ , -1 and -3. PIAS $\alpha$  and PIAS1 can function as E3 enzymes for the AR; however, PIAS3, - $\gamma$  and - $\beta$  do not. Furthermore, PIAS interaction with the E2, Ubc9, is necessary for E3 ligase activity (Nishida & Yasuda 2002).

The E3 protein PIAS $\alpha$  has been found to interact with the AR to modulate its activity, for which its E3 ligase activity and AR SUMO acceptor sites are essential. Additionally, this protein interacts with Ubc9 and SUMO1 and has been found to enhance the *in vitro* sumoylation of AR and c-Jun (Kotaja *et al.* 2002). As PIAS is a nuclear protein, the AR must translocate to the nucleus to be sumoylated, an idea that is supported by the observation that AR ligand binding is required in order for sumoylation to occur (Nishida & Yasuda 2002).

Interestingly, PIAS1 and PIAS $\alpha$  can have differing effects on AR activity depending on the presence of SUMO1. When SUMO1 is present, AR activity is repressed, but in the absence of SUMO1, PIAS $\alpha$  and to a lesser extent PIAS1 can activate the AR (Zheng *et al.* 2006).

PIASy can also repress the transcriptional activity of the AR, yet it plays no role in AR-Su. This protein binds the AR DBD via its RING finger-like domain; however, even in the absence of its E3 ligase activity and mutation of the AR SUMO acceptor sites, it can still inhibit the transcriptional activity of the AR. Within the PIASy protein, there are two repression domains, RD1 and RD2, of which only RD1 is required for inhibition of the AR. RD1 has been shown to bind to HDAC1 and HDAC2, two proteins that have been previously shown to inhibit AR activity via their enzymatic activity (Gaughan *et al.* 2005). In the case of PIASy, HDAC activity of these two proteins is required in order for PIASy to inhibit AR transcriptional activity (Gross *et al.* 2004).

A PIAS-like protein termed, human zinc finger containing, MIZ1, PIAS-like protein on chromosome 10 (hZIMP10) has also been found to interact with the AR specifically in comparison with other steroid receptors. This particular protein has been found to reside at cell cycle-regulated DNA replication foci during S phase, suggesting a role for this protein in the replication process. hZIMP10, like PIAS1 and PIAS $\alpha$ , can enhance the sumoylation of the AR and therefore the authors suggested that there may be a role for the AR and SUMO1 in DNA replication and chromatin modification (Sharma *et al.* 2003). From the same laboratory, a homologue of hZIMP10 has been identified, hZIMP7. Similarly, this protein was found not only to associate with the AR and DNA replication foci, but also to interact with the SWI/SNF complex, further supporting a role in chromatin modification (Huang *et al.* 2005).

AR–Su is a reversible process (Poukka *et al.* 2000) achieved by a family of SUMO proteases termed Sentrin/SUMO-specific proteases (SENPs). There are six described SENPs: -1, -2, -3, -5, -6 and -7. SENP1 resides in the nucleus, whereas SENP2 is associated with the nuclear envelope. SENP3 and SENP5 are nucleolar and display a preference for SUMO-2/3. SENP6 and -7 are also nuclear but currently their function is unknown (Cheng *et al.* 2006).

SENP1 was the first of these proteases to be described as an enhancer of AR-mediated transcription, although its activity was not directed at the AR itself but towards its co-repressor protein HDAC1 to reduce its deacetylase activity (Cheng *et al.* 2004). However, a more recent study declared that intact SUMO acceptor sites are required on the AR for SENP1 to have its activating effect on the AR (Kaikkonen *et al.* 2009). Yet both studies clearly show that SENP1 can indeed desumoylate the AR. Furthermore, SENP1 has been found to be overexpressed in prostatic intraepithelial neoplasia (PIN) and PC, and transgenic mice overexpressing SENP1 in the prostate were found to have early PIN development (Cheng *et al.* 2006).

## AR PTMs and Kennedy's disease

Early studies into the importance of the glutamine repeat sequence (CAG repeat) found that deletion of this region results in a single form of the AR being observed instead of a 110 and a 112 kDa doublet, suggesting that the phosphorylation responsible for this doublet is unable to occur. Furthermore, if this repeat region is further expanded, the upshift was enhanced (Jenster *et al.* 1994). Increased CAG repeat length is also associated with an increased rate of Ser94 phosphorylation (Choong *et al.* 1998).

