CDK5 and MEKK1 mediate pro-apoptotic signalling following endoplasmic reticulum stress in an autosomal dominant retinitis pigmentosa model

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Chronic stress in the endoplasmic reticulum (ER) underlies many degenerative and metabolic diseases involving apoptosis of vital cells. A well-established example is autosomal dominant retinitis pigmentosa (ADRP), an age-related retinal degenerative disease caused by mutant rhodopsins¹,². Similar mutant alleles of Drosophila Rhodopsin-1 also impose stress on the ER and cause age-related retinal degeneration in that organism³. Well-characterized signalling responses to ER stress, referred to as the unfolded protein response⁴ (UPR), induce various ER quality control genes that can suppress such retinal degeneration⁵. However, how cells activate cell death programs after chronic ER stress remains poorly understood. Here, we report the identification of a signalling pathway mediated by cdk5 and mekk1 required for ER-stress-induced apoptosis. Inactivation of these genes specifically suppresses apoptosis, without affecting other protective branches of the UPR. CDK5 phosphorylates MEKK1, and together, they activate the JNK pathway for apoptosis. Moreover, disruption of this pathway can delay the course of age-related retinal degeneration in a Drosophila model of ADRP. These findings establish a previously unrecognized branch of ER-stress response signalling involved in degenerative diseases.

Three branches of the UPR are particularly well characterized in mammals and conserved in Drosophila⁴. In brief, these pathways involve transmembrane proteins ATF6, IRE1 and PERK, respectively, that can sense stress in the ER lumen. ATF6 is a transcription factor anchored to the ER membrane that translocates to the nucleus after ER stress triggers its proteolysis, and IRE1 is an endonuclease that activates the transcription factor XBP1 through an unconventional messenger RNA splicing mechanism. PERK is an ER-stress responsive kinase that mediates the translational activation of the transcription factor ATF4. The predominant effect of these pathways is to reduce stress in the ER and help the cells return to their normal physiological state. Consistently, the main targets of these transcription factors include genes that encode ER chaperones, anti-oxidant proteins and those involved in misfolded protein degradation⁶–⁸.

Our in vivo model for ER-stress-induced apoptosis is based on a mutant Drosophila Rhodopsin-1 (Rh-1) allele, Rh-1°⁵⁶, which is similar in nature to human rhodopsin mutants that undergo retinal degeneration in ADRP (refs 9,10). Although the endogenous allele causes late-onset retinal degeneration without affecting the external eye morphology, overexpression of this encoded protein in larval eye imaginal discs (during photoreceptor differentiation) led to an easily identifiable adult eye phenotype by eclosion (Fig. 1a,b and Supplementary Fig. S1b). The adult eye was abnormally small, indicative of massive cell loss, and the surviving eye tissue showed a glassy surface that was devoid of ommatidial structures. The effect of Rh-1°⁵⁶ overexpression can be attributed to excessive ER stress for the following reasons. The Rh-1°⁵⁶ overexpression phenotype was suppressed by the co-expression of Drosophila hrd1 (Supplementary Fig. S1c), which encodes an E3 ubiquitin ligase dedicated to degrading misfolded ER proteins⁵. In addition, we detected signs of ER stress using two independent reporters. One is the XBP1–EGFP reporter, which expresses EGFP in frame only when ER stress stimulates IRE1-dependent xbp1 mRNA splicing⁷. This reporter was activated in Rh-1°⁵⁶ mis-expressing imaginal discs but not active in control tissues (Supplementary Fig. S1d,e). We were also able to detect signs of ER stress through an antibody against Drosophila ATF4. This protein is encoded in the cryptocephal (crc) locus¹¹. As in mammals¹², we found that Drosophila ATF4 expression was induced after ER stress (Supplementary Fig. S1f–h). Expression of Rh-1°⁵⁶ in eye imaginal discs also increased the level of endogenous superoxides as evidenced by dihydroethidium labelling (Supplementary Fig. S1j,k), consistent with previous reports of elevated ROS levels in stressed ER (refs 13–17). Co-expressing Hrd1 suppressed such induction of ATF4 and ROS

