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MicroRNA Pathways Modulate Polyglutamine-Induced Neurodegeneration

Short Article

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Summary

Nine human neurodegenerative diseases are due to expansion of a CAG repeat- encoding glutamine within the open reading frame of the respective genes. Polyglutamine (polyQ) expansion confers dominant toxicity, resulting in neuronal degeneration. MicroRNAs (miRNAs) have been shown to modulate programmed cell death during development. To address whether miRNA pathways play a role in neurodegeneration, we tested whether genes critical for miRNA processing modulated toxicity induced by the spinocerebellar ataxia type 3 (SCA3) protein. These studies revealed a striking enhancement of polyQ toxicity upon reduction of miRNA processing in *Drosophila* and human cells. In parallel genetic screens, we identified the miRNA *bantam* (*ban*) as a potent modulator of both polyQ and tau toxicity in flies. Our studies suggest that *ban* functions downstream of toxicity of the SCA3 protein, to prevent degeneration. These findings indicate that miRNA pathways dramatically modulate polyQ- and tau-induced neurodegeneration, providing the foundation for new insight into therapeutics.

Introduction

Human polyQ diseases are due to the expansion of a CAG repeat within the open reading frame of the respective genes (Zoghbi and Orr, 2000). The polyQ expansion confers dominant toxicity, resulting in progressive neuronal dysfunction and loss. In such diseases, the pathogenic protein accumulates, typically in nuclear inclusions. Although it is unclear how these accumulations contribute to disease pathogenesis, several lines of evidence support the idea that a conformational change occurs in the disease protein associated with reduced solubility, enhanced oligomerization, and toxicity (Muchowski and Wacker, 2005). Studies with animal models and patient tissue indicate that the protein accumulations contain chaperones and components of the ubiquitin-proteasome system, suggesting that the toxic, misfolded protein triggers a stress response to lower the amount of disease protein. A number of suppressors of neurodegeneration in animal models have coupled reduced protein accumulation, or modulation of ability to handle misfolded protein, to mitigation of degeneration (Bilen and Bonini, 2005).

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Although modifiers that implicate protein misfolding pathways have been defined, cellular pathways that link polyQ toxicity and degeneration of the cell are less clear. Morphological analysis of degenerating neurons reveals that they appear to undergo condensation of the nucleus and cytoplasm; however, other hallmarks of programmed cell death, such as apoptotic bodies and fragmentation of nuclear DNA are not observed (Jackson et al., 1998; Turmaine et al., 2000). Studies in *Drosophila* have shown that the baculoviral protein P35, which potently inhibits caspase activity, is largely ineffective at mitigating polyQ-induced degeneration (Jackson et al., 1998; Warrick et al., 1998). Autophagy, which couples reduced protein accumulation with cell survival, is thought to be involved, as are dApaf-1/Dark activities for the Huntington's disease protein (Ravikumar et al., 2004; Sang et al., 2005).

miRNA pathways have been implicated in developmental apoptosis, normal developmental processes, and cancer (Ambros, 2004; Bartel, 2004; Caldas and Brenton, 2005). In *Drosophila*, the miRNAs *ban, mir-14*, and select other miRNAs have been shown to modulate programmed cell-death genes (Brennecke et al., 2003; Leaman et al., 2005; Xu et al., 2003). SCA3 is one of the polyQ diseases and is among the most common dominantly inherited ataxias (Zoghbi and Orr, 2000). To address a possible role for miRNAs in neuronal maintenance, we tested whether compromising miRNA processing modulated polyQ pathogenesis. Our findings extend the processes modulated by miRNAs to human neurodegenerative diseases.

Results

Reduced miRNA Processing Dramatically Enhances Ataxin-3- and tau-Induced Neurodegeneration in *Drosophila*

We tested whether miRNAs modulated polyQ pathogenesis in *Drosophila* by downregulating miRNA processing with the *dicer* mutation and then determining the effect on Ataxin-3-induced neurodegeneration. Dicer activity is essential for miRNA processing in flies and vertebrates (Bernstein et al., 2001; Hutvagner et al., 2001). *Drosophila* has two *dicer* genes: *dicer-1* (*dcr-1*) is critical for maturation of miRNAs, whereas *dcr-2* modulates generation of small interfering RNAs (siRNAs) (Lee et al., 2004). Flies were generated that expressed the pathogenic polyQ protein and were also mutant for *dcr-1* activity in the eye.

