Intersectin enhances huntingtin aggregation and neurodegeneration through activation of c-Jun-NH$_2$-terminal kinase

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Huntington’s disease is a progressive neurodegenerative disease arising from expansion of a polyglutamine (polyQ) tract in the protein huntingtin (Htt) resulting in aggregation of mutant Htt into nuclear and/or cytosolic inclusions in neurons. Mutant Htt affects multiple processes including protein degradation, transcription, signal transduction, fast axonal transport and endocytosis [reviewed in Ross, C.A. and Poirier, M.A. (2005) Opinion: what is the role of protein aggregation in neurodegeneration? Nat. Rev. Mol. Cell. Biol., 6, 891–898]. Here, we report that the endocytic and signal transduction scaffold intersectin (ITSN) increased aggregate formation by mutant Htt through activation of the c-Jun-NH$_2$-terminal kinase (JNK)-MAPK pathway. Conversely, silencing ITSN or inhibiting JNK attenuated aggregate formation. Using a Drosophila model for polyQ repeat disease, we observed that ITSN enhanced polyQ-mediated neurotoxicity. A reciprocal relationship was observed between ITSN and Htt. While ITSN enhanced Htt aggregation and toxicity, Htt, in turn, inhibited the cooperativity between ITSN and the epidermal growth factor receptor signal transduction pathway. Finally, we observed that ITSN overexpression enhanced aggregation of polyQ-expanded androgen receptor (AR) as well as wild-type versions of both Htt and AR suggesting a broader involvement of ITSN in neurodegenerative diseases through destabilization of polyQ-containing proteins.

INTRODUCTION

Huntington’s disease (HD) is an autosomal dominant neurological disease characterized by a progressive neurodegeneration of striatal neurons resulting in chorea, progressive dementia and eventually death (1). In 1993, the gene responsible for this disease was identified and found to contain an expansion of a CAG trinucleotide repeat in the first exon resulting in a polyglutamine tract (polyQ) of variable length in the encoded protein product referred to as huntingtin (Htt) (2). Although mutant Htt is widely expressed, the associated neuropathology is restricted to specific regions of the brain. In particular, there is a dramatic loss of medium spiny neurons in the striatum and large neurons in layer IV of the cerebral cortex with moderate atrophy observed in the thalamus, hypothalamus and amygdala as well (reviewed in 3).

PolyQ expansion underlies a number of diseases including spinobulbar muscular atrophy (Kennedy’s disease) (4), spinocerebellar ataxia-3 (Machado–Joseph disease) (5) and dentatorubral–pallidoluysian atrophy (6). The presence of a polyQ tract in Htt renders the resulting mutant protein unstable, resulting in the proteolytic cleavage to release an NH$_2$-terminal fragment containing the polyQ expansion. Through an undefined series of steps (7), this truncation product eventually forms insoluble aggregates within cells. Expansion of the polyQ tract inversely correlates with age of disease onset (8). Furthermore, polyQ expansion correlates with increased density of inclusions in HD patients (9) and increased aggregation of the protein in both in vitro and cell culture models (10, 11). Indeed, the introduction of polyQ peptides capable of forming amyloid fibrils in vitro resulted in cellular toxicity when these peptides were localized to the nucleus (12).
Conversely, treatment of animal models for polyQ disease with agents that inhibited aggregation in cell culture models resulted in a delayed onset of pathological phenotype (13–16). Together, these data have led to the conclusion that polyQ aggregates represent a toxic burden in the cell.

Recent studies, however, have called into question the toxicity of polyQ aggregates. The presence of aggregates, per se, does not necessarily correlate with toxicity (10) and may in fact protect cells from death (17). Furthermore, treatment of cells expressing mutant Htt or α-synuclein with a compound that enhanced aggregation formation resulted in decreased toxicity of these mutant proteins (18). Regardless, the toxicity/pathogenicity of mutant Htt increases with increasing length of the polyQ tract. These data suggest a model in which formation of aggregates is a multi-step process and an intermediate species in the formation of aggregates is the toxic component. In this model, the aggregates themselves are protective end products of the process. This model suggests that the process of aggregate formation is pathogenic and that identification of agents that modulate aggregate formation by either preventing the formation of the toxic intermediate or by enhancing the clearance of this intermediate by promoting aggregate production may lead to potential therapeutic treatment of HD (18).