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Figure 1 CDK5 and its regulatory subunit p35 (CDK5p35) are required for Rh-1G69D-induced apoptosis. (a-c) External adult eye phenotypes caused by overexpressing Rh-1G69D, together with lacZ (a) or with inverted-repeat (IR) transgenes to knockdown lacZ (b) and cdk5 (c). Note a partial recovery of eye size on cdk5 knockdown. (d-f) cdk5 knockdown does not affect the rough eye phenotype caused by p53 overexpression (e,f). d shows a control eye fly with normal morphology. (g-j) Apoptosis in larval eye discs assessed through TUNEL labelling (magenta). Mis-expression of Rh-1G69D led to massive apoptosis (g), which was suppressed by knocking down cdk5 (h). Rh-1G69D-triggered apoptosis (i) was also suppressed in a p35Vcdk5xN background (j). (k-m) cdk5 knockdown does not affect the degree of ATF4 protein induction (red) in response to Rh-1G69D mis-expression. Shown are: a control eye disc (genotype, gmrGal4, eyp/+; UASRh-1G69D), a disc mis-expressing Rh-1G69D together with a control lacZ transgene (l) or with a cdk5-IR transgene (m). (n-p) cdk5 knockdown does not affect the degree of XBP1 pathway activation, as assessed through the XBP1EGFP reporter (green). Shown are: a control eye disc expressing XBP1-EGFP alone (n), or together with Rh-1G69D and lacZ (o) or with Rh-1G69D and cdk5-IR (p). (q-r) Rh-1G69D-induced apoptosis in atf4 (crc)-/- discs. TUNEL labelling (magenta) shows Rh-1G69D-triggered apoptosis in crc+ (q) and crc-/- discs (r). (s,t) Rh-1G69D-induced apoptosis (magenta) in ire1/- clones (marked by the absence of green). The image is a magnified view of the region overexpressing Rh-1G69D. TUNEL-positive cells are found within the ire1-/- - clones. (u,v) The level of CDK5 is not affected by mis-expression of Rh-1G69D in eye discs. (a) A representative western blot of eye imaginal disc extracts mis-expressing Rh-1G69D through the gmr-Gal4 driver. The upper gel shows bands corresponding to CDK5, and the lower gel shows anti-Profilin bands as a loading control. The middle gel shows an anti-Rh-1 blot. (v) Comparison of the normalized CDK5 band intensity, with the value from the control gmr+/+ set at 1. The scale bar in g represents 100 µm for g-r and that in s represents 20 µm for s,t. Genotypes: gmrGal4, UAS-Rh-1G69D/UAS-lacZ; UAS-dicer2/+ (a,g,l), gmrGal4, UAS-Rh-1G69D/UAS-lacZ-IR;UAS-dicer2/+ (b), gmrGal4, UAS-Rh-1G69D/UAS-cdk5-IR; UAS-dicer2/+ (c,h,m), gmrGal4/+ (d), gmrGal4, UAS-p53/+;UAS-dicer-2/+ (e), gmrGal4, UAS-p53/UAS-cdk5-IR; UAS-dicer2/+ (f), gmrGal4/UAS-Rh-1G69D/+;+/+ (i), gmrGal4,F(p35)f2;UAS-Rh-1G69D.Df(p35)64 (j), y w (k), gmrGal4/UAS-xbp1EGFP/+ (n), gmrGal4, UAS-Rh-1G69D/UAS-lacZ; UAS-dicer2/UAS-xbp1-EGFP (o), gmrGal4, UAS-Rh-1G69D/UAS-cdk5-IR;UAS-dicer2/UAS-xbp1-EGFP (p), gmrGal4/UAS-Rh-1G69D (q), gmrGal4, crc64/crc, UAS-Rh-1G69D (r), gmrGal4, ey-FLP++; UAS-Rh-1G69D/+; FRT82, ire11201/FRT82, ubi-GFP (s,t). Uncropped images of blots are shown in Supplementary Fig. S6.

(Supplementary Fig. S1i,j), indicating that these markers appear as a result of misfolded protein overload in the ER.