Expression of truncated pathogenic Ataxin-3 induces degeneration, which is characterized by partial loss of pigmentation and retinal structure (Figure 1A; Warrick et al., 1998). *dcr-1* mutation dramatically enhanced degeneration due to the pathogenic protein, such that the eye was now severely degenerate with complete loss of pigmentation (Figure 1B). Because loss of *dcr-1* alone affects eye morphology (Figure 1C; Lee et al., 2004), we also examined the effects of a second gene critical for miRNA maturation, *R3D1/loquacious*. R3D1 is a dsRNA binding protein that is required for the

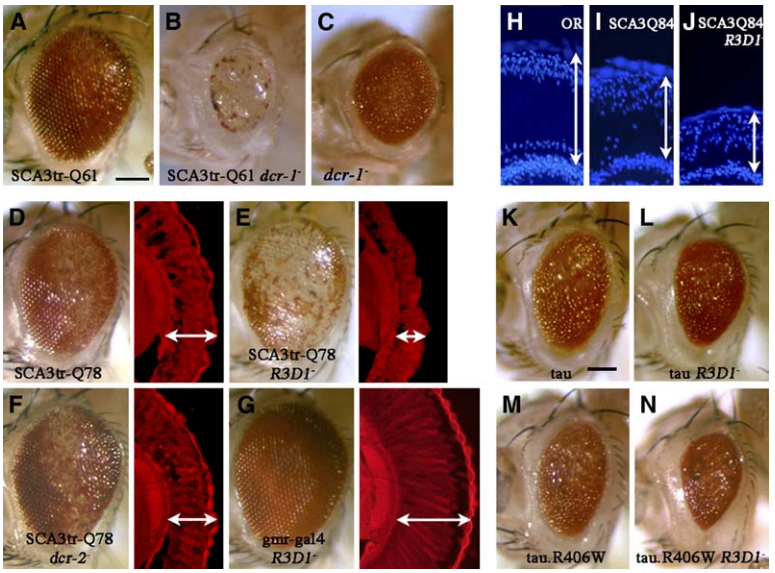

Figure 1. Reducing miRNA Processing Enhances polyQ and tau Toxicity in *Drosophila*

(A–C) External eyes of 1 day flies. (A) SCA3tr-Q61 normally shows weak degeneration. Eye genotype: *ey-FLP; gmr-gal4 UAS-SCA3tr-Q61/+; FRT82B*. (B) SCA3tr-Q61 degeneration in *dcr-1* is dramatically enhanced. Eye genotype: *ey-FLP; gmr-gal4 UAS-SCA3tr-Q61/+; FRT82B dcr-1*^{Q11147X}. (C) Loss of *dcr-1* activity alone. Eye genotype: *ey-FLP;; FRT82B dcr-1*^{Q11147X}.

(D–G) External eye and retinal sections. Arrow highlights retinal thickness, which reflects severity of degeneration. (D) SCA3tr-Q78 causes partial degeneration. Genotype *gmr-gal4 UAS-SCA3tr-Q78/+*. (E) Loss of *R3D1* enhances degeneration, with less pigmentation and little retinal tissue compared to control (D). Genotype: *R3D1*^{f00791}*/R3D1*^{f00791}*; gmr-gal4 UAS-SCA3tr-Q78/+*. (F) Loss of *dcr-2*, which modulates siRNA production, has no effect on polyQ toxicity. Eye genotype: *FRT42D dcr-2*^{L811fsX}*; gmr-gal4 UAS-SCA3tr-Q78/ey-gal4 UAS-FLP*. (G) Control flies bearing driver line alone with loss of *R3D1* have normal retinal morphology. Genotype: *R3D1*^{f00791}*/R3D1*^{f00791}*; gmr-gal4/+*.

(H–J) Hoechst staining of retinal sections of 1 day flies. (I) Expression of full-length pathogenic Ataxin-3 causes mild retinal degeneration, seen as reduced retinal depth compared to (H) normal. (J) Reducing miRNA processing by mutation of *R3D1* enhances degeneration, seen as the dramatically reduced retinal thickness. Genotypes: (H) Oregon-R, (I) *gmr-GAL4/ UAS-SCA3-Q84*, and (J) *R3D1*^{f00791}*/R3D1*^{f00791}*; gmr-GAL4/ UAS-SCA3-Q84*.

(K–N) Normal or mutant (R406W) human tau causes degeneration that is enhanced by loss of *R3D1*. Genotypes: *gmr-gal4* in *trans* to *UAS-tau* or *UAS-tau.R406W* with or without *R3D1*^{f00791}*/R3D1*^{f00791}.

Bar in (A), 100 μm for eyes in (A)–(G); bar in (K), 100 μm for (K)–(N).

activity of Dcr-1 in miRNA processing (Forstemann et al., 2005; Jiang et al., 2005). We confirmed that flies homozygous for the hypomorphic allele *R3D1*^{f00791} had reduced miRNA processing and that *R3D1* mutation had no effect on transcription from the Gal4-UAS system (Figures S1A and S1B in the Supplemental Data available with this article online). Loss of *R3D1* alone had minimal effects on the eye (Figure 1G). However, reduction of *R3D1* activity dramatically enhanced SCA3tr-Q78-induced neural degeneration (Figures 1D and 1E). This confirmed that miRNA pathways normally play a protective role in polyQ-induced neurodegeneration.

We considered whether reduction of miRNA processing enhanced SCA3 pathogenesis through the same type of degeneration pathways that normally occur upon pathogenic polyQ protein expression or whether reduction of miRNA processing modulated programmed cell death, which then enhanced the phenotype. To address this, we determined whether we could detect features of programmed cell death when expressing the disease protein alone or upon enhanced degeneration. TUNEL assays and western immunoblots for activated caspase failed to reveal an effect in polyQ pathology or with reduced *R3D1* activity, and coexpression of the baculoviral anticaspase protein P35 had minimal effect (Figure S2). These data support the idea that reduction of miRNA activity enhances degeneration by modulating pathways that normally contribute to polyQ toxicity.

