Protein Arginine Methyltransferase 6 Enhances Polyglutamine-Expanded Androgen Receptor Function and Toxicity in Spinal and Bulbar Muscular Atrophy

Highlights

- PRMT6 is a coactivator of AR whose function is enhanced by polyglutamine expansion

- PRMT6 methylates the AR at the two Akt consensus site motifs RXRXXS

- AR arginine methylation by PRMT6 and phosphorylation by Akt are mutually exclusive

- PRMT6 enhances mutant AR toxicity in spinobulbar muscular atrophy cells and flies

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In Brief

The relationship between polyglutamine protein structure/function and neurodegeneration is poorly understood. Using SBMA as a model of polyglutamine diseases, Scaramuzzino et al. show that protein arginine methyltransferase 6 enhances polyglutamine androgen receptor function and toxicity through direct modification of the disease protein.

Protein Arginine Methyltransferase 6 Enhances Polyglutamine-Expanded Androgen Receptor Function and Toxicity in Spinal and Bulbar Muscular Atrophy

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SUMMARY

Polyglutamine expansion in androgen receptor (AR) is responsible for spinobulbar muscular atrophy (SBMA) that leads to selective loss of lower motor neurons. Using SBMA as a model, we explored the relationship between protein structure/function and neurodegeneration in polyglutamine diseases. We show here that protein arginine methyltransferase 6 (PRMT6) is a specific co-activator of normal and mutant AR and that the interaction of PRMT6 with AR is significantly enhanced in the AR mutant. AR and PRMT6 interaction occurs through the PRMT6 steroid receptor interaction motif, LXXLL, and the AR activating function 2 surface. AR transactivation requires PRMT6 catalytic activity and involves methylation of arginine residues at Akt consensus site motifs, which is mutually exclusive with serine phosphorylation by Akt. The enhanced interaction of PRMT6 and mutant AR leads to neurodegeneration in cell and fly models of SBMA. These findings demonstrate a direct role of arginine methylation in polyglutamine disease pathogenesis.

INTRODUCTION

Polyglutamine diseases are neurodegenerative disorders caused by expansion of CAG trinucleotide repeats encoding polyglutamine tracts in specific genes (Orr and Zoghbi, 2007). The family of polyglutamine diseases includes spinal and bulbar muscular atrophy (SBMA), Huntington's disease (HD), dentatorubral-pallidoluysian atrophy (DRPLA), and spinocerebellar ataxia (SCA) type 1, 2, 3, 6, 7, and 17. These disorders are caused by glutamine expansions in androgen receptor (AR), huntingtin, atrophin-1, ataxin-1, ataxin-2, ataxin-3, CACNA1A, ataxin-7, and the TATA-box binding protein (TBP), respectively. One unsolved question in the field of polyglutamine diseases is why the same mutation in different genes causes the dysfunction and death of specific populations of neurons in the CNS, leading to different clinical disease manifestations. The selective pattern of neuronal degeneration in the CNS contrasts with the widespread distribution or housekeeping function displayed by the disease proteins. This indicates that expansion of polyglutamine tracts is necessary, but not sufficient to cause disease. Evidence has been obtained that intrinsic protein features play a critical role in dictating the initiation and progression to cellular dysfunction and degeneration (Graham et al., 2006; Katsuno et al., 2002; Klement et al., 1998; Tsuda et al., 2005), suggesting a mechanistic link between expanded polyglutamine-induced toxicity and protein structure/function (Parodi and Pennuto, 2011).

SBMA clinical and pathological features clearly illustrate the relevance of protein context to disease pathogenesis. SBMA is an X-linked motor neuron disease characterized by selective degeneration of lower motor neurons (Kennedy et al., 1968). In the family of polyglutamine diseases, SBMA is unique in that the disease fully manifests only in males. The hormone-dependent nature of SBMA is well recapitulated in animal models of disease, including the fruit fly *Drosophila melanogaster* (Pandey et al., 2007; Takeyama et al., 2002). The sex specificity of SBMA

and the toxicity of polyglutamine-expanded AR result from binding to its natural ligand, testosterone, or its more potent derivative, dihydrotestosterone (DHT). Upon hormone binding, AR translocates to the nucleus, undergoes a conformational change that leads to amino/carboxy-terminal (N/C) interactions, binds DNA at androgens response elements (AREs), and recruits specific transcription co-factors, including chromatin remodeling factors, to regulate the expression of androgen-responsive genes. Although most of these hormone-induced post-translational events have been associated with disease pathogenesis (Katsuno et al., 2003, 2005; Lieberman et al., 2002; Montie et al., 2009; Nedelsky et al., 2010; Orr et al., 2010), the mechanism through which hormone binding converts mutant AR into a toxic species is an important open question.

In response to hormone binding, AR acquires numerous post-translational modifications (Pennuto et al., 2009), most of which play a critical role in disease pathogenesis (Parodi and Pennuto, 2011). We previously demonstrated that phosphorylation of polyglutamine-expanded AR by Akt at serines 215 and 792, which lie in the Akt consensus site motif RXRXXS (where R is arginine, S serine, and X any amino acid), reduces hormone binding and AR transactivation and protects from neurodegeneration (Palazzolo et al., 2007; Palazzolo et al., 2009). Similar to phosphorylation, arginine methylation is a post-translational modification with major impact on protein structure and function (Bedford and Clarke, 2009). Arginine methylation is catalyzed by a family of enzymes known as protein arginine methyltransferases (PRMTs), which differ in their activity, substrate specificity and subcellular localization. Mammalian cells express at least 11 PRMTs. Apart from the most recently identified PRMT10 and 11, all the other PRMTs have catalytic activity and are classified as type I or II depending of the type of methylated arginine generated. Type I includes PRMT1, 2, 3, 4, 6, and 8 and catalyzes the addition of two methyl groups to one of the two ω-guanidino nitrogen atoms of arginine, thereby generating asymmetric dimethylarginine. Type II includes PRMT5, 7, and 9 and catalyzes the addition of one methyl group to each ω-guanidino nitrogen atoms to generate symmetric dimethylarginine. PRMTs target histones and non-histone proteins (Di Lorenzo and Bedford, 2011; Wei et al., 2014) and have been shown to act as co-factors of AR and other nuclear hormone receptors (Lee et al., 2005; Meyer et al., 2007; Sun et al., 2014). However, nothing is known as to whether PRMTs target polyglutamine proteins and play a role in polyglutamine disease pathogenesis.

To address this question, we used SBMA as a model of polyglutamine diseases. Our results show a key role for arginine methylation in the pathogenesis of polyglutamine diseases and provide evidence that a causative link between primary structure and function of polyglutamine protein is the underlying mechanism in the pathogenesis of polyglutamine diseases.

RESULTS

PRMT6 Is a Co-Activator of Polyglutamine-Expanded AR
To determine whether arginine methylation contributes to polyglutamine-induced neurodegeneration, we investigated the role of PRMT function on SBMA pathogenesis. First, we as-

sessed whether eight mammalian PRMTs (PRMT1–8) colocalize and interact with mutant AR. We analyzed the subcellular distribution of the PRMTs and mutant AR in COS1 cells expressing polyglutamine-expanded AR with 65 glutamine residues (AR65Q) and the PRMTs tagged to enhanced GFP (EGFP). Consistent with previous reports (Herrmann et al., 2009), PRMT1, 3, and 4 localized predominantly to the cytosol, PRMT2 and 7 localized to the nucleus and cytosol, PRMT6 was present almost exclusively in the nucleus, PRMT5 formed cytosolic aggregates, and PRMT8 had plasma membrane localization due to myristoylation (Figure S1A). AR localized mostly to the cytosol in vehicle-treated cells and to the nucleus in DHT-treated cells. AR subcellular localization and nuclear translocation in response to hormone treatment were not affected by the overexpression of any of these PRMTs. In the DHT-treated cells, mutant AR co-localized with PRMT2, 6, and 7 in the nucleus. Similar results were obtained with non-expanded AR (data not shown). By immunoprecipitation assay in DHT-treated HEK293T cells co-expressing Flag-tagged AR together with either soluble EGFP or the EGFP-tagged PRMTs, normal and polyglutamine-expanded AR specifically formed a complex with PRMT2, 6, and 7 (Figure 1A and Figure S1B). To test whether the PRMTs act as transcription co-factors of AR, we measured AR transactivation by transcriptional assay in HEK293T cells transfected with either normal AR (AR24Q) or AR65Q under the control of the Cytomegalovirus promoter, and using as reporter the luciferase gene whose expression was driven by an ARE, as previously described (Palazzolo et al., 2007). Among the PRMTs tested here, only PRMT6 significantly increased the transcriptional activity of AR (Figure 1B). The effect of PRMT6 was hormone dependent, indicating that PRMT6 acts as a co-activator of AR rather than as a general transcription activator, and it did not result from altered AR expression (Figure S1C). Importantly, PRMT6 increased the transactivation of AR24Q by 3.3-fold and that of AR65Q by 5.3-fold, indicating that the effect of PRMT6 on AR transactivation is enhanced by polyglutamine expansion. Similar results were obtained by expressing non-expanded and polyglutamine-expanded AR under the control of an elongation factor 1 promoter (Figure S1D). These results show that PRMT6 is a specific AR co-activator, whose function is enhanced by polyglutamine expansion.