Intersectin (ITSN) is a unique, multi-domain scaffolding protein that regulates endocytosis and signal transduction (19). The protein comprises two NH2-terminal Eps15 homology (EH) domains followed by a coiled-coil (CC) region and five Src homology 3 (SH3) domains (Fig. 1A). Each of these domains promotes the interaction of ITSN with its various target proteins (19). The EH domains bind to Asn—Pro—Phe motifs on proteins such as epsin, whereas the SH3 domains bind to Pro-rich sequence in proteins such as Sos, Cbl, dynamin and synaptojanin. The CC domain promotes the oligomerization with other CC proteins such as Eps15 and ITSN (20). It has been suggested that ITSN may be associated with Htt through its ability to regulate actin dynamics (21). Here, we demonstrate that ITSN interacts with several proteins that are associated with Htt and may function in aggregation of polyQ-expanded Htt suggesting that this scaffolding protein may influence Htt aggregation. We present evidence that ITSN associates with Htt in cells and plays an important role in regulating aggregation of mutant Htt through a c-Jun-NH2-terminal kinase (JNK)-dependent signaling pathway. The involvement of ITSN in polyQ aggregation does not appear limited to Htt as we demonstrate that ITSN also enhances aggregation of the androgen receptor (AR), which undergoes polyQ expansion in Kennedy’s disease. Using a *Drosophila* model for polyQ-expansion diseases such as HD and Kennedy’s disease, we provide in vivo evidence that ITSN enhances the neurodegenerative effects of a polyQ-expanded protein such as Htt. These studies provide the first clear evidence that ITSN may play an important role in Htt-induced neurodegeneration.

**RESULTS**

**Yeast two-hybrid screen revealed link of ITSN to Htt**

To identify potential targets of ITSN, we performed a high-throughput yeast two-hybrid screen (Y2H) that resulted in a large number of unique ITSN-binding proteins. Interestingly, we identified three classes of candidate ITSN partners that suggested a possible link to HD (Table 1). These included known Htt-interacting proteins (arfaptin2, HIP1, Beclin1 and Nwk), proteins that bind Htt-interacting proteins (FBP-4, SAPAP1 and β-adaptin) and proteins that were homologous to known Htt-interacting proteins but have not themselves been described as binding to Htt (FBP-17 and PACSIN-3). Given the involvement of ITSN in regulating endocytosis and JNK activation coupled with the link of Htt with these same processes, these Y2H results suggested a potential link between ITSN and Htt.

**ITSN co-localized with both wild-type and mutant Htt proteins**

To explore the possible interaction between Htt and ITSN, we co-expressed YFP-tagged mouse ITSN (YFP-ITSN) (Fig. 1A) with a CFP-tagged fragment of Htt encoded by the first exon of either wild type Htt (Htt-Q23-CFP) or polyQ-expanded Htt (Htt-Q65-CFP) in HEK293T cells (Fig. 1B). YFP-ITSN exhibited a punctate localization consistent with previous immunofluorescence data on endogenous ITSN (22). Although wild-type Htt-Q23-CFP exhibited a diffuse, cytosolic localization, Htt-Q65-CFP was frequently found in dense, well-defined, brightly fluorescent structures which have been referred to as aggresomes (23) (Fig. 1B and Supplementary Material, Fig. S1A). When co-expressed, YFP-ITSN co-localized with both forms of Htt-CFP. In the case of Htt-Q65-CFP, YFP-ITSN was recruited into the dense Htt aggregates with little punctate localization remaining. In the case of Htt-Q23-CFP, YFP-ITSN also co-localized with Htt into what we referred to as ‘pseudoaggregates’ (Fig. 1B and Supplementary Material, Fig. S1A). These structures were less well defined and did not have the characteristic bright fluorescence as seen with the Htt-Q65 aggregates. However, in cells containing these Htt-Q23 pseudoaggregates, we observed a re-localization of YFP-ITSN from its normal punctate pattern to these Htt-Q23-CFP structures. Since the nuclei of HEK293T cells occupy the majority of the cytoplasmic volume, it was not possible to discern cytoplasmic versus nuclear localization of the Htt aggregates. However, expression of these proteins in COS cells (Fig. 1C) revealed that the majority of aggregates appeared cytosolic.

