Supporting Information

Suzuki et al. 10.1073/pnas.0809819106

SI Experimental Procedures

Fly Stocks. Fly culture and crosses were carried out at room temperature. All general fly stocks, *dE2f* deletion mutant lines (*E2f* ^{07172} and *E2f* ^{i2}) and the *UAS-dE2f,UAS-dDp* line were obtained from the Bloomington Fly Stock Center. *UAS-AR(wt)* and *UAS-AR(Q52AF-1)* transgenic flies were described (1–3). The *UAS-Q127* line was a generous gift from Dr. Kazemi-Esfajani (4). The *gmr-Rbf* line was a generous gift from Dr. Du (5). Dr. Bonini kindly provided the *UAS-SCA3trQ78* line (6). GS strains were described in ref. 7. *gmr-GAL4/+;UAS-AR(Q52AF-1)/+* females were crossed to males from the collection of GS strains, and the resulting progeny were screened by the degree of rough eye phenotype. The rough eye phenotype was observed in the *UAS-AR(Q52)* line and the *UAS-AR(Q52)* line in *trans* to the *gmr-Rbf* line (both of which were cultured on media containing 10^{-5} M DHT during early stages of development), in the *UAS-AR(Q52 AF-1)* line in *trans* to *gmr-Rbf* line (grown on normal medium).

Plasmids. The AR(wt), AR(Q52), or AR(Q92) expression vectors, and the ARE-tk-Luc reporter vectors were constructed as described in refs. 1 and 8. The E2F1 site x6-TATA-Luc reporter vectors were kindly provided by Dr. Hatakeyama (9). Expression vectors carrying FlagHA-E2F1 and FlagHA-E2F1ΔC, FlagHA-Rb, FlagHA-Rb(A/B/C), AR[(wt)A/B], AR[(Q52)A/B], and AR(C/D/E) were amplified by PCR (PCR) and cloned into pcDNA3 (Invitrogen). Expression vectors carrying AR[(wt) C562A] and AR[(Q52) C562A] were constructed by PCR mutagenesis. GST-Rb and GST-Rb truncated mutants, carrying the N terminus (amino acids 1–379), or the A/B pocket domain (amino acids 379–772), or the C domain (amino acids 772–930), were constructed by PCR and inserted into pGEX4T-1 (Amersham).

Immunoprecipitation. For immunoprecipitation, 293T cells were seeded in 100-mm dishes and transfected at 70–80% confluence using Lipofectin (Invitrogen) in Opti-MEM (Gibco-BRL). After transfection of 293T cells with 5 μg of pcDNA3-AR(wt), or pcDNA3-AR(Q52), they were grown for 24 h in the absence or presence of 10^{-8} M DHT. Whole cell lysate supernatants in TNE buffer [10 mM Tris·HCl (pH 7.8), 1 mM EDTA, 0.15 M NaCl, 0.1% Nonidet P-40] containing protease inhibitors were immunoprecipitated with anti-Rb (IF8) antibody (Santa Cruz) or anti-E2F1(C-12) antibody (Santa Cruz) and then added to protein G-Sepharose beads. After successive washes in TNE buffer, proteins were resolved by SDS/PAGE and Western blotted using either anti-Rb (IF8) antibody, anti-AR(N-20) antibody, anti-E2F1(C-12) antibody, or anti-HDAC1(C-11) antibody (all from Santa Cruz).

For immunoprecipitation (Fig. S1 *B* and *C*), 5 μg of pcDNA3-[AR(wt) C562A], pcDNA3-[AR(Q52) C562A], pcDNA3-FlagHA-Rb or pcDNA3-FlagHA-Rb (A/B/C) were transfected to 293T cells. In the case of Fig. S2*C*, 5 μg of pcDNA3-FlagHA-E2F1 or pcDNA3-FlagHA-E2F1ΔC were transfected to 293T cells. Whole cell lysate supernatants in TNE buffer were immunoprecipitated with ANTI-FLAG M2 Affinity Gel (Sigma). After being washed, immunoprecipitated proteins were analyzed by SDS/PAGE and Western blotting using anti-Flag antibody from rabbit (Sigma) or anti-AR (N-20) antibody (Santa Cruz).

Cell Culture and Transfection. Dulbecco's modified Eagle's medium (DMEM) with high-glucose (Sigma) supplemented with 10% FBS (FBS) was used to maintain 293T cells. SH-SY5Y cells were maintained in a 1:1 mixture of Eagle's Minimum Essential Medium (EMEM) (Nissui) with non-essential amino acids (Gibco-BRL) and F12 Medium (Nissui) supplemented with 10% FBS. The cells were seeded in 12 well plates and transfected at 70–80% confluence using PolyFect (Qiagen) in Opti-MEM (Gibco-BRL) medium. For luciferase assays of AR transactivation, SH-SY5Y cells were cotransfected with 250 ng of ARE-tk luc as a reporter, 50 ng of pcDNA3-AR(wt) or pcDNA3-AR(Q52) and 200 ng of pcDNA3-myc-his-Rb or pcDNA3-FlagHA-E2F1. Cells were harvested after 12 h in the absence or presence of 10^{-8} M DHT and assayed using the Dual-luciferase reporter assay system according to the manufacturer's instructions (Promega) (10). For luciferase assays of E2F1 transactivation, SH-SY5Y cells were cotransfected with 200 ng of pGL3TATAbasic-6xE2F as a reporter, and 200 ng of pcDNA3-AR(wt) or pcDNA3-AR(Q52). The transfectants were cultured for 48 h with 0.5% FBS in the absence or presence of 10^{-8} M DHT or 10^{-7} M HF and assayed using the Dual-Luciferase reporter assay system according to the manufacturer's instructions (Promega). For knockdown of Rb, siRNA against Rb (Santa Cruz) and a control siRNA (Thermo) were transfected in 293T cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions.

GST Pull-Down Assays. Full length Rb and its mutants were expressed as GST-fusion proteins in *E. coli* strain BL21. The expression of proteins was then monitored by SDS/PAGE. GST pull-down assays using [^{35}S]-methionine-labeled AR(wt), AR(Q52), AR[(wt) A/B domain], AR[(Q52) A/B domain], AR (C/D/E domain), AR[(wt) C562A] and AR[(Q52) C562A] were performed as described in ref. 11. GST proteins were incubated with [^{35}S]-methionine-labeled proteins in TNE buffer [300 mM NaCl, 50 mM Tris·HCl (pH 7.5), 5 mM EDTA (pH 7.9), 0.5% Nonidet P-40] for 30 min at 4 °C. After washing five times in wash buffer [500 mM NaCl, 50 mM Tris·HCl (pH 7.5), 5 mM EDTA (pH 7.9), 0.5% Nonidet P-40], samples were separated by SDS/PAGE and visualized by autoradiography.

RT-PCR. TRIzol (Invitrogen) was used to isolate total RNA from 50 pairs of adult eyes or SH-SY5Y cells that had been transfected with pcDNA3-AR(wt) or pcDNA3-AR(Q52) using PolyFect (Qiagen). Reverse transcription was performed by oligo dT-primed RT from 1 μg of total RNA with Super Script II (Invitrogen), as described in ref. 12. cDNA from the reverse transcription was aliquoted and directly PCR amplified. PCR products were separated on a 1.5% agarose gel. The primers used for PCR were as follows: *dPCNA*, 5′-cca cca tcc tga aga aga-3′ and 5′-gaa gtc tgt ctc cgg tat-3′; *Actin5C*, 5′-agc aag cgt ggt atc ctc ac-3′ and 5′-acg tcc aca tct gct gga ag-3′; *Cyclin E*, 5′-cct cca aag ttg cac cag-3′ and 5′-ctg tgg gtc tgt atg ttg-3′; *GAPDH*, 5′-acc aca gtc cat gcc atc ac-3′ and 5′-tcc acc acc ctg ttg ctg ta-3′.

Chromatin Immunoprecipitation. Chromatin immunoprecipitation was performed using a ChIP assay kit (Upstate Biotechnology), as described in ref. 13. Lysates of intact adult eyes were immunoprecipitated with one of the following: anti-dE2f monoclonal antibody (Hao4) kindly provided by Dr. Dyson (5, 14), anti-AR (441) (Santa Cruz), anti-acetyl-histone H3 (Upstate), or rabbit anti-mouse whole serum (non-specific antibody). PCR products were visualized on 1.5% agarose/TAE gels. The primers used for

PCR were as follows: *dPCNA*, 5′-gca gag ctg cgt gga atg att aaa gc-3′ and 5′-ttg acc cag gcg tgc ctc gaa cat at-3′.

In the case of mammalian cells, 5 μg of pcDNA3-AR(wt) or pcDNA3-AR(Q52) were transfected into SH-SY5Y cells. Cells were grown with 0.5% FBS for 24 h in the absence or presence of 10^{-8} M DHT for 24 h. Cell lysates were immunoprecipitated with anti-E2F1 (C-20), anti-AR (441), anti-Rb1 (C-15), anti-HDAC1 (C-11) (Santa Cruz), or anti-acetyl-histone H3 (Upstate) antibodies, or with rabbit anti-mouse whole serum (nonspecific antibody). PCR products were visualized on 1.5% agarose/TAE gels. The primers used for PCR were as follows: *Cyclin E*, 5′-gga cgg gct ctg ggt ccc-3′ and 5′-cgt cca gtc ccg gca cgg-3′.

1. Takeyama K, et al. (2002) Androgen-dependent neurodegeneration by polyglutamine-expanded human androgen receptor in *Drosophila*. *Neuron* 35:855–864.
2. Furutani T, et al. (2005) Human expanded polyglutamine androgen receptor mutants in neurodegeneration as a novel ligand target. *J Pharmacol Exp Ther* 315:545–552.
3. Murata T, et al. (2008) RNA-binding protein hoip accelerates polyQ-induced neurodegeneration in *Drosophila*. *Biosci Biotechnol Biochem* 72:2255–2261.
4. Kazemi-Esfarjani P, Benzer S Genetic suppression of polyglutamine toxicity in *Drosophila* (2000) *Science* 287:1837–1840.
5. Du W, Vidal M, Xie JE, Dyson N (1996) RBF, a novel RB-related gene that regulates E2F activity and interacts with cyclin E in *Drosophila*. *Genes Dev* 10:1206–1218.
6. Warrick JM, et al. (1998) Expanded polyglutamine protein forms nuclear inclusions and causes neural degeneration in *Drosophila*. *Cell* 93:939–49.
7. Toba G, et al. (1999) The gene search system. A method for efficient detection and rapid molecular identification of genes in *Drosophila melanogaster Genetics* 151:725–737.
8. Zhao Y, et al. (2008) A TFTC/STAGA module mediates histone H2A and H2B deubiquitination, coactivates nuclear receptors, and counteracts heterochromatin silencing. *Mol Cell* 29:92–101.
9. Yamada M, et al. (2002) Role of pRB-family/E2F complex in the inhibition of IL-3-dependent lymphoid cell proliferation. *Cytokine* 17:91–97.
10. Ito S, et al. (2004) *In vivo* potentiation of human oestrogen receptor alpha by Cdk7-mediated phosphorylation. *Genes Cells* 9:983–992.
11. Takada I, et al. (2007) A histone lysine methyltransferase activated by non-canonical Wnt signalling suppresses PPAR-gamma transactivation. *Nat Cell Biol* 9:1273–1285.
12. Fukuda T, et al. (2007) DEAD-box RNA helicase subunits of the Drosha complex are required for processing of rRNA and a subset of microRNAs. *Nat Cell Biol* 9:604–611.
13. Kitagawa H, et al. (2003) The chromatin-remodeling complex WINAC targets a nuclear receptor to promoters and is impaired in Williams syndrome. *Cell* 113:905–917.
14. Bosco G, Du W, Orr-Weaver TL (2001) DNA replication control through interaction of E2F-RB and the origin recognition complex. *Nat Cell Biol* 3:289–295.

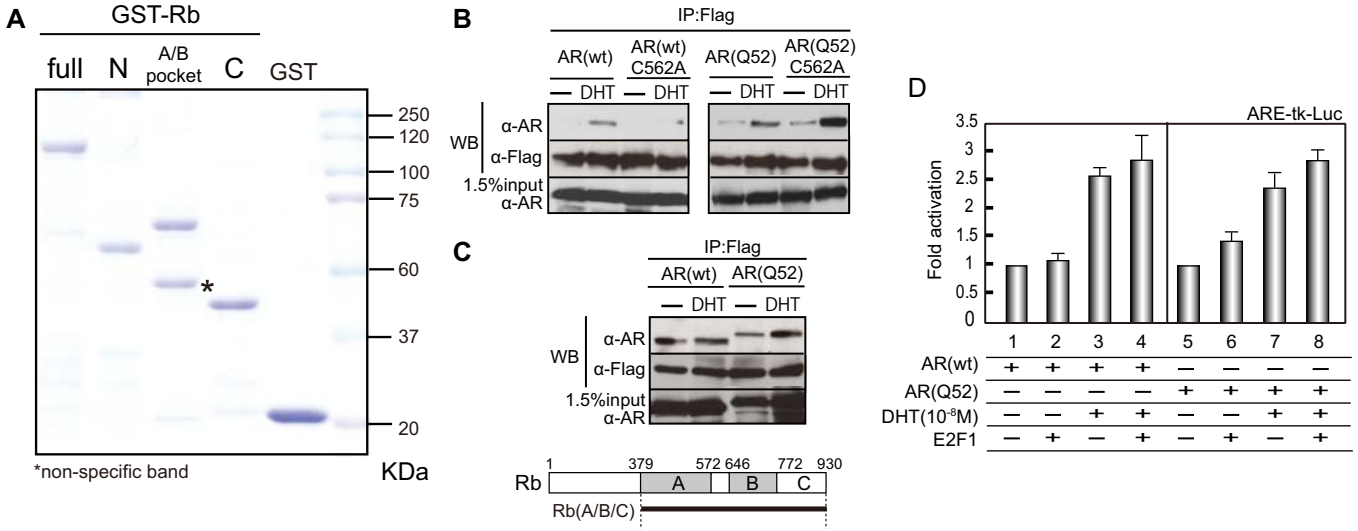

Fig. S1. Aberrant association of polyQ-AR with Rb. (*A*) CBB staining of purified GST fusion proteins expressed in *E. coli*. GST-Rb full length (full) (lane 1), GST-Rb N-terminal (lane 2), GST-Rb A/B pocket domain (lane 3), GST-Rb C domain (lane 4), GST only (lane 5). Asterisk (*) indicates non-specific band. (*B*) Co-IP of Flag-Rb with AR mutants in 293T cells. (*C*) Co-IP of Flag-Rb mutant that contains A/B pocket and C domain [Rb(A/B/C)] with ARs in 293T cells. Diagram of Rb(A/B/C) is shown under figure. (*D*) Effect of E2F1 on wild-type and polyQ-AR transactivation. ARE-dependent reporter was cotransfected with AR(wt) or AR(Q52) with or without E2F1 expression plasmids in SH-SY5Y cells that were treated with vehicle or 10^{-8} M DHT. Results are given as means ± SD for at least 3 independent experiments.

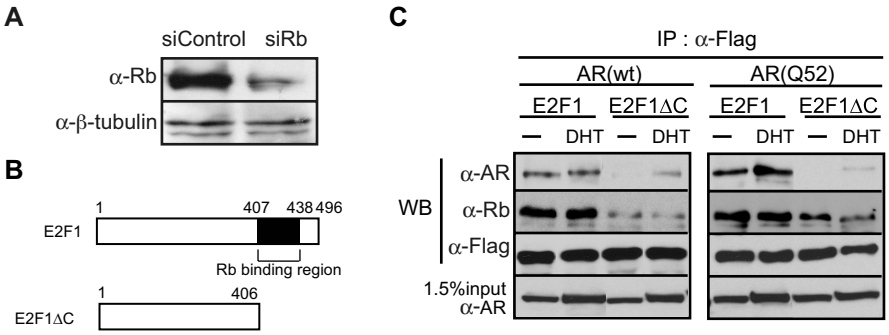

Fig. S2. Rb mediates association of polyQ-AR mutant with E2F1. (*A*) Knock-down of Rb by siRNA in 293T cells. (*B*) Diagram of wild type and mutant E2F1 structure. Rb binding region is indicated in black. The E2F1 ΔC mutant includes amino acids 1–406, and lacks the C terminus that contains the Rb-binding region. (*C*) Co-IP of Flag-E2F1 with Rb or ARs in 293T cells.

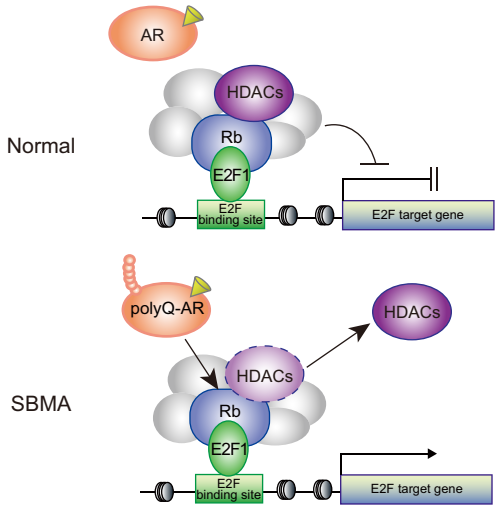

Fig. S3. Aberrant E2F1 activation by a polyQ-AR mutant. Under normal conditions, Rb represses E2F1-mediated transcription through formation of an Rb/HDACs complex. In the presence of polyQ-AR, HDACs' recruitment to Rb may be impaired, leading to aberrant E2F1 activation.