Polyglutamine Atrophin provokes neurodegeneration in Drosophila by repressing fat

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Large alterations in transcription accompany neurodegeneration in polyglutamine (polyQ) diseases. These pathologies manifest both general polyQ toxicity and mutant protein-specific effects. In this study, we report that the fat tumour suppressor gene mediates neurodegeneration induced by the polyQ protein Atrophin. We have monitored early transcriptional alterations in a Drosophila model of Dentatorubral-pallidoluysian Atrophy and found that polyQ Atrophins downregulate fat. Fat protects from neurodegeneration and Atrophin toxicity through the Hippo kinase cascade. Fat/Hippo signalling does not provoke neurodegeneration by stimulating overgrowth; rather, it alters the autophagic flux in photoreceptor neurons, thereby affecting cell homeostasis. Our data thus provide a crucial insight into the specific mechanism of a polyQ disease and reveal an unexpected neuroprotective role of the Fat/Hippo pathway.

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Introduction

Polyglutamine (polyQ) diseases are a family of dominantly inherited neurodegenerative diseases, caused by an expanded CAG repeat tract resulting in polyQ stretches in the encoded protein (Ross, 2002).

For some time, the leading view in the field has been that the proteins affected by polyQ expansion misfold and accumulate in large aggregates, which are toxic for neurons and lead to cell death and organism pathology (Ross, 2002). However, this model has not been able to account for the striking cell-specific degeneration of polyQ diseases. More recent hypotheses hold that toxicity of polyQ proteins would be a mix of common intrinsic polyQ properties and specific negative effects given by the protein context, which would be responsible for the differences observed among various syndromes (Williams and Paulson, 2008).

It has been suggested in particular that polyQ diseases are transcriptionopathies in which toxicity first arises from large-scale alterations of transcription. These are major effects of polyQ toxicity and different models have been proposed to explain how they may arise (Wood et al, 2000; Nucifora et al, 2001; Luthi-Carter et al, 2002; Schaffar et al, 2004; Riley and Orr, 2006). In some cases it has been demonstrated that reinstating appropriate transcription factors can revert polyQ toxicity (Nucifora et al, 2001; Taylor et al, 2003). However, in most studies, it is difficult to sort out primary from secondary effects and thus whether transcriptional deregulation causes neurodegeneration or vice versa. In late-stage transcriptional analysis it is also harder to identify gene regulations linked to specific misfunctions of the proteins affected, rather than to common polyQ toxicity.

Dentatorubral-pallidoluysian Atrophy (DRPLA) is a human polyQ disease caused by the expansion of a CAG stretch in the atrophin-1 (at-1) gene. In all vertebrates, a second atrophin gene, at-2, is present and encodes a related protein but void of polyQ tracts. Atrophins take part in several cellular processes and have been shown to function as bimodal transcriptional cofactors that are recruited to regulatory elements by a number of transcription factors (Wang et al, 2006; Shen et al, 2007).

Mouse knockouts exist for both atrophin genes, revealing that at-2 has essential roles in the development of the nervous system, somites and limbs (Zoltewicz et al, 2004; Vilhais-Neto et al, 2010), whereas at-1 is entirely redundant, possibly because a shorter form of At-2 (At-2S) may compensate for At-1 loss (Shen et al, 2007). Two different mouse models for DRPLA have been generated and both recapitulate important neurological and cytopathological features of human disease (Schilling et al, 1999; Sato et al, 2009). Major transcriptional alterations have been detected in DRPLA mice and these have also been compared with Huntington mouse models to reveal common alterations and also specific effects (Luthi-Carter et al, 2002; Sato et al, 2009).

In the fruitfly Drosophila melanogaster there is one conserved Atrophin (Atro) gene. Mutations in Atro reveal that it is required for diverse processes such as planar cell polarity and some forms of cell adhesion/cell affinities leading to defects in embryonic segmentation and leg and eye development (Erkner et al, 2002; Zhang et al, 2002; Fanto et al, 2003). Atro contains all functional domains of vertebrate atrophins, including two polyQ stretches, and is ubiquitously expressed. We have generated Drosophila models for DRPLA and described both polyQ and Atrophin-specific events that modulate cell and organism toxicity (Nisoli et al, 2010).

Because of the molecular function of Atrophins, DRPLA is a disease with a straightforward link to transcriptional...
activity. To understand to what extent transcriptional alterations cause neurodegeneration and are linked to the normal functions of Atrophin, we carried out a genome-wide transcriptional profiling in our Drosophila models, focusing on primary events that precede neurodegeneration. Our data suggest that polyQ Atro causes metabolic stress and loss of terminal differentiation markers. Importantly, polyQ Atro represses transcription of the fat tumour suppressor gene, the function of which in this system protects from degeneration and Atrophin toxicity. In fat mutants, neurons undergo progressive degeneration with autophagic hallmarks. We also show that the Hippo pathway downstream of fat is necessary for correct neuronal homeostasis and mediates autophagic degeneration by Fat and polyQ Atrophins. Thus, our data uncover a specific mechanism of toxicity of a polyQ disease and reveal for the first time an unexpected neuroprotective role of the conserved Fat/Hippo tumour suppressor pathway.