To determine which domains of ITSN were required for co-localization with Htt-Q65, we examined the localization of YFP-tagged ITSN truncation mutants with Htt-Q65-CFP. Given the larger nuclei and small rounded morphology of HEK293T cells, we switched to COS cells which exhibit a flatter morphology and smaller nuclei more conducive to confocal analysis. The EH-Coil and Coil regions were sufficient for interaction with Htt-Q65, whereas neither the EH nor SH3 domains interacted with Htt-Q65 (Fig. 1C).

**ITSN expression increased Htt aggregate formation**

PolyQ expansion in Htt resulted in aggregation of the mutant protein and the formation of visible aggregates in cells (Fig. 1). Co-expression of ITSN [either YFP-tagged (Fig. 2A) or HA-tagged (Fig. 2B)] resulted in a statistically significant
increase in the percentage of CFP-positive cells containing visible aggregates (Fig. 2). This increase was seen with full-length ITSN as well as the EH-Coil and Coil regions; however, neither the isolated EH nor SH3 regions affected Htt aggregation. Although ITSN overexpression increased the formation of pseudoaggregates of Htt-Q23, the difference did not rise to the level of statistical significance ($P = 0.056$).

To determine whether the effect of ITSN on Htt aggregation was specific to Htt or more generally applicable to polyQ-expanded proteins, we examined the effect of ITSN expression on aggregation of an NH$_2$-terminal fragment of the AR. Expansion of the polyQ-repeat region of AR occurs in Kennedy’s disease. As observed with Htt, co-expression of ITSN with a non-pathogenic AR fragment (AR-Q25-CFP) or a polyQ-expanded pathogenic AR fragment (AR-Q65-CFP) resulted in enhanced aggregate formation of both AR proteins (Fig. 2C). Although the effect of ITSN on Htt-Q23 did not rise to statistical significance, the effect on AR-Q25 was statistically
significant \( (P = 0.0002) \). Furthermore, the enhancement of AR-Q65-CFP aggregation was also statistically significant \( (P = 0.002) \). Thus, ITSN overexpression enhances aggregation of both Htt as well as AR fragments. Given the lack of a Pro-rich region in AR, these results also suggest that ITSN’s effect on Htt aggregation is independent of this region and does not involve interaction with ITSN’s SH3 domains. This conclusion is consistent with the observation that the SH3 domains of ITSN are not necessary for enhancement of Htt aggregation.

To determine whether endogenous ITSN was involved in Htt aggregation, we co-transfected Htt-Q65-CFP along with short interfering RNAs (siRNA) to ITSN and determined the consequence to aggregate formation. Diminishing ITSN expression decreased the percentage of CFP cells with visible aggregates supporting the premise that ITSN contributes to Htt aggregation (Fig. 2D).

**ITSN activation of JNK stimulates Htt aggregation**

Given the involvement of ITSN in Htt aggregation, we next sought to determine the mechanism by which ITSN enhanced this process. Aggregation of Htt is augmented through stress-activated signaling pathways (24). Given that ITSN activates a JNK-dependent signaling pathway (25), we examined whether ITSN stimulated Htt aggregation through JNK activation. Co-expression of a dominant-negative JNK inhibited aggregation of Htt-Q65 and also blocked ITSN enhancement of Htt-Q65 aggregation (Fig. 3A). This JNK DN did not affect the expression of either ITSN or Htt-CFP (Supplementary Material, Fig. S2A). Additionally, treatment of cells with a pharmacological inhibitor of JNK (SP600125) decreased ITSN’s enhancement of aggregate formation (Fig. 3B) without affecting expression of either ITSN or Htt-CFP (Supplementary Material, Fig. S2B). Finally, expression of ITSN, EH-Coil or Coil resulted in activation of JNK, whereas expression of the EH or SH3 domains did not ([25] and Supplementary Material, Fig. S3]. These data indicate that ITSN enhancement of aggregate formation required JNK activity. These data also reveal a correlation between the ability of ITSN (and ITSN truncation mutants) to interact with Htt aggregates and the activation of JNK by these ITSN proteins.

