The Innate Immune Response Transcription Factor Relish Is Necessary for Neurodegeneration in a *Drosophila* Model of Ataxia-Telangiectasia

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ABSTRACT Neurodegeneration is a hallmark of the human disease ataxia-telangiectasia (A-T) that is caused by mutation of the *A-T mutated (ATM)* gene. We have analyzed *Drosophila melanogaster ATM* mutants to determine the molecular mechanisms underlying neurodegeneration in A-T. Previously, we found that *ATM* mutants upregulate the expression of innate immune response (IIR) genes and undergo neurodegeneration in the central nervous system. Here, we present evidence that activation of the IIR is a cause of neurodegeneration in *ATM* mutants. Three lines of evidence indicate that *ATM* mutations cause neurodegeneration by activating the Nuclear Factor- κ B (NF- κ B) transcription factor Relish, a key regulator of the Immune deficiency (Imd) IIR signaling pathway. First, the level of upregulation of IIR genes, including Relish target genes, was directly correlated with the level of neurodegeneration in *ATM* mutants. Second, *Relish* mutations inhibited upregulation of IIR genes and neurodegeneration in *ATM* mutants. Third, overexpression of constitutively active Relish in glial cells activated the IIR and caused neurodegeneration. In contrast, we found that *Imd* and *Dif* mutations did not affect neurodegeneration in *ATM* mutants. *Imd* encodes an activator of Relish in the response to gram-negative bacteria, and *Dif* encodes an immune responsive NF- κ B transcription factor in the Toll signaling pathway. These data indicate that the signal that causes neurodegeneration in *ATM* mutants activates a specific NF- κ B protein and does so through an unknown activator. In summary, these findings suggest that neurodegeneration in human A-T is caused by activation of a specific NF- κ B protein in glial cells.

ATAXIA-TELANGIECTASIA (A-T) is a rare human disease characterized by progressive degeneration of Purkinje and granule neurons in the cerebellum (Sedgwick and Boder 1960; Bunday 1994; McKinnon 2004). A-T is caused by recessive mutation of the *A-T mutated (ATM)* gene, which encodes a protein kinase (Savitsky *et al.* 1995). The molecular mechanisms underlying neurodegeneration in A-T are not well understood but probably involve events that go awry in neurons and glial cells due to inadequate phosphorylation of ATM substrate proteins. ATM phosphorylates hundreds of proteins in response to DNA damage, most notably proteins that initiate cell cycle checkpoints, DNA damage repair mechanisms, and apoptotic pathways (Bakkenist and

Kastan 2003; Matsuoka *et al.* 2007; Derheimer and Kastan 2010; Bhatti *et al.* 2011).

To understand the molecular mechanisms underlying neurodegeneration in A-T, we have used *Drosophila melanogaster* as an experimental system. Since *ATM* is essential in flies, we have used a temperature-sensitive *ATM* allele (*ATM⁸*) and *ATM* RNA interference (RNAi) to conditionally inactivate *ATM* (Silva *et al.* 2004; Rimkus *et al.* 2008; Pedersen *et al.* 2010; Petersen *et al.* 2012). *ATM⁸* homozygous flies cultured at 25° are pupal lethal but when cultured at 18° they are viable (Silva *et al.* 2004). *ATM⁸* homozygous flies cultured at 18° and shifted to 25° as adults have negligible ATM kinase activity, as assayed by phosphorylation of serine 137 (pS137) in histone H2Av in response to ionizing radiation (IR)-induced DNA damage (Petersen *et al.* 2012). *ATM⁸/+* heterozygous flies cultured at 18° and shifted to 25° as adults have an intermediate level of ATM kinase activity, relative to wild-type and *ATM⁸* homozygous flies. *Repo-ATMi* flies have partially reduced *ATM* expression in glial cells due to *ATM* knockdown by RNAi. *Repo-ATMi* flies

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contain two transgenes, a target transgene that expresses an *ATM* short hairpin RNA (shRNA) under transcriptional control of GAL4 UAS sequences (*ATMi*) and a *Repo-GAL4* driver transgene that expresses the GAL4 transcription factor only in glial cells and drives expression of the target transgene (Brand and Perrimon 1993; Xiong *et al.* 1994; Sepp *et al.* 2001; Rimkus *et al.* 2008). Based on phenotype, the level of *ATM* in glial cells of *Repo-ATMi* flies is comparable to the level in *ATM* heterozygous null mutant flies (Supporting Information, Figure S1).