Results

An experimental design aimed at early transcriptional responses to polyQ Atro

The eye is the most accessible part of the nervous system of the fly and is dispensable for life; therefore, it has been widely used to model neurodegeneration in Drosophila. To detect the earliest possible alterations in transcription resulting from the expression of polyQ Atro, we used a robust and reliable system to control the induction of expression (McGuire et al., 2004). Atro transgenes have been expressed in all retinal cells, using the combination of the GMR–Gal4 driver with a temperature-sensitive mutant of the Gal80 repressor, expressed ubiquitously. When the flies are raised at 18°C, the transgenes are not expressed. Shifting the flies to 29°C results in Gal80 inactivation and Gal4-dependent transgene expression (Figure 1A).

Using this experimental design, expression of no transgene, wt Atro or two previously reported polyQ mutants, Atro66QC and Atro75QN (Nisoli et al., 2010), was induced. In all cases, RNA from whole heads has been isolated after 0, 2 and 14 days (d) of ageing at 29°C.

The first time point (in which flies are kept at 18°C) is a negative control for transgene expression to standardise possible differences in the genetic backgrounds. The other two time points have been selected on the basis of the extent of degeneration. After 2 days, no degeneration is detectable in the tangential eye section (Figure 1B). Minimal loss of photoreceptor neurons (~10% of the total) is present after 14 days in the retinas expressing the polyQ mutants (Figure 1B). However, if aged longer, flies expressing all forms of Atro develop more severe neurodegeneration with more dramatic vacuolisation, enhanced loss of the regular structure of the retinae, and, in the case of polyQ mutants, collapse of tissue (Supplementary Figure 1). Therefore, all Atro-overexpressing flies show signs of progressive degeneration, albeit at different speed.

Thus, this protocol focuses on early events in the neurodegenerative process and allows the characterisation of the initial transcriptional alteration, as the analysis terminates at a stage of degeneration that has often been the starting point in similar studies (Nelson et al., 2005).
Microarray analysis of transcriptional profiles and validation of results
Following Drosophila 2.0 Affymetrix arrays hybridisation and scanning, results were normalised and filtered. Data normalisation was carried out with two algorithms, RMA (Irizarry et al., 2003) and VSN (Huber et al., 2002). To focus on the impact of Atro mutations, all changes due to temperature shift and ageing, which are independent of Atro expression, were filtered out. No fold change threshold has been considered in our statistical filtering. Using this conservative protocol, 269 probe sets were called differentially expressed after 2 days in both normalisations, and 390 after 14 days. This indicates that a significant transcriptional response is set from very early on. The full list of detected alterations is shown in Supplementary Table 1.

Given the substantial agreement of both normalisation protocols, the more stringent VSN set was used for further global analysis. Most genes are downregulated by all forms of Atro, and the downregulating activity of polyQ Atro, but not of wt Atro, increases with time (Supplementary Figure 2). Within a given genotype, there are many changes over time, indicating significant progression in transcriptional responses despite marginal or no phenotypic alteration. Importantly, transcriptional response to Atro wt expression is stable between 2 and 14 days, whereas the polyQ mutants produced a more dynamic alteration, scattered in time, that suggests an intracellular conflict, resulting in opposed trends displayed over time by the same genes (Supplementary Figure 3).

Pattern clustering according to fold changes shows that the effects of the different Atro mutants branch mainly according to time points (Figure 2A and Supplementary Figure 4), matching phenotypic strength (Figure 1B and Supplementary Figure 1) and highlighting a convergent common polyQ effect. Venn diagrams (Figure 2B) illustrate that the degree of overlap between the different mutants increases.

Figure 2 Global analysis of transcript profiling. (A) Heat maps for three subsets of the pattern clustering analysis generated using the Euclidean Distance metric. Upregulated genes are in red and downregulated genes are in green. The downregulating transcriptional activity increases with time. The main branch in clustering of the different Atro mutants is along time points. See also Supplementary Figure 4. (B) Venn diagrams for the subset of genes affected at the two time points for the different Atro versions with respect to control flies. The overlap between all Atro forms and the two polyQ versions increases with time. The genes in common for all Atro versions or between the polyQ Atro mutants at both times are listed.

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with time, particularly between the two polyQ mutants. The increased convergence, however, appears to affect only the downregulated genes, whereas for upregulated genes the overlap remains constant (Supplementary Figure 5). We speculate that this may be because the downregulating activity rests on common properties of all Atro forms, whereas upregulation diverges more, perhaps because it reflects more indirect events. All these results are in agreement with the description of Atro as a co-repressor (Erkner et al., 2002; Zhang et al., 2002; Haecker et al., 2007).

Gene ontology (GO) annotation and clustering of the probe sets carried out with the most commonly used tool, the DAVID 6.7 algorithm (Huang da et al., 2009), reveals that the vast majority of clusters deal with general metabolic processes, indicating a status of metabolic stress, the effects of which on neurodegeneration could be two-fold and will require further analysis (Supplementary Table 2 and Supplementary Figure 5).

Many genes affected are involved in the specialised function of phototransduction (cluster no. 2). These clusters are significantly altered by Atro75QN from the very early stages, whereas the effect of wt Atro is more delayed (Supplementary Table 2 and Supplementary Figure 5). Because our transcriptional profiling reflects events that take place well before severe degeneration (Figure 1B), these data do not result from cell and tissue loss. Rather, they suggest loss of terminal differentiation markers in photoreceptor neurons, which may result in early functional impairment.

Two other clusters include genes important for cell cycle and mitosis (nos. 27 and 40). This could result from either an attempt at cycle re-entry by post-mitotic neurons undergoing de-differentiation or compensatory proliferation by another cell population. Some clusters (nos. 34 and 38) are linked to programmed cell death (Supplementary Table 2 and Supplementary Figure 5), which appears to be repressed at the transcriptional level, as some key genes like the caspase dream are significantly downregulated (Supplementary Table 1).