To further test the specificity of miRNA pathways in neurodegeneration, we determined whether reduction of siRNA activity affects polyQ toxicity. In contrast to loss of *dcr-1* and reduction of *R3D1*, loss of *dcr-2*, which reduces siRNAs, had little or no effect on polyQ-induced neurodegeneration (Figures 1D and 1F). These data indicated that siRNA-dependent pathways do not modulate polyQ toxicity; rather, protective activity appears specific to miRNA-dependent pathways.

We then addressed the broader role of miRNA pathways in neurodegeneration. We first determined that reduction of *R3D1* activity enhanced not only pathogenicity of truncated forms of Ataxin-3 but also of the full-length pathogenic protein (Figures 1H–1J). To extend these findings beyond polyQ disease, we then examined the effect on tau. Abnormal tau accumulation is associated with Alzheimer's disease and frontotemperal dementia (Lee et al., 2001). In *Drosophila*, expression of normal or pathogenic tau induces severe neurodegeneration, reflected by a severely disrupted eye phenotype (Figures 1K and 1M; Wittmann et al., 2001). Reduction of *R3D1* activity dramatically enhanced tau toxicity (Figures 1L and 1N). These data indicate that the miRNA pathway not only modulates polyQ toxicity but also toxicity of other proteins associated with human neurodegenerative disease.

Blocking miRNA Processing Dramatically Enhances Ataxin-3 Toxicity in Human Cells

Given these observations in flies, we addressed whether these findings extended to human cells. To do this, we expressed normal and pathogenic full-length Ataxin-3 protein in human HeLa cells in culture, in the presence of normal or reduced Dicer activity. HeLa cells were treated with siRNA directed to the *dicer* sequence; this treatment lowered *dicer* activity, as shown by reduced expression and processing of endogenous Dicer targets

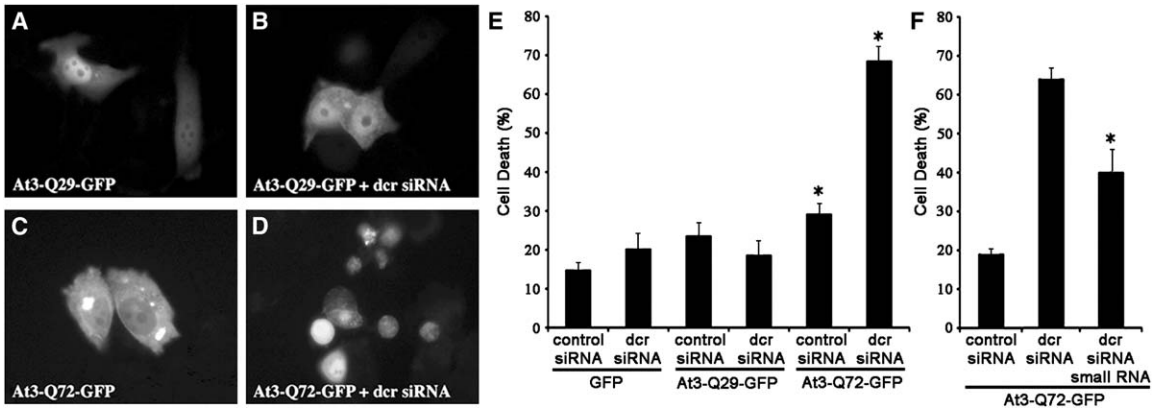

Figure 2. Reducing miRNA Processing Enhances Ataxin-3 Toxicity in Human Cells

(A–D) Human HeLa cells expressing At3-Q29-GFP or At3-Q72-GFP and treated with control siRNA or siRNA to *dicer*. (A and B) Cells expressing the control Ataxin-3 have similar viability with or without *dicer* siRNA treatment. (C and D) Cells expressing pathogenic Ataxin-3 normally (C) show little toxicity by 24 hr but (D) show dramatically enhanced death, reflected in condensed cells with altered morphology, upon *dicer* knockdown.

(E) Cell death of GFP-positive cells after 24 hr detected by uptake of propidium iodide; mean ± SEM (n = 3 independent experiments; *$p < 0.001$ compared to GFP or At3-Q29-GFP treated with control or *dicer* siRNA; At3-Q72-GFP with *dicer* siRNA is also significantly different [$p < 0.001$] from At3-Q72-GFP with control siRNA).

(F) Increased cell death induced by At3-Q72-GFP with *dicer* knockdown is mitigated by transfection of the purified small RNA fraction back into HeLa cells. (*$p < 0.002$ compared to At3-Q72-GFP with *dicer* siRNA).

(Figures S1C and S1D). Reduction of Dicer had no effect on cells expressing a control protein (GFP) or on cells expressing wild-type Ataxin-3 (At3-Q29-GFP) over the time course of the studies (Figures 2A and 2B). This is similar to previous studies showing that reduction of Dicer in vertebrate cells in culture has minimal effects over short time frames (Fukagawa et al., 2004).