Our analyses of *ATM*⁸/+, *ATM*⁸, and *Repo-ATMi* flies indicate that *ATM* mutation in glial cells causes neurodegeneration (Petersen *et al.* 2012). These flies have phenotypes that typify neurodegeneration: reduced longevity, reduced climbing ability, increased death of neurons and glial cells in the brain, and vacuolar-like holes in the brain (Lessing and Bonini 2009; Petersen *et al.* 2012). Furthermore, gene expression analysis of adult heads indicates that these flies activate the innate immune response (IIR) in glial cells (Petersen *et al.* 2012). Activation of the IIR causes photoreceptor cell neurodegeneration in fly models of human retinal degeneration and Alzheimer's disease (Tan *et al.* 2008; Chinchore *et al.* 2012; Petersen and Wassarman 2012). Moreover, activation of a glial cell IIR occurs in Alzheimer's disease and Parkinson's disease and is thought to exacerbate the process of neurodegeneration (Amor *et al.* 2010). These findings suggest a causative link between activation of the IIR and neurodegeneration in A-T.

The IIR is primarily known to combat pathogens. In *Drosophila*, there are two major IIR pathways, the Toll pathway and the Imd (Immune deficiency) pathway (Lemaitre and Hoffmann 2007). These pathways activate different Nuclear Factor- κ B (NF- κ B) transcription factors to promote the expression of IIR genes. The Toll pathway responds to eukaryotic pathogens and gram-positive bacteria. Extracellular pathogens are recognized by peptidoglycan recognition proteins (PGRPs) and facilitate cleavage of the cytokine Spätzle, which binds to and activates the Toll receptor. Toll activation stimulates degradation of the Inhibitor of κ B (I κ B) protein Cactus in the cell cytoplasm, releasing the NF- κ B transcription factor Dif to allow it to enter the nucleus. In the nucleus, Dif promotes the transcription of IIR genes, including antimicrobial peptide (AMP) genes that encode secreted proteins. The Imd pathway, which is homologous to the mammalian tumor necrosis factor- α (TNF α) pathway, recognizes gram-negative bacteria through membrane-bound PGRP proteins. PGRP activation signals through the Imd protein to activate the NF- κ B protein Relish. Relish contains an N-terminal NF- κ B motif and a C-terminal I κ B motif. Activation of the Imd pathway causes Relish cleavage, releasing the NF- κ B motif from its inhibitory I κ B motif. Activated Relish translocates to the nucleus and promotes the transcription of IIR genes, including AMP genes. In support of this mechanism, overexpression of the Relish NF- κ B motif in the fat body, a major immune-responsive tissue, is sufficient to stimulate Relish target gene expression (DiAngelo

Figure 1 Schematic diagram of the countercurrent climbing apparatus. A description of the countercurrent assay is provided in *Materials and Methods*. Shaded circles indicate flies that began in vial 1. Indicated are the numbered vials that contained poor, moderate, and good climbers after the seven steps of the assay.

et al. 2009; Wiklund *et al.* 2009). Finally, NF- κ B-independent pathways activate portions of the IIR, but these pathways are primarily involved in tissue-specific constitutive expression of IIR genes rather than inducible pathogen-mediated expression (Han *et al.* 2004; Ryu *et al.* 2004; Peng *et al.* 2005; Senger *et al.* 2006; Tzou *et al.* 2010).

Here, we present studies that tested whether activation of the IIR is necessary for neurodegeneration in the brain of *ATM* mutant flies. Specifically, we tested whether the level of IIR gene expression correlates with neurodegeneration, whether components of canonical IIR pathways are required for activation of IIR gene expression and neurodegeneration, and whether activation of the IIR in glial cells is sufficient to cause neurodegeneration.

Materials and Methods

Drosophila stocks and crosses

Flies were maintained on standard molasses medium at 25° unless otherwise stated. All experiments involving *ATM*⁸ flies were performed on flies raised at 18° throughout development and transferred to 25° at 0–3 days of adulthood. The *pWiz-ATM* RNAi construct is described by Rimkus *et al.* (2008). *Repo-ATMi* flies contained a recombined chromosome with both the *Repo-GAL4* and the *pWiz-ATM*^{T4} transgenes. For *ATM*⁸ experiments with the *Rel*^{E20} and *Rel*^{E38} mutations, recombination was used to combine the mutations onto a single chromosome. *ATM*³, *ATM*⁸, *Rel*^{E20}, *Rel*^{E38}, *Imd*^{EY08573}, *Repo-GAL4*, and *UAS-Relish* flies were obtained from the Bloomington Stock Center (Bloomington, IN). *Dif*¹ and *UAS-Rel-49* flies were provided by Barry Ganetzky (University of Wisconsin, Madison), *Imd*^{SDK} and *Imd*¹⁰¹⁹¹ flies were provided by Mimi Shirasu-Hiza (Columbia University, New York), *UAS-RelD* flies were provided by Sara Cherry (University of Pennsylvania, Philadelphia), and *UAS-GFP* flies were provided by Morris Birnbaum (University of Pennsylvania).

