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# Androgen-Dependent Neurodegeneration by Polyglutamine-Expanded Human Androgen Receptor in *Drosophila*

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## Summary

**Spinal and bulbar muscular atrophy (SBMA) is an X-linked, adult-onset, neurodegenerative disorder affecting only males and is caused by expanded polyglutamine (polyQ) stretches in the N-terminal A/B domain of human androgen receptor (hAR). Although no overt phenotype was detected in adult fly eye photoreceptor neurons expressing mutant hAR (polyQ 52), ingestion of androgen or its known antagonists caused marked neurodegeneration with nuclear localization and structural alteration of the hAR mutant. Ligand-independent toxicity was detected with a truncated polyQ-expanded A/B domain alone, which was attenuated with cytosolic trapping by coexpression of the unliganded hAR E/F ligand binding domain. Thus, our findings suggest that the full binding of androgen to the polyQ-expanded hAR mutants leads to structural alteration with nuclear translocation that eventually results in the onset of SBMA in male patients.**

## Introduction

Spinal and bulbar muscular atrophy (SBMA), or Kennedy's disease, is a rare degenerative disease of the motor neurons, characterized by progressive muscle atrophy and weakness in male patients, usually beginning at 30–50 years of age (Kennedy et al., 1968). Mapping studies and functional analyses of SBMA cases revealed expansions in the number of trinucleotide CAG repeats in the first exon of the *androgen receptor* (*AR*) gene, generating expanded polyQ stretches in the A/B domain of the AR protein (La Spada et al., 1991; Choong and Wilson, 1998; Merry and Fischbeck, 1998). These repeats encode polyglutamine (polyQ) stretches, and it has been found that disease onset occurs when the stretches contain more than 40 glutamine residues, compared to a range of 8 to 34 polyQ stretches in normal individuals. SBMA patients often suffer mild androgen insensitivity, indicating impaired AR function due to the expanded polyQ stretches (Pinsky et al., 1992). However, it appears unlikely that motor neuronal cell death is caused simply by the loss of AR function, as neurodegeneration is not observed in severe testicular feminization (Tfm) patients that completely lack AR function (Brown et al., 1988; Yong et al., 1994). Like other neurodegenerative diseases involving polyQ stretches, such as Huntington's disease (HD), dentatorubral and pallidoluysian atrophy (DRPLA), and spinocerebellar ataxia atrophy (SCA), formation of mutant protein aggregates is observed in SBMA patients with a loss of selected neuronal populations (Kim and Tanzi, 1998). Abnormal folding of polyglutamine-expanded proteins may cause neural death through a common mechanism, as evidenced by the production of aggregates in these diseases (Ross, 1997). While polyQ-expanded hAR protein expressed in cultured cells has been shown to have reduced transactivation function, ligand binding is indispensable for aggregate formation (Stenoien et al., 1999).

AR is a member of the nuclear receptor superfamily and acts as a ligand-inducible factor to control transcription of a particular set of target genes (Mangelsdorf et al., 1995; Glass and Rosenfeld, 2000). Members of the steroid/thyroid hormone family share common structural features, with distinct functional domains, referred to as domains A to E/F. The highly conserved middle region (C domain) acts as a DNA binding domain (DBD), while the ligand binding domain (LBD) is located in the less well-conserved C-terminal E/F domain. The LBDs of most nuclear receptors, including AR, have been analyzed and are comprised of 12 $\alpha$ helices that form a pocket to capture cognate ligands (Shiau et al., 1998; Poujol et al., 2000). Upon ligand binding, the C-terminal $\alpha$ helix 12 (H12) in the LBD shifts position to create a space, with helices 3 to 5 serving as the key interface following dissociation of corepressor complexes and association of coactivator complexes (Freedman, 1999; Glass and Rosenfeld, 2000; Mckenna and O'Malley, 2002; Yanagisawa et al., 2002). During ligand-induced transactivation, the two N-terminal domains A/B and the steroid receptor LBD act as interacting regions for the coactivator complexes (He et al., 1999; Watanabe et al., 2001; Shang et al., 2002). The autonomous activation function-1 (AF-1) within the A/B domain is ligand independent, while the AF-2 in the LBD is induced upon ligand binding (Kato et al., 1995). Unliganded LBD appears to suppress the function of AF-1, while ligand binding to the LBD is thought to evoke the function of LBD and to restore the A/B domain function through an as yet undescribed intramolecular alteration of the entire steroid receptor structure. As SBMA occurs in men rather than women, we reasoned that a critical step in

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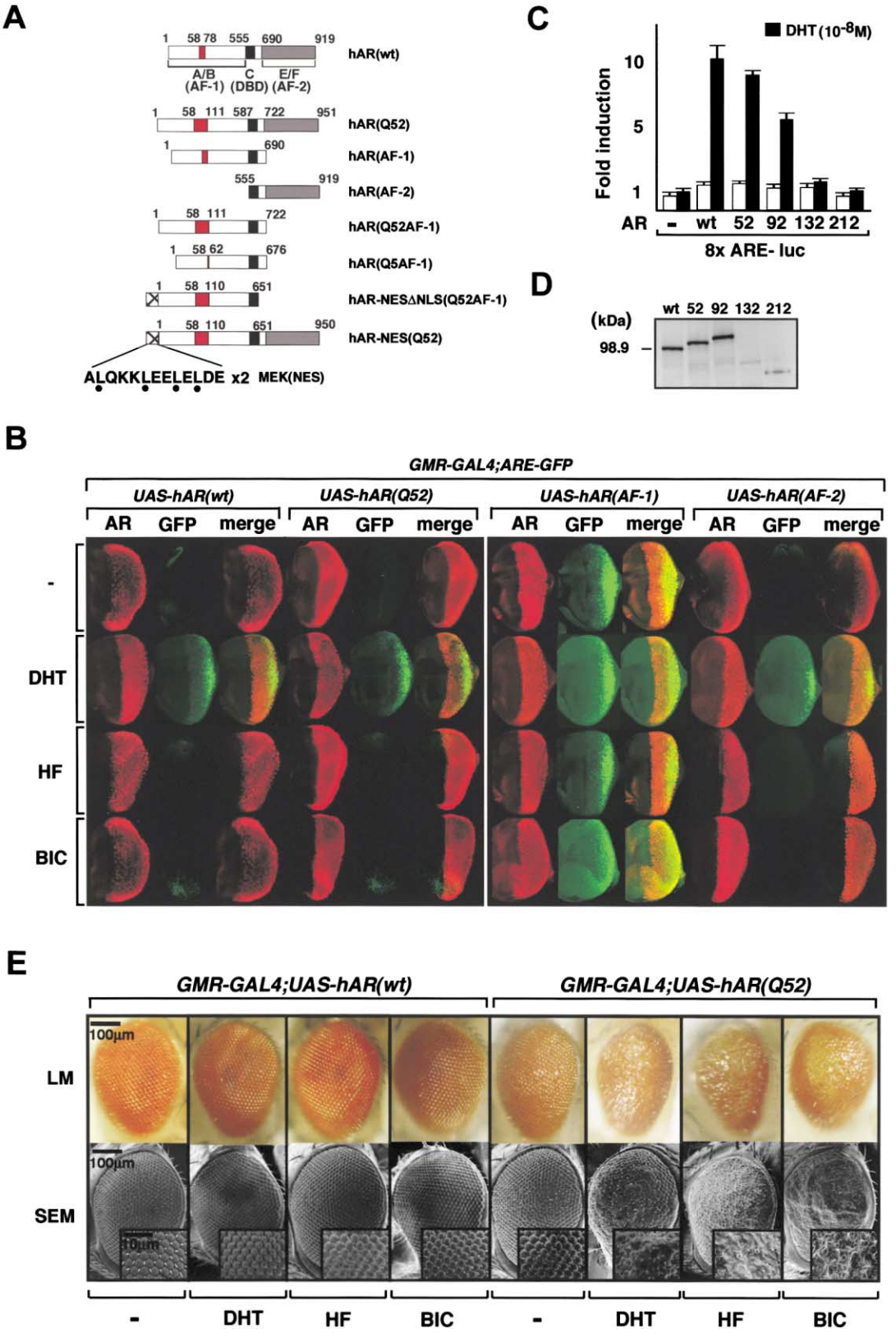