We then examined the effect of reducing Dicer activity in the presence of pathogenic Ataxin-3. These studies revealed dramatically enhanced cell loss due to pathogenic Ataxin-3 when Dicer activity is reduced: whereas normally, after 24 hr, there was only a modest effect of the pathogenic protein on cell viability, with loss of *dicer*, 70%–80% of the cells were dead (Figures 2C–2E). The enhancement was specific to *dicer* siRNA treatment, as treatment with a control siRNA of random sequence had no effect (Figure 2E). We confirmed that reduction of Dicer had no effect on expression level of the transgenes or Ataxin-3 protein (Figure S1E). Moreover, the enhanced cell loss was mitigated upon transfecting cells with the purified small RNA fraction that contains total HeLa cell miRNAs (Figure 2F). Taken together, these studies demonstrated a striking effect of compromising miRNA processing on cell loss induced by pathogenic polyQ protein in *Drosophila* in vivo and in human cells.

The *ban* miRNA Suppresses SCA3 Degeneration

In parallel to these studies, we performed an overexpression EP screen for modifiers of the degenerative eye phenotype of flies expressing pathogenic human Ataxin-3 protein. We then tested if any of these modifiers also affected other biological processes, to define additional activities of the modifier genes. Among the identified modifiers, several also affected Hid-induced programmed cell death. Genetic analysis indicated that five of these modifiers mapped to the same genomic region; subsequent molecular analysis revealed these to be upregulation alleles of the miRNA *ban* (Figure 3A).

Whereas, normally, strong expression of pathogenic SCA3tr-Q78 protein induces severe degeneration, upregulation of *ban* strikingly suppressed external pigmentation loss and restored internal retinal structure (Figures 3B–3D). Remobilization of the EP elements from the *ban* gene to generate precise excision ban^{ex} alleles reverted the suppression (Figure 3E). We also determined if various *UAS-ban* transgenic lines (Brennecke et al., 2003) had the ability to modulate polyQ toxicity. Expression of a construct bearing only a 100 bp region containing the *ban* miRNA suppressed degeneration, restoring external and internal eye structures toward normal (*UAS-banD*, Figure 3F). However, a control UAS construct, driving a 6.7 kb genomic fragment that encompassed the *ban* region but lacked the predicted *ban* miRNA hairpin, did not suppress (*UAS-banB*, Figure 3G). Further control studies confirmed that *ban* had no effect on the level of *UAS-transgene* expression from the GAL4-UAS system (Figure 4I). Taken together, these data indicate that upregulation of the *ban* miRNA suppresses neurodegeneration induced by pathogenic SCA3tr-Q78.

The *ban* miRNA functions in growth as well as programmed cell death (Brennecke et al., 2003). We noted that *ban* upregulation not only suppressed polyQ toxicity but also caused a large eye phenotype (Figure 3D). To rule out the possibility that simply enlarging the eye can suppress polyQ degeneration, we tested other genes that enlarge the eye. These genes had no effect on SCA3tr-Q78-induced neurodegeneration (Figure S3), indicating that suppression of polyQ degeneration was specific to *ban*.

To further rule out developmental effects of *ban*, we examined the ability of *ban* to suppress neurodegeneration in the adult. To do this, we used a *rhodopsin1* promoter GAL4 line, which directs expression to photoreceptor neurons (PR), initiating at terminal stages of retinal formation. When expressed in this manner, upregulation of *ban* alone had no effect on eye structure, cell