**PolyQ-expanded Htt inhibits cooperative signaling between RTK and ITSN**

The above results indicate that ITSN influences Htt aggregation; however, we examined the reciprocity of this relationship by determining whether Htt might influence ITSN function. Given Htt’s role in regulating signaling pathways (26) and endocytosis (27), we examined the consequences of polyQ-expanded Htt on ITSN signaling (25,28–30). Although Myc-tagged Htt-Q103 did not appear to block ITSN’s effect in this assay, transcriptional activation by EGF was inhibited by 41% (Fig. 4). Furthermore, in the presence of ITSN, Htt-Q103 resulted in a 61% reduction in EGF-induced transcriptional activation. These data indicate that ITSN enhancement of aggregate formation required JNK activity. In addition, these results demonstrate that Htt-Q103 blocks ITSN’s ability to enhance EGFR signaling.
ITSN overexpression enhances neurodegeneration in an in vivo model of polyQ disease

The results of our cell-based experiments indicate that ITSN contributes to the aggregation of polyQ-expanded Htt as well as polyQ-expanded AR. To discriminate between a potential protective versus pathogenic effect for ITSN’s enhancement in polyQ-protein aggregation, we utilized a Drosophila model to measure in vivo polyQ-induced neurodegeneration. In previous studies, expression of polyQ-expanded proteins in the Drosophila photoreceptor cells resulted in neurodegeneration that increased with expansion of the polyQ region and negatively impacted on photoreceptor function (31,32). Here, we asked whether the photoreceptor degeneration caused by overexpression of a polyQ peptide of 48Qs was modified by concurrent overexpression of the Drosophila ITSN homolog, Dap160 (33,34). Given that ITSN enhances aggregation of both Htt and AR fragments which only share the common feature of an expanded polyQ repeat (AR does not contain a comparable Pro-rich region as found in Htt), this Drosophila polyQ model represents a valid in vivo model in which to examine the effect of ITSN overexpression. At the level of light microscropy, we observed that polyQ overexpression caused disorganization of the photoreceptors in the compound eye characterized by a reduction in size of the phototransduction organelles, rhabdomeres (data not shown). At this resolution, we could not determine whether concurrent overexpression of Dap160 with polyQ modified the degenerative effect of the polyQ peptide. Hence, we performed electroretinograms to determine whether Dap160 modified the effect of polyQ at the functional level. In electroretinograms, amplitudes of depolarization in response to pulses of light serve as a measure of phototransduction. Retinal expression of a polyQ protein of 48Qs decreased the amplitude of depolarization by 55% (11.8 ± 0.53 versus 5.3 ± 0.70 mV, t-test: \( P < 0.0001 \)) (Fig. 5) consistent with the neurodegenerative effect of polyQ. This effect was not due to heterologous expression of proteins in the eye as overexpression of neither Dap-160 nor EGFP caused significant reductions in depolarization amplitudes (\( P > 0.05 \)) (Fig. 5). However, co-expression of Dap-160 with polyQ enhanced