Figure 1. Ligand-Induced Degeneration in Photoreceptor Neurons by hAR Mutants with Expanded PolyQ Stretches

(A) Diagram of the AR constructs. Location of the polyglutamine (polyQ) region (red boxes) in relation to the DNA binding domain (DBD) (black boxes). Transactivation function 1 (AF-1) region is localized within the N-terminal A/B domain, and transactivation function 2 (AF-2) region is

the onset of SBMA could be the structural alteration and nuclear translocation of mutant AR upon binding of significant amounts of androgen.

To test this hypothesis, we investigated the role of hAR mutants with expanded polyQ stretches in neurodegeneration. To this end, we established a *Drosophila* model that ectopically overexpressed a mutated AR in photoreceptor neurons. Although the fly eye has proved to be an effective model in which to observe neuronal degeneration through the expression of other mutant proteins containing polyQ stretches (Jackson et al., 1998; Warrick et al., 1998), no abnormalities were found in eyes expressing a mutant hAR that contained an expanded 52 stretch polyQ (Q52). However, dietary ingestion of dihydroxytestosterone (DHT) induced marked degeneration of the photoreceptor neurons, along with apoptosis, although the mutant hAR still retained reduced transactivation function. Neurodegeneration was induced even in the absence of DHT when only the A/B domain, which harbors the 52 polyQ stretch, was expressed but was abrogated by coexpression of unliganded LBD domain. Surprisingly, known androgen antagonists failed to suppress the DHT-induced neurodegeneration in the Q52 line. Trapping the polyQ-expanded receptor mutants in the cytosol prevented neurodegeneration. Thus, our results suggest that hormone binding and subsequent structural alteration of hAR mutants with nuclear localization appears to be critical for SBMA onset. Furthermore, they reveal that the fly eye model may be useful for the development of novel therapeutic approaches to SBMA.

## Results

### Targeted Expression of Functional Human Androgen Receptor in *Drosophila*

We investigated the role of hAR mutants that contain expanded polyQ stretches by ectopic expression in the *Drosophila* eye (Figure 1A). The fly eye has proven to be an effective model to observe neuronal degeneration through the expression of other mutant proteins that also contain polyQ stretches (Jackson et al., 1998; Warrick et al., 1998). We first expressed wild-type and mutated hARs (Figure 1A) in photoreceptor neurons and accessory pigment cells in developing eye discs under a *glass multimer reporter* (*GMR*) gene promoter (Moses and Rubin, 1991), using the *Drosophila melanogaster* GAL4-UAS system (Brand and Perrimon, 1993). Using this system, targeted expression of hARs was also achieved in the anterior portion of embryonic segments by a *patched* (*ptc*) gene promoter and to the anterior-posterior boundary area in developing wing discs under a *decapentapregic* (*dpp*) gene promoter (data not shown) (Tanimoto et al., 2000). Northern blot analysis from different tissues of DHT-treated and untreated transgenic flies (data not shown) suggests that ectopic expression of hAR did not affect the expression of endogenous nuclear receptor genes (e.g., *Eip75B*, *ecdysone receptor*) (White et al., 1997). To monitor the ligand-induced transactivation function of hAR, hAR-expressing flies were further crossed to fly lines bearing a *GFP* reporter gene, such that GFP expression could be induced by ligand-bound AR that recognized the consensus androgen response element (*ARE*) in the *GFP* promoter (Yamamoto et al., 2000). Expressed hARs were then detected as red fluorescence in situ using an immunofluorescent antibody. Dietary ingestion of androgen (dihydrotestosterone/DHT) for 5 days from hatching induced remarkable and targeted GFP expression observed as green fluorescence in eye discs of third instar larva by the *GMR* promoter (Figure 1B) and in the other tissues by the *ptc* and *dpp* promoters (data not shown). Two independent transactivation functions (AF-1 in the A/B and AF-2 in the E/F domain of AR) were detected in eye discs (Figure 1B), as observed in cultured mammalian cells (Yamamoto et al., 2000; Ikonen et al., 1997). These observations indicate that ectopic expression of hARs translocated into the nuclei upon DHT binding and activated transcription in the tissues examined.

### Ligand-Induced Neurodegeneration in the Fly Line Expressing Polyglutamine-Expanded Human Androgen Receptor

We then characterized the expanded polyQ hAR mutants. The reduction in the hormone-induced transactivation function of hAR mutants in COS-1 cells was dependent on the length of polyQ stretches in the A/B domain (Figures 1A and 1C). An hAR mutant that contained a 52 polyQ stretch [hAR(Q52)] exhibited only a slight reduction in DHT-induced transactivation (Figure 1C) but showed normal translation efficiency as estimated by in vitro translation (Figure 1D), while expres-

localized within the C-terminal E/F domain (gray boxes) containing the ligand binding domains (LBD). Nuclear export signals (NES) derived from MEK (Toyoshima et al., 1998) were tagged at the N terminus.