Figure 2 Longevity correlated with climbing ability in *ATM⁸* and *Repo-ATMi* flies, and cell death in the brain correlated with climbing ability in *ATM⁸* flies. (A and B) Graphed is the longevity of (A) *ATM⁸* and (B) *Repo-ATMi* flies that were countercurrent separated into poor, moderate, and good climber groups. For *ATM⁸* flies, the analysis was performed on 409 poor climbers, 178 moderate climbers, and 122 good climbers. For *Repo-ATMi* flies, the analysis was performed on 625 poor climbers, 231 moderate climbers, and 197 good climbers. Error bars indicate standard errors of the mean. Dashed lines indicate the median survival for each group. The difference between *ATM⁸* poor and good climbers is significant ($P < 0.01$), and the difference between *Repo-ATMi* poor, moderate, and good climbers is significant for all comparisons ($P < 0.01$). (C) Graphed is the number of CaspAct-positive cells counted in the brains of *ATM⁸* flies that were countercurrent separated into poor or good climbers. Flies were assayed either 0 or 4 days after counter-current separation. Each circle represents a single brain. Horizontal lines indicate the average. * $P < 0.05$ based on one-way ANOVA analysis.

Countercurrent climbing assay

The countercurrent apparatus is diagrammed in Figure 1 and was based on an apparatus developed by Benzer (1967). The apparatus consisted of two sets of four fly vials that were taped together. One hundred flies were loaded into the first vial of the bottom set, with the other set inverted and staggered on top of this vial. Flies were tapped to the bottom of the vial and allowed to climb for 1 min, after which time the top set of vials was shifted over one vial. Flies that were able to climb into the top vial were tapped to the bottom, resulting in flies in the first and second bottom vials. This process was repeated seven times. Vials that were not engaged with another vial were plugged with cotton to prevent flies from escaping. Flies in vials 1, 2–4, and 5–8 were designated poor, moderate, and good climbers, respectively.

Western blot analysis

ATM⁸ and *w¹¹¹⁸* adult flies were collected at 18° and shifted to 25° for 3 days. After 3 days, *ATM⁸* flies were subjected to countercurrent separation. *w¹¹¹⁸* flies were not separated because very few were poor or moderate climbers. IR exposure and Western blot analysis were performed as described in Petersen *et al.* (2012).

Neurodegeneration assays

The longevity, cell death, and brain morphology assays were performed as described in Petersen *et al.* (2012). Statistical

analysis for the longevity assay was either log-rank analysis via the chi-square statistic (Figure 2, A and B) or one-way ANOVA with Bonferroni posttest (Figure 4). Statistical analyses of the cell death data were performed using one-way ANOVA with Bonferroni posttest, except for Figure 2C, which was analyzed using Student's *t*-test. Statistical analyses were performed using Prism software (Graphpad).

RNA isolation and qPCR

RNA isolation and qPCR was performed according to Petersen *et al.* (2012). Sequences of primer sets for *Cactus*, *Relish*, and *Dif* are provided in Table S1. Sequences of other primer sets are provided in Petersen *et al.* (2012). Statistical analyses of the qPCR data were performed using one-way ANOVA with Bonferroni posttest.

Results

Climbing ability correlates with neurodegeneration in *ATM* mutant flies

Previously, we found that *ATM⁸* and *Repo-ATMi* flies are heterogeneous in their ability to climb (Petersen *et al.* 2012). When tapped to the bottom of a vial, flies normally respond by climbing to the top, a behavior called negative geotaxis. About 50% of *ATM⁸* or *Repo-ATMi* flies have impaired climbing ability and are unable to climb beyond the bottom quarter of a vial within 30 sec of being tapped to the bottom. In contrast, ~15% of *ATM⁸* or *Repo-ATMi* flies have

Figure 3 The expression of IIR genes correlated with climbing ability in *ATM⁸* and *Repo-ATMi* flies. (A) Western blot analysis for H2Av-pS137 in adult head extracts from control *w¹¹¹⁸* (lanes 1 and 2) and *ATM⁸* (lanes 3–8) flies exposed (+) or not exposed (–) to IR. *w¹¹¹⁸* flies were not separated by the countercurrent assay, and *ATM⁸* flies were separated by the countercurrent assay into poor, moderate, and good climbers. As a loading control, the same membrane was probed for α-tubulin (bottom). (B–E) Graphed is qPCR analysis depicting the fold change in expression of the indicated genes in (B and C) *ATM⁸* flies relative to unseparated control *w¹¹¹⁸* flies or in (D and E) *Repo-ATMi* flies relative to unseparated control *Repo-GAL4* flies. Note that the scale is different for each panel. Error bars indicate standard errors of the mean. Asterisks above a bar indicate a significant difference relative to the control and asterisks above a line that spans poor and good climber bars indicate a significant difference between poor and good climber groups. **P* < 0.05, ***P* < 0.01 based on one-way ANOVA analysis.

normal climbing ability and are able to climb to the top quarter of a vial within 30 sec of being tapped to the bottom. We reasoned that if climbing ability is a reflection of neurodegeneration, then the variability in climbing ability among *ATM⁸* and *Repo-ATMi* flies provides an opportunity to identify the molecular causes of neurodegeneration.