We next focused on the set of genes commonly regulated by at least two Atro forms for validation of microarray data through two different qPCR approaches. Some genes were validated using standard real-time PCR and a larger scale confirmation was sought by analysing the pattern of expression of 17 genes with the Universal Probe Library approach, which couples qPCR with probe detection. We selected a spectrum of genes displaying different patterns and significance in the statistical analysis and in most cases the result of the qPCR confirmed the trend of the microarray analysis (Supplementary Figure 6). Interestingly, because of its greater sensitivity, this analysis revealed significant deregulation after 2 days as well and by wt Atro for some genes, including fat (ft).

**ft is transcriptionally regulated by Atrophins and is a direct target of Atro**

The most intriguing single-gene alteration is the downregulation of the ft gene (Mahoney et al., 1991), a tumour suppressor that codes for a gigantic cadherin the cytoplasmic domain of which interacts with Atro to regulate planar polarity (Fanto et al., 2003). Real-time qPCR validated the microarray result for ft and further establishes the fact that wt Atro also significantly downregulates this gene, albeit at a much lower degree than the polyQ forms, which also affect this gene early on at 2 days (Figure 3A). Fat is an attractive candidate in light of the current knowledge on Atrophins. In addition, its multiple roles as a signalling molecule and cadherin link it potentially to many other processes highlighted in our analysis and make it of outstanding interest for further investigations.

The effect of wt Atro on ft suggests that its downregulation is linked to an Atrophin function, which is altered by polyQ expansion. In agreement with this hypothesis, downregulation of Atro by RNA interference leads to a significant upregulation of ft (Supplementary Figure 7). On the contrary, human Htt-exon-1, wt or with 93Q, does not affect ft transcription, indicating that this is not a common effect of polyQ proteins (Supplementary Figure 7).

Interestingly, overexpression of wt human Atrophin-1 up-regulates ft (Supplementary Figure 7). This is consistent with the description of At-1 mainly as an activator of transcription (Shen et al., 2007) and its dominant-negative effects on Atro in flies (Charroux et al., 2006). However, polyQ expansion in At-1 interferes with the At-1 effect and downregulates ft with respect to control flies (Supplementary Figure 7). This suggests that the regulation of ft transcription is due to a conserved property of Atrophins and may be relevant to a human context as well.

To address whether Atro regulates ft transcription directly we carried out chromatin immunoprecipitation, followed by qPCR analysis of different regions that putatively regulate transcription immediately upstream of ft or in its first intron, in areas reported to be bound by RNA PolIII in the modENCODE project (Celniker et al., 2009). In untreated S2 cells, we detected a mild but significant enrichment of a putative enhancer region 3.9 kb upstream of the ft transcriptional start site (Supplementary Figure 7). To confirm this result in a neuronal context and to establish its dependence on the levels of Atro, we used a clone of BG3 Drosophila neuronal cells. These cells express low levels of Atro endogenously and have been previously validated by us for the pathological response to polyQ Atro (Nisoli et al., 2010). We have stably transfected BG3 cells with exogenous Atro under inducible control and, although we were unable to select a pure clonal population, we enriched the transfected fraction with time, particularly between the two polyQ mutants. The increased convergence, however, appears to affect only the downregulated genes, whereas for upregulated genes the overlap remains constant (Supplementary Figure 5). We speculate that this may be because the downregulating activity rests on common properties of all Atro forms, whereas upregulation diverges more, perhaps because it reflects more indirect events. All these results are in agreement with the description of Atro as a co-repressor (Erkner et al., 2002; Zhang et al., 2002; Haecker et al., 2007).

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ft mediates Atro neurodegeneration and is required for neuronal homeostasis

To address the functional significance of ft downregulation for neurodegeneration induced by Atrophins, we tested genetic interactions in Drosophila between the two mutants. Two independent and molecularly characterised (Matakatsu and Blair, 2006) loss of function alleles of ft (ft and ftGrv) in heterozygosity enhance photoreceptor loss due to Atro75QN expression with Rhodopsin-1-Gal4 (Figure 3C). When wt or
polyQ Atro is expressed with GMR–Gal4, the retina collapses progressively (Nisoli et al., 2010) and this is enhanced by heterozygosis for ft^{ld} (Supplementary Figure 8). On the contrary, overexpression of an exogenous Ft construct leads to significant suppression of neuronal cell loss, retinal collapse and premature organism lethality, which arises when polyQ Atrophins are expressed in glial cells with RepoGal4 (Nisoli et al., 2010; Figure 3D and Supplementary Figure 8). These results indicate that regulation of ft levels is functionally relevant for Atrophin-mediated neurodegeneration, also outside the context of retinal neurons.

This suggests that ft itself may be required for neuronal homeostasis. Strikingly, homozygous mutant clones for ft^{ld} in the eye bring about progressive retinal degeneration when the flies are aged through our standard protocol. Although the ft mutant tissue is normal after eclosion from the pupal case, photoreceptors start degenerating in aged flies and finally the whole ft mutant tissue falls apart (Figure 4A). A quantification of the severity of the degeneration shows significant and remarkable progression with age (Figure 4B). A similar, albeit weaker, phenotype is displayed by another independent ft allele, ft^{82}, but not by a wt clone (Supplementary Figure 9). To
assess whether the temperature used (29°C) in the ageing protocol made a critical contribution to the emergence of neuronal degeneration, ftcl1 clones were aged at 25°C. At this temperature, neurodegeneration in ft clones progresses more slowly on an absolute time scale (Supplementary Figure 9). However, if the difference in lifespan at the two temperatures is taken into consideration (14 days represent 40% of the mean survival for ut flies at 29°C, whereas 21 days at 25°C represent 33.3% of mean survival), neurodegeneration due to lack of ft at 25°C is as strong as, if not stronger than, at 29°C.