(B) hAR mutant expression and transactivation function in eye discs. Expression of hAR(wt), hAR(Q52), hAR(AF-1), and hAR(AF-2) in third instar eye imaginal discs driven by *GMR-GAL4* was detected using anti-hAR N-20 and/or C-19 antibodies (left panel). Transactivation function of hAR mutants was assessed using GFP expression (middle panel). A merged image is shown in the right panel.

(C) Reduction in the transactivation function of hAR mutants is dependent on polyQ length. COS-1 cells were cotransfected with 1 μg ARE-tk-luc and 0.1 μg AR expression vector (wt, Q52, Q92, Q132, or Q212), and $10^{-8}$ M DHT was added to the medium 6 hr after transfection (black boxes). After 18 hr, firefly luciferase activity (from ARE-tk) was measured to obtain the transfection efficiency, as previously described for Renilla luciferase activity (from pRL-CMV) (Yamamoto et al., 2000).

(D) In vitro translated hARs. Wild-type and mutant hARs were produced by in vitro translation (TNT-coupled in vitro translation system, Promega) in the presence of [$^{35}$S]methionine. Labeled proteins were separated by SDS-PAGE and analyzed by autoradiography. No stable protein can be produced for hAR(Q132) or (Q212).

(E) Rough-eye phenotype induced in hAR(Q52) lines by DHT or AR antagonists. Light microscopic (LM) and scanning electron microscopic (SEM) images of adult eyes from 5-day-old flies treated as larval with or without $10^{-5}$ M DHT, HF, or BIC, respectively (closed arrow in Figure 2A). Genotypes are *UAS-hAR(wt)* or *UAS-hAR(Q52) in trans* to *GMR-GAL4*. While no degeneration is detectable after expression of the hAR(wt) protein, severe degeneration was observed in hAR(Q52) lines after treatment with the ligands. Scale bar: whole eye, 100 μm; eye inset, 10 μm.

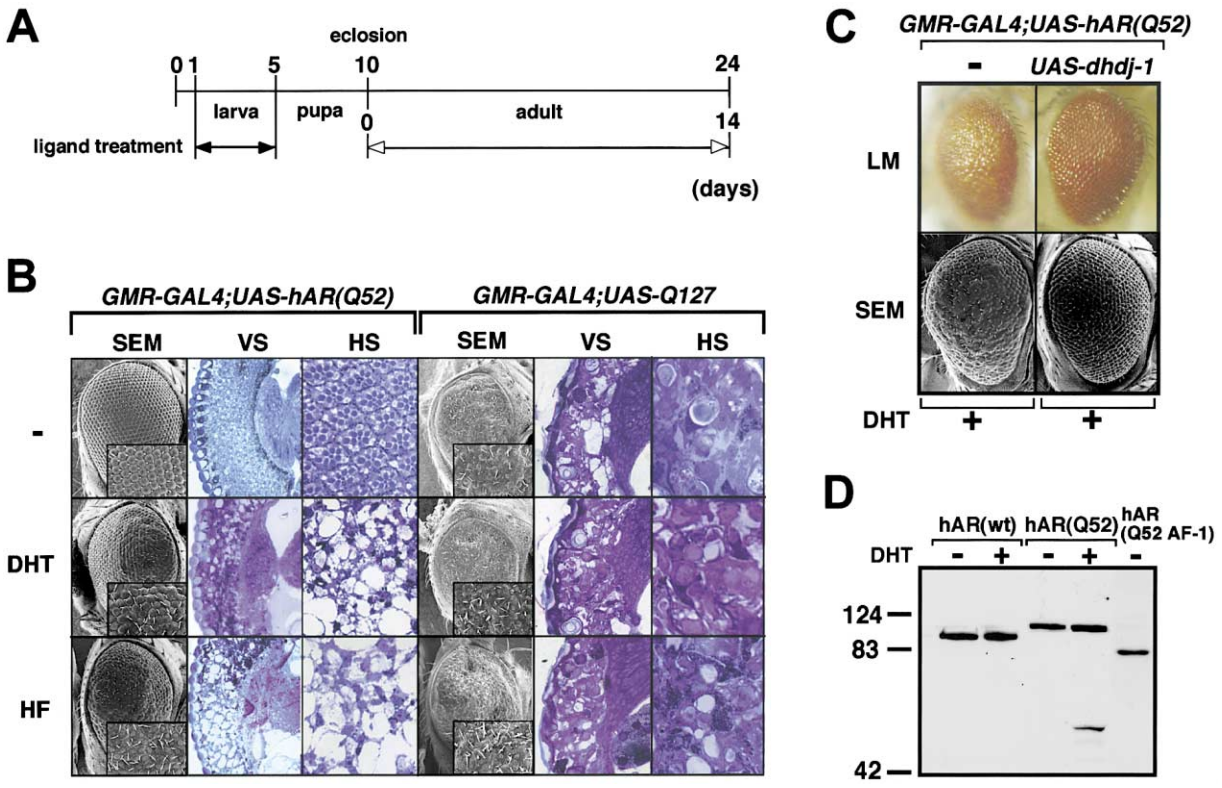


Figure 2. Enhanced Neurodegeneration in Photoreceptor Neurons of hAR(Q52) Lines by Ligand Ingestion

(A) Experimental schedule for ligand treatment. The ligands were given for 5 days after hatching (closed arrow) or for 2 weeks to adult flies after eclosion (opened arrow).

(B) Ligands induced neurodegeneration during adulthood. Genotypes are *UAS-hAR (Q52)* or *UAS-Q127 in trans* to *GMR-GAL4*. Adult transgenic flies were kept for 2 weeks on medium containing vehicle or ligands ($10^{-5}$ M DHT or HF). Scanning electron microscopic images (SEM) of vertical (VS) and horizontal (HS) toluidine blue-stained sections show ligand-dependent degeneration in the hAR(Q52) line but not in the Q127 line. Eyes from six flies were analyzed for each genotype after 2 weeks with or without $10^{-5}$ M DHT or $10^{-5}$ M HF from eclosion. Degeneration was also observed in two other independent transgenic lines.

(C) Genetic suppression of ligand-induced neurodegeneration in hAR(Q52) lines by the chaperone component Hsp40. Genotypes are *GMR-GAL4;UAS-hAR(Q52) in trans* to *UAS-dhdj-1(dhsp-40)*. The analysis of fly eyes in the presence of $10^{-5}$ M DHT by LM and SEM shows a reduction of the pigmentation and rough-eye phenotype by expression of Hsp40 in fly eye.