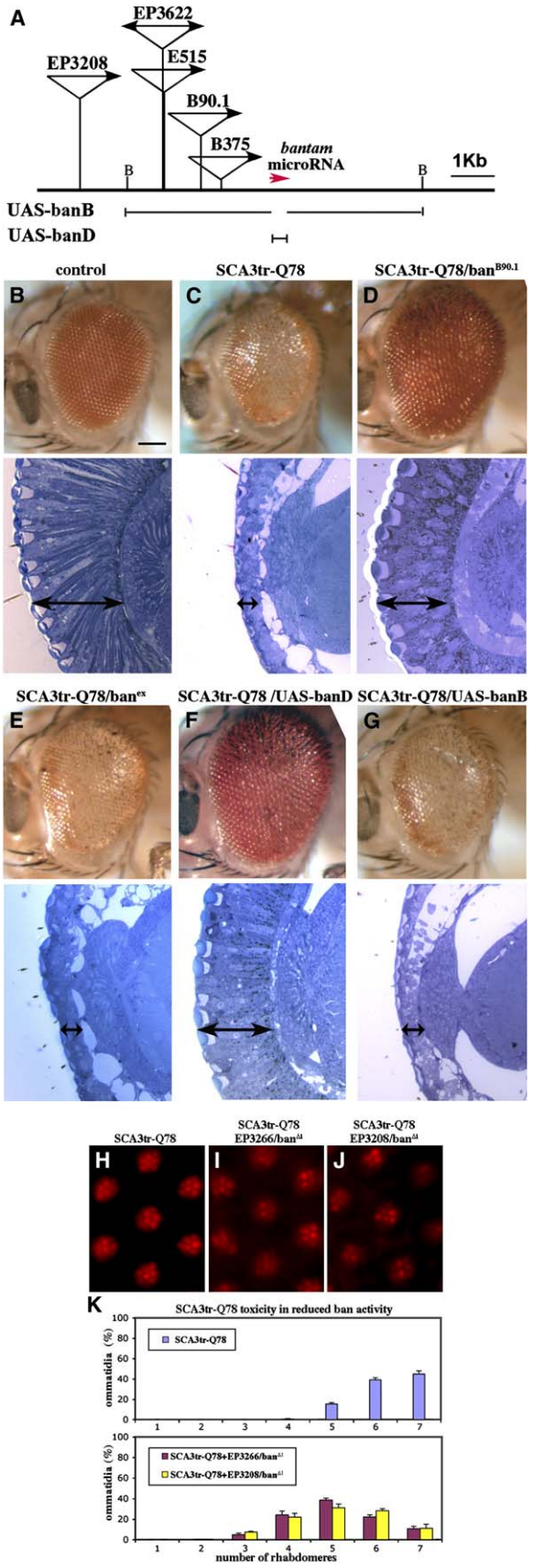

Figure 3. The miRNA *ban* Modulates Neurodegeneration Induced by Pathogenic Ataxin-3

proliferation, or growth but still strikingly mitigated SCA3-induced neural degeneration (Figure S4). These data indicate that *ban* is a potent suppressor of polyQ neurodegeneration in adult stages.

We then tested whether *ban* was effective at mitigating pathogenicity of other proteins associated with human neurodegenerative disease. These studies revealed that *ban* mitigated degeneration induced by not only truncated but also full-length, pathogenic forms of Ataxin-3 (Figure S5). Moreover, upregulation of *ban* suppressed tau-induced degeneration (Figure S5). These findings indicate that *ban* mitigates neurotoxicity induced by multiple proteins associated with human disease, including pathogenic Ataxin-3 and tau.

Loss of *ban* Enhances SCA3-Associated Neurodegeneration

These studies raised the question whether the *ban* miRNA normally plays a role in pathological conditions and therefore could be one of the miRNAs that functions to protect against polyQ toxicity, which is affected by reduction of *dcr-1* and *R3D1* gene function. Because complete loss of function of *ban* is lethal, we reduced *ban* activity by placing hypomorphic alleles in *trans* to a *ban* deletion line (ban^{EP3208} or ban^{EP3622}, in *trans* to $ban^{\Delta 1}$). Whereas these alleles upregulate *ban* activity in the presence of a GAL4 driver line, in the absence of GAL4, these alleles decrease endogenous *ban* function (Hipfner et al., 2002). We then analyzed retinal degeneration of flies expressing pathogenic SCA3tr-Q78 protein directly by the *glass multiple reporter* (*gmr*) promoter, rather than using the GAL4-UAS system. Flies bearing *gmr-SCA3tr-Q78* were born with a slightly degenerate internal retinal structure (Figures 3H and 3K). Reduction of *ban* activity enhanced SCA3tr-Q78-induced degeneration, such that flies were now born with more severe neuronal loss (Figures 3I–3K). These findings indicated that not only does upregulation of *ban* activity mitigate

(A) Genomic region of *ban* with insertion sites of EP alleles and *ban* transgenes noted. *UAS-banB* and *UAS-banD* from Brennecke et al. (2003). B, BamH1 site.

(B–G) Suppression of polyQ toxicity by upregulation of *ban*. External eyes (top) and internal retinal sections (bottom) of 1 day flies. (B) Control fly bearing only the driver line. (C) SCA3tr-Q78 induces severe retinal degeneration with loss of pigmentation and collapse of retinal structure. (D) Coexpression of $ban^{B90.1}$ with SCA3tr-Q78 gives normal pigmentation and improved retinal structure. (E) Reversion of EP element reverts suppression, such that flies now show severe degeneration. (F) Coexpression of *UAS-banD*, a transgene bearing the *ban* miRNA (Brennecke et al., 2003), improves retinal structure. (G) Coexpression of *UAS-banB*, a genomic fragment encompassing the region, but deleted for the *ban* miRNA (Brennecke et al., 2003), gives severe degeneration. Genotypes: (B) *gmr-gal4/+*, (C) *gmr-GAL4 UAS-SCA3tr-Q78/+*, and *gmr-GAL4 UAS-SCA3tr-Q78* in *trans* to (D) $ban^{B90.1}$, (E) ban^{ex}, (F) *UAS-banD*, or (G) *UAS-banB*.

(H–K) Loss of *ban* function enhances Ataxin-3 degeneration. (H) Normally, flies expressing *gmr-SCA3tr-Q78* have mild degeneration by pseudopupil assay. (I and J) With reduction of endogenous *ban*, neuronal loss is enhanced. Genotypes: *gmr-SCA3tr-Q78* in (H) a normal background, with (I) $ban^{EP3266}/ban^{\Delta 1}$ or (J) $ban^{EP3208}/ban^{\Delta 1}$. (K) PR quantitation of flies expressing SCA3tr-Q78 with normal (top, average 6.3 ± 0.1 PR) or reduced levels of endogenous *ban* (bottom, average 5.1 ± 0.3 PR for both genotypes, values significantly different from control, $p < 0.001$). Mean ± SEM (n = 3 independent experiments).