**Figure 2.** Aggregation of polyQ-expanded Htt is stimulated by ITSN. (A) YFP-ITSN was co-transfected into HEK293T cells with Htt-Q23-CFP or Htt-Q65-CFP. The number of CFP-positive cells containing one or more aggregates was then counted visually with the observer blinded to the identities of the samples while counting. At least 500–1000 CFP-positive cells were scored for the presence of aggregates under each condition. The results are the average of two independent experiments ± SEM performed in duplicate. The difference in Htt-Q65 aggregation in the presence of ITSN versus YFP was statistically significant by Student’s \( t \)-test. However, the difference in Htt-Q23 aggregation in the presence of YFP-ITSN versus YFP alone did rise to the level of statistical significance (\( P = 0.0505 \)). (B) Htt-Q65-CFP was co-transfected along with various HA-tagged ITSN mutants and their effects on aggregation were determined as in (A). The asterisks indicates that differences in Htt-Q65 aggregation induced by expression of ITSN, EH-Coil and Coil alone compared with vector were statistically significant by a Student’s \( t \)-test (\( P = 0.03, 0.006 \) and 0.002, respectively). (C) AR-Q25-CFP or AR-Q65-CFP was co-transfected with either empty vector or an HA-tagged ITSN expression vector and scored for aggregates as in (A). The results are the average of three independent experiments ± SEM. The difference in the percent of cells containing aggregates in the presence of ITSN was statistically significant by Student’s \( t \)-test. (D) HEK293T cells were transfected with scramble or ITSN-specific small interfering RNA (siRNA) and scored for aggregate formation as described in (A). ITSN silencing was confirmed by western blot analysis of cell lysates (data not shown). The results are the average of three independent experiments ± SEM in which >950 cells were counted for each condition in each experiment. The reduction in percent aggregates is statistically significant by Students \( t \)-test (\( P = 0.01 \)).
the inhibitory effect of polyQ on the amplitude of depolarization (5.3 ± 0.7 mV polyQ alone versus 3.5 ± 0.36 mV polyQ + Dap160, t-test: P = 0.03), whereas expression of EGFP did not influence polyQ-mediated inhibition of the amplitude of depolarization (P > 0.05). Thus, in Drosophila photoreceptors Dap160 overexpression enhanced the toxicity of polyQ suggesting that ITSN enhances the toxicity of mutant polyQ-expanded Htt in vivo.

**DISCUSSION**

ITSN has emerged as a multi-functional protein that participates in the regulation of several cellular processes (19). While many studies have focused on the role of ITSN in endocytosis, increasing evidence points to a role for this unique, scaffolding protein in the direct regulation of signaling pathways as well. ITSN associates with the Ras exchange factor Sos leading to Ras activation on a subset of intracellular vesicles (25). ITSN also cooperates with receptor tyrosine kinases in the activation of signaling and in cellular transformation (28), and this cooperativity may involve regulation of receptor ubiquitylation and trafficking (22). These results reveal that ITSN not only regulates signal transduction pathways but that it also stimulates the compartmentalized activation of these signaling pathways.

In this current study, we provide the first demonstration that ITSN overexpression is involved in neurodegeneration. Our Y2H screen revealed several potential connections between ITSN and HD (Table 1). Furthermore, Y2H analysis with Dap160, revealed that Drosophila ITSN interacts with two proteins that possess polyQ tracts, Drongo and CG5053 (35). Our Y2H analysis also identified another polyQ-containing protein, angiomotin-like 1, as an ITSN-binding protein (unpublished data). Given our current findings that ITSN...
in vivo regulate this class of proteins and AR proteins (wild-type and polyQ-expanded), the association of ITSN with polyQ proteins suggests that ITSN may regulate this class of proteins in vivo. Elevation in ITSN levels in a Drosophila model for polyQ expansion diseases such as HD and Kennedy’s disease resulted in enhanced neurodegeneration, suggesting that ITSN’s effect on Htt and AR aggregation results in a pathological response. Although recent studies provide compelling evidence that polyQ aggregates are not the toxic species responsible for the pathologic effects of polyQ expansion (10,17,18), our data demonstrate that the ITSN-mediated increase in aggregation in polyQ expanded Htt and AR nonetheless correlates with increased neurodegeneration in vivo. But how can these findings be reconciled? Formation of polyQ aggregates is a multi-step process beginning with a nucleation step leading to formation of protofibrils followed by mature fibrils and eventually visible aggregates (7). One or more of these intermediates in the process of aggregate formation may represent the pathogenic species in vivo. Given that ITSN promoted the coalescence of wild-type Htt and AR into pseudoaggregates, we propose that ITSN overexpression enhances the nucleation of polyQ-expanded proteins resulting in increased levels of some toxic intermediate and thus enhanced pathogenicity of polyQ.