(D) Expression of the hAR(wt) and hAR mutant proteins in transgenic fly eyes. The intact adult eyes of hAR (wt), hAR(Q52), or hAR(Q52 AF-1), treated with or without DHT for 2 weeks, were dissected and analyzed by Western blotting using an N-terminal-specific anti-hAR antibody (anti-hAR N20). Molecular weights (kDa) are indicated on the left.

sions of hAR(Q132) and hAR(Q212) proteins appeared at very low levels, judging from their translation efficiency (Figure 1D). Transgenic fly lines that expressed hAR(Q52) in the eye under the *GMR* promoter showed nearly normal eye morphology (Figures 1B and 1E) by light microscopy (LM) and scanning electron microscopy (SEM) throughout the life span of the fly, and no other tissues examined appeared to be affected (data not shown). However, we found that ingestion of DHT for 5 days after hatching (Figure 2A) induced marked disruption of the eye, including severely reduced ommatidia numbers and loss of pigmentation with thinned retinas in all lines tested at adult day 0 (see "DHT" treatment in right panels of Figure 1E). Notably, hAR(Q52) expression levels in the eye discs appeared to be unaffected compared to those of hAR(wt), both with and without DHT ingestion (Figure 1B). Despite the marked neurodegeneration in the adult eyes, DHT-induced transactivation function of hAR(Q52) in the eye disc nuclei was still detected (Figure 1B), as seen in COS-1 cells (Figure 1C). We then tested whether androgen antagonists could antagonize hAR(Q52) function and prevent eye disruption. As observed in other tissues, hydroxyflutamide (HF) and bicalutamide (BIC), as expected, depressed hAR(Q52) and hAR(wt) transactivation function but did not affect receptor protein expression levels in the fly eye discs (Figure 1B). Although the antagonists potently blocked DHT-induced hAR transactivation functions (data not shown), surprisingly, both antagonists not only failed to prevent but, indeed, appeared to potentiate eye disruption, leading to even more extreme phenotypes with increased loss of red pigmentation with retinal degeneration as compared to that observed using DHT alone (Figure 1E). The antagonists themselves appeared not to be toxic to the fly eye, as no phenotypic abnormalities were induced in either developing or adult eyes in wild-type hAR lines (Figure 1E) or in any of the tissues examined from normal flies (data not shown).

However, it remained unclear whether the rough-eye phenotype induced by hAR ligand treatment at larval

stages was due to impaired eye formation during development or to neuronal degeneration. To address this issue, AR ligands were given to adult fly lines for 2 weeks following eclosion (Figure 2A). DHT treatment in adults led to severe rough-eye phenotypes in the hAR(Q52) lines in 14 day adults, with loss of photoreceptor neurons, retina, and red pigmentation as observed by SEM and LM with vertical and horizontal sections (VS and HS) (left panels in Figure 2B), clearly indicating that the eye disruption induced by DHT reflects neurodegeneration. The androgen antagonist HF also induced eye disruption in the hAR(Q52) lines (left panels in Figure 2B). The rough-eye phenotype we observed looked similar to that of flies expressing other polyQ mutant proteins (Jackson et al., 1998; Warrick et al., 1998). As expected, neurodegeneration was induced by expressing a fragment with 127 polyQ (right panels in Figure 2B) (Kazemi-Esfarjani and Benzer, 2000). However, no ligand treatments potentiated the rough-eye phenotype (Figure 2B), suggesting that the AR ligands themselves were not enhancing polyQ-induced degeneration. Furthermore, the DHT effect was blocked by coexpression of Hsp40 (dhdj-1), a common suppressor of neurodegeneration by polyQ-expanded proteins (Figure 2C) (Kazemi-Esfarjani and Benzer, 2000; Warrick et al., 1999). The DHT treatment did not appear to affect the expression levels of hAR(Q52) or hAR(wt) as observed by Western blotting with an antibody against the N-terminal end of hAR (Figure 2D). Most notably, an N-terminal fragment containing the expanded polyQ repeats was generated in a DHT-dependent manner in the adult eyes of the hAR(Q52) line (Figure 2D). No eye disruption was induced in the adult hAR(wt) lines by the hAR ligand treatment (data not shown). Thus, the function of hAR(Q52) in the induction of the rough-eye phenotype, which is induced by ligand binding, cannot distinguish between DHT and androgen antagonists. Again, the features of DHT-dependent neurodegeneration in hAR(Q52) lines appeared to strongly resemble the neural abnormalities in the male SBMA patients, and it would be interesting to compare the effect of anti-androgen treatment in these patients. However, no such clinical data are available.

### Nuclear Localization Is Necessary for Expression of the Toxicity by the PolyQ-Expanded hAR A/B Domain

To explore the molecular mechanism of androgen dependency of hAR(Q52)-induced neurodegeneration, we first examined the effect of a truncated polyQ-expanded A/B domain construct, hAR(Q52AF-1) (Figure 1A). A chimeric hAR(Q52AF-1) protein fused only to the nuclear localization signal (NLS) of hAR was sufficient to induce marked toxicity (Figure 3A) with relevant expression levels to those of hAR(Q52) (Figure 2D), along with AF-1 transactivation function, even in the absence of ligand (right panels in Figure 1B). hAR(Q52AF-1) protein was predominantly localized to the nucleus, in agreement with a ligand-independent and constitutive transactivation function (Figure 3B), while nuclear localization of hAR(Q52) was observed only in the presence of a ligand (Figure 3B). The wild-type A/B domain [hAR(AF-1)] alone exhibited slight toxicity due to the wild-type 21 polyQ stretches (Figure 3A), presumably together with more sensitivity of the photoreceptor neurons to polyQ stretch, as expected from previous reports that wild-type disease proteins with normal polyQ stretches could cause neurodegeneration in the fly eyes (Fernandez-Funez et al., 2000). Indeed, a shortening of the polyQ stretches to five repeats in the A/B domain [hAR(Q5AF-1)] resulted in loss of toxicity (Figure 3A). However, we cannot exclude the possibility that the expressed hAR(Q52AF-1) and hAR(AF-1) proteins are in altered structures, which exhibit toxicity more than those of full-length hARs. Notably, the coexpression of unliganded LBD domain [hAR(AF-2)] attenuated the neurodegeneration induced by hAR (Q52AF-1) (Figure 3C), presumably by trapping the hAR(Q52AF-1) in the cytosol. However, DHT treatment aborted this attenuation (Figure 3C). These results indicated that unliganded LBD domain masks the toxic effects of the polyQ stretches as well as their transactivation function in the A/B domains. However, upon ligand binding, the toxic and transactivation functions of the polyQ-expanded A/B domain may be restored, accompanied with translocation into the nuclei.