To determine whether climbing ability is a reflection of neurodegeneration, we used a countercurrent assay to separate flies based on climbing ability (Benzer 1967). Flies were separated into three groups: poor climbers were unable to climb beyond the top of a vial in any of four 1-min trials (vial 1 in Figure 1), moderate climbers were able to climb beyond the top of a vial between one and three times in four to seven 1-min trials (vials 2–4), and good climbers were able to climb beyond the top of a vial four times in seven 1-min trials (vials 5–8). For *ATM⁸* and *Repo-ATMi* flies, 55% and 70% were poor climbers, 30% and 16% were moderate climbers, and 15% and 14% were good climbers, respectively. In contrast, for control *w¹¹¹⁸* flies, 13% were either poor or moderate climbers and 87% were good climbers. Since the percentage of flies in the poor, moderate, and good climber groups was similar to the percentage of

flies in the bottom, middle, and top of the vial in the 30-sec climbing assay, respectively, we concluded that the counter-current method accurately separates flies based on climbing ability (Petersen *et al.* 2012).

To determine whether differences in climbing ability are due to differences in the severity of neurodegeneration, we subjected counter-current-separated flies to two assays for neurodegeneration. First, flies were assayed for longevity. Commonly, neurodegeneration shortens the longevity of flies (Lessing and Bonini 2009). For both *ATM⁸* and *Repo-ATMi* flies, poor climbers had significantly shorter longevity than good climbers (*P* < 0.01) and moderate climbers had intermediate longevity (Figure 2, A and B). Second, *ATM⁸* flies were assayed for cell death in the brain. To identify cells undergoing apoptosis, brains were stained with an antibody to the activated form of Caspase-3 (Casp^{Act}) and analyzed by immunofluorescence microscopy (Kamada *et al.* 2005; Fan and Bergmann 2010; Petersen *et al.* 2012). When *ATM⁸* flies were assayed immediately after counter-current separation, poor climbers relative to good climbers exhibited a significantly higher average number of Casp^{Act}-positive cells (24.0 ± 5.0 and 12.6 ± 2.6 cells per brain, respectively

Table 1 Effects of IIR gene mutations on gene expression

	<i>w¹¹¹⁸</i>	<i>Rel^{E20}</i>	<i>Rel^{E38}</i>	<i>Dif^l</i>	<i>Imd^{EY08573}</i>	<i>Imd^{SDK}</i>	<i>Imd¹⁰¹⁹¹</i>
WT							
<i>AttC</i>	1.0 ± 0.2	0.7 ± 0.1	40.0 ± 12.0*	4.5 ± 1.2	0.3 ± 0.4	2.8 ± 0.4	0.7 ± 0.2
<i>CecA1</i>	1.0 ± 0.2	0.5 ± 0.2	0.4 ± 0.4	1.3 ± 0.6	0.5 ± 0.2	1.1 ± 0.2	0.2 ± 0.01**
<i>DiptB</i>	1.0 ± 0.4	0.4 ± 0.1	1.1 ± 0.3	2.0 ± 0.3*	0.6 ± 0.1	1.4 ± 0.1	0.1 ± 0.02**
<i>Mtk</i>	1.0 ± 0.2	0.9 ± 0.4	12.8 ± 2.8*	1.4 ± 0.4	1.1 ± 0.2	2.8 ± 0.5	0.8 ± 0.1
<i>PGRP-SC2</i>	1.0 ± 0.3	0.8 ± 0.2	0.5 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	0.4 ± 0.1**	0.2 ± 0.1**
<i>ATM^{8/+}</i>							
<i>AttC</i>	39.4 ± 13.1	1.7 ± 0.6**	3.6 ± 0.9**	223.1 ± 43.6*	3.0 ± 0.9**	7.4 ± 1.5**	12.0 ± 4.2**
<i>CecA1</i>	36.0 ± 13.5	0.8 ± 0.3**	0.1 ± 0.1**	158.3 ± 38.0*	1.46 ± 0.4**	2.3 ± 0.7**	8.7 ± 3.0**
<i>DiptB</i>	11.5 ± 2.7	1.0 ± 0.2**	1.5 ± 0.5**	54.7 ± 10.3*	1.2 ± 0.3**	2.5 ± 0.9**	6.8 ± 1.8
<i>Mtk</i>	50.9 ± 10.1	1.1 ± 0.4**	9.7 ± 1.7**	88.0 ± 26.1*	10.1 ± 2.1**	4.1 ± 1.3**	24.7 ± 5.0**
<i>PGRP-SC2</i>	1.3 ± 0.4	1.0 ± 0.2	0.4 ± 0.1	8.6 ± 4.3*	0.7 ± 0.3	0.4 ± 0.04	0.6 ± 0.2
<i>ATM⁸</i>							
<i>AttC</i>	156.5 ± 48.0	0.6 ± 0.2**	4.8 ± 1.7**	161.5 ± 59.7	149.7 ± 11.0	115.8 ± 29.7	189.5 ± 37.9
<i>CecA1</i>	65.4 ± 19.4	0.2 ± 0.1**	0.1 ± 0.01**	109.5 ± 29.4	141.3 ± 30.1	127.2 ± 58.6	45.0 ± 5.6
<i>DiptB</i>	45.5 ± 13.1	0.7 ± 0.3**	0.5 ± 0.1**	29.1 ± 8.7	41.8 ± 8.9	39.8 ± 7.4	51.8 ± 11.3
<i>Mtk</i>	134.9 ± 31.7	0.8 ± 0.4**	11.1 ± 3.2**	60.6 ± 11.6	132.3 ± 27.0	53.6 ± 6.9	134.2 ± 20.4
<i>PGRP-SC2</i>	5.7 ± 2.1	0.5 ± 0.1**	0.4 ± 0.04**	7.4 ± 3.0	20.0 ± 4.6	8.0 ± 3.4	4.1 ± 1.6