Polyglutamine Expansion Enhances the Interaction of AR with PRMT6 in Neuronal and Patient-Derived Cells
PRMT6 is expressed to a similar extent in control and SBMA cells, including rat PC12 cells stably expressing either normal AR with 10 glutamine residues (AR10Q) or mutant AR with 112 glutamine residues (AR112Q) (Walcott and Merry, 2002), mouse motor neuron-derived MN-1 cells stably expressing either AR24Q or AR65Q (Brooks et al., 1997), as well as human primary fibroblasts and induced pluripotent stem cells (iPSCs) derived from normal subjects and SBMA patients (Grunseich et al., 2014) (Figure S2A). Upon hormone treatment, normal and polyglutamine-expanded AR colocalized with endogenous PRMT6 in the nucleus of control and SBMA PC12 cells (Figure 1C and Figure SB). AR112Q forms intranuclear inclusions in response to hormone binding in the PC12 cells (Walcott and Merry,
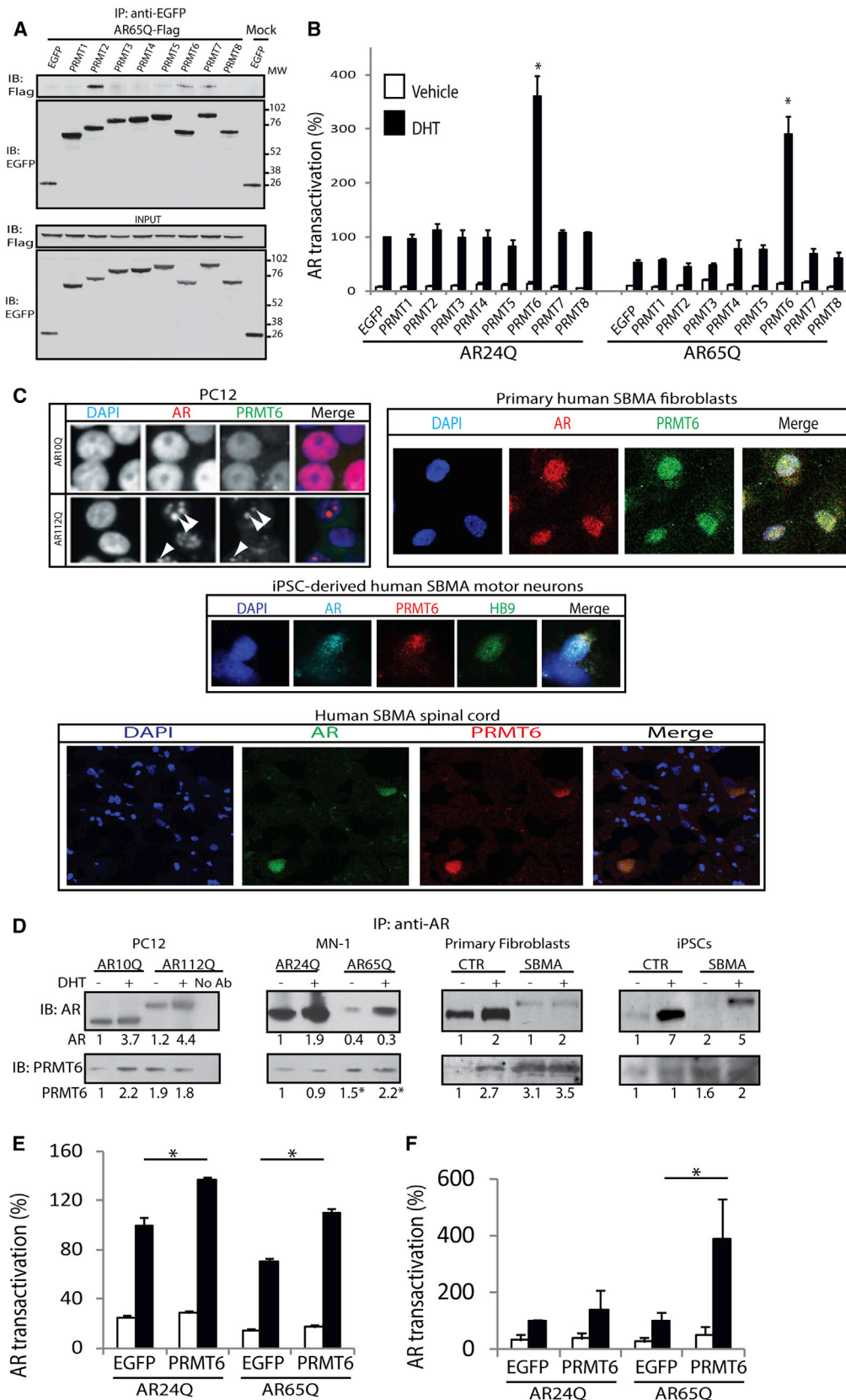
(legend on next page)

2002), and PRMT6 localized to AR-positive intranuclear inclusions in these cells (Figure 1C, arrows). AR and PRMT6 co-localized in the nucleus of control and SBMA human primary fibroblasts as well as iPSCs reprogrammed to motor neurons. Notably, PRMT6 was expressed and co-localized with polyglutamine-expanded AR in the ventral horn motor neurons of spinal cord autopsy specimens derived from an SBMA patient (Figure 1C and Figure S2B). By immunoprecipitation assays, both normal and polyglutamine-expanded AR formed a complex with endogenous PRMT6 in PC12 and MN-1 SBMA cells (Figure 1D and Figure S2C). Furthermore, endogenous AR and PRMT6 formed a complex in control and SBMA human primary fibroblast cells and iPSCs. The AR/PRMT6 interaction occurred both in the absence and presence of hormone and was significantly enhanced by polyglutamine expansion in the MN-1 cells. By transcriptional assay, overexpression of PRMT6 increased the transactivation of normal AR by 3.3-fold and that of polyglutamine-expanded AR by 5.7-fold in the MN-1 cells (Figure 1E). In mouse primary cortical neurons, PRMT6 increased the transactivation of normal AR by 1.4-fold, but this difference was not significant (Figure 1F). Rather, in primary neurons PRMT6 significantly increased the transactivation of polyglutamine-expanded AR by 3.8-fold. Taken together, these results indicate that polyglutamine expansion enhances the structural and functional interaction of AR with its co-activator PRMT6 in neuronal and patient-derived cells.

Transactivation of AR by PRMT6 Requires the Catalytic Activity and LXXLL Motif of PRMT6 and the "Activating Function 2" Surface of AR

We then investigated the mechanism through which PRMT6 transactivates AR. The interaction between AR and co-regulators can be inhibited by the anti-androgen 5-hydroxy-1,7-bis(3,4-dimethoxyphenyl)-1,4,6-heptatriene-3-one (ASC-J9), which has been shown to rescue the phenotype of a mouse model of SBMA (Yang et al., 2007). In a transcriptional assay, ASC-J9 significantly and dose dependently decreased the transcriptional activity of polyglutamine-expanded and non-expanded AR induced by PRMT6 (Figure 2A and Figure S3A). To address whether the catalytic activity of PRMT6 is required to transactivate AR, we performed transcriptional assays in cells treated with the pan-PRMT inhibitor adenosine dialdehyde (Adox). Adox reduced polyglutamine-expanded AR transactivation induced by DHT by 30%, and it completely abolished the effect of PRMT6 on AR transactivation (Figure 2B and Figure S3B). Similar results were obtained using the PRMT inhibitor 7,7'-carbonylbis(azanediyl)bis(4-hydroxynaphthalene-2-sulfonic acid)

(AMI-1). Because Adox and AMI-1 are not specific inhibitors of PRMT6, we generated a PRMT6 methylation-deficient mutant by substituting valine 86 with lysine and aspartate 88 with alanine (PRMT6-V86K,D88A, Figure 2C) (Boulanger et al., 2005). PRMT6-V86K,D88A retained its capability to bind polyglutamine-expanded and normal AR (Figure 2B and Figure S3B, insets), but it failed to transactivate AR (Figure 2B and Figure S3B), indicating that PRMT6 requires its catalytic activity to transactivate AR.