Our results suggest that ITSN enhancement of Htt aggregation requires the JNK-MAPK pathway. We previously demonstrated that ITSN activates JNK through the EH region (25). The importance of this ITSN-JNK signaling pathway for Htt toxicity is underscored by previous studies linking JNK to polyQ-expansion diseases. Both aggregation and toxicity of polyQ-expanded proteins are enhanced by the cellular stress response, and inhibition of the JNK pathway attenuates these responses (24,27,36). Although JNK activation enhances phosphorylation of DRPLA (37), the polyQ-containing protein product of the dentatorubral–pallidoluysian atrophy disease gene, JNK does not appear to play a role in the direct phosphorylation of Htt (38). Thus, the mechanism by which JNK activation enhances Htt aggregation remains to be defined. Although the CC domain of ITSN does not appear to activate a JNK-dependent signaling pathway (28), we speculate that the enhanced aggregation observed upon overexpression of this domain with Htt may be due to the inherent ability of CC domains to aggregate thus providing a catalyst for the aggregation of Htt.

The connection between JNK and polyQ aggregation appears to be a two-way street. PolyQ-expanded Htt activates the JNK pathway both in cell lines as well as primary neurons (39–42). Furthermore, JNK mediates the pathogenic effects of polyQ-expanded AR on fast axonal transport (43).

However, the inhibitory effects of Htt-Q103 on EGFR signaling are not likely due to exclusive effects on the JNK pathway. Htt-Q103 did not stimulate transcriptional activation in our reporter assays even though polyQ-expanded Htt stimulates JNK activation as noted earlier. ITSN’s stimulation of this transcriptional reporter is JNK-dependent (25). These results suggest that the pool of JNK that is activated by Htt-Q103 may not be capable of stimulating transcription, possibly through sequestration of activated JNK in the cytoplasm. Rather, we propose that ITSN stimulates Htt-Q103 aggregation which then inhibits pathways such as the ERK-MAPK as previously described (44,45), thereby resulting in a decrease in transcriptional activation. Such a mechanism is consistent with our previous findings that the ERK-MAPK pathway is necessary for cooperativity between ITSN and EGF in stimulating transcriptional activation (28).

Our findings also provide potential insight into the involvement of ITSN in Down’s Syndrome (DS) (46–48). The gene for ITSN is located on human chromosome 21 in the region commonly referred to as the Down’s Syndrome Critical Region or DSCR. Analysis of brains from patients with DS revealed that expression of ITSN mRNA was elevated (48). In further support of this finding, analysis of the Ts65Dn mouse model for DS demonstrated that ITSN protein levels were elevated in mutant mice compared with normal control litter mates (47). Like DS patients, the Ts65Dn mice develop an age-related neurodegeneration of the basal forebrain cholinergic neurons and impairment in spatial learning and memory (reviewed in 49). These findings, coupled with ITSN’s involvement in JNK signaling (25) and endocytosis (22), have led to the notion that ITSN overexpression may contribute to the development of a subset of the DS phenotypes (46,50). This study demonstrates that elevation in ITSN expression may enhance nucleation of wild-type polyQ containing proteins (e.g. Htt and AR) leading to increased levels of a neurotoxic intermediate. While this effect may be rather modest, the cumulative effect over the lifetime of an individual may negatively impact neuron function and survival. Our observation that ITSN overexpression resulted in the coalescence of both wild-type Htt (Htt-Q23) and AR (AR-Q25) into ‘pseudo-aggregates’ supports this possibility.

Our study demonstrates a physical and pathological link between ITSN and HD. Overexpression of ITSN enhanced aggregation of polyQ-expanded Htt leading to defects in both cellular signaling and neuron function. While ITSN expression has not been reported to be altered in HD, these results suggest that further studies may be necessary to explore the connections between alteration in ITSN expression or function and polyQ-mediated neurodegeneration.

MATERIALS AND METHODS

Cells and transfection

HEK293T and COS1 cells were maintained in Dulbecco’s Modified Eagle Medium supplemented with 10% fetal bovine serum and 1 mM sodium pyruvate. Standard calcium phosphate precipitation protocol was used to transfect HEK293T cells. COS1 cells were transfected with LipofectAMINE reagent (Invitrogen) as recommended by the manufacturer.