* $P < 0.05$, significant increase in expression relative to the control; ** $P < 0.05$, significant reduction in expression relative to the control. All values were normalized to the expression level in *w¹¹¹⁸* flies.

($P = 0.04$)) (Figure 2C). However, when *ATM⁸* flies were assayed 4 days after countercurrent separation, the number of Casp^{Act}-positive cells was not significantly different between poor and good climbers ($P = 0.67$). Thus, immediately after separation, climbing ability directly correlated with cell death, but this difference was lost over time. In summary, the longevity and cell death data indicate that climbing ability reflects the severity of neurodegeneration: poor-climbing flies have more neurodegeneration than good-climbing flies.

Climbing ability correlates with the expression of IIR genes in *ATM* mutant flies

The variability in neurodegeneration among *ATM⁸* and *Repo-ATMi* flies could be due to differences in ATM kinase activity. To test this possibility, we analyzed the ability of flies to carry out ATM-mediated phosphorylation of H2Av in response to IR (Petersen *et al.* 2012). *ATM⁸* flies that were separated by climbing ability and control *w¹¹¹⁸* flies that were not separated were exposed to 50 Gy of IR and Western blot analysis was used to detect H2Av-pS137 in head extracts. As expected, no H2Av-pS137 was detected in flies not exposed to IR, and a high level of H2Av-pS137 was detected in *w¹¹¹⁸* flies exposed to IR (Figure 3A). In contrast, irrespective of climbing ability, H2Av-pS137 was not detected in *ATM⁸* flies exposed to IR. These data indicate that the variability in neurodegeneration is not due to differences in ATM kinase activity.

Alternatively, our prior studies indicated that the variability in neurodegeneration among *ATM⁸* and *Repo-ATMi* flies could be due to differences in the expression of IIR genes. To test this possibility, we used quantitative real-time PCR (qPCR) to determine the level of IIR gene expression in the heads of countercurrent-separated flies. Genes that were

examined included AMPs [*Attacin C* (*AttC*), *Cecropin A1* (*CecA1*), *Diptericin B* (*DiptB*), and *Metchnikowin* (*Mtk*)] and *PGRP-SC2* that are targets of both the Imd and the Toll pathways, *Relish* that is specific to the Imd pathway, and *Cactus* and *Dif* that are specific to the Toll pathway (Ten *et al.* 1992; Sun *et al.* 1993; Lemaitre and Hoffmann 2007). For *ATM⁸* flies, *AttC*, *CecA1*, *DiptB*, *Mtk*, *PGRP-SC2*, and *Relish* were upregulated in all of the separated groups (except for good-climbing flies in the case of *PGRP-SC2*), but *Cactus* and *Dif* were not upregulated in any of the separated groups (Figure 3, B and C). These data suggest that loss of ATM kinase activity upregulates the Imd pathway but not the Toll pathway. Furthermore, poor climbers had significantly higher AMP gene expression than good climbers and moderate climbers had an intermediate level of expression (Figure 3B). Thus, the level of AMP gene expression directly correlates with the severity of neurodegeneration in *ATM⁸* flies. Similar trends were observed for *Repo-ATMi* flies, but only *CecA1*, *DiptB*, and *Relish* had significantly higher expression in poor climbers relative to good climbers (Figure 3, D and E). Taken together, these data indicate that the variability in neurodegeneration could be due to differences in the expression of Imd IIR pathway genes in glial cells.

Relish upregulates the expression of IIR genes in *ATM⁸* and *ATM^{8/+}* flies

To determine whether the Imd pathway is necessary for activation of the IIR, we examined the expression of IIR genes in *ATM^{8/+}* and *ATM⁸* flies that carried mutations in *Relish* (*Rel*) or *Imd*. The *Rel^{E20}* and *Rel^{E38}* alleles result from imprecise excision of a *P* element (Hedengren *et al.* 1999). Both alleles eliminate the *Relish* translation start codon, suggesting that they are null alleles. The *Imd^{EY08573}* allele

Figure 4 *Relish* mutations suppressed the longevity defect of *ATM⁸* flies. (A and B) Graphed is the median survival in days of 200 flies of the indicated genotype in (A) a wild-type or (B) an *ATM⁸* background. Note that the scale is different for A and B. Complete longevity graphs are presented in Figure S2. For statistical analysis of A, mutant flies were compared to control *w¹¹¹⁸* flies, and for statistical analysis of B, mutant flies were compared to control *ATM⁸* flies. ***P* < 0.01, based on one-way ANOVA analysis.

contains a *P*-element insertion within the 5'-UTR, and the *Imd¹⁰¹⁹¹* and *Imd^{SDK}* alleles have unknown lesions. However, all three *Imd* alleles inhibit activation of the IIR by gram-negative bacteria (Taylor and Kimbrell 2007; Costa *et al.* 2009). To determine whether effects on the expression of IIR genes are specific to the *Imd* pathway, we examined *ATM⁸/+* and *ATM⁸* flies that carried a mutation in the Toll pathway gene *Dif*. The *Dif^f* allele contains a missense mutation that is thought to inhibit the ability of Dif to interact with DNA (Rutschmann *et al.* 2000).

qPCR analysis of RNA isolated from fly heads revealed that, relative to control *w¹¹¹⁸* flies, both *ATM⁸/+* and *ATM⁸* flies had significantly higher expression of IIR genes, with *ATM⁸/+* flies having an intermediate level of expression (Table 1). This is consistent with the intermediate level of ATM kinase activity in *ATM⁸/+* flies relative to *w¹¹¹⁸* and *ATM⁸* flies (Petersen *et al.* 2012). The *Dif^f* mutation significantly increased the expression of IIR genes in *ATM⁸/+* flies but had no effect in *ATM⁸* flies, and the *Imd^{EY08573}*, *Imd^{SDK}*, and *Imd¹⁰¹⁹¹* mutations significantly reduced the expression of IIR genes in *ATM⁸/+* flies but had no effect in *ATM⁸* flies. At this point, it is unclear why *Dif* and *Imd* mutations affected the expression of IIR genes in the context of intermediate but

Figure 5 *Relish* mutation reduced cell death in the brain of *ATM⁸* flies. (A–C) Graphed is the number of Casp^{Act}-positive cells in the brains of flies of the indicated genotype in (A) wild-type, (B) *ATM⁸/+*, or (C) *ATM⁸* background. Note that the scale is different for each panel. Each circle represents a single brain. Horizontal lines indicate the average. For statistical analysis of A, mutant flies were compared to control *w¹¹¹⁸* flies. For statistical analysis of B, mutant flies were compared to control *ATM⁸/+* flies. For statistical analysis of C, mutant flies were compared to control *ATM⁸* flies. ***P* < 0.01, based on one-way ANOVA analysis.

Table 2 Effects of Relish overexpression in glial cells or neurons on gene expression

	Repo-GAL4				Elav-GAL4	
	UAS-GFP	UAS-Relish	UAS-Rel-49	UAS-RelD	UAS-GFP	UAS-RelD
<i>Attc</i>	1.0 ± 0.4	1.1 ± 0.5	0.5 ± 0.2	84.1 ± 32.3*	1.0 ± 0.3	1.1 ± 0.5
<i>CecA1</i>	1.0 ± 0.3	0.6 ± 0.1	1.0 ± 0.3	442.4 ± 70.7*	1.0 ± 0.5	0.6 ± 0.3
<i>DiptB</i>	1.0 ± 0.3	0.2 ± 0.1	0.2 ± 0.1	31.5 ± 7.9*	1.0 ± 0.3	0.4 ± 0.1
<i>Mtk</i>	1.0 ± 0.3	0.7 ± 0.3	0.7 ± 0.1	70.1 ± 19.1*	1.0 ± 0.2	1.9 ± 0.6
<i>Rel</i>	1.0 ± 0.1	8.2 ± 1.7**	0.9 ± 0.2	20.8 ± 4.8**	1.0 ± 0.1	4.5 ± 1.8**

Values were normalized to UAS-GFP expression for each GAL4 construct. * $P < 0.01$, significant increase compared to Repo-GAL4,UAS-GFP and Elav-GAL4,UAS-RelD; ** $P < 0.05$, significant increase compared to the corresponding UAS-GFP control.

not negligible ATM kinase activity. In contrast, *Relish* mutations significantly reduced the expression of IIR genes in both *ATM*^{8/+} and *ATM*⁸ flies. For almost all of the IIR genes tested, the *Rel*^{E20} and *Rel*^{E38} mutations reduced expression to near normal levels. These data indicate that Relish is necessary for transcription upregulation of IIR genes in *ATM* mutants.