Mitotic clones are generated during larval development; this raises the possibility that ft mutations may lead neurons to degenerate through an effect in development. To address whether loss of ft specifically in adult post-mitotic cells is sufficient to cause neurodegeneration, an RNAi construct against Atrophin was expressed in the adult photoreceptors with GMRGal4 and G428D2, as for the transcrip- tional profiling. Also in this case, photoreceptor cells are progressively and significantly lost (Supplementary Figure 10) despite a completely normal development of the retina. The effect of the RNAi is much weaker than that of mutant clones and does not entirely rule out a contribution of developmental abnormalities to the neurodegeneration observed in ft mutant clones. Given the weak efficacy of this RNAi line (as judged by the mild polarity phenotypes recovered when expressed in development, data not shown), this limited effect could be expected; however, this result indicates that Ft is also required specifically in adult neurons for their homeostasis.

Ft is a multifunctional protein that takes part in several cellular processes and many hypotheses could be put forward as to the mechanism through which it affects neuronal survival. A first possibility is that the Ft planar polarity pathway, in which Atro is also involved, could mediate neurodegeneration. However, genetically wt ommatidia outside the clones, the polarity of which is non-autonomously affected by neighbouring ft mutant cells, are not susceptible to degeneration (Figure 4A). In addition, at a stage of neurodegeneration in which half of ft mutant cells (not...
pigmented) are degenerating in mosaic ommatidia, the neighbouring wt cells (pigmented) are almost totally unaffected (Figure 4C). This analysis reveals that degeneration by loss of ft is strictly cell autonomous, in contrast to the ft planar polarity phenotypes (Fanto et al., 2003).

Ft is also a cadherin that can putatively affect cell adhesion, and loss of cell contacts could potentially lead to degeneration. However, electron microscopy (EM) analysis reveals that adherent junctions are preserved in cells at an advanced degeneration stage (Supplementary Figure 11), indicating that loss of cell adhesion is not driving degeneration. In addition, driving an RNAi transgene against shotgun (coding for the fly E-cadherin), specifically in the adult retina through the Gal4/Gal80ts system, leads to disorganisation of the distribution of pigment cells and orientation of the ommatidia but not to degeneration or loss of neurons (Supplementary Figure 11). Thus, it is unlikely that loss of a potential role of Ft in cell adhesion would be required for the degenerative phenotype.

The Hippo tumour suppressor pathway controls neurodegeneration

It has been shown that Ft controls tissue growth and proliferation cell autonomously through the Hippo tumour suppressor pathway, although the phenotype of ft mutants does not entirely overlap with those of the other components of the pathway (Harvey and Tapon, 2007). We analysed the possibility that the Hippo pathway mediates neurodegeneration and, in agreement with this hypothesis, homozygous mutant clones for the core components of the pathway (Tapon et al., 2002), warts (wts), salvador (sav) and hippo (hpo), display significant progressive neuronal photoreceptor degeneration (Figure 5 and Supplementary Figure 12) very similar to that of ft.

In mutants of the Hippo pathway there is also a massive overgrowth of lattice cells, which results in increased ommatidial spacing as previously described (Tapon et al., 2002). Lattice cells are also sensitive to ageing and, despite appearing intact in newly enclosed flies, they rapidly degenerate, leading to dramatic tissue collapse (Figure 5 and Supplementary Figure 12).

The Hippo cascade represses the transcriptional co-activator Yorkie (Yki; Huang et al., 2005). Overexpression of Yki in flip-on clones, which causes loss of Hippo signalling, also brings about progressive neuronal degeneration (Supplementary Figure 13), albeit at a much weaker level if compared with loss of Ft or of the hippo core genes.

To address whether Hippo signalling is required specifically in adult neurons for its neuroprotective function, as is the case for ft, we expressed Yki in adult retinae only with GMR-Gal4 and Gal80ts and detected unequivocal, albeit weak, neuronal cell loss (Figure 6A and B).

These data establish that the Hippo pathway is required for cell homeostasis specifically in adult neurons.

The Hippo pathway partially mediates neurodegeneration by ft and polyQ–Atro

Our analysis so far suggests the hypothesis that neurodegeneration by loss of ft and overexpression of Atro is therefore mediated by the Hippo pathway. It has been shown that Ft regulates the Hippo cascade through repression of the unconventional myosin Dachs, a negative regulator of the

pathway (Cho et al., 2006). Consistent with our hypothesis, mutations in dachs (d) strikingly rescue neurodegeneration due to loss of ft (Figure 4A and B). d single mutant clones remain wt (Supplementary Figure 12), as expected by the fact that Dachs is inhibited by Ft (Cho et al., 2006), and therefore its loss of function mimics Ft activation rather than loss.