Bar in (B), 100 μm for eyes in (B)–(G).

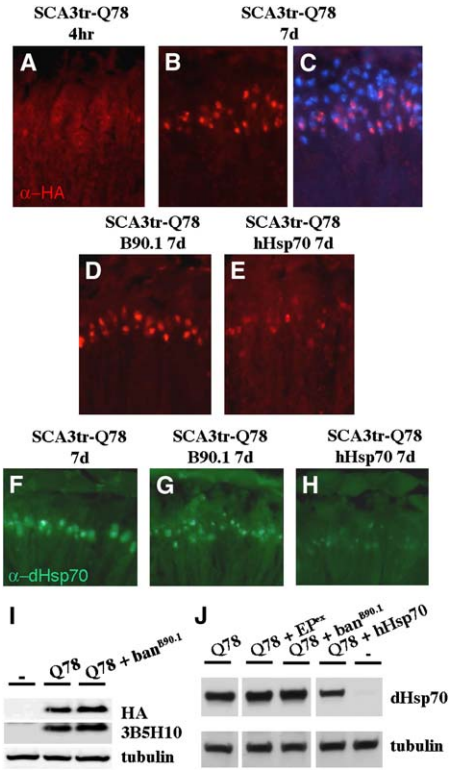

Figure 4. *ban* Has No Effect on Protein Level or Endogenous Stress Response

Immunostaining of the retina for polyQ protein (A–E, anti-HA, red) or stress-induced Hsp70 (F–H, anti-Hsp70, green) of (A) 4 hr and (B–E and F–H) 7 day flies expressing SCA3tr-Q78 in the adult. (A) Control flies expressing SCAtr-Q78 at 4 hr after eclosion of the adult show diffuse protein. (B and C) By 7 days, the pathogenic protein has accumulated in NI ([C], overlay of protein and Hoechst [blue] for nuclei). (D) Coexpression of $ban^{B90.1}$ has no effect on NI. ImageJ analysis confirmed no significant effect on size of inclusions (1.0 ± 0.1 arbitrary units for control; 1.0 ± 0.1 for *ban* allele) or intensity of immunostaining (1.00 ± 0.02 arbitrary units for control; 1.00 ± 0.12 for *ban* allele). (E) Coexpression of human *hsp70* reduces accumulation, such that fewer and more weakly immunostained inclusions are seen (ImageJ indicates a decrease to ~60% of control). (F–H) Immunostaining for stress-induced Hsp70 in 7 day flies expressing SCA3tr-Q78 alone, with *ban*, or with human Hsp70. Flies of genotype *UAS-SCA3tr-Q78/+*; *rh1-GAL4/+* in *trans* to +, $ban^{B90.1}$, or *UAS-human-Hsp70*. (I) Western immunoblot of control flies, flies expressing pathogenic protein alone (Q78), and flies coexpressing pathogenic protein with *ban*. Anti-HA is the total level of pathogenic protein, 3B5H10 is sensitive to the level of protein in a toxic conformation (Brooks et al., 2004). Flies of genotype: *UAS-SCA3tr-Q78/+*; *rh1-GAL4/+ in trans* to + or $ban^{B90.1}$. ImageJ analysis confirmed no significant effect of *ban* on protein level. (J) Western immunoblot for stress-induced Hsp70 (dHsp70). Flies expressing pathogenic protein alone (Q78), with ban^{ex} (Q78 + ban^{ex}), or with $ban^{B90.1}$ (Q78 + $ban^{B90.1}$) show similarly increased levels of dHsp70. This indicates a minimal change in the level of endogenous stress response to disease protein with (94% ± 3%, mean ± SEM, three independent experiments) or without *ban* (set to 100%). There is no significant effect on dHsp70 with *ban,* but dHsp70 is reduced by human Hsp70 (Q78 + hHsp70) to 66% of Q78 levels.

Ataxin-3 toxicity but that endogenous *ban* activity also normally helps protect against polyQ-induced neurodegeneration. The enhancement of degeneration upon reduction of miRNA processing was more severe than with reduction of *ban* alone (Figure S6), indicating that miRNAs in addition to *ban* modulate polyQ toxicity.

The *ban* miRNA Suppresses Degeneration Downstream of polyQ Protein Toxicity

To reveal further insight into the biological activity of *ban* in polyQ suppression, we determined whether *ban* upregulation modulated the accumulation, level, or inherent toxicity of the pathogenic polyQ protein. First, we asked whether *ban* suppression was associated with a reduction in level or accumulation of the pathogenic protein into inclusions. We used the adult-onset disease model in these studies, where inclusion formation takes place over days, allowing sensitive analysis of protein level and accumulation. When expressed in this manner, the pathogenic protein was initially diffuse at 1 day but then accumulated into nuclear inclusions over 7 days (Figures 4A–4C). Upregulation of *ban* did not alter the level of the pathogenic protein compared to controls, nor did *ban* affect protein accumulation into inclusions—the inclusions that formed in the presence of added *ban* were similar to those in the absence of added *ban* (Figure 4D). In contrast, the suppressor Hsp70 decreased accumulation of the pathogenic protein into inclusions (Figure 4E). Thus, *ban* suppression was not associated with a change in the level or accumulation of the pathogenic protein.