Transcriptional regulation by steroid receptors involves the interaction with co-factors bearing LXXLL (where L is leucine and X is any amino acid) or FXXLF (where F is phenylalanine) motifs (Heery et al., 1997). In search for FXXLF and LXXLL motifs in the PRMT family, we analyzed the sequence of the 11 known PRMTs (Wolf, 2009). None of these PRMTs have the FXXLF motif, while only PRMT6 contains the LXXLL motif ^{353}LRVLL^{357} (Figure 2C and Figure S4). To establish whether the ^{353}LRVLL^{357} motif of PRMT6 is necessary for AR transactivation, we substituted leucines 356 and 357 with alanines, thereby generating PRMT6-LXXAA mutant (Heery et al., 1997). Mutation of the LXXLL motif decreased the interaction with polyglutamine-expanded and normal AR (Figure 2D and Figure S3C, inset) and abolished AR transactivation (Figure 2D and Figure S3C), indicating that the ability of PRMT6 to enhance AR transcriptional activity is dependent on the integrity of the ^{353}LRVLL^{357} motif.

Steroid receptor primary structure is composed of an amino-terminal domain (NTD), a DNA-binding domain (DBD), and the ligand-binding domain (LBD) (Figure 2C). Transactivation of steroid receptors by co-factors bearing the LXXLL motif occurs through interaction with the "activating function 2" (AF-2) surface in the LBD, which provides a hydrophobic surface flanked by two conserved opposing charged amino acids, lysine 720 (K720) and glutamic acid 897 (E897) (Trapman and Dubbink, 2007). To test whether the interaction between PRMT6 and AR occurs through the AF-2 surface, we performed transcriptional assay in cells expressing AR variants in which either K720 was substituted with alanine (K720A) to reduce interaction with co-factors or E897 was substituted with lysine (E897K) to disrupt binding to co-factors (Figure 2E). As controls, we tested mutation of alanine 574 to aspartate (A574D) to prevent DNA binding, and mutation of glycine 21 to glutamic acid (G21E) to abolish the N/C interactions without altering interaction with co-regulators. As expected, the A574D mutation did not respond to hormone stimulation or to PRMT6 transactivation. The G21E mutation had no effect on AR transactivation induced by PRMT6. The K720A mutation decreased the effect of PRMT6 on AR transactivation by

Figure 1. PRMT6 Is a Co-Activator of AR whose Function Is Augmented by Polyglutamine Expansion in Neuronal and Patient-Derived Cells

(A) Immunoprecipitation assay in HEK293T cells transfected with Flag-tagged AR65Q and soluble EGFP or EGFP-tagged PRMT1-8 and treated with DHT (10 nM, 24 hr). Shown is one experiment out of three. IP, immunoprecipitation; IB, immunoblotting; MW, molecular weight.

(B) Transcriptional assay in HEK293T cells transfected with AR24Q and AR65Q, EGFP, EGFP-tagged PRMTs, and the androgen-responsive element (ARE)-luciferase reporter vector and treated as in (A). Graph, mean ± SEM, n = 3, *p = 0.001.

(C) Immunofluorescence analysis treated as in (A). Intranuclear inclusions in PC12 cells treated with DHT (10 μM, 48 hr) are indicated by arrowheads. AR, PRMT6, and HB9 were immunostained with specific antibodies and nuclei with DAPI.

(D) Immunoprecipitation assay from cells treated as in (C). Quantification (average, n = 3–4) of AR and PRMT6 is shown at the bottom of each panel, and values were normalized to the levels of normal AR and PRMT6 in vehicle-treated cells, *p = 0.05.

(E and F) Transcriptional assays in motor neuron-derived MN-1 cells (E) and mouse primary cortical neurons (F) treated with DHT (10 nM, 24 hr). Graph, mean ± SEM, n = 3, (E) *p = 0.001; (F) *p = 0.01.
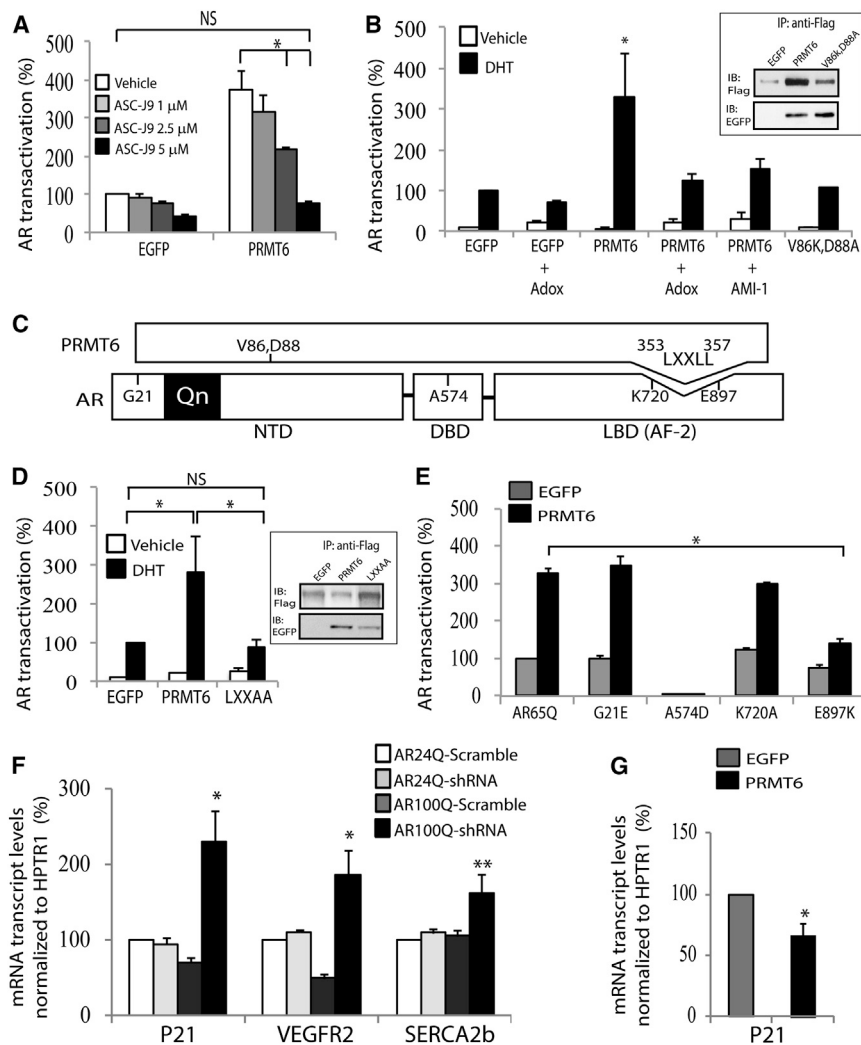
Figure 2. AR-PRMT6 Functional Interaction Requires PRMT6 Catalytic Activity and Is Mediated by the LXXLL Motif of PRMT6 and the AF-2 Surface of AR

(A) Transcriptional assay in HEK293T cells transfected with AR65Q together with EGFP and EGFP-tagged PRMT6, treated with DHT (10 nM, 24 hr) and either vehicle or ASC-J9. Graph, mean ± SEM, n = 3, *p = 0.0003, NS, nonsignificant.

(B) Transcriptional assay in HEK293T cells transfected with AR65Q and soluble EGFP, EGFP-tagged PRMT6, or the catalytically inactive PRMT6-V86K,D88A mutant, and treated with vehicle, DHT, and the PRMT inhibitors Adox (10 μM) and AMI-1 (100 μM) for 24 hr. Graph, mean ± SEM, n = 3, *p = 0.02. Inset: immunoprecipitation assay in HEK293T cells transfected as indicated. Shown is one experiment representative of four.

(C) Schematic of PRMT6 and AR functional domains.

(D) Transcriptional assay in HEK293T cells expressing AR65Q together with EGFP, PRMT6, or PRMT6-LXXAA mutant and treated as indicated. Graph, mean ± SEM, n = 3, *p = 0.003; NS, nonsignificant. Inset: immunoprecipitation assay.

(E) Transcriptional assay performed in HEK293T cells transfected with the indicated polyglutamine-expanded AR mutants together with soluble EGFP or EGFP-tagged PRMT6 and treated with DHT. Graph, mean ± SEM, n = 3, *p = 0.00001.

(F) Real-time PCR analysis of P21, VEGFR2 and SERCA2b, and reference gene, HPRT1, in MN-1 cells stably transfected with vector expressing either AR24Q or AR100Q and vector expressing scramble or PRMT6 shRNAs and treated with DHT. Graph, mean ± SEM, n = 4, *p = 0.001, **p = 0.01.

(G) Real-time PCR analysis in AR100Q-expressing MN-1 cells transfected with either EGFP or PRMT6 and treated with DHT. Graph, mean ± SEM, n = 5, *p = 0.01.

10%, whereas the E897K mutation reduced it by 60%, indicating that the AR-PRMT6 interaction requires an intact AF-2 surface.