Heterozygous mutations for yki also suppress the degeneration inside ft mutant clones (Figure 4A and B). Despite statistical significance, the weak rescue did not allow a complete assessment of the role of Yki in this pathway, which was also questioned by the weaker phenotypes...
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Figure 6 Uncoupling of neurodegeneration and overgrowth. (A) Regression analysis of the overgrowth versus the neurodegeneration observed in loss of function mutants (left) and overexpression of transgenics (right). Overgrowth quantification is shown in Supplementary Figure 14. For loss of function mutant, the neurodegeneration was quantified at 14 days in clones (as shown in Figures 4 and 5). For UAS-overexpression transgenics neurodegeneration was quantified at 14 days when expressed with GMRGal4; UbiGal80ts. For Cyd–Cd4, these specific data are missing and have not been plotted; however, Supplementary Figure 13 shows that these mutants do not cause neurodegeneration in a different setup in which they are expressed during both development and adult life. In both cases the regression coefficient r^2 is extremely low (0.21 and 0.03), indicating an absence of correlation between the two processes. (B) Tangential eye sections through the eyes of flies expressing with GMR–Gal4, Ubi–Gal80ts either UAS–EGFP or UAS–Yki or UAS–Yki;GMRGal4;Ubi–Gal80ts and aged 1 or 28 days. Arrows point at missing or degenerated photoreceptors. The UAS–Yki;GMRGal4;Ubi–Gal80ts is so effective that even the very low expression leaking out with this system is enough to affect development and generate mild eye roughness and overproliferation of lattice cells. Histograms showing the quantification of cell loss in eyes shown in (A). A statistically significant rescue is obtained both for the number of wt ommatidia (*P < 0.05 in two-tailed t-test) and for all other categories of ommatidia (r^2 = 0.21, P < 0.001 for 2 degrees of freedom).

experiments established that downregulation of yki dramatically suppressed the neurodegenerative phenotype due to loss of ft (Figure 4D).

As polyQ Atro overexpression results in downregulation of ft at the transcriptional level, and ft mediates part of the Atro toxicity, the Ft/Hippo pathway may also be required for polyQ Atro. Importantly, neuronal photoreceptor degeneration by Atro7SQN is significantly suppressed inside d clones (Figure 7), indicating that the d-dependent branch of the Ft pathway mediates part of the polyQ Atro degeneration.

Furthermore, mild Ga4-independent expression of wts suppresses neurodegeneration brought about by expression of Atro7SQN with Rh–Gal4 (Figure 3C), whereas heterozygosity for saw enhances it. Heterozygosity for yki or wts did not display an effect in this particular setup; however, Ga4-dependent overexpression of Wts, through an EP element insertion, or Hpo or an RNAi against yki, suppressed the degenerative phenotype caused by adult expression of Atro7SQN in the retina with GMR–Gal4 and Gal80ts

Figure 7 Neurodegeneration by polyQ Atro partially requires dachs. Tangential eye sections through d^21,13 clones (marked by the absence of yellow pigment) either in control flies or those expressing Atro7SQN with the Rhodopsin1 driver and aged 28 days. Mask panels below each section exemplify clonal boundaries and PR no. for the ommatidia. Early signs of degeneration indicated by reduced complement of photoreceptors are detected specifically in the pigmented area (arrowhead). At this stage, inside d mutant clones all ommatidia are unaffected and display a wt arrangement (arrows). (B) Histograms showing the quantification of cell loss in eyes shown in (A). A statistically significant rescue is obtained both for the number of wt ommatidia (*P < 0.05 in two-tailed t-test) and for all other categories of ommatidia (r^2 = 0.21, P < 0.001 for 2 degrees of freedom).

obtained through Yki overexpression. To further dissect the ft–yki interaction we took advantage of the MARCM technique (Lee and Luo, 2001) to express with Tub–Gal4 an RNAi construct against Yki specifically inside ft clones. These
(Figure 3D and Supplementary Figure 8). In particular the effect of Wts was dramatic and comparable to the rescue caused by Ft overexpression.

Taken together, these data establish that the Hippo pathway mediates neurodegeneration due to loss of ft and, at least partially, also the degeneration caused by polyQ Atro expression.

**Neurodegeneration is not a secondary effect of overproliferation**

The Hippo pathway is a well-known tumour suppressor pathway that controls organ size in *Drosophila* and mammals (Harvey and Tapon, 2007). It may be hypothesised that neurodegeneration could arise as a secondary consequence of excessive proliferation or as a stimulus for neurons to re-enter the cell cycle, as also suggested by a set of data in the microarray analysis. We thus set out to tackle this possible mechanism for neurodegeneration. Stimulating growth and cell cycle independently of the Hippo signalling by overexpression of CyclinD in combination with Cyclin-dependent-kinase 4 (Datar et al., 2000) did not bring about any significant cellular degeneration (Supplementary Figure 13). Likewise, mutant clones for expanded, a tumour suppressor gene linked through multiple mechanisms to the Hippo pathway (Feng and Irvine, 2007; Badouel et al., 2009) that is however outside the pathway core and does not recapitulate all hippo pathway mutant phenotypes (e.g., lattice cells excess), caused overgrowth but no discernible loss of neurons even after 21 days at 29°C (Supplementary Figure 12). These two experiments suggest that effective overgrowth during development does not necessarily result in adult neurodegeneration.