To further address *ban* function, we determined whether the cellular response to the polyQ protein, revealed by antibodies that selectively detect the stress-induced form of endogenous Hsp70 (dHsp70), was altered upon upregulation of *ban*. Both immunohistochemistry and western immunoblot analysis indicated that the endogenous stress response was not altered by *ban* (Figures 4F–4H and 4J). We also tested the reactivity of the polyQ protein to a monoclonal antibody (3B5H10) that detects pathogenic forms of polyQ protein (Brooks et al., 2004). This analysis revealed similar immunoreactivity in control flies compared to those with degeneration suppressed by *ban* (Figure 4I). These data suggest that *ban* modulates cellular pathogenicity downstream of protein accumulation and inherent protein toxicity.

As noted, *ban* upregulation suppresses Hid-induced cell death (Brennecke et al., 2003). Thus, one possibility was that *hid* was a target of *ban* in SCA3 degeneration. To test this, we generated flies not only homozygous mutant for *hid* but also expressing pathogenic polyQ. Flies lacking *hid* showed no change in neurodegeneration (Figure S7). Further testing of other proteins of apoptotic pathways (P35, DIAP1, DIAP2, and dApaf-1/Dark) showed that none of these manipulations modulated SCA3 neurodegeneration (Figure S7). We also determined that upregulation of *ban* does not modulate autophagy (Table S1), which has been implicated in cell survival in polyQ toxicity (Ravikumar et al., 2004). These data indicate that targets of *ban* or other miRNAs that modulate polyQ degeneration are distinct from *hid* and other genes tested that regulate programmed cell death pathways. Together these data suggest that *ban* activity defines distinct targets that modulate neurodegeneration.

Discussion

Our study reveals a striking role for miRNA-regulated pathways in modulation of polyQ toxicity in both flies and human cells. Notably, reduction of genes that affect

miRNA processing, but not siRNA processing, in *Drosophila* modulates polyQ degeneration, underscoring the specificity to miRNA pathways. In *Drosophila*, one of these miRNAs is *ban*, which modulates cell survival upon polyQ- and tau-induced neurodegeneration. Moreover, reduction of miRNA processing in human cells also strikingly enhanced polyQ toxicity, indicating that miRNAs also play a protective role in human cells. These data suggest that *ban* and potentially additional miRNAs are involved in mitigating polyQ- and tau-induced neurodegeneration.

miRNA Pathways Modulate polyQ and tau Neurodegeneration

Given the role of miRNAs in modulation of developmental programmed cell death, we tested whether miRNA pathways modulated neurodegeneration. We addressed this by reducing miRNA processing in flies and in human cells in the presence of pathogenic polyQ protein. In flies, loss of *dcr-1* or *R3D1* had striking effects to enhance SCA3-induced neurodegeneration. In contrast, loss of *dcr-2*, which is specific to siRNA pathways, had little effect in the situation tested. These studies indicate that miRNA-regulated activities, and not siRNA-regulated activities, are critical to neurodegeneration in vivo. In human cells, reduction of *dicer* activity also dramatically enhanced cell toxicity induced by pathogenic Ataxin-3. Although, in vertebrate cells in culture, *dicer* activity affects both miRNA- and siRNA-regulated activities, siRNA-dependent activities like heterochromatic silencing do not become disrupted until later time periods (Fukagawa et al., 2004). The effect of reducing *dicer* in human cells could be rescued in part by complementing the treated cells with a fraction containing miRNAs, indicating that the enhanced cell loss was likely due to reduction in one or more miRNAs. As miRNAs could affect many cellular processes, we confirmed that enhanced degeneration with polyQ is unlikely due to sensitizing cells to programmed cell death but rather resembles normal polyQ degeneration; identification of miRNAs and target genes will further define the pathways involved. There are hundreds of miRNAs in humans, with a subset expressed in both human brain and HeLa cells (Bentwich et al., 2005). Focus on these common miRNAs, coupled with our demonstrated ability to rescue polyQ toxicity associated with *dicer* deprivation, promises to reveal genes with a critical role in neuroprotection from polyQ-induced degeneration. We also extended our findings with *ban* and genes of the miRNA pathway beyond polyQ toxicity to modulation of tau; these findings suggest a broader role for miRNA regulated pathways in neuroprotection.

The miRNA *ban* Modulates polyQ Degeneration

A genetic modifier screen in *Drosophila* revealed that one miRNA that functions to modulate Ataxin-3 degeneration is *ban*. *ban* is a critical regulator in that both loss of activity and upregulation modulated degeneration. *ban* mitigated not only degeneration induced by polyQ protein but also by tau, an unrelated neurodegenerative disease protein. Although *ban* mitigates programmed cell death through *hid*, our studies indicate that *hid* is not involved in modulation of degeneration induced by pathogenic Ataxin-3.