Relish activation causes neurodegeneration in *ATM*^{8/+} and *ATM*⁸ flies

To determine whether Relish is necessary for neurodegeneration in *ATM* mutant flies, we examined the effect of *Relish* mutations on longevity and cell death in the brain. In the longevity assay, the number of days that it took for 50% of the flies to die (i.e., median survival) was used as a measure of longevity. The longevity of *ATM*⁸ flies was not affected by *Dif* or *Imd* mutations, but it was significantly increased from 16.1 to 24.3 or 24.9 days by the *Rel*^{E20} and *Rel*^{E38} mutations, respectively (Figure 4). The complete longevity graphs revealed that *Relish* mutations delayed the onset of death in *ATM*⁸ flies, suggesting that Relish functions to initiate neurodegeneration (Figure S2). These data indicate that Relish is necessary for neurodegeneration in *ATM*⁸ flies.

In the cell death assay, the number of Casp^{Act}-positive cells per adult brain was used as a measure of cell death. *Rel*, *Dif*, and *Imd* mutant flies had a similar level of cell death to that of control *w*¹¹¹⁸ flies (Figure 5A). However, *ATM*^{8/+} and *ATM*⁸ flies had a significantly higher level of cell death than control flies, with *ATM*^{8/+} flies having an intermediate level (Figure 5, B and C). The *Dif*¹, *Imd*^{EY08573}, *Imd*^{SDK}, and *Imd*¹⁰¹⁹¹ mutations did not affect the level of cell death in *ATM*^{8/+} and *ATM*⁸ flies. In contrast, the *Rel*^{E20} and *Rel*^{E38} mutations significantly reduced the level of cell death in *ATM*^{8/+} and *ATM*⁸ flies ($P < 0.01$). These data indicate that Relish is necessary for neurodegeneration in *ATM*^{8/+} and *ATM*⁸ flies.

Expression of constitutively active Relish in glial cells is sufficient to activate the IIR and to cause neurodegeneration

The data presented thus far indicate that Relish activity in glial cells is required to activate the IIR and cause neurodegeneration in *ATM* mutant flies. To determine whether Relish activation in glial cells is sufficient to produce these outcomes, we characterized *Repo-GAL4,UAS-RelD* flies that overexpressed the constitutively active Relish NF-κB motif

only in glial cells (Sepp *et al.* 2001; DiAngelo *et al.* 2009). To control for effects due to protein overexpression, we characterized *Repo-GAL4,UAS-GFP* (Green fluorescent protein) flies. To control for effects due to Relish expression that are independent of Relish activation, we characterized *Repo-GAL4,UAS-Relish* and *Repo-GAL4,UAS-Rel-49* flies, which express full-length Relish and the Relish IκB domain, respectively, and do not activate the transcription of Relish target genes (Hedengren *et al.* 1999; Wiklund *et al.* 2009). To control for the cell type specificity of the effects, we used *Elav-GAL4* to drive overexpression of these UAS transgenes only in neurons (Luo *et al.* 1994; Yao and White 1994).

qPCR was used to determine the level of expression of IIR genes in the heads of flies that overexpressed Relish proteins. This analysis revealed that overexpression of constitutively active Relish, RelD, in glial cells was sufficient to activate the expression of IIR genes (Table 2). In contrast, expression of full-length Relish or the Relish IκB domain in glial cells had no effect on the expression of IIR genes. Furthermore, expression of RelD in neurons had no effect on the expression of IIR genes, indicating that glial cells are specifically competent to respond to Relish activation. These data indicate that Relish activation in glial cells is sufficient to upregulate the expression of IIR genes.

The Casp^{Act} cell death assay was used to determine the level of neurodegeneration in the heads of flies that overexpressed Relish proteins. This analysis revealed that only overexpression of constitutively active Relish, RelD, in glial cells caused a significant increase in the level of neurodegeneration (Figure 6A). In addition, RelD expression in glial cells, but not in neurons, caused vacuolar-like holes in the lamina (Figure 6, B and C). A similar phenotype was observed in *Repo-ATMi* flies (Figure 6D). Vacuolar-like holes in the adult brain also occur in *ATM*⁸ flies and are a common feature of neurodegeneration in flies (Lessing and Bonini 2009; Petersen *et al.* 2012). Thus, Relish activation in glial cells is sufficient to cause neurodegeneration.