Next, we sought to investigate the biological significance of AR transactivation by PRMT6. To elucidate this aspect, we tested whether arginine methylation alters the expression of genes regulated by AR and PRMT6 in motor neuron-derived MN-1 cells. We focused on genes that are known targets of PRMT6 and AR or that have previously been implicated in SBMA pathogenesis, including cyclin-dependent kinase inhibitor 1 (p21CIP/WAF1, hereafter referred to as p21),vascular endothelial growth factor receptor 2 (VEGFR2), and sarco(endo)plasmic reticulum Ca(2+) ATPase 2b (SERCA2b) (Montague et al., 2014; Sopher et al., 2004). We generated stable MN-1 clones expressing either AR24Q or AR100Q (Figures 5A and 5B). By real-time PCR analysis, we found that upon DHT treatment, p21 and VEGFR2 mRNA transcript levels were downregulated in mutant cells (Figure 2F). We infected the cells with lentiviruses expressing either scramble or shRNA against PRMT6 (Phalke et al., 2012). Expression of shRNA against PRMT6 (#2) reduced PRMT6 expression by 40% (Figure 5A),

and significantly increased the expression of p21, VEGFR2, and SERCA2b specifically in the mutant cells (Figure 2F). On the other hand, overexpression of PRMT6 had the opposite effect on p21 expression (Figure 2G). These results support the idea that gene expression is altered by the interaction of polyglutamine-expanded AR and PRMT6.

Polyglutamine-Expanded AR Is a Substrate of PRMT6

The observation that PRMT6 catalytic activity is required to activate AR prompted us to determine whether the effect of PRMT6 occurs through direct modification of AR. In an in vitro methylation assay, incubation of polyglutamine-expanded AR with PRMT6, but not PRMT6-V86K,D88A, in the presence of the methyl donor [³H]S-adenosylmethionine ([³H]-SAM) increased methylated AR by 4-fold (Figure 3A and Figure S5A). Consistent with previous findings that PRMT6 undergoes auto-methylation (Frankel et al., 2002), methylated PRMT6 was also increased (Figure S5B). To determine whether full-length AR is methylated, we immunoprecipitated Flag-tagged AR expressed with either soluble EGFP or EGFP-tagged PRMT6 in HEK293T cells and
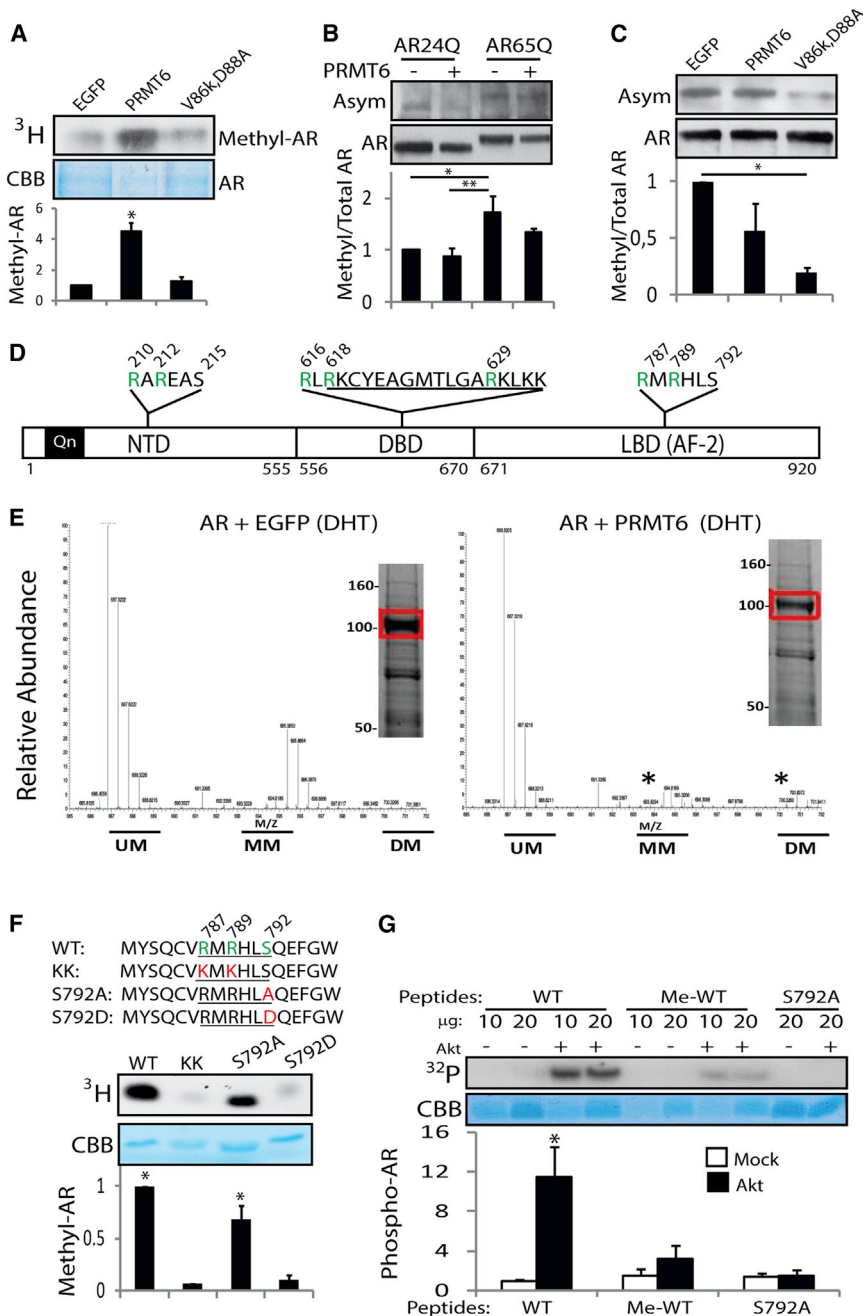
Figure 3. PRMT6 Methylates AR at the Akt Consensus Site RXRXXS and GAR Motifs

(A) EGFP, PRMT6, or PRMT6-V86K,D88A tagged to EGFP and Flag-tagged AR65Q were pulled down from HEK293T cells and incubated together in the presence of [³H]-SAM to perform in vitro methylation assay. Top: fluorography. Bottom: Coomassie brilliant blue (CBB) staining. Graph, mean ± SEM, n = 3, *p = 0.001.

(B) Normal and mutant AR were expressed in HEK293T cells with EGFP or EGFP-tagged PRMT6 in the presence of DHT (10 nM, 24 hr) and immunoprecipitated with anti-AR antibody. Methylated arginine was detected with anti-asymmetrically dimethylated arginine antibody (asym). Graph, mean ± SEM, n = 4, *p = 0.035, **p = 0.017.

(C) Dimethyl arginine was analyzed as in (B). Graph, mean ± SEM, n = 3, *p = 0.004.

(D) Schematic of the RXR and GAR motifs present in AR (NM_000044). Nuclear localization signal is underlined.

(E) Mass-spectrometry (MS) analysis of AR expressed in HEK293T cells with either EGFP or EGFP-tagged PRMT6 in the presence of DHT. Coomassie-stained gel lanes are shown as insets, where boxes depict the excised band analyzed. The position of ion series indicative of unmodified (UM), mono-methyl (MM), and di-methyl (DM) arginine at position 629 is shown. *Methylated residues identified by MS fragmentation data.