An easily detectable adult eye phenotype allowed us to conduct an in vivo RNA interference (RNAi) screen to identify genes required for Rh-1G69D-induced toxicity. We specifically focused on kinases and phosphatases that could serve as signalling proteins potentially linking the distressed ER and the apoptotic machinery. Of the 196 protein kinases and 66 protein phosphatases encoded in the Droso phila genome8, we were able to target 119 kinases and 39 phosphatases through RNAi-mediated knockdown, using a total of 276 inverted-repeat transgenes available from the Vienna Drosophila RNAi Center (Supplementary Table S1). We found three lines that strongly suppressed the adult eye phenotype, two of which (VDRC35855 and VDRC35856) targeted Drosophila cdk5 (Fig. 1c). CDK5 is an atypical cyclin-dependent kinase with established roles in differentiated post-mitotic cells, such as neurons, adipose tissue and pancreatic beta islet cells19-22. In mammals, CDK5 is reportedly activated by various stress conditions, including those that disrupt ER function. Excessive activation of CDK5 contributes to neurotoxicity in Alzheimer’s and Parkinson’s diseases models24,25. We found that cdk5 knockdown did not affect an independent cell death phenotype caused by p53 overexpression in the eye (Fig. 1e,f). These results indicate that cdk5 mediates a specific signalling response to mutant Rh-1, rather than affecting the general cell death machinery. When eye imaginal discs were inspected, we noticed a marked reduction of TUNEL-positive cells, indicating that cdk5 is required for apoptosis in this assay (Fig. 1g,h).
To determine whether CDK5 has a conserved role in mammals, we used mouse Min6 cells, which readily succumb to apoptosis when treated with tunicamycin (Supplementary Fig. S2), a compound that inhibits protein glycosylation and causes stress in the ER (ref. 26). Knockdown of CDK5 strongly suppressed tunicamycin-induced apoptosis, as assessed through TUNEL labelling (Supplementary Fig. S2). CDK5 levels did not change in response to Rh-1-G69D expression (Fig. 1u,v), indicating that the protein may be regulated by post-transcriptional mechanisms. In fact, CDK5 activity is often regulated through its regulatory subunit, p35 (also known as CDK5-R28). In a loss-of-function p35 (cdk5c) background, the amount of apoptosis induced by Rh-1-G69D expression was significantly reduced (Fig. 1i,j), further confirming the role of CDK5 in apoptosis.

To determine whether the cdk5 knockdown condition suppresses apoptosis by reducing the overall stress levels in the ER, we labelled imaginal discs with the anti-ATF4 antibody. The degree of ATF4 induction in Rh-1-G69D-overexpressing eye discs was not affected by cdk5 knockdown (Fig. 1k–m). We also assessed the extent of IRE1/XBP1 pathway activation, using the XBP1-EGFP reporter. Again, knockdown of cd5 did not affect the degree of this ER-stress reporter activation in response to Rh-1-G69D expression (Fig. 1n–p). These observations indicate that cdk5 mediates Rh-1-G69D-induced apoptosis without affecting the overall levels of misfolded protein load in the ER. To further determine whether the ATF4 and IRE1/XBP1 pathways contribute to Rh-1-G69D-induced apoptosis, we examined the degree of cell death in mutants that disrupt these pathways. In the loss-of-function ATF4 condition, crc –/– (ref. 11), the degree of Rh-1-G69D-induced apoptosis was similar to those of the crc+ background (Fig. 1q,r). In the icr1 –/– mosaic clones, the degree of Rh-1-G69D-induced apoptosis was increased (Fig. 1s,t). Overall, these results show that ATF4 and IRE1 are not required for Rh-1-G69D expression to induce apoptosis.

Independently, we carried out a gene-overexpression screen with EPgy2 lines28 for modifiers of the gmr-Gal4-driven Rh-1 overexpression phenotype (Supplementary Fig. S3). Although a wild-type Rh-1 transgene was used in this experimental set-up, the system drives the expression of Rh-1 beyond the folding capacity of the imaginal disc cells, as indicated by the activation of ER stress reporters5. We specifically screened 400 lines with insertions in the third chromosomes that were associated with genes with annotated function and scored a total of six suppressors. Among these suppressors were expected ones, including a line associated with hrd1 (P[EPgy2] sip3[EY11980]), whose effect on the Rh-1-G69D mis-expression phenotype was independently validated in Supplementary Fig. S1. Another expected suppressor line was P[EPgy2] th[EY00710], with the P[EPgy2] element inserted upstream of the anti-apoptotic gene encoding Drosophila IAP1 (Dia1; ref. 29), indicating that excessive apoptosis contributes to the Rh-1-overexpression phenotype.

We also identified an enhancer of the Rh-1-overexpression phenotype, EY02276, associated with the mekk1 locus. Previous studies have characterized mekk1 as an osmotic-stress response gene that lies upstream of JNK and p38 kinases30,31. This line did not show any overexpression-associated phenotype on its own, but enhanced the Rh-1-overexpression phenotype when co-expressed (Supplementary Fig. S3). Conversely, the Rh-1-G69D mis-expression phenotype was sup-