Our studies suggest that *ban* modulates survival of cells to pathogenic polyQ protein downstream of protein accumulation, cellular stress response, and inherent protein toxicity. In the presence or absence of added *ban*, the pathogenic polyQ protein was present at similar levels and elicited a similar stress response. This indicates that *ban* regulates progression of degeneration downstream of these events. Taken together, these results suggest that *ban* may modulate the survival of cells. Indeed, *ban* may modulate cell survival in multiple situations: after initiation of programmed cell death, as well as in response to neurodegenerative disease proteins, including polyQ and tau. Our studies also suggest a role for additional miRNAs, due to the stronger enhancement of polyQ degeneration upon reduction of miRNA processing compared to reduction of *ban* function alone. This suggests that miRNAs in addition to *ban* in *Drosophila* likely play a role in regulating neurodegeneration. Moreover, although loss of miRNA processing results in an overall enhancement, specific miRNAs may be protective, whereas others promote degeneration. It is also possible that proteins involved in miRNA processing could themselves be targets of polyQ toxicity.

These findings expand the role of miRNA function from programmed cell death pathways, developmental processes, and cancer to suggest a striking role in protection from cellular degeneration associated with human neurodegenerative disease proteins. Further identification of the miRNAs and their targets will reveal new insight into mechanisms and therapeutics for the treatment of polyQ, tau-associated, and potentially other neurodegenerative diseases.

Experimental Procedures

Drosophila Techniques

Fly lines were grown on standard cornmeal molasses agar medium with dry yeast at 25°C. *ban* alleles $ban^{E515.1}$, $ban^{EB5\text{-}1}$, $ban^{B90.1}$, and $ban^{B375.1}$ were isolated in an overexpression screen for modifiers of SCA3tr-Q78 degeneration. Fly line *EP55* (Rorth, 1996) was crossed to a line with transposase (*w; TM6, Dr/Sb*Δ*2-3*), and progeny males crossed to *w; gmr-GAL4 UAS-SCA3tr-Q78*. Progeny with restored pigmentation were backcrossed to *w; gmr-GAL4 UAS-SCA3tr-Q78*, then suppressor EP insertion lines were isolated and balanced. *ban* excision lines ($ban^{E515.1ex}$ and $ban^{EP3622ex}$) were generated by crossing $ban^{E515.1}$ and ban^{EP3622} to flies with transposase and screening for loss of the EP w^+ marker. To test interactions with *dcr-1* and *dcr-2*, FRT clones in the eye in polyQ disease background were generated, using stocks *ey-FLP;; FRT82B CL gmr-hid CL/TM3, Sb* and *gmr-gal4 UAS-SCA3tr-Q61/CyO; FRT82B (dcr-*1^{Q1147X} *or dcr-*1^{d102}*)/TM6, Tb* and *FRT42D gmr-hid CL/CyO; ey-gal4 UAS-FLP* and *FRT42D dcr-*$2^{L811fsX}$*/CyO; gmr-gal4 UAS-SCA3tr-Q78/TM6, Tb*.

Mammalian Cell Culture

HeLa cells were maintained in DMEM with 10% fetal calf serum. For *dicer* knockdown, siRNA duplex targeting *dicer* mRNA was synthesized 5′-GCUCGAAAUCUUACGCAAAUA/UAUUUGCGUAAGAUUUCGAGC-3′ (Invitrogen, Carlsbad, CA). Scrambled siRNA sequence was 5′-CGUUACCGCGGAAUACUUCGA/UCGAAGUAUUCCGCGGUAACG-3′. Cells were transfected with 100 nM *dicer* siRNA duplex with lipofectamine 2000 (Invitrogen, Carlsbad, CA), followed 48 hr later by a second round of transfections with siRNA duplex and/or GFP or AT3-GFP or, for rescue experiments, an additional 25 nM *dicer* siRNA duplex with or without 200 ng small RNA fraction purified from HeLa cells. The number of GFP-positive cells that stained with propidium iodide 24 hr after transfection was scored to determine percentage cell loss. In each experiment, over 200 GFP-positive cells from randomly selected fields were scored.

Data are presented as the mean ± SEM of three independent experiments.

Histological and Immunochemical Analysis

Epon sections, cryosections, and paraffin sections of adult heads were performed as described (Auluck et al., 2002; Chan et al., 2000; Warrick et al., 1998). Pseudopupil analysis was performed on flies of the indicated genotypes by using the corneal optical neutralization technique (Franceschini, 1972). Twenty eyes/genotype were scored, and ten ommatidia/eye were counted for each of three independent experiments, and data are presented as mean ± SEM. For immunochemistry, primary antibodies were: rabbit anti-HA (Y-11, 1:50, Santa Cruz Biotechnology) and rat anti-Hsp70 (7FB, 1:1000; [Velazquez and Lindquist, 1984]). Primary antibodies for westerns were rat anti-Hsp70 (7FB, 1:2000), mouse anti-tubulin (E7, 1:2000, Developmental Studies Hybridoma Bank), and mouse anti-3B5H10 (1:120,000; [Brooks et al., 2004]). ImageJ analysis (National Institutes of Health, USA) was used for comparison of protein by western and immunohistochemistry (http://rsb.info.nih.gov/ij/).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, seven figures, and one table and can be found with this article online at http://www.molecule.org/cgi/content/full/24/1/157/DC1/.

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