PKA activation and PKA inhibition have been found to inhibit and activate the activity of the AR respectively. Interestingly, following mutation of AR–Ac acceptor sites, manipulation of PKA activity had no effect on AR activity (Fu *et al.* 2004).

Phosphorylation of Ser515 has been found to be critical in Kennedy's disease. In this disease, the AR has an extended N-terminal polyglutamine (CAG) tract, which leads to proteolytic cleavage resulting in cell death. This expansion results in activation of three kinase pathways: ERK, p38 and SAPK/JNK, suggesting that phosphorylation could be important. Upon mutation of the Ser515 residue to an alanine, AR-induced cell death and cleavage products were reduced, suggesting that Ser515 phosphorylation promotes caspase 3-mediated AR cleavage to yield the cytotoxic AR fragments (LaFevre-Bernt & Ellerby 2003).

Akt, when activated by IGF1, can also phosphorylate AR with expanded polyglutamine tracts. Interestingly, using phosphorylation mimic mutants (Ser213 and Ser791), one study found that phosphorylation by Akt can reduce the toxicity of the mutant protein (Palazzolo *et al.* 2007) by

enhancing the destruction of the AR via ubiquitination (Palazzolo *et al.* 2009).

Links between acetylation and the AR have also been demonstrated in the study of Kennedy's disease. In this disease, the presence of an expanded polyglutamine region causes the AR to aggregate. It appears that the expanded polyglutamine mutant AR protein binds CBP, thereby quenching the acetylase activity, which can result in altered protein acetylation within the cell (McCampbell *et al.* 2001). Furthermore, ARs with expanded CAG repeats have been found to undergo PTM, including phosphorylation and acetylation, in the absence of ligand (Lieberman *et al.* 2002). Interestingly, AR mutated at both Lys632 and Lys633 was reported to misfold and aggregate, thereby mimicking the effects of an expanded CAG repeat, with effects on receptor trafficking also being reported. This double lysine mutant can also interact with HSP40 and -70 and the E3 ligase, CHIP, to result in inhibited proteasome function (Thomas *et al.* 2004).

Enhanced sumoylation of the AR has been suggested as a treatment for Kennedy's disease to alleviate the ligand-dependent misfolding and subsequent AR aggregation, which results in neurotoxicity. Sumoylation of the AR greatly reduces AR aggregation without altering the transcriptional activity or overall AR levels (Mukherjee *et al.* 2009).

## PTM interplay and future research

The AR methyl and acetyl acceptor sites, Lys630 and Lys632, suggest that there will be crosstalk between these two types of modification. Many studies that investigate the effects of AR–Ac have used mutant AR proteins. Now that AR–Me has been described at the same sites as acetylation, it can no longer be concluded that all the effects seen are due to the lack of AR–Ac; perhaps some are due to lack of Me. One important observation is that acetylation appears to be important for AR translocation to the nucleus (Shiota *et al.* 2010) and Me should occur in the nucleus based on the cellular localisation of most methyltransferase enzymes, including Set9. Therefore, acetylation should precede Me upon stimulation with androgens. Furthermore, deacetylation will be required before Me. SIRT1 is a deacetylase enzyme that can repress DHT-mediated AR expression by binding to and deacetylating AR. This deacetylation event has been found to be neuroprotective and can protect against proteotoxicity in Kennedy's disease (Montie *et al.* 2011). It can also reduce the AR N/C terminal interaction and inhibit contact-independent cell growth. This particular deacetylase enzyme is a member of the class III deacetylase enzymes, which are dependent on NAD levels within the cell. Interestingly, it has been reported that there is an increase in lactate within cells during PC progression, which is predicted to inhibit the action of these deacetylase enzymes, resulting in enhanced AR function. It appears that AR Lys630 is more susceptible to SIRT1 (Fu *et al.* 2006), which is supported by Lys630Ala