(F) In vitro methylation assay of peptide spanning the Akt consensus site motif located in the AF-2 surface (WT peptide), and peptides with arginine-to-lysine (KK peptide), serine 792-to-alanine (S792A), or serine 792-to-aspartate (S792D) substitutions incubated with PRMT6 in the presence of [³H]-SAM. Top: fluorography. Bottom: CBB staining. Graph, mean ± SEM, n = 3, *p = 0.001.
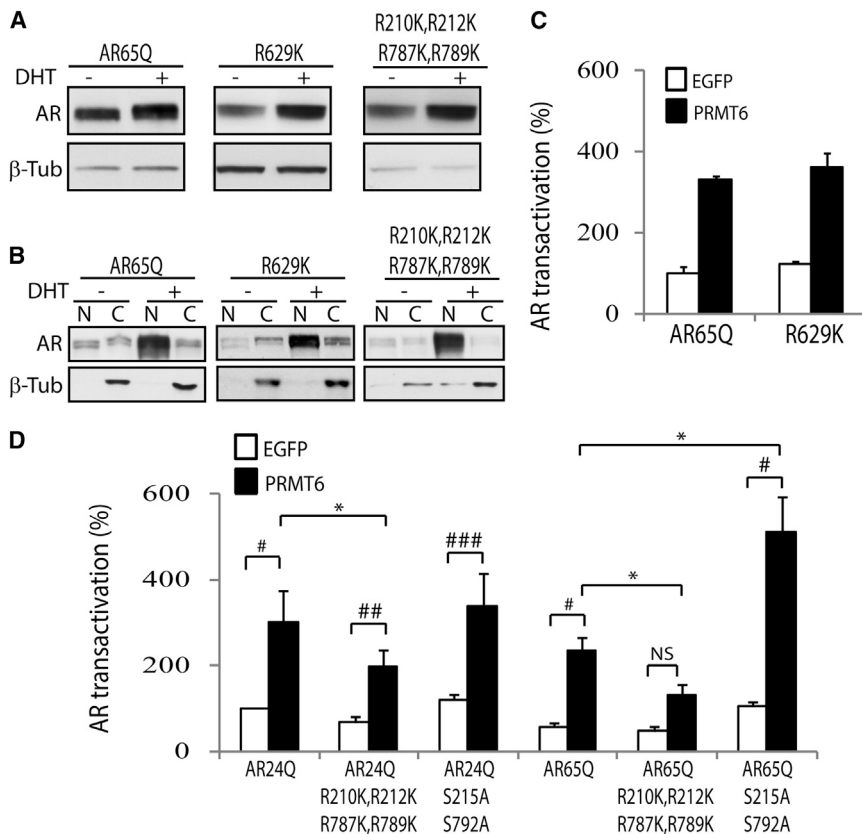
(G) In vitro phosphorylation assay of WT peptide, methylated WT peptide (Me-WT), and S792A peptide incubated with [³²P]-γATP and purified Akt. Top: autoradiography. Bottom: CBB staining. Graph, mean ± SEM, n = 3, *p = 0.02.

analyzed samples using an antibody that specifically recognizes asymmetrically dimethylated arginine (Figure 3B). Polyglutamine expansion increased the methylation status of AR by 1.7-fold compared to normal AR. Overexpression of PRMT6 did not change arginine dimethylation of both normal and mutant AR, suggesting that endogenous PRMT6 is sufficient to methylate AR. On the other hand, overexpression of catalytically inactive PRMT6 (V86K,D88A) reduced the methylation of polyglutamine-expanded AR by 40% (Figure 3C), probably reflecting a dominant-negative action of mutant PRMT6 (Herrmann et al., 2005).

Next, we sought to identify the arginine residues of AR that were methylated by PRMT6. Most PRMTs target arginine residues within GAR, RXR, and RGG motifs (Feng et al., 2013; Wada et al., 2002). AR does not have RGG motifs, while it has one ^{627}GAR^{629} motif and the three RXR motifs ^{210}RAR^{212}, ^{616}RLR^{618}, and ^{787}RMR^{789} (Figure 3D). ^{627}GAR^{629} motif and R618 are part of the nuclear localization signal (Figure 3D, underlined). ^{210}RAR^{212} and ^{787}RMR^{789} are part of the two Akt consensus site motifs RXRXXS of AR, one located in the NTD and the other in the AF-2 surface. By mass spectrometry analysis, R629, but not R618, was methylated by PRMT6 in response to hormone treatment (Figure 3E and Figure S5C). For technical reasons, we could not resolve the arginines at the Akt consensus sites by mass spectrometry analysis. Because of the relevance of these sites in SBMA pathogenesis (Palazzolo et al., 2007,

Figure 4. PRMT6 Requires Intact Akt Consensus Site Motifs for Full AR Transactivation

(A) Western blotting analysis of AR expression levels in HEK293T cells transfected with the indicated polyglutamine-expanded arginine methylation-defective AR variants and treated with DHT (10 nM, 24 hr). β-Tub served as loading control. Shown is one experiment out of three.

(B) Nuclear (N) and cytoplasmic (C) fractions of HEK293T cells transfected as indicated and treated with vehicle or DHT were analyzed by western blotting. β-Tub was used as loading control.

(C) Transcriptional assay in HEK293T cells expressing either AR65Q or the arginine methylation-defective AR65Q-R629K together with EGFP or EGFP-tagged PRMT6 and treated with DHT. Graph, mean ± SEM, n = 3.

(D) Transcriptional assay in HEK293T cells expressing the indicated arginine methylation-defective or phosphorylation-defective AR variants together with EGFP and EGFP-tagged PRMT6 and treated with DHT. Graph, mean ± SEM, n = 5, *p = 0.03; #p = 0.001; ##p = 0.008; ###p = 0.004; NS, nonsignificant.

2009), we used an in vitro methylation assay to test whether PRMT6 methylates the arginines of the Akt consensus site motifs of AR (Figure 3F and Figure S5D). Peptides spanning these Akt consensus site motifs (WT peptides) were incubated with purified PRMT6 in the presence of [^{3}H]-SAM. As control, we made conservative substitutions of arginines with lysines (KK peptides). We found that WT but not KK peptides were methylated by PRMT6. Collectively, these results suggest that AR is methylated by PRMT6 at arginines 210, 212, 629, 787, and 789.

Arginine Methylation and Serine Phosphorylation at the Akt Consensus Site Motifs of AR Are Mutually Exclusive

Similar to AR, also forkhead box O (FOXO) transcription factors, which play a critical role in neurodegeneration (Mojsilovic-Petrovic et al., 2009), are arginine-methylated at Akt consensus site motifs (Yamagata et al., 2008). Importantly, arginine methylation of FOXO was shown to block phosphorylation by Akt, but not vice versa. To investigate the potential relationship between arginine methylation and serine phosphorylation at the AR Akt consensus site motifs, we performed in vitro methylation assay using peptides in which S215 and S792 were substituted with either phospho-defective alanine (S215A and S792A peptides) or phospho-mimetic aspartate (S215D and S792D peptides) (Figure 3F and Figure S5D), as we previously described (Palazzolo et al., 2007). S215A and S792A peptides were methylated by PRMT6, suggesting that loss of phosphorylation does not affect methylation. On the other hand, phospho-mimetic substitution of S215 and S792

abolished methylation, suggesting that phosphorylation prevents methylation.

To determine whether methylation affects phosphorylation, we conducted an in vitro phosphorylation assay by incubating WT peptide spanning the Akt consensus site within the AF2 surface and chemically modified by arginine methylation (Me-WT peptide) with recombinant Akt in the presence of [^{32}P]-γATP (Figure 3G). As negative control, we used the S792A peptide. As expected (Palazzolo et al., 2007), S792A was not phosphorylated, indicating that Akt specifically targets serine 792. Incubation of Me-WT peptide with Akt reduced the incorporation of [^{32}P]-γATP by 80%. Similar results were obtained with prior incubation of WT peptide with PRMT6 and [^{3}H]-SAM, and subsequent incubation with Akt and [^{32}P]-γATP (Figure S5E). Together, these results suggest that arginine methylation of the RXRXXS motif by PRMT6 prevents phosphorylation by Akt and vice versa.

AR Transactivation by PRMT6 Occurs through Arginine Methylation of RXRXXS Motifs and Is Regulated by Phosphorylation

Major events occurring upon hormone binding are protein stabilization, nuclear translocation, and transactivation of gene expression (Parodi and Pennuto, 2011). To investigate the functional significance of arginine methylation at the ^{627}GAR^{629} and the Akt consensus site motifs, we generated the methylation-defective AR variants, AR65Q-R629K and AR65Q-R210K, R212K,R787K,R789K. Loss of arginine methylation at these sites neither altered accumulation of monomeric AR in response to hormone binding (Figure 4A), nor reduced hormone-induced nuclear translocation (Figure 4B). In transcriptional assays, loss of
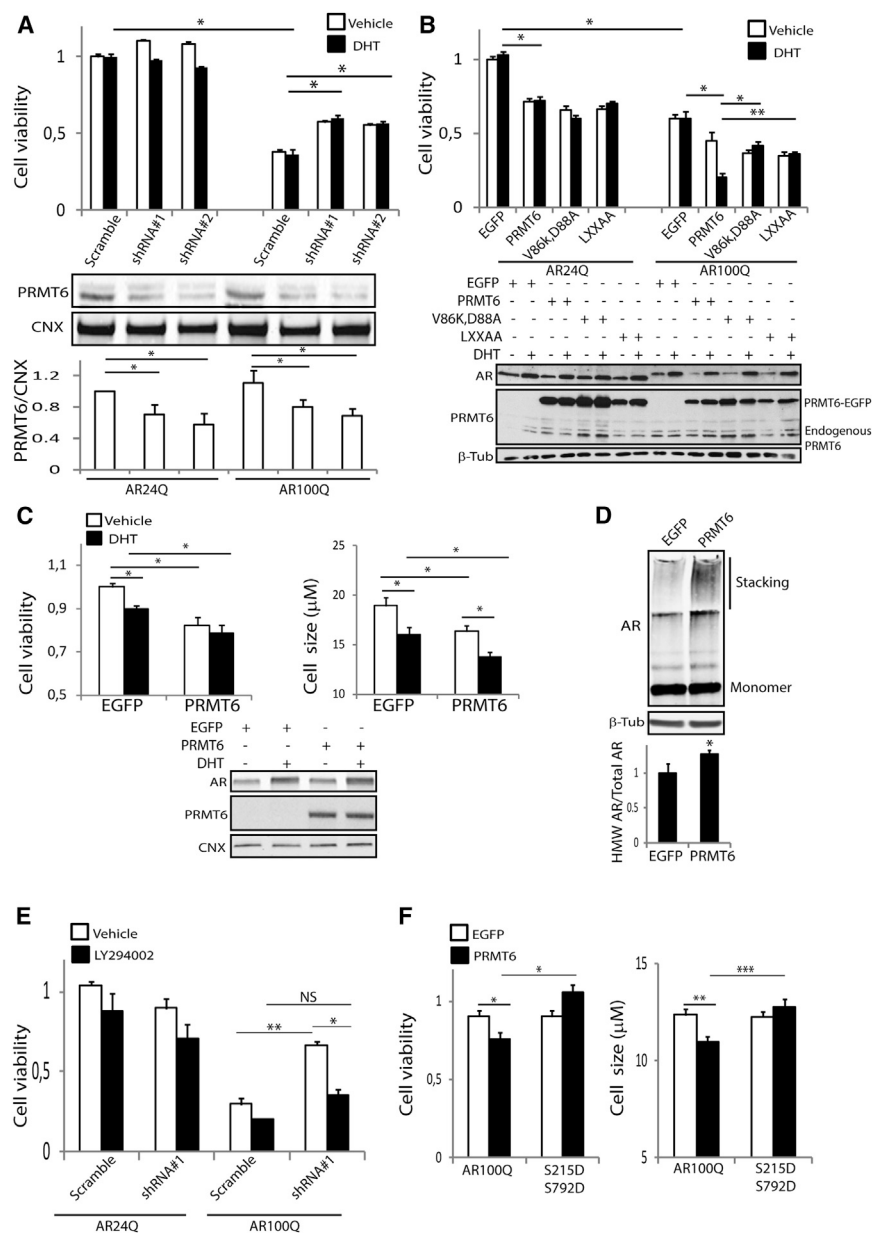
Figure 5. PRMT6 Exacerbates the Toxicity of Polyglutamine-Expanded AR in Motor Neuron-Derived MN-1 Cells and PC12 Cells

(A) XTT assay in AR24Q and AR100Q MN-1 cells transfected with scramble shRNA or two different shRNAs against PRMT6, and treated with vehicle and DHT (10 μM, 48 hr). Graph, mean ± SEM, n = 3, *p = 0.0001. Western blotting analysis and quantification of PRMT6 expression levels are shown at the bottom; loading control: calnexin (CNX); graph, mean ± SEM, n = 3, *p = 0.01.

(B) XTT assay in AR24Q and AR100Q MN-1 cells transfected with EGFP or wild-type and mutant (V86K,D88A and LXXAA) PRMT6 and treated with vehicle and DHT. Graph, mean ± SEM, n = 9, *p = 0.0001, **p = 0.0004. AR and PRMT6 expression levels are shown at the bottom. Beta-Tubulin (β-Tub) was used as loading control.

(C) Trypan blue assay in AR112Q PC12 cells transfected with EGFP or PRMT6 and treated with vehicle or DHT (50 μM, 48 hr). Graph, mean ± SEM, n = 10, *p = 0.001. AR and PRMT6 expression levels are shown at the bottom. Calnexin (CNX) was used as loading control.

(D) Western blotting analysis of high molecular weight (HMW) species and monomeric AR112Q upon PRMT6 overexpression in PC12 cells treated with DHT. Quantification of HMW species is shown at the bottom. Graph, mean ± sem, n = 6, *p = 0.03.

(E) XTT assay in AR100Q MN-1 cells transfected with scramble or PRMT6 shRNA and treated with DHT and vehicle or LY294002 (10 μM, 24 hr). Graph, mean ± SEM, n = 3, *p = 0.001, **p = 0.0001.
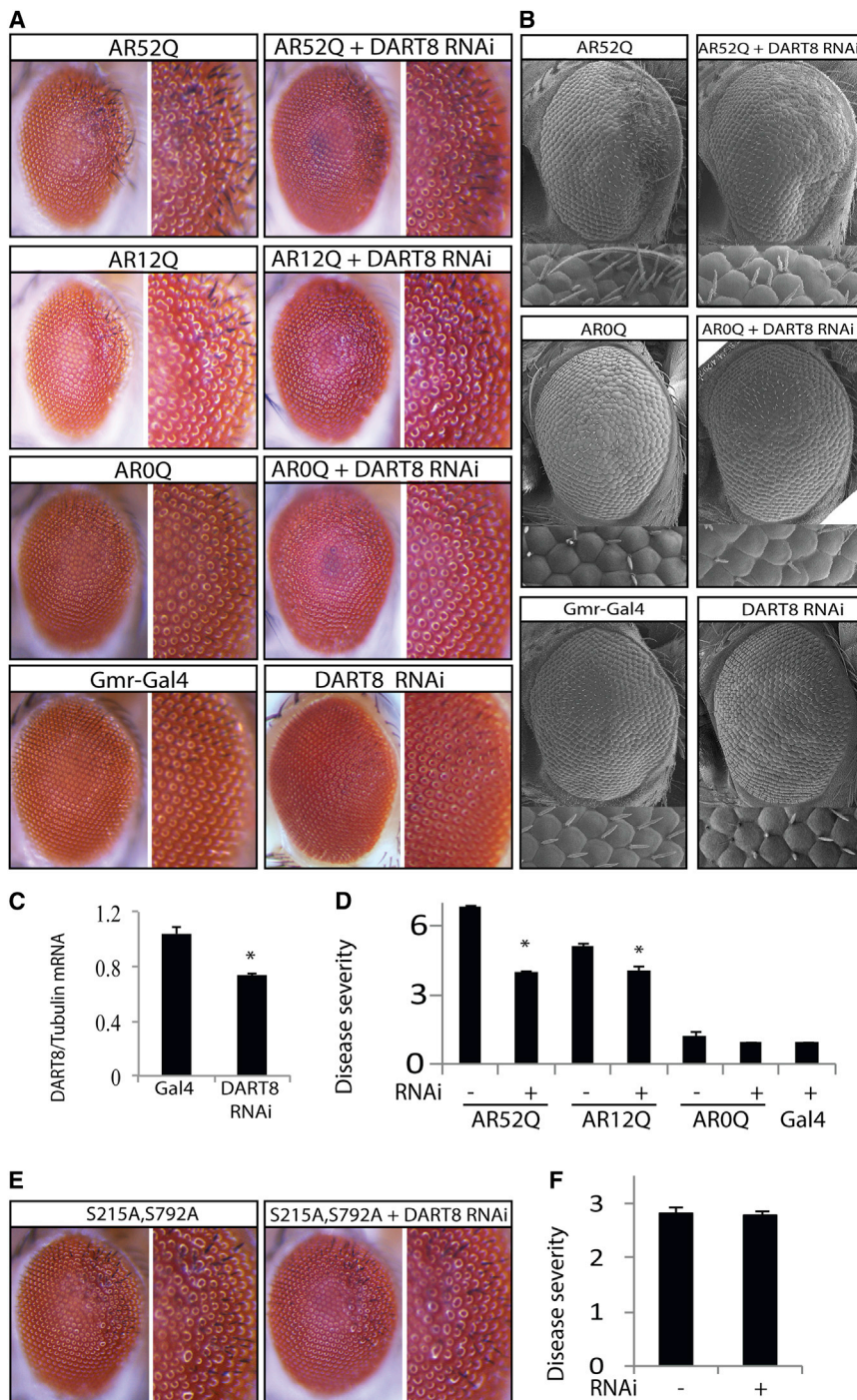
(F) Trypan blue assay in PC12 cells transiently transfected as indicated and treated with DHT. Graph, mean ± SEM, n = 7–11, *p = 0.001, **p = 0.05, ***p = 0.001.

methylation at R629 did not affect AR transactivation by PRMT6 (Figure 4C). On the other hand, loss of arginine methylation at the Akt consensus site motifs reduced the transactivation of normal AR by 34% and of mutant AR by 45%, respectively (Figure 4D). These observations indicate that PRMT6 requires the arginine residues at the Akt consensus site motifs in order to fully transactivate polyglutamine-expanded AR. To assess whether PRMT6-induced AR transactivation is modulated by phosphorylation at the Akt consensus sites, we investigated the effect of PRMT6 transactivation on phosphorylation-defective (S215A,S792A) AR variants (Figure 4D). PRMT6 did not alter the transactivation of phosphorylation-defective non-expanded AR, but it enhanced that of polyglutamine-expanded AR by 2.1-fold.

PRMT6 Is a Modifier of Polyglutamine-Expanded AR Toxicity In Vitro and In Vivo

Because pharmacologic (ASC-J9) or genetic (AR mutation E897K) inhibition of the interaction between mutant AR and its co-factors suppresses toxicity (Nedelsky et al., 2010; Yang et al., 2007), and reduces the transactivation of AR by PRMT6, we hypothesized that AR-PRMT6 interaction is pathogenetic in SBMA. To test this hypothesis, we undertook both a loss- and a gain-of-function approach to target PRMT6-AR interaction. The SBMA MN-1 cells that we generated showed reduced cell viability compared to normal cells, although this difference was not hormone dependent (Figure 5A). Silencing endogenous PRMT6 by 30%–40% with two different shRNAs increased cell viability by 1.5-fold in the mutant cells. Conversely, overexpression of wild-type PRMT6 as well as PRMT6-V86K,D88A and -LXXAA mutants decreased cell viability by 30% in both normal and mutant cells, indicating that the effect of PRMT6 overexpression on the survival of these cells was independent of its ability to transactivate AR

Figure 6. PRMT6 Is a Modifier of SBMA Pathogenesis In Vivo

(A) Light microscopy representative images of flies expressing AR52Q, AR12Q, and AR0Q with and without DART8 RNAi.

(B) Scan electron microscopy representative images of SBMA flies with and without DART8 RNAi.

(C) Real-time PCR analysis of DART8 transcript levels. Graph, mean ± SEM, n = 5, *p = 0.001.

(D) Quantification of disease severity for (A). Graph, mean ± SEM, n = 50, *p = 0.001.

(E) Light microscopy representative images of flies expressing phosphorylation-defective AR65Q-S215A,S792A with and without DART8 RNAi.

(F) Quantification of disease severity for (E). Graph, mean ± SEM, n = 21–33.

cells, respectively. In these cells, DHT treatment also reduced cell size by 15%, which was further reduced by PRMT6 overexpression by 14% and 27% in the absence and presence of DHT, respectively (Figure 5C). A hallmark of polyglutamine diseases is the accumulation of mutant proteins in forms of aggregates or micro-oligomers, which can be revealed as high molecular weight species that accumulate in the stacking portion of polyacrylamide gels (Palazzolo et al., 2009). Overexpressing PRMT6 significantly enhanced by 1.26-fold polyglutamine-expanded AR aggregation, further supporting a toxic gain-of-function effect of PRMT6 on mutant AR (Figure 5D). Treatment of the cells with the PI3K/Akt signaling inhibitor LY294002 for 24 hr, a condition that does not elicit overt toxicity (Palazzolo et al., 2007), reduced the protective effect of PRMT6 knockdown on cell viability, suggesting that PRMT6 enhances polyglutamine-expanded AR toxicity by counteracting its phosphorylation by Akt (Figure 5E). Consistent with this model, PRMT6 decreased neither the viability nor the size of PC12 cells expressing the phospho-mimetic AR variant AR100Q-S215D, S792D (Figure 5F).

To test whether PRMT6 modifies the SBMA phenotype in vivo, we used fly models of SBMA. Overexpression of polyglutamine-expanded AR (AR52Q) in the eye caused degeneration of the posterior side ommatidia (Figures 6A and 6B) (Nedelsky et al., 2010; Pandey et al., 2007). Also AR with a 12 glutamine-long tract (AR12Q) resulted in mild neurodegeneration when overexpressed in this system, whereas AR without a polyglutamine tract (AR0Q) was not toxic. We crossed the SBMA flies with flies in which the PRMT6 *Drosophila* ortholog, DART8, was knocked down by

(Figure 5B). Notably, in the mutant cells overexpression of wild-type, but not mutant (V86K,D88A and LXXAA), PRMT6 reduced cell viability by 66% in a hormone-dependent manner, indicating that PRMT6 gain of function in mutant cells enhances the toxicity of mutant AR in a hormone-dependent fashion. PC12 cells expressing AR112Q showed reduced cell viability when treated with hormone (Figure 5C). Overexpression of PRMT6 decreased cell viability by 17% and 21% in the vehicle- and DHT-treated

31% by specific RNA interference (Figure 6C). Knocking down endogenous DART8 itself did not cause any obvious ommatidial degeneration in flies (Figures 6A, 6B, and 6D). However, knockdown of DART8 suppressed polyglutamine-expanded AR-induced neurodegeneration, without altering AR expression (Figure S6A). Notably, DART8 knockdown also suppresses the phenotype caused by overexpression of non-expanded AR. Knockdown of the PRMT8 *Drosophila* ortholog, DART2, did not suppress the degenerative eye phenotype in this SBMA fly model, suggesting that the effect of DART8 knockdown is specific (Figure S6B). Consistent with the idea that silencing PRMT6 ameliorates phenotype because it results in increased phosphorylation at the Akt consensus site motifs, DART8 knockdown did not modify the phenotype of flies expressing phosphorylation-defective AR65Q-S215A,S792A (Figures 6E and 6F). Overexpression of PRMT6 in the eye of SBMA flies did not modify phenotype, suggesting that the endogenous fly ortholog of PRMT6 is sufficient to cause neurodegeneration (Figures S6C and S6D). Collectively, these results indicate that PRMT6 is a modifier of mutant AR toxicity in vivo.

DISCUSSION

Here, we show that PRMT6 co-localizes and forms a complex with AR and that the resulting transactivation of the receptor is significantly enhanced by polyglutamine expansion. We had previously shown that AR is phosphorylated by Akt at the RXRXXS motifs. Here, we report that PRMT6 methylates the AR at arginine residues spanning the Akt consensus site motifs and that arginine methylation and serine phosphorylation at these sites are mutually exclusive. Importantly, inhibition of PRMT6 suppressed the toxicity of mutant AR in vitro and in vivo, whereas overexpression of PRMT6 enhanced toxicity. Our findings establish a key role for arginine methylation and PRMT6 in the pathogenesis of polyglutamine diseases.

Emerging evidence in the field of polyglutamine diseases supports the idea that the toxic gain of function conferred by polyglutamine expansion arises from alteration of the normal, native function(s) of the mutant protein (Orr, 2012). Although the physiological function(s) of several polyglutamine proteins is not known, there is evidence from proteins of known function that glutamine expansions act by enhancing the normal function of the disease protein, and finally cause neurodegeneration (McMahon et al., 2005; Mo et al., 2010). Indeed, amplification of protein function by overexpression of AR and ataxin-1 with non-pathogenic repeat lengths leads to a neurodegenerative phenotype similar to that caused by polyglutamine expansion (Fernandez-Funez et al., 2000; Monks et al., 2007; Nedelsky et al., 2010). Consistent with this concept, knockdown of PRMT6 not only suppressed the toxicity of polyglutamine-expanded AR, but it also ameliorated the phenotype of flies overexpressing normal AR, further supporting that PRMT6 contributes to toxicity by enhancing the native function of AR. Moreover, there is evidence that polyglutamine expansion leads to amplification of interaction with native cellular partners, as reported for a variety of polyglutamine proteins, including ataxin-1 (Lim et al., 2008), AR (Nedelsky et al., 2010), and TBP (Friedman et al., 2007). Expanding this idea, we here present

evidence that PRMT6 acts as a co-activator of AR whose function is enhanced by polyglutamine expansion.

In SBMA, pathogenic interactions occur through the AR cofactor interaction surface, AF-2, and an intact AF-2 domain is indeed required for toxicity (Nedelsky et al., 2010). We show here that the interaction between polyglutamine-expanded AR and PRMT6 is mediated by the AF-2 surface of AR and the steroid receptor interaction motif, LXXLL, of PRMT6. Hormone binding induces a conformational change in the LBD that leads to generation of a hydrophobic pocket that initially binds to a hydrophobic helix in the NTD of AR, thereby generating intra- and inter-molecular N/C interactions. N/C interactions occur before and are lost upon DNA binding (van Royen et al., 2007). Subsequently, the hydrophobic pocket mediates binding to LXXLL motifs in transcriptional co-factors (Heery et al., 1997). Pharmacologic (ASC-J9) or genetic (AR mutation E897K) intervention to disrupt the interaction of AR with co-regulators suppresses the toxicity of mutant AR in mouse and fly models of SBMA (Nedelsky et al., 2010; Yang et al., 2007). A mutation abolishing DNA binding (A574D), which occurs before binding to co-regulators, as well as a mutation that disrupts binding to co-regulators (E897K) suppress the eye degenerative phenotype caused by polyglutamine-expanded AR in flies. Substitution of K720 with alanine (K720A), which reduces interaction with co-factors, partially attenuated neurodegeneration. On the other hand, a mutation of glycine 21 to glutamic acid (G21E) that abolishes the N/C interactions without altering interaction with co-regulators had no effect on toxicity. Consistent with these observations, ASC-J9 as well as mutations K720A and E897K, but not G21E, reduced transactivation of AR by PRMT6.