We further tested whether nuclear localization is necessary for the expression of the toxicity by the expanded polyQ stretches in the hAR A/B domain by hAR mutants with a nuclear export signal (NES) (Toyoshima et al., 1998) (see Figure 1A), which is expected to constitutively retain the cognate protein in the cytosol. The addition of an NES to hAR(AF-1) lacking the D domain harboring the nuclear localization signal (NLS) (Zhou et al., 1994) [hAR-NES$\Delta$NLS (Q52 AF-1)] resulted in a predominant localization in the cytosol, and the DHT-induced nuclear localization of hAR(Q52) was prevented by the tagged-NES [hAR-NES(Q52)] (Figure 3B). In agreement with their cytosolic localization and lack of GFP induction by transactivation functions of hARs (Figure 3B), the mutants tagged with the NES exerted no toxicity in the fly eyes (Figure 3D), clearly establishing that nuclear localization is a prerequisite for the onset of neurodegeneration by the hAR mutants.

### Ligand Binding Induces Toxicity of the PolyQ-Expanded hAR A/B Domain with Structural Alteration

We then directly analyzed androgen-induced alterations in hAR structure using a GST pull-down assay. While androgen-dependent interactions between the A/B and E/F domains were observed for hAR(wt) (Figure 4A, lane 3), hAR(Q52) exhibited ligand-dependent dissociation (Figure 4A, lane 5). This indicated that structural alterations took place upon ligand binding for both wild-type hAR and hAR(Q52), irrespective of the distinct structures of unliganded hAR(Q52) compared to hAR(wt). The ligand-induced alterations in receptor structure were further visualized using a trypsin digestion assay. Again, it was evident from the digestion patterns that structural alterations induced by DHT binding for hAR(wt) and hAR(Q52) were not identical (Figure 4B), in agreement with the observations in the adult eyes of hAR(Q52) lines that DHT treatment induced the generation of a fragment containing the polyQ repeats (Figure 2D). Thus, these results suggest that structural alterations of hAR mutants by ligand binding rendered the polyQ-expanded A/B domain more accessible to proteolysis, resulting in

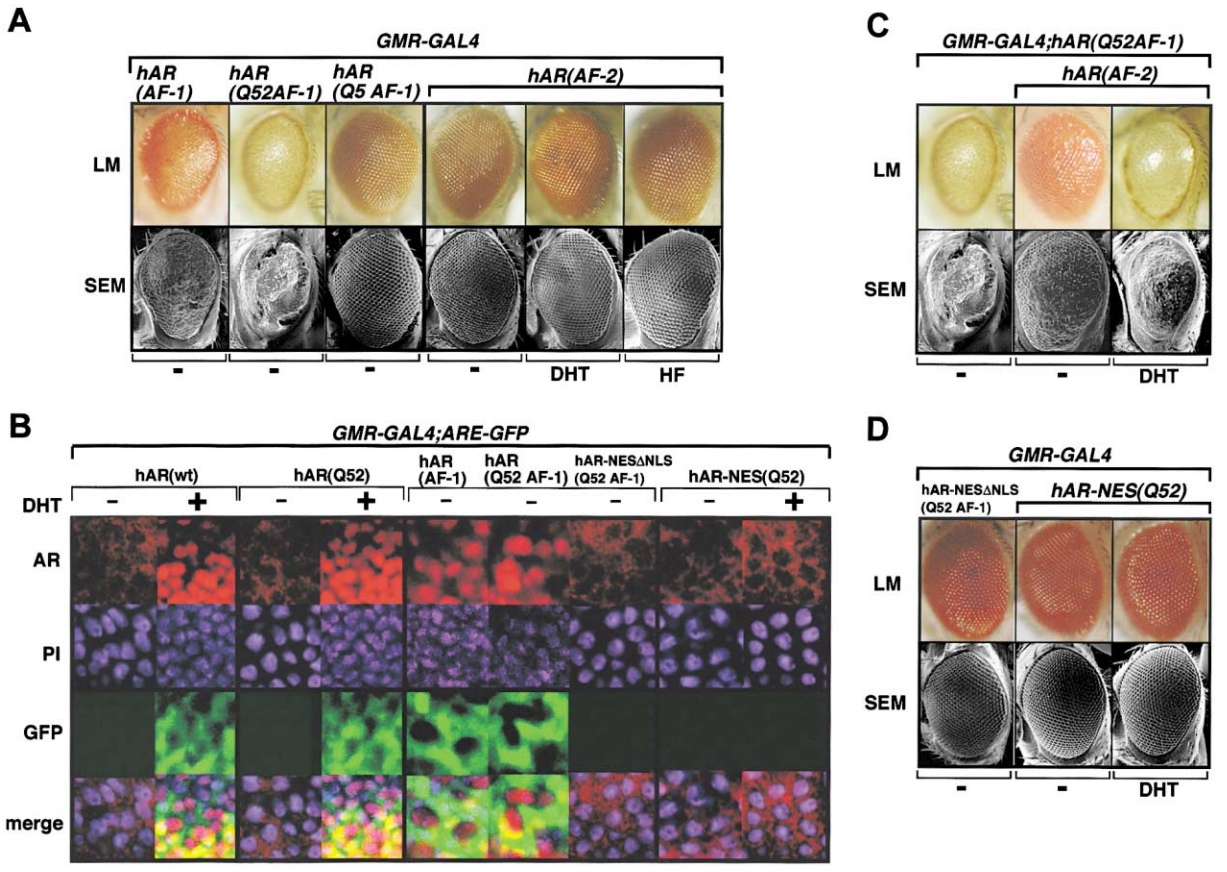


Figure 3. PolyQ-Expanded hAR AF-1 Alone Is Sufficient to Cause Neurodegeneration

(A) Ligand-independent neurodegeneration by hAR (Q52 AF-1). Genotypes are *UAS-hAR (AF-1)*, *UAS-hAR (Q52 AF-1)*, *UAS-hAR (Q5 AF-1)*, or *UAS-hAR (AF-2) in trans* to *GMR -GAL4*. Transgenic flies were kept on medium containing vehicle or ligands ($10^{-5}$ M DHT or HF). While hAR(AF-1) and hAR(Q52 AF-1) induced degeneration even without ligand treatment, expression of hAR(Q5 AF-1) did not induce detectable degeneration, even after the treatment of ligands.