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**Figure 2** *Drosophila* MEKK1 is required for Rh-1-G69D to trigger apoptosis. (a–c) External adult eyes. A control adult eye with wild-type morphology is shown in a. The degree of eye ablation as a result of Rh-1-G69D mis-expression (b) was suppressed in a mekk1+/–/+ background (c). (d–f) Apoptosis in eye discs as assessed through TUNEL labelling (magenta). A control eye disc shows little apoptosis (d). Massive apoptosis caused by Rh-1-G69D mis-expression (e) is strongly suppressed in a mekk1+/–/+ background (f). (g–i) The degree of ER stress as estimated through the XBP1-EGFP reporter (green). Control eye discs show little signs of ER stress (g). The degree of XBP1-EGFP reporter activation by Rh-1-G69D mis-expression is similar between mekk1+/- discs (h) and mekk1+/–/+ discs (i). (j–l) Anti-ATF4 antibody labelling (red) is not affected by mekk1. j shows a control disc. ATF4 is induced in Rh-1-G69D-expressing discs (k), and is not affected in a mekk1+/–/+ background (l). (m) mekk1 mutants are more resistant to tunicamycin feeding. Four- to five-day-old male flies (20–25 flies in each vial) were allowed to feed for 7 days on standard cornmeal medium supplemented with 5µg ml⁻¹ tunicamycin. The percentage indicates the number of flies surviving after feeding on tunicamycin (n = 3, P = 0.0062). The scale bar in d represents 100 µm for all panels. Error bars show ± s.e.m. Genotypes: gmr-Gal4/+ (a,j), gmr-Gal4/UAS-Rh-1-G69D;+/+(b,e,k), gmr-Gal4/UAS-Rh-1-G69D; mekk1+/–/+ (c,f,l), y w, gmr-Gal4/UAS-xbp1-EGFP;+/+(g), gmr-Gal4, UAS-Rh-1-G69D/UAS-xbp1-EGFP;+/+(h), gmr-Gal4, UAS-Rh-1-G69D/UAS-xbp1-EGFP; mekk1+/–/+ (i).
MEKK1 and CDK5 mediate JNK signalling activation in response to stress. (a) Thapsigargin (Tg) treatment induces CDK5 phosphorylation in a manner that is dependent on cdk5 and mekk1. Cells pre-treated with dsRNA against cdk5 or mekk1 reduces the level of JNK phosphorylation, whereas dsRNAs against ire1, traf4, atf6 and perk do not have obvious effects. (b) The role of cdk5 was further assessed by pre-treating cells with the CDK5 inhibitor roscovitine (DMSO; lanes 13). (c) To determine the role of MEKK1 and CDK5 mediate JNK signalling activation in response to stress in the ER, we assessed the degree of XBP1 expression in cells pre-treated with dsRNAs either against mekk1 or against EGFP (lanes 1–3) or against cdk5 or against EGFP (lanes 1–3) or against cdk5 or against EGFP (lanes 4–6). The anti-CDK5 blot (middle panel) shows the degree of CDK5 knockdown by RNAi. (d) Quantification of phospho-JNK bands 1 h after H2O2 treatment, from cells pre-treated with dsRNAs against either EGFP or against cdk5, shows a statistically significant change (n = 3, P = 0.018). (e) H2O2-induced JNK phosphorylation in cells pre-treated with dsRNAs against either EGFP (lanes 1–3) or mekk1 (4–6). The anti-MEKK1 blot (middle panel) shows the degree of MEKK1 knockdown by RNAi. (f) Quantification of phospho-JNK bands 1 h after H2O2 treatment, from cells pre-treated with dsRNAs against either EGFP or mekk1, shows a statistically significant difference (n = 3, P = 0.0023). (g–i) Rh-1G69D expression (green) activates JNK signalling in eye imaginal discs, as evidenced by the JNK reporter puc–lacZ (magenta; h,i). h shows the anti-beta-Gal single channel of i. g is a negative control without Rh-1G69D expression. (j–l) The requirement of bsk (Drosophila JNK) in Rh-1G69D-induced apoptosis. A control bsk+ disc expressing Rh-1G69D shows many TUNEL-positive cells (magenta; j), the number of which is suppressed in discs with bsk–/– nuclei (k,l). bsk–/– clones are marked by the absence of GFP (green). The scale bar in g represents 100 μm for all panels. Genotypes: gmr–Gal4/+; pucCΔE69D/+ (g), gmr–Gal4, UAS Rh-1G69D/+, pucCΔE69D/+ (h,i), gmr–Gal4, ey-flp/UAS–Rh-1G69D/ubi–GFP, FRT40A, +/+/ (j), gmr–Gal4, ey-flp/+;bskΔE69D, FRT40A, UAS–Rh-1G69D/ubi–GFP, FRT40A, +/+/ (k,l). Uncropped images of blots are shown in Supplementary Fig. S6.

As previous studies have placed mekk1 genetically upstream of JNK (ref. 30), we examined the relationship between JNK, MEKK1 and the ER stress response.
and CDK5. For this, we exposed *Drosophila* S2 cells to thapsigargin, a SERCA inhibitor that is widely used to cause ER stress in cells32. Phospho-JNK appