Correction

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The authors note that Fig. 4 appeared incorrectly. The corrected figure and its legend appear below. These errors do not affect the conclusions of the article.

www.pnas.org/cgi/doi/10.1073/pnas.1214509109

Fig. 4. PolyQ-AR activates E2F1 target gene expression in a ligand-dependent manner. (A) RT-PCR of dE2f target gene, dPCNA, expression in AR(wt) or AR (Q52) Drosophila in the absence or the presence of ligand. Genotypes: gmr-GAL4/+; UAS-AR(wt)+ and gmr-GAL4/+; UAS-AR(Q52)+. (B) RT-PCR of the human E2F1 target gene, human Cyclin E (hCyclin E) expression in SH-SY5Y cells transfected with AR(wt) or AR(Q52) expression plasmids and treated with vehicle or $10^{-8}$ M DHT. (A, B) Graphs show fold induction of the indicated mRNAs compared with their levels in the absence of ligand. Results are given as means ± SD for at least 3 independent experiments. (C) ChIP analysis of dE2F1, Rbf, and ARs, acetylated histone H3 (AcH3), and Rpd3 at the E2F response elements of the dE2f target gene, dPCNA, promoter in Drosophila in the presence of DHT. Genotypes: gmr-GAL4/+; UAS-AR(wt)+ or gmr-GAL4/+; UAS-AR(Q52)+. (D) ChIP analysis of E2F1, Rb, ARs, AcH3, and HDAC1 at the E2F response elements in the promoters of endogenous human E2F1 target gene, hCyclin E, in SH-SY5Y cells transfected with AR(wt) or AR(Q52) together with empty vector or Rb expression plasmids and treated with vehicle or $10^{-8}$ M DHT.

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Spinal and bulbar muscular atrophy (SBMA) is a neurodegenerative disorder caused by a polyglutamine repeat (polyQ) expansion within the human androgen receptor (AR). Unlike other neurodegenerative diseases caused by abnormal polyQ expansion, the onset of SBMA depends on androgen binding to mutant human polyQ-AR proteins. This is also observed in Drosophila eyes ectopically expressing the polyQ-AR mutants. We have genetically screened mediators of androgen-induced neurodegeneration caused by polyQ-AR mutants in Drosophila eyes. We identified Rbf (Retinoblastoma-family protein), the Drosophila homologue of human Rb (Retinoblastoma protein), as a neuroprotective factor. Androgen-dependent association of Rbf or Rb with AR was remarkably potentiated by aberrant polyQ expansion. Such potentiated Rb association appeared to attenuate recruitment of histone deacetyltransferase 1 (HDAC1), a corepressor of E2F function. Either overexpression of Rbf or E2F deficiency in fly eyes reduced the neurotoxicity of the polyQ-AR mutants. Induction of E2F function by polyQ-AR-bound androgen was suppressed by Rb in human neuroblastoma cells. We conclude that abnormal expansion of polyQ may potentiate innate androgen-dependent association of AR with Rb. This appears to lead to androgen-dependent onset of SBMA through aberrant E2F transactivation caused by suppressed histone deacetylation.

Transcriptional regulation | Neurodegenerative disease | Retinoblastoma protein

Aberrant E2F activation by polyglutamine expansion of androgen receptor in SBMA neurotoxicity

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Edited by David J. Mangelsdorf, The University of Texas Southwestern Medical Center, Dallas, TX, and approved January 7, 2009 (received for review October 1, 2008)

Rbf Overexpression Rescues PolyQ-AR-Induced Neurodegeneration.

We reported that neurodegeneration induced by the full-length human polyQ-AR mutant [AR(Q52)] was ligand-dependent, but...
the N-terminal domain of the polyQ-AR mutant [AR(Q52 AF-1)] was constitutively and sufficiently potent to induce neurodegeneration as a rough eye phenotype (Fig. 1A and B) (4). This ligand-independent neurodegeneration by AR(Q52 AF-1) is advantageous for genetic screening to identify modifiers of polyQ-AR-mutant-dependent neurodegeneration. To understand the molecular basis of polyQ-AR-induced neurodegeneration, we carried out genetic screening for modifiers of eye degradation. We crossed GS lines (which misexpressed unknown genes driven by the GAL4 protein) with UAS-AR(Q52 AF-1) lines driven by gmr-GAL4. With gmr-GAL4, gene expression could be specifically targeted to the eye. After screening ≈2,000 fly strains, several candidate genes capable of modifying the rough eye phenotype were identified. To verify reproducibility of the phenotype, putative modifiers selected in the initial screen were further tested by crossing with different independent strains expressing AR(Q52) in the presence of an active androgen [dihydrotestosterone (DHT)]. One of the candidate lines, GS-5173, was found to rescue neurodegeneration by AR(Q52 AF-1) and androgen (DHT)-bound AR(Q52) (Fig. 1B).

With the Drosophila Gene Search Project (DGSP) database, analysis of genomic sequences downstream of the P element in the GS-5173 line led to identification of the Rbf gene. Thus, the gmr-GAL4-driven GS-5173 line was expected to be an Rbf overexpression mutant. We verified Rbf function by crossing a UAS-AR(Q52 AF-1) line with an Rbf overexpression line, gmr-Rbf. Rbf overexpression clearly prevented the rough eye phenotype induced by either AR(Q52 AF-1) or AR(Q52) in the presence of DHT (Fig. 1B).

To determine the specificity of Rbf as a modifier of the neurodegenerative phenotype, Rbf overexpression lines were then crossed with a strain expressing the isolated polyQ127 repeat alone, the UAS-polyQ-127 line or the UAS-polyQ-ataxin3 line (SCA3tr-Q78 line), a Drosophila model of spinocerebellar ataxia type 3 (12). Notably, Rbf overexpression was unable to rescue neurodegeneration by either polyQ127 or SCA3tr-Q78 (Fig. 1C and D). These results suggested that Rbf genetically and selectively interacts with polyQ-AR mutants in the adjacent regions and not through the polyQ repeats themselves in the AR mutants.

**Androgens Stimulate the Interaction of PolyQ-AR Mutants with Rb**

For detailed analysis of the phenotypic effects of overexpression of Rbf, we tested possible interactions between AR and endogenous Rb in human embryonic kidney 293T cells transfected with either AR(wt) or AR(Q52) expression constructs. By co-immunoprecipitation (Co-IP) and Western blot (WB) assays, Rb was shown to weakly interact with AR(wt). Interaction appeared to depend on binding of DHT but not hydroxyflutamide (HF), the antagonist for AR(wt) (Fig. 2A). In contrast, the interaction of AR(Q52) with Rb was remarkably pronounced with both DHT and HF. Surprisingly, HF served as an agonist in the association. This observation supports our previous findings that HF serves as an agonist for polyQ-AR-dependent neurodegeneration (4). To examine whether the potential association of AR with Rb was mediated through the expanded polyQ repeats, a similar Co-IP experiment was performed in 293T cells expressing ataxin-3, which contains a polyQ60 repeat similar to that of the polyQ-AR mutants. Consistent with the inability of Rb to rescue the polyQ-expanded ataxin-3-induced neurodegeneration phenotype (Fig. 1D), no association of Rb with ataxin-3(Q60) was detected in the Co-IP assay with anti-Rb antibodies (Fig. 2B). These results support our hypothesis that Rb interacts selectively with polyQ-AR mutants.

We then performed GST pull-down assays to determine whether the functional interaction of the polyQ-AR mutant with Rb is direct, using recombinant proteins consisting of AR deletion mutants and GST-fused Rb. Rb physically interacted with the AR A/B domain containing polyQ [AR(Q52) A/B] but not with its wild-type [AR(wt) A/B] (Fig. 2C). The AR C/D/E/F mutant reportedly associates with Rb (14). Because the AR C domain bears a “LXCXE”-like motif (LICGDE), a
PolyQ-AR Counteracts Rb for E2F Repression. The Rb protein negatively regulates the G1/S transition by repressing the transcriptional activity of the E2F transcription factors. Rbf also represses dE2F-dependent transcription and suppresses the phenotype generated by ectopic expression of dE2F and dPcp in the developing *Drosophila* eye (Fig. 3A). Because Rbf overexpression prevents polyQ-AR-induced neurodegeneration in the fly eye similarly to dE2F/dPcp1-induced neurodegeneration, we reasoned that E2F transcriptional activity may mediate polyQ-AR-induced neurodegeneration. To test this idea, we crossed the *AR(Q52 AF-1)* line with dE2F-deficient mutant lines. In 2 independent *Drosophila* mutant lines deficient in dE2F, *E2f*^Q772^ and *E2f*^Q12^, the action of AR(Q52 AF-1) in the rough eye was partially reduced when compared with that in the control fly (Fig. 3B). Partial rescue of the rough eye phenotype by the dE2F mutant was also prevented by Rbf overexpression (Fig. 3C). These results indicate that E2F transcriptional activity is enhanced in the *Drosophila* eye model of SBMA.

Next, we examined whether polyQ-AR influences the transcriptional function of E2F1 in the human neuroblastoma cell line SH-SY5Y. An E2F-dependent reporter plasmid with a cassette of 6 E2F binding sites derived from the E2F1 gene promoter was cotransfected with expression vectors for AR, Rb, or both. Considerably weaker transcriptional activation of endogenous E2F1 was observed with AR(wt) activated by DHT (Fig. 3D). However, AR(Q52) activated by DHT significantly enhanced E2F1 transcriptional activity (Fig. 3D). The AR antagonist, HF, also enhanced E2F1 transcriptional activity. Furthermore, coexpression of Rb abrogated the enhancement of E2F1 transcriptional activation by AR(Q52) (Fig. 3D).

We then studied the molecular basis of the functional association of the polyQ-AR mutant with E2F1. With Co-IP with anti-E2F1 antibody (Fig. 3E), we observed DHT-induced association of endogenous E2F1 with AR(Q52). The association was undetectable when Rb was knocked down by siRNA (Fig. 3E and Fig. S2A). Thus, it appears that Rb bridges E2F1 to the AR(Q52) mutant. This idea was further confirmed by the finding that the AR(Q52) mutant was not coimmunoprecipitated with an E2F1 mutant (E2F1ΔC) lacking its Rb-interacting domain (Fig. S2B and C). Because the polyQ-AR mutant inhibited the transrepressive function of Rb for E2F1, we reasoned that the polyQ-AR mutant might attenuate recruitment of the Rb corepressor, HDAC1, through association of the Rb transrepressive domain (A/B pocket domain). We found that HDAC1’s association with Rb was unaffected by AR(wt)-bound DHT. However, binding of DHT to AR(Q52) indeed induced HDAC1 dissociation (Fig. 3F).
hyperacetylation of histone H3 in SH-SY5Y cells (Fig. 4).

The retinoblastoma tumor suppressor Rb and the related factors for the neurodegenerative (rough eye) phenotype in Drosophila radishila are negative regulators of cell proliferation. Rb functions at major G1 checkpoints, thereby inhibiting S-phase entry and progression. They also promote terminal differentiation by inducing both cell-cycle exit and tissue-specific gene expression (18, 19).

Discussion

In this report, we used genetic screening for neuroprotective factors for the neurodegenerative (rough eye) phenotype in Drosophila radishila and identified Rbf/Rb as a candidate (Fig. 1). The retinoblastoma tumor suppressor Rbf and related factors, such as mammalian p107 and p130 or Drosophila Rbf, are negative regulators of cell proliferation. Rb functions at major G1 checkpoints, thereby inhibiting S-phase entry and progression. They also promote terminal differentiation by inducing both cell-cycle exit and tissue-specific gene expression (18, 19).

Consistent with the neuroprotective action of Rbf, we found that expansion of polyQ repeats in AR mutants augmented the physical association of Rbf and Rb with the polyQ-AR protein. Because Rb was unable to counteract the neurodegenerative activity of the expanded polyQ repeat on its own (Fig. 1C), Rb/Rbf thus appears to be a selective neuroprotective factor for SBMA among hereditary polyglutamine diseases. We attribute the selective Rb/Rbf activity to its enhanced association with polyQ-AR mutants. This idea is consistent with the recent findings that abnormal polyQ expansion in spinocerebellar ataxia type 1 protein potentiates native protein interactions, thus leading to neuropathology (20, 21).

It is widely accepted that Rb and related factors function through their effects on the transcription of genes regulated by the E2F proteins. E2F binding sites are found in the promoters of many genes essential for cell-cycle progression and cell proliferation. Most Rb regulatory functions are associated with the repressor activities of Rb/E2F complexes located on the promoters of cell-cycle regulatory genes such as Cyclin E and the E2F transcription factor (22–24). Moreover, many E2F target genes regulate apoptosis, development, and differentiation. Overexpression of E2F1 in mammalian cells results in the induction of apoptosis (25–27). Similarly, ectopic expression of Drosophila E2f E2f induces apoptosis in imaginal discs and, if expressed in the eye discs, causes the rough eye phenotype (28). Significantly, in our genetic analysis, E2f-deficient Drosophila mutants partially reversed the polyQ-AR-induced rough eye phenotype (Fig. 3B). Expression of Rb abrogated E2f transactivation by polyQ-AR (Fig. 3D). The reporter expression assay data were further confirmed by observations of polyQ-AR-induced expression of endogenous E2f target genes in Drosophila eye disc and cultured mammalian cells (Fig. 4A and B). These findings suggest that polyQ-AR activates endogenous E2f proteins in neuronal cells in a ligand-dependent manner. Although it was reported that Rb coactivates AR through its physical association (14, 16), no association of the other 8 polyQ-expanded proteins with Rb have been mentioned. In the present study, we also could not detect the interaction of polyQ-ataxin-3 with Rb (Fig. 2B). Thus, we presume that, among the polyQ-expanded proteins, only polyQ-AR selectively enhances E2f transactivation via Rb.

The transrepression function of Rb/Rbf for E2f requires HDAC1 to deacetylate histone for chromatin inactivation (18). As expected, wild-type AR did not affect the function of Rb or E2f. However, supporting our in vitro observations that the polyQ-AR mutant competed with HDAC1 in Rb associ-