(B) Localization of hAR mutants in the third instar eye imaginal discs. hAR(AF-1) and (Q52 AF-1) predominantly localized to nuclei irrespective of DHT ingestion, but the nuclear localization of hAR(wt) and hAR(Q52) required DHT. Expression of hAR(wt), hAR(Q52), hAR(AF-1), hAR(Q52 AF-1), hAR-NESΔNLS(Q52 AF-1), and hAR-NES(Q52) in third instar eye imaginal discs driven by *GMR -GAL4* were detected using anti-hAR N-20 (left panel), and chromosomal DNA was stained with propidium Iodide (PI), transactivation function of hAR mutants assessed using GFP expression (GFP), and merged image are shown in the right panel.

(C) Attenuation of hAR(Q52 AF-1)-induced neurodegeneration by unliganded hAR(AF-2). Genotypes are *GMR-GAL4;UAS-hAR(Q52 AF-1) in trans* to *UAS-hAR(AF-2)*. Without DHT, the expression of hAR(AF-2) suppressed the degeneration induced by hAR(Q52 AF-1). Treatment with DHT ($10^{-5}$ M) abolished this effect.

(D) No toxicity of hAR mutants in the cytosol. Genotypes are *GMR-GAL4;UAS-hAR NES*ΔNLS*(Q52 AF-1)* or *UAS-hAR NES(Q52)*. Transgenic flies were kept on medium containing vehicle or ligands (DHT $10^{-5}$ M). The addition of a nuclear export signal to hAR(Q52 AF-1) or (Q52) abolished the toxic effect.

the generation of fragments that could potentially be toxic.

## Discussion

### The Transactivation Function of hAR Expressed in *Drosophila* Is Maintained without Affecting the Endogenous Nuclear Receptor System

*Drosophila melanogaster* possesses a number of endogenous nuclear receptors that are functionally homologous to members of the vertebrate nuclear receptor superfamily (White et al., 1997). Of the fly nuclear receptors, the physiological role of the ecdysone receptor (EcR) has been well-documented (Bender et al., 1997). Like vertebrate steroid hormone receptors, the transactivation function of EcR is completely dependent upon ligand binding. Specific DNA elements that bind EcR and other fly nuclear receptor molecules are thought to be composed of a directly repeated 5′-AGGTCA-3′ core motif (DRs), whereas vertebrate steroid hormone receptor homodimers bind a pair of core motifs arranged as inverted core motifs (IRs). The opposite orientation between DRs and IRs is thought to render ectopic expression of vertebrate steroid receptors in *Drosophila* unable to compete with endogenous fly receptors in DNA binding. However, as there are functional similarities in gene regulation between vertebrate and insect nuclear receptors, it is speculated that there may be a common coregulatory system that supports the transactivation function of nuclear receptors. Indeed, two

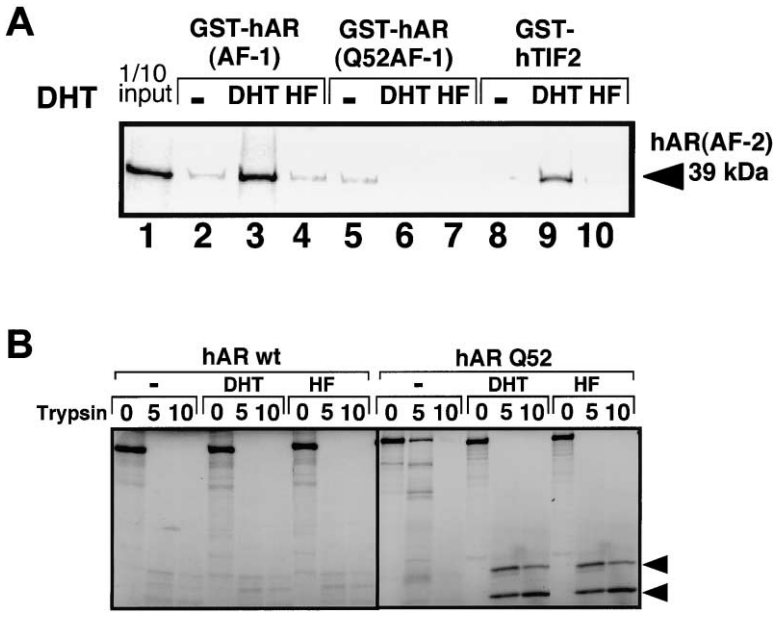


Figure 4. Structural Alteration Induced by Ligand Binding Differs between Wild-Type and Mutant hARs

(A) Ligand-induced dissociation of hAR(AF-2) and hAR(Q52 AF-1) in vitro. Interaction was assessed by incubating a GST fusion protein with either hAR(AF-1) [GST-AR(AF-1)], mutant hAR(AF-1) with Q52 [GST-AR(Q52 AF-1)], or GST-hTIF2 as a positive control, with in vitro translated [$^{35}$S]methionine-labeled hAR LBD by pcDNA3-hAR 560-919. A ligand-induced interaction between hAR LBD [hAR(AF-2)] and hAR A/B domain [hAR(AF-1)] was observed, while a ligand-depedent dissociation is seen for the hAR A/B domain mutant hAR(Q52 AF-1).

(B) Different structural alterations of hAR(wt) and hAR(Q52) induced by ligand binding. In vitro translated hAR(wt) and hAR(Q52) were incubated with or without DHT or HF ($10^{-5}$ M) and were digested with 0, 5, or 10 ng of trypsin for 10 min at 32°C with (right panel) or without (left panel) recombinant hAR LBD expressed in *E. coli*. Two hAR (Q52)-specific fragments (arrow bands) can be detected by autoradiography after ligand treatment and digestion.

homologs to mammalian nuclear receptor coactivators CBP and AIB1 have been recently identified in *Drosophila* (Akimaru et al., 1997; Bai et al., 2000).

Ectopic expression of hAR targeted to particular tissues by specific promoters appeared functional in ligand-induced transactivation in all tissues tested, including the eye. Both unliganded and liganded hAR(wt) were nontoxic in the transgenic flies under all conditions studied, such that no phenotypic abnormalities were observed in any of the tissues. Also, the activities of ingested AR ligands agreed well with results obtained using mammalian cultured cells and intact mammals. Thus, it is clear that the fly system will be a useful tool in helping to dissect the function of vertebrate steroid hormone nuclear receptors and for the genetic screening of coregulators and chromatin remodeling factors that are essential for the ligand-induced transactivation function of steroid receptors.

### Polyglutamine-Expanded Human Androgen Receptor Induces Degeneration of Photoreceptor Neurons

Ligand binding induces the transactivation function of AR, including the structural alteration required to activate the transcription of target genes via direct binding to specific DNA promoter elements (Freedman, 1999; Poujol, et al., 2000). However, it is unlikely that the ligand-induced neurodegeneration caused by polyQ-expanded AR mutants is directly related to the binding of mutant AR to the promoters of genes involved in neuronal cell death. First, the androgen antagonists did not induce transactivation function of the human AR mutants (Figure 1B) but induced neurodegeneration (Figure 2B). Second, the features of late-onset neurodegeneration in the eyes of Q52 transgenic fly lines appeared indistinguishable from transgenic flies expressing other polyQ-expanded disease proteins (Warrick et al., 1998; Jackson et al., 1998). Third, previous studies that extensively analyzed common events in those transgenic fly lines suggested that aggregate formation may cause neurodegeneration, which also appears to occur in Q52 lines. These neurodegenerate events are thought to be mediated through factors associated with the expanded polyQ stretches in the aggregates and not through any innate function of the disease proteins. This hypothesis is further supported by the fact that no phenotypic abnormalities in the fly eye expressing wild-type hAR were observed, even in the presence of ligand. Thus, ligand-induced neurodegeneration caused by AR mutants is most likely directly due to the expanded polyQ stretches in the hAR A/B domain and not to indirect alterations in hAR function.

### Onset of Neurodegeneration by hAR Mutants Is Hormone Dependent

The androgen receptor is one of several neurodegenerative disease proteins which harbors an expanded polyQ stretch. However, in sharp contrast to other neurodegenerative disease-associated mutant proteins, SBMA develops only in men (Kennedy et al., 1968; Choong and Wilson, 1998). In the present study, we clearly show in an intact animal model that the onset of neurodegeneration is completely dependent on androgen binding to mutant hAR and nuclear translocation. Moreover, the mutated hAR A/B domain (Q52 AF-1) alone, when transported into nuclei, was sufficient to promote androgen-independent toxicity as well as transactivation, whereas coexpression of unliganded LBD abrogated the neurodegeneration induced by the A/B domain mutant, presumably by trapping the A/B domain in the cytosol. Although the structures of unliganded and liganded hAR mutants are likely to differ from androgen-bound wild-type hAR, polyQ-expanded hAR A/B domains appeared to be functionally exposed, like the wild-type hAR A/B domain, only upon androgen binding (Yamamoto et al., 2000; Ross, 1997; Watanabe et al., 2001). These findings

strongly indicate that the polyQ-expanded AR A/B domains in the SBMA patients are functionally and physically masked by the unliganded ligand binding domain in the cytosol. Androgen binding to mutant hARs induced structural alterations and translocation into the nuclei that resulted in toxicity. Thus, together with the fact that serum androgen levels in adult men are 10 to 20 times higher than in women, the androgen-dependent onset of the neurodegeneration in the fly eye may explain why only men suffer SBMA.

### Nuclear Localization of PolyQ-Expanded hAR Mutants Depends on Ligand Binding

The nuclear localization of hAR mutants, like huntingtin and spinocerebellar ataxia type 1 (SCA1) mutants (Klement et al., 1998; Saudou et al., 1998), appears to be critical for the onset of neurodegeneration, since the toxicity of the hAR mutants was abolished when the polyQ-expanded hAR mutants were trapped in the cytosol by tagged-NES, even in the presence of ligand (Figures 3C and 3D). Moreover, the polyQ-expanded A/B domain mutant [hAR(Q52AF-1)], which constitutively localizes in the nuclei (Figure 3B) with the autonomous transactivation function (Figure 1B), caused ligand-independent neurodegeneration (Figure 3A). Nevertheless, the neurodegeneration induced by hAR(Q52AF-1) was attenuated by coexpression of the unliganded LBD E/F domain [hAR(AF-2)] (Figure 3C), which appears to trap the A/B domain mutant in the cytosol. More interestingly, known androgen antagonists clinically applied in androgen-dependent prostate cancer (Ruijter et al., 1999) failed to attenuate the DHT-induced neurodegeneration in the hAR(Q52) line. Although these antagonists were effective in blocking transactivation function of hAR mutants in the fly eyes (Figure 1B), nuclear translocation of the polyQ-expanded hAR mutants was unlikely to be inhibited, as antagonist-induced nuclear translocation of hAR has been previously reported (Tomura et al., 2001). These findings of the nuclear events are further supported by the recent report that neurodegeneration induced by polyQ repeats in huntingtin in the adult fly eyes required a transcriptional cofactor, CBP (Steffan et al., 2001).

### Ligand Binding Causes Structural Alteration of hAR Mutants to Expose the PolyQ-Expanded A/B Domain

Ligand binding to nuclear receptors induces structural alterations, dissociation of corepressor complexes, and association of coactivator complexes for ligand-dependent transactivation (Freedman, 1999; Glass and Rosenfeld, 2000; Mckenna and O'Malley, 2002; and Yanagisawa et al., 2002). Crystallographic analyses of the structural changes in LBDs of many nuclear receptors, including AR, revealed that H12 is drastically shifted, while other helices are also repositioned upon ligand binding (Poujol et al., 2000). The angle of H12 movement is ligand-type dependent and determines the agonistic/antagonistic action of the ligand. Improper H12 shifting and impaired pocket formation of the other helices by antagonist binding may result in the lack of recruitment of coactivator complexes to the ligand-bound LBD (Shiau et al., 1998). These findings suggest a general molecular basis by which structural alterations caused by agonist or antagonist binding modulate AF-2. In contrast, due to technical limitations of structurally analyzing the whole nuclear receptor, little is known of the structural basis of A/B domain structural alteration upon ligand binding and subsequent AF-1 induction. However, it is evident that an intramolecular structural alteration involving the entire receptor molecule takes place after ligand binding that exposes the A/B domain and allows AF-1 activation (Kato et al., 1995; Watanabe et al., 2001; Kitagawa, et al., 2002). The A/B domain may be exposed upon ligand binding, such that coactivator complexes are recruited after dissociating from the ligand-bound LBD domain. Alternatively, it is also possible that coactivator complexes recruited to the LBD upon ligand binding form a bridge to the A/B domain by releasing an inhibitory factor that suppresses AF-1.

While mutated hAR A/B domains appear to be functionally exposed upon ligand binding, the resultant structural alterations in hAR mutants are likely to differ from those of ligand-bound wild-type hAR. While known androgen antagonists are capable of inactivating the transactivation function of mutant hAR structures, the structurally altered mutant hAR still appears to be in a position to exhibit the toxicity. Judging from expression levels in fly eyes treated with and without DHT, it is unlikely that the half-life of the hAR(Q52) protein is significantly affected by ligand binding. It has been previously demonstrated that some mutant polyQ proteins are alternatively cleaved by proteases (Merry et al., 1998; Stenoien et al., 1999). It has been suggested that these truncated proteins are toxic (Ross, 1997; Kim and Tanzi, 1998). We find that ligand binding of the mutant hAR receptor results in the generation of an N-terminal fragment (see Figure 2D). It is therefore possible that ligand binding results in a conformational change of the hAR, making it more accessible to proteolysis, which allows for the generation of a potentially toxic polyQ-containing fragment.

### A Clue to Rescue SBMA/Kennedy Disease

All together, these findings suggest that nuclear localization with the ligand-dependent structural alteration is critical for the onset of neurodegeneration by hAR. Although cellular formation of aggregates by polyQ-expanded hAR mutants in cultured cells (Stenoien et al., 1999; Simeoni et al., 2000) and subsequent inhibition of aggregate formation in the cytosol by antagonists (Becker et al., 2000) have been previously reported, it appears likely that the nuclear events caused by the polyQ-expanded hAR mutants are required for SBMA pathogenesis. If the mutated receptors could be trapped in the cytosol by a novel ligand, the toxicity of mutant hAR might be prevented or at least reduced. For this reason, androgen antagonists that still permit nuclear localization would not be useful therapeutically for the treatment of SBMA. Thus, the hAR(Q52) *Drosophila* line is a useful SBMA model for drug development and for genetic screening for factors involved in androgen-induced neurodegeneration. In conclusion, we propose that SBMA may be treated by giving patients novel hAR ligands that prevent nuclear translocaton of hAR.

## Experimental Procedures

### Transactivation Assay

COS-1 cells were maintained in Dulbecco's modified Eagle's medium without phenol red, supplemented with 5% fetal-calf serum stripped with dextran-coated charcoal (Kato et al., 1995). COS-1 cells were cotransfected with 1 μg ARE-tk-luc and 0.1 μg AR expression vector (wt, Q52, Q92, Q112, or Q212). Cells were incubated for 18 hr in the absence or presence of $10^{-8}$ M DHT and then assayed for luciferase activity as previously described.

### In Vitro Translation System

Wild-type and mutant AR proteins were produced by in vitro translation of respective cDNAs in pSG5 in the presence of [$^{35}$S]methionine (Promega) (Takeyama et al., 1997).

### *Drosophila* Stocks and Generation of Transgenic Flies

All general fly stocks and the *ptc-GAL4* line were obtained from the Bloomington *Drosophila* Stock Center. Transgenic constructs together with pπ 25.7 wc transposase were microinjected into 5–30 min old $w^{1118}$ embryos reared at 18°C, using a micromanipulator (Leica). Several transgenic lines were generated (Tsuneizumi et al., 1997). The AR mutant cDNAs in *pCaSpeR3* (see Figure 1A) and an *ARE-GFP* reporter construct (*GFP-TT* in *pCaSpeR3* with a consensus ARE in its promoter) were constructed specifically for microinjection into *Drosophila*. Plasmid rescue and sequencing were performed to confirm the presence of AR mutants in the transgenic lines. Target chromosomes were separated from those carrying the *GAL4*-driver by crossing with flies harboring second and third balancer chromosomes CyO and TM3. *GAL4*-driver lines used were as follows: *GMR-GAL4* line, expressing GAL4 in the retina driven by the glass multimer reporter (Moses and Rubin, 1991); *dpp-GAL4* line, in the anterior-posterior boundary area in developing wing disc; and *ptc-GAL4* line, in the anterior portion of embryonic segments. The *UAS-dhdj-1* and *UAS-Q127* lines were the generous gift of Dr. Kazemi-Esfajani (Kazemi-Esfarjani and Benzer, 2000).

### Histology

Tissues were dissected and fixed for 20 min in 4% formaldehyde (Tanimoto et al., 2000) and incubated with a primary antibody, hAR (N-20), to recognized the N-terminal A/B domain of AR (Santa Cruz Biotechnology, Inc). Cy5-conjugated AffinityPure donkey anti-rabbit IgG (Jackson Immunoresearch) was used as the secondary antibody for immunofluorescence staining. Toluidine blue stainings of adult eyes was performed on 1 μm thick vertical or horizontal serial sections (VS or HS) (Kanuka et al., 1999). Chromosomal DNA was stained with propidium Iodide (PI). Confocal microscopy was carried out on a Zeiss confocal laser scanning system 510, and results were assessed with Adobe Photoshop 5.0 (Adobe). For scanning electron microscopy (SEM) images, whole flies were dehydrated in ethanol, critical-point dried, and analyzed with a JEOL JSM 6100 microscope.

### Western Blot Analysis

To detect hAR proteins and fragments containing the polyQ-expanded N-terminal regions, cell lysates of intact adult eyes with or without ligand were separated by 7.5% SDS-PAGE and detected with hAR (N-20) antibody (Santa Cruz Biotechnology, Inc).

### GST Pull-Down Assay

Human AR A/B domain (AF-1) and its Q52 mutant (Q52 AF-1) were expressed as GST fusion proteins [GST-AR(AF-1) and GST-AR(Q52 AF-1), respectively] in *E. coli*, as previously described, and bound to glutathione-Sepharose 4B beads (Pharmacia Biotech). The $^{35}$S-labeled AR deletion mutant together with DNA and ligand binding domains CDE/F were incubated with beads bound with either GST-AR(AF-1) or GST-AR(Q52 AF-1) in the absence or presence of $10^{-6}$ M DHT or HF in NET-N buffer (0.5% Nonidet P-40, 20 mM Tris-HCl [pH 7.5], 200 mM NaCl, 1 mM EDTA) with 1 mM PMSF. Bound proteins were separated by 7.5% SDS-PAGE and lightly stained with Coomasie brilliant blue to verify equal protein loading and then visualized by autoradiography.

### Trypsin Digestion Assay

Labeled translation mixtures were incubated at 25°C with or without $10^{-6}$ M DHT or HF for 30 min. Limited trypsinization was performed by addition of trypsin solutions (5 μg/ml or 10 μg/ml trypsin) at 32°C and stopped with equivalent amounts of 2× SDS sample buffer.

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