Discussion

Insights into why neurodegeneration occurs in A-T

Our data indicate that a glial cell IIR promotes neurodegeneration in *ATM* mutant flies. Furthermore, our data indicate that the level of activation of the IIR affects the severity of neurodegeneration. Flies with the same *ATM*

Figure 6 Overexpression of a constitutively active form of Relish, RelD, in glial cells but not in neurons caused neurodegeneration. (A) Graphed is the number of Casp^{Act}-positive cells in the brains of flies of the indicated genotype. (B–D) Images of paraffin sections of the optic lobe and retina of flies of the indicated genotypes. In B, the optic lobe (OL), lamina (LA), and retina (RE) are labeled. In C and D, open arrowheads indicate vacuolar-like holes in the lamina.

mutation activated the IIR to different extents and had correspondingly different severities of neurodegeneration (Figures 1–3). This suggests that there are environmental or genetic factors that modulate the IIR in *ATM* mutant flies. Analogous factors may underlie variability in the severity of neurodegeneration in A-T patients. For example, suppressing factors may explain why some individuals with undetectable levels of ATM have mild clinical phenotypes (Lavin *et al.* 2006).

Relish activation in the process of neurodegeneration

Our data indicate that the NF- κ B transcription factor Relish is necessary for activation of the IIR and neurodegeneration in *ATM* mutant flies. Likewise, Chinchore *et al.* (2012) have also shown that Relish is necessary for activation of the IIR and neurodegeneration in a fly model of human retinal degeneration. However, in neither case is it known what factor substitutes for the pathogen to initiate the IIR or what pathway of events is required to trigger Relish activation. We found that mutation of *Imd*, a canonical activator of Relish in response to pathogens, had variable effects on the expression of IIR genes and no effect on neurodegeneration (Figures 4 and 5 and Table 1). Likewise, Chinchore *et al.* (2012) found that mutation of *Imd* had no effect on neurodegeneration. This suggests that a noncanonical pathway activates Relish to cause neurodegeneration. The unknown pathway may be specific to Relish since mutation of another NF- κ B protein Dif did not affect neurodegeneration in *ATM* mutant flies (Figures 4 and 5). This does not mean that Dif activa-

tion cannot cause neurodegeneration. In fact, Dif has been implicated in causing neurodegeneration in a fly model of Alzheimer's disease (Tan *et al.* 2008). Therefore, future efforts will focus on determining the mechanisms that regulate Relish activation in *ATM* mutant cells.

Relish transcriptional targets that cause neurodegeneration

Our data indicate that proteins encoded by transcriptional targets of Relish cause neurodegeneration in *ATM* mutant flies. Indeed, constitutive activation of Relish in glial cells was sufficient to upregulate the expression of IIR genes and to cause neurodegeneration (Table 2 and Figure 6). Proteins encoded by AMP genes are obvious candidates because their expression was highly increased in *ATM* mutant flies and correlated with the severity of neurodegeneration (Figure 3 and Table 1). AMPs are small secreted peptides that contribute to the elimination of pathogens through mechanisms that remain largely unknown. However, it is generally believed that positively charged AMPs bind negatively charged membranes of pathogens, leading to increased membrane permeability and death (Zaiou 2007). Thus, neurodegeneration may be caused by AMP-mediated disruption of neuron membranes. Arguing against a role for AMPs in neurodegeneration is our finding that neurodegeneration was not affected in *ATM*^{8/+} flies that have a *Dif* or *Imd* mutation, despite the fact that the level of AMP gene expression was significantly affected (Figure 5B and Table 1). Therefore, knowledge of the genome-wide transcriptional changes

caused by *Rel* mutations in *ATM* mutant flies may help identify gene targets that are relevant to neurodegeneration.

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Supporting Information

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The Innate Immune Response Transcription Factor Relish Is Necessary for Neurodegeneration in a *Drosophila* Model of Ataxia-Telangiectasia

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Figure S1 Graphed is the expression of IIR genes in *ATM³/+* and *Repo-ATMi* flies. Expression in *ATM³/+* flies was normalized to expression in *w¹¹¹⁸* flies and expression in *Repo-ATMi* flies was normalized to expression in *Repo-GAL4* flies. Error bars indicate standard errors of the mean.

Figure S2 Graphed is the percent survival at days post-eclosion for the indicated mutants in (A and C) a wildtype or (B and D) an *ATM⁸* background. 200 flies were analyzed for each genotype. Dotted lines indicate the median survival for each group.

Table S1 Primer sequences for qPCR

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
<i>Cactus</i>	CCGTCGAAAGCCATTTGGTCAGTC	GCTGTGGAGGATTGAACCTTGCTG
<i>Relish</i>	GGCATCATACACACCGCCAAGAAG	GTAGCTGTTTGTGGGACAACTCGC
<i>Dif</i>	CAGTTTGCTACGACCGGAGAGCTA	GAATATCCGCCAGTTGCAGAGTGC