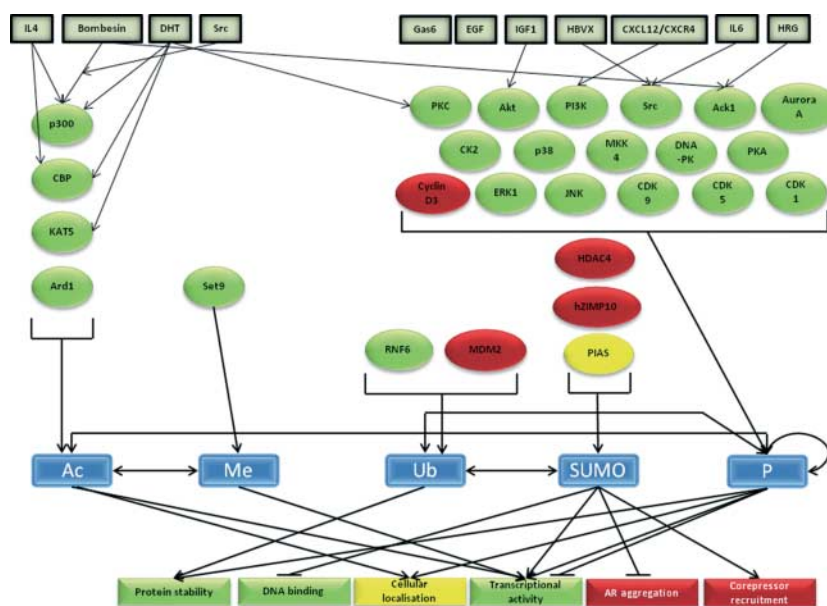
substitution also demonstrating reduced N/C terminal interaction (Ko *et al.* 2011), raising the question as to whether acetylation and Me can functionally interact via the action of SIRT1. As AR-Me is not currently well documented, it may emerge that many other KMTs can methylate the AR. Furthermore, the role of demethylation has not been investigated at this stage. As many KDMs can influence AR activity, e.g. LSD1 and KDM4A, further research is required to elucidate whether Me is also a reversible process similar to other PTMs. It will be interesting to determine how a flux in Me state can be influenced by other PTMs such as acetylation and indeed how Me changes can affect other PTMs of the AR.

Functional relationships between acetylation and phosphorylation of the AR have been proposed. Efficient regulation of the AR by AKT, PKA and JNK requires functional acetylation sites within the AR. Indeed, mutation of acetyl acceptor sites within the AR reduced the ability of the AR to become phosphorylated. Similarly, point mutations at phosphorylation sites reduced the ability of TSA to stimulate the AR, for example Ser94Ala results in decreased transactivation by p300 (Fu *et al.* 2004). Co-occurrence of phosphorylation and acetylation within the same protein is widely seen with the modifications occurring at interaction interfaces. As phosphorylation of Ser94 and Ser213 is stimulated by androgens similar to AR-Ac, functional interplay may be occurring here. Similarly, due to the identification of AR-Me sites being shared with AR-Ac sites, there may also be potential for interaction between phosphorylation and Me.

Sumoylation has been suggested as a competitor for ubiquitination as the same residues are often targeted within proteins. At present, there is no evidence to suggest that this is the case for AR. However, there is minimal information relating to AR Ub sites, with the functional outcome and types of Ub modifications being poorly understood at present.

As mentioned previously, AR-P at Ser515 is required for MDM2-mediated AR-Ub. When Ser515 cannot be phosphorylated, the AR interacts with an alternative E3 ligase, CHIP (Chymkowitch *et al.* 2011). Association with CHIP can be promoted by AR-P at Ser650 to result in increased AR-Ub (Narayanan *et al.* 2010). Interestingly, Ser515 phosphorylation mediated by EGF at low androgen concentrations can influence phosphorylation of Ser650 (Wong *et al.* 2004). This would suggest that the AR would be less stable in response to this modification; however, phosphorylation at Tyr534 can be observed under these conditions to result in reduced AR-CHIP interaction and an increase in AR levels (DaSilva *et al.* 2009). Phosphorylation of Ser515 is reduced upon mutation of Ser578 to result in nuclear retention of the AR (Ponguta *et al.* 2008), which may be due to inhibition of MDM2-mediated AR-Ub at androgen-regulated genes.

In summary, this highlights the complexity of PTM interactions within one molecule to regulate its activity (Fig. 2). This will only increase in scale as all types of modification are better characterised in the coming years. Furthermore, with respect to the AR itself, the complexity of its regulation further emphasises its biological importance. Moreover, there is potential for PTMs on different proteins to



**Figure 2** Summary of enzymes and molecules involved in regulation of androgen receptor (AR) post-translational modifications and their effects on AR function. The AR can be acetylated (Ac), methylated (Me), sumoylated (Su), ubiquitinated (Ub) and phosphorylated (P) by a number of enzymes to have either positive effects on the AR (shown in green) or negative effects on the AR (shown in red).



interact, an idea that is being developed between histone proteins and transcription factors, further expanding the complexity of this interaction pathway.

### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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