Plasmids

YFP/GST tagged wild-type and truncated mutants of ITSN have been described previously (25). Htt-CFP was a kind gift from Peter Reinhart (Duke), and AR-CFP was the kind gift of Marc Diamond (UCSF). Myc-tagged Htt-Q103 was a gift of Marc Diamond (UCSF). Myc-tagged Htt-Q103 was a
Yeast two-hybrid

Analysis was performed through a contract with Myriad Genetics essentially as described previously (51) except using the various individual domains of mouse ITSN as bait.

Confocal microscopy

COS1 cells were plated on glass bottom plates and transfected with appropriate plasmid DNA using Lipofectamine reagent (Invitrogen Life Technologies). Forty-eight hours post-transfection, confocal images of cells were taken using Zeiss LSM510 NLO microscope.

Htt aggregate formation assay

Forty-eight hours following transfection of Htt-Q65-CFP or AR-CFP and the various ITSN expression constructs (either HA- or YFP-tagged), HEK293T cells were photographed using Olympus IX70 microscope. Five-hundred to thousand randomly selected CFP-positive cells were scored for aggregate formation. Cells were scored positive if they contained one or more visible aggregates either in the cytoplasm or nucleus. There was no size restriction for aggregates. Furthermore, there was no distinction between cells that contained one aggregate versus several aggregates. Percent aggregates formed in each transfected cell line represents the percentage of the CFP-positive cells that contain visible aggregates.

Western blot analysis

Cells were washed with PBS prior to lysis with buffer containing 50 mM HEPES, 10% glycerol, 150 mM NaCl, 1% TritonX-100, 1 mM EGTA, 1.5 mM MgCl2, 100 mM NaF and protease inhibitor cocktail. All debris were spun down, and the protein concentration in cleared lysate was determined using BCA kit (Pierce). Equal amounts of cell lysate were resolved on SDS–PAGE, transferred to PVDF and then probed using the following primary and secondary antibodies: mouse anti-HA antibody (Babco, 1:1000); rabbit anti-GFP (ClonTech, 1:100); sheep anti-mouse antibody, HRP conjugated (Amersham, 1:5000); Protein-A, HRP conjugated (Amershams, 1:5000).

Transcriptional reporter assays

JNK involvement was determined using a transient transcriptional reporter assay as previously described (25). For each experiment, relative activation was normalized to the vector control determined by dividing the RLU/µg protein for each experimental point by the RLU/µg for unstimulated vector transfected samples.

Electroretinograms

We used the GMR-Gal4 driver (P12) (52) to co-overexpress Dap160 with a poly-glutamine peptide in Drosophila photoreceptors. Male flies carrying w; P[GAL4-ninaE.GMR]12; P[UAS-Dap160]/9.1 were crossed to females carrying w; UAS-Q48.myc/flag/42 (53). 1-day-old w; UAS-Q48.myc/flag/42/w; P[GAL4-ninaE.GMR]12; P[UAS-Dap160]/9.1/+ female adults were subjected to electroretinogram recordings as described previously (54). Briefly, flies were immobilized with a non-toxic glue to allow for the insertion of a sharp glass reference electrode in the thorax and the gentle impalement of a sharp recording electrode in the eye. The electrodes were filled with 3 M NaCl. Light flashes of 1 s were delivered using a halogen lamp. In addition, the following genotypes were used for comparison: (i) w; P[GAL4-ninaE.GMR]12/+; (ii) w; P[GAL4-ninaE.GMR]/12/+; P[UAS-Dap160]/9.1/+; (iii) w; UAS-Q48.myc/flag/42/w; P[GAL4-ninaE.GMR]12/+; (iv) w; P[GAL4-ninaE.GMR]/12 P[UAS-mCD8::GFP,L]LL5/+; (v) w; UAS-Q48.myc/flag/42/w; P[GAL4-ninaE.GMR]12 P[UAS-mCD8::GFP,L]LL5/+.

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