Post-translational modifications are critical regulators of protein function and are modifiers of polyglutamine protein toxicity (Pennuto et al., 2009). Methylation, together with phosphorylation, is a major post-translational modification occurring in mammalian cells, with about 2% of cellular proteins containing dimethylated arginine residues (Bedford and Clarke, 2009). We have previously shown that phosphorylation of polyglutamine-expanded AR by Akt suppresses toxicity (Palazzolo et al., 2007, 2009). Importantly, we demonstrate here that arginine methylation prevents phosphorylation by Akt, and vice versa, thereby implying that arginine methylation and serine phosphorylation at these sites are mutually exclusive in AR. Other polyglutamine proteins have RXRXXS motifs. Phosphorylation of polyglutamine-expanded huntingtin at serine 421 by Akt has been shown to be protective in striatal neurons (Humbert et al., 2002), whereas phosphorylation of polyglutamine-expanded ataxin-1 at serine 776 enhances toxicity (Emamian et al., 2003). Our findings indicate the existence of an additional important level of regulation of phosphorylation at the RXRXXS motif by arginine methylation, which may also play a critical role in polyglutamine diseases other than SBMA.

Arginine methylation has been recently implicated in the pathogenesis of other neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS). The ALS-linked fused in sarcoma (FUS) undergoes extensive arginine methylation, and this modification alters its subcellular localization and toxicity (Dormann et al., 2012; Scaramuzzino et al., 2013; Tradewell et al., 2012). Moreover, arginine methylation of spliceosomal proteins affects
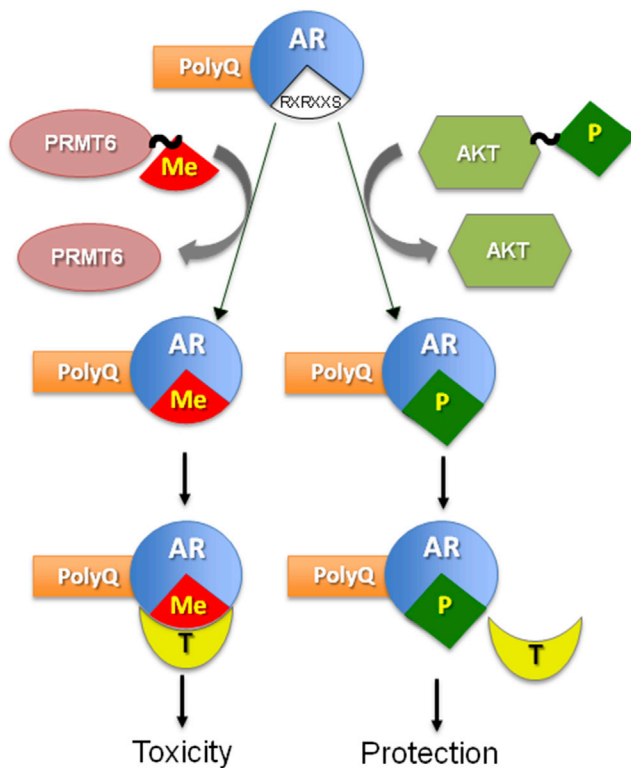
Figure 7. Model of Polyglutamine-Expanded AR Methylation and Phosphorylation

Arginine methylation of polyglutamine-expanded AR by PRMT6 at the Akt consensus site motif RXRXXS enhances function and toxicity leading to neurodegeneration, whereas phosphorylation by Akt prevents binding to testosterone (T), thereby protecting neurons from degeneration.

interaction with survival of motor neuron (SMN), another protein involved in motor neuron disease (Brahms et al., 2001). However, the mechanism through which arginine methylation affects neuronal survival in pathological conditions is poorly understood. Given the role of the PRMTs as major modifiers of histones, the interaction between AR and PRMT6 may alter histone epigenetic marks, thereby contributing to the transcription abnormalities that characterize SBMA neuron and muscle cells (Lieberman et al., 2002; Mo et al., 2010). Consistent with this idea, we found that polyglutamine-expanded AR and PRMT6 suppress the expression of specific genes in motor neuron-derived cells, further supporting that PRMT6 contributes to disease by altering AR function and transcription regulation. The enhanced interaction between polyglutamine-expanded AR and PRMT6 may also lead to sequestration of PRMT6 away from active chromatin sites, thereby causing a loss of PRMT6 function. However, the observation that loss of PRMT6 function in mice does not cause any overt phenotype (Neault et al., 2012), whereas gain of PRMT6 function causes premature death (Di Lorenzo et al., 2014), argues against this idea. Rather, this evidence supports our hypothesis that a gain of functional interaction between PRMT6 and its native partners/substrates, e.g., polyglutamine-expanded AR as shown here, is the mechanism underlying cell dysfunction and degeneration. In conclusion,

we propose a model in which polyglutamine-expanded AR is phosphorylated by Akt, an event that blocks binding to hormones, protects from toxicity, and prevents methylation (Figure 7) (Palazzolo et al., 2007, 2009). On the other hand, the interaction between polyglutamine-expanded AR and PRMT6 leads to arginine methylation of the AR with enhancement of protein function and toxicity.

EXPERIMENTAL PROCEDURES

Additional details are provided in the Supplemental Information section.

Cell Cultures and Transfections
MN-1, PC12, COS1, HEK293T, mouse-, and patient-derived cells were cultured as previously described (Basso et al., 2012; Grunseich et al., 2014; Palazzolo et al., 2007; Walcott and Merry, 2002).

Immunocytochemistry and Microscopy
Human spinal cord slides were fixed with 4% PFA, then placed in blocking solution (10% NGS, 0.3% Triton X-100 in PBS) for 45 min at room temperature. Primary antibody staining was done at 4°C overnight in PBS containing 5% NGS and 0.1% Triton X-100, using AR and PRMT6 antibodies. Slides were then incubated with secondary antibody and then washed before drying and adding Vectashield/DAPI stain (Vector Lab). For AR staining in IPSC-derived motor neurons, slides were treated with 100 mM glycine after fixation and blocked in PBS with 3% BSA. Antibody staining was performed in PBS with 3% BSA and 0.1% Tween overnight with 0.1% Tween/PBS used for all washes. HB9 was used at 1:200 (DSHB). Coverslips were mounted with permamount (Thermo).

Western Blotting, Immunoprecipitation, Nuclear-Cytosolic Fractionation
Cells were processed as previously described (Palazzolo et al., 2007, 2009). For analysis of AR aggregation, cell lysates were collected in lysis buffer (150 mM NaCl, 6 mM Na2HPO4, 4 mM NaH2PO4, 2 mM EDTA [pH8], 1% Na-DOC, 0.5% Triton X-100, SDS 0.1%). Quantifications were done using ImageJ 1.45 software.

Transcriptional and Cytotoxicity Assays
Transcriptional assays were performed using the Dual-Luciferase assay kit (Promega), according to manufacturers' instructions. Cell toxicity was measured by XTT assay in MN-1 cells and trypan blue assay in PC12 cells.

Quantitative Real-Time PCR Analysis
Total RNA was extracted with Trizol (Invitrogen). 2 μg RNA were retro-transcribed using the SuperScript III First-Strand Synthesis System kit (Invitrogen). Gene expression was measured by quantitative real-time PCR using 7900 HT Fast Real-Time PCR System (Applied Biosystems). The level of each transcript was measured with the threshold cycle (Ct) method. Values were normalized to the mean of the cells expressing AR24Q or AR100Q, which were assigned as 100%.

In Vitro Methylation and Kinase Assays
For in vitro methylation assays, immunoprecipitated Flag-tagged AR65Q was incubated with immunoprecipitated EGFP, EGFP-tagged PRMT6, or PRMT6-KLA in the presence of S-adenosyl-L-[methyl-^{3}H] methionine (Perkin Elmer). 20 μg of indicated peptides (United Biosystem) were incubated with immunoprecipitated EGFP or EGFP-tagged PRMT6 and S-adenosyl-L-[methyl-^{3}H] methionine. After washing the beads, the reaction products were analyzed by fluorography and CBB staining. For in vitro phosphorylation assays, the indicated peptides were incubated in phosphorylation buffer (20 mM NaF, 20 mM MOPS [pH 7.2], 25 mM beta-glycerolphosphate, 10 mM MgCl$_2$, 25 μM ATP, 1 mM Na$_3$VO$_4$, 5 mM EGTA, 1 mM DTT), followed by the addition of 50 ng recombinant Akt (Upstate) and [γ-^{32}P] ATP (Perkin Elmer). After 30 min of incubation, samples were subjected to SDS-PAGE, transferred on PVDF membrane. Signals were detected by autoradiography.

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