In contrast, the most complete phosphomutant Yki (Yki S111A,S168A,S250A), which cannot be inactivated by Wts (Oh and Irvine, 2009), brings about dramatic overproliferation and significant loss of adult photoreceptor neurons (Supplementary Figure 13). However, the malformation of the tissue and the altered recruitment of photoreceptors into clusters, present as a result of incorrect development, make it difficult to compare the effect of this mutant with that of the wt Yki protein. When expressed specifically in adult retina, however, Yki S111A,S168A,S250A causes significant but weak neurodegeneration, indistinguishable from the one caused by wt Yki (Figure 6B). This indicates that the function of Yki (and of the Hippo pathway) in adult neuronal homeostasis can be separated by that in proliferation and does not appear to be affected through phosphorylation of those residues known to be critical in proliferation.

This conclusion applies to all other mutants, as no correlation can be observed between the degree of overgrowth caused by several different loss of function or overexpressing mutants in the eye imaginal discs and the degree of neurodegeneration observed in the same neuroepithelium in adult stages (Figure 6A and Supplementary Figure 14).

It is in particular worth noting that Atro75QN does not cause any significant overgrowth. BrdU incorporation experiments confirm that expression of this polyQ protein drives some cells into cell cycle re-entry and DNA replication, as suggested by the microarray analysis; however, these are exclusively non-neuronal cells (Supplementary Figure 15).

In conclusion, these results indicate that overgrowth and neurodegeneration are not similarly affected and rule out excessive developmental proliferation and cell cycle re-entry as the mechanism through which Atro, Fat and the Hippo pathway affect neuronal degeneration.

**Ft and Hippo components cause neurodegeneration through defective autophagy**

We have previously reported that neurodegeneration due to polyQ–Atro arises through defective autophagy (Nisoli et al., 2010) and the Hippo pathway has been shown to affect autophagy during salivary gland degradation (Dutta and Baehrecke, 2008). Therefore, we also sought to establish the contribution of this cellular mechanism for neurodegeneration caused by loss of the Ft/Hippo signalling pathway.

EM analysis of ft mutant photoreceptors reveals autophagic vacuoles filled with unstructured partially degraded material and mitochondrial damage inside mutant photoreceptors (Figure 8A). Autophagosomes accumulate moderately but significantly with age in ft mutant neurons, in comparison with wt neurons (Figure 8B). Staining for GFP::Atg8a, a marker for autophagosomes, confirms the presence of these organelles in ft mutant cells (Figure 8C), in agreement with the EM analysis. The p62 protein, a multifunctional scaffold protein that marks ubiquitinated protein aggregates destined to autophagic degradation (Bjorkoy et al., 2005; Nezis et al., 2008), accumulates specifically inside ft mutant cells early on and increases dramatically with the progression of degeneration (Figure 8D). In addition, as already reported in the case of polyQ Atro (Nisoli et al., 2010), block of autophagy induction via mutations in *atg1* results in stronger or faster degeneration, as clones double mutant for *ft* and *atg1* are more degenerated than *ft* clones at the same age (Supplementary Figure 16) and *atg1* single mutants are wt at this stage.

Finally, EM analysis of sav mutant cells reveals a statistically significant accumulation of autophagic vesicles with partially degraded content identical to the vesicles detected in *ft* mutants (Figure 9A and B). Taken together, these data indicate that mutations in *ft* and the Hippo pathway genes deregulate autophagy in photoreceptor neurons and that autophagy is functionally relevant to neurodegeneration, as already shown for polyQ Atro (Nisoli et al., 2010).

**Discussion**

Polyglutamine diseases have been proposed to be ‘transcriptionopathies’ and transcriptional alterations are a major effect of polyQ toxicity (Riley and Orr, 2006). However, in most cases, it is difficult to sort out primary from secondary effects and thus whether transcriptional deregulation causes neurodegeneration or vice versa. We designed an experimental setup to unequivocally establish the chain of events by taking advantage of an effective inducible system of expression and analysing early time points at which no or very little degeneration is present. The mild phenotypes guarantee that detected alterations are caused neither by massive cell loss nor by severe degeneration; rather, they are early events that precede, and may give rise to, neurodegeneration.

Our results demonstrate conclusively the presence of robust transcriptional alterations from very early phases and that some of these alterations progress to later stages and are functionally relevant to neurodegeneration, like in...
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Figure 8 Autophagic modifications in ft mutants. (A) EM scan of a wt (left) and ftfd mutant ommatidium (right) of a ftfd clone after 14 days. Scale bar: 1 μm. High-magnification panels (far right, top to bottom) display autophagosomes with undigested debris, damaged mitochondria and forming phagophores (arrowheads), found in ftfd mutant cells. Scale bar: 0.2 μm for zoom-in panels. (B) Graphs of the quantification of autophagic vesicles (AV) per photoreceptor found in EM sections of 7-day (left)- and 14-day (right)-old ftfd clones. Significant accumulation of AV is found in mutant cells (not pigmented) with respect to genotypically wt (pigmented) cells. **p<0.01 and ***p<0.001 in one-tailed t-test. N=12 pigmented cells versus 20 non-pigmented cells for the 7-day graph and 13 pigmented cells versus 21 non-pigmented cells for the 14-day graph. (C) Confocal pictures of whole-mount retinae of a ftfd clone aged 7 days and expressing GFP::Atg8a ubiquitously with Tub–Gal4. Red is phalloidin marking rhabdomeres, green is GFP and the clone is marked by the absence of β-gal staining (blue). Small GFP::Atg8a dots accumulate inside ft mutant cells (arrow). (D) Confocal pictures of whole-mount retinae of a ftfd clone aged 7 days (left) and 14 days (right). Red is phalloidin marking rhabdomeres, blue is p62 and the clone is marked by the absence of GFP staining (green). p62 starts to gather in small dots specifically inside ft mutant cells (arrow) and then accumulates massively (arrow) in the ft mutant clones as many cells degenerate.

the case of ft. Although a time-course analysis cannot identify whether transcriptional changes are a result of direct Atrophin function or indirect events, the genes affected early on, also by wt Atro, are more likely to be linked to direct Atrophin transcriptional regulation, as is the case for ft, whereas genes affected only later on are more likely to be indirect responses to ongoing processes in the cell.

Global scale analysis of alterations leads to three major conclusions. First, the activity of Atro and its polyQ mutants is mostly a repressive one, in agreement with the corepressor function attributed to Atro (Erkner et al, 2002; Zhang et al, 2002; Haecker et al, 2007). Second, the polyQ effect builds a significant share of its toxicity upon an existing Atro toxicity by altering the transcription of genes affected by wt Atro as well. This confirms at a genome-wide level previous conclusions drawn from genetic and phenotypic examination (Nisoli et al, 2010) and is in agreement with recent discoveries that also link polyQ expansion to gain of normal protein functions (Duvick et al, 2010; Nedelsky et al, 2010). Third, functional clustering analysis revealed a complex set of transcriptional alterations, which hint at many different components: some, like oxidative and metabolic stress, commonly found in polyQ toxicity or retinal degeneration in Drosophila (Xu et al, 2004; Nelson et al, 2005), others previously unreported, like loss of terminal differentiation markers linked to the specialised phototransduction process. Importantly, we also do not find evidence of induction of apoptosis by Atrophin at the transcriptional level, which is quite the opposite, as some key genes are significantly downregulated.

The different approaches, neuronal tissues and statistical treatments used make it difficult to compare our result with that of previous transcriptional profiling in DRPLA mouse models (Luthi-Carter et al, 2002; Sato et al, 2009). A detailed comparison has also not been attempted between the two mouse models. However, we find some commonalities in the prevalence of downregulations, and the marked effects on metabolism and signal transduction.

Although only over 80% of the candidate genes have been confirmed by qPCR, the few negative results, which include highly statistically significant candidates, stress the requirement for independent validation of functional genomic data before drawing conclusions about biological relevance. Thus, it is fundamental to establish whether such transcriptional...
Despite different effects of the wt forms of fly and human Atrophins on ft transcription, which are consistent with the differences in proteins structure and with their known effects on transcription (Zhang et al., 2002; Wang et al., 2006; Shen et al., 2007), in both cases polyQ expansion results in potentially toxic downregulation of ft. This mechanism is, however, not a general property of all polyQ proteins; rather, it is specific for Atrophin toxicity, as indicated by the absence of any effect of a Huntingtin mutant on ft. The basis of this is likely to rely on Atro directly regulating the ft gene, as we show that Atro is recruited to a specific enhancer region 3.9 kb upstream of the ft transcriptional start site and increased Atro levels lead to increased Atro recruitment at the site. As Atro does not bind DNA directly, it must be recruited by a currently unknown transcription factor. This will be the subject of further investigations in the future.

Although polyQ Atrophins, as polyQ proteins, also elicit many detrimental effects that are not dependent on Ft, like misfolding and aggregation (Nisoli et al., 2010), part of their toxicity is accomplished through an alteration of the Ft/Hippo signalling cascade that we identify as a crucial pathway for post-mitotic cellular homeostasis. In the context of DRPLA, the role of ft downregulation is to be considered as a modulatory event, specifically due to the Atrophin protein context, which is added to the many other polyQ toxic effects (Figure 9C).

A straightforward hypothesis would be that neurodegeneration in ft/hippo mutants arises as a consequence of apoptosis inhibition and growth stimulation, which would put the cells under considerable metabolic stress. Downregulation of Ft/Hippo signalling may push neuronal cells to attempt re-entering cell cycle, known to be a detrimental event in other neurodegenerative conditions (Herrup and Yang, 2007). This may be consistent with some of our microarray results, which report a repression of apoptosis and a moderate stimulation of cell proliferation; however, both categories are only mildly enriched in GO clustering and we demonstrate that cell cycle re-entry, as visualised by BrdU incorporation, only affects non-neuronal cells and is therefore to be interpreted as compensatory proliferation, probably linked to an attempt at tissue repair and scar formation.

Furthermore, we demonstrate that the effects on overgrowth and on neuronal homeostasis of the different Hippo pathway components are clearly uncoupled. This is exemplified by the strikingly different effects on proliferation and neurodegeneration achieved by Yki in its wt or phosphomutant form. Whereas YkiS111A,S168A,S250A has an effect on proliferation and neurodegeneration, our DRPLA model is partially mediated by loss of Ft expression, which is a direct target of Atro, downregulated at the transcriptional level. Because mutations in Atro phenocopy mutations in ft in ommatidial polarity (Fanto et al., 2003), and overexpressed Atro also with polyQ expansion acts as a gain of function in planar polarity and wing vein establishment (data not shown), transcriptional downregulation of ft represents a negative feedback loop in Ft/Atro signalling rather than the main pathway stream.
regulates the correct Rhodopsin expression in R7/R8 cells (Mikeladze-Dvali et al., 2005) independently of growth regulation. It has also been recently shown that mutations in the Hippo pathway block induction of autophagy in the digestion of salivary glands during pupal stages, independently of Yki (Berry and Baehrecke, 2007; Martin et al., 2007; Dutta and Baehrecke, 2008). The alteration of autophagy is an attractive alternative cellular mechanism through which the Ft/Hippo pathway could mediate Atrophin toxicity. We previously reported that polyQ Atro induces neurodegeneration through a critical block in the autophagic flux (Nisoli et al., 2010). Interestingly Atro, also called Gug, has also been identified as a molecule important for autophagic digestion of salivary glands (Martin et al., 2007).

Our data also confirm the role of Hippo signalling in regulating autophagy. Indeed, the effect of Hippo signalling in autophagic cell death of the salivary glands is consistent with our observations, with some remarkable difference. We note unequivocal accumulation of autophagosomes in ft and sav mutant cells. This suggests that the block in adult neurons is in clearance, rather than in induction, of autophagic vacuoles as it is in the salivary glands (Dutta and Baehrecke, 2008). The discrepancy is also likely to reflect the different role and basal level of autophagy in the two tissues. Whereas in the salivary glands autophagy is massively induced as a cell killing mechanism, in post-mitotic neurons expressing polyQ Atrophins autophagy is a rescue attempt, which fails because of a block at the level of lysosomal digestion (Nisoli et al, 2010). The double mutant ft,atg1 clones support the protective nature of endogenous levels of autophagy, also for ft-induced degeneration.

In addition, although more weakly than ft or other Hippo pathway components, Yki is capable of phenocopying neurodegeneration and its downregulation is able to suppress degeneration caused by loss of ft. The effects of yki in the retina could be due to a fundamental difference with salivary glands and are best interpreted by a model in which Yki is necessary but not sufficient to mediate all the functions of Hippo signalling in neuronal homeostasis, differently from growth control.

The data reported in this study suggest that degeneration by ft is due to autophagic stress and phenocopies many, but not all, aspects of polyQ Atrophin toxicity. The effect of ft mutations is more similar to the overexpression of wt Atro, in which, as in ft, at the ultrastructural level there is accumulation of autophagosomes but not of massive undigested autophagolyosomes (Nisoli et al., 2010). In addition, overexpression of wt Atro, as for loss of ft, leads to considerable accumulation of p62 with little increase in Atg8 punctae, whereas polyQ versions of Atrophins induce a much greater accumulation of Atg8. This would be consistent with a model (Figure 8C) in which Ft mediates an Atrophin-specific part of the toxicity, distinct from the general polyQ toxicity (i.e. protein misfolding, aggregation and resulting metabolic stress), and yet significantly affected by polyQ expansion in Atro.

The detailed molecular mechanism through which Ft and the Hippo pathway affect autophagy awaits further investigations, both in the context of development and in post-mitotic adult neurons. Nevertheless, it is likely that any effect on autophagy by the Ft pathway would be of crucial importance for its role in tumour suppression as well, given the relevance of autophagy in tumours and the wealth of common mechanisms shared by cancer and ageing (Finkel et al., 2007).

Finally, at least four Ft orthologues have been identified in mammals and are widely expressed throughout the mouse CNS (Tanoue and Takeichi, 2005), and Ft1 has recently been shown to interact with mouse Atrophins (Hou and Sibinga, 2009), indicating an evolutionarily conserved relation. Transcriptional profiling analyses on DRPLA mouse models (Luthi-Carter et al., 2002; Sato et al., 2009) have not detected downregulation in any of the ft mouse genes. However, the array platform used in both studies (Affymetrix Mu 11K) did not include any mouse ft. It is thus possible that the regulation of Ft-like molecules and of the Hippo tumour suppressor cascade in mammals is important for neuroprotection and has a key role in DRPLA in humans.

Materials and methods

Genetics

The following mutant fly stocks have been used: dEAA1-Gal4, GMR-Gal4, Rhodopsin1-Gal4, Repo-Gal4, Tub-Gal4, GMR > UAS > Gal4, UAS-EGFP, UAS-Flp, UAS-Hpo, UAS-Sav, UAS-WtsEGFPDM, UAS-Yki, UAS-Ykif36, UAS-Ykif36d, UAS-Ykif36e, UAS-CyD3, UAS-CDk4, UAS-GFP::Atg8a, Ubi-Gal80d, Tub-Gal80, ftΔp62Δd, ftfsΔykiΔd, atglΔs1Δd, dΔΔΔ-savΔd, hpo1Δ3, wtsMGH1, wtsMGH1. All clones have been generated with the Fyp/FRT system using either evy-flp or hs-flp.

Histology and EM

Semi-thin retinal sections were obtained as previously reported (Montrasio et al., 2007). EM samples were processed as in Nisoli et al, 2010.

Immunohistochemistry

Immunostaining procedures of adult retina were carried out with whole-mount preparation as previously reported (Nisoli et al, 2010). The following antibodies were used: mouse anti-f-l-galactosidase (1:1000) (Promega), rabbit anti-GFP (1:500, Molecular Probe), rabbit anti-p62 (1:2000) (a gift from Didier Contamine), mouse anti-Ellav (1:500) (DHSB) and rat anti-BrdU (1:100) (AbCam). Secondary fluorescence-conjugated antibodies from Molecular Probes or Jackson Laboratories were used at 1:200 dilutions. Samples were viewed with BioRad and Zeiss LSM confocal microscopes.

The microarray data set is available at GEO (www.ncbi.nlm.nih.gov/geo/) with the accession ID: GSE26246. See Supplementary data for further Materials and methods.

Supplementary data

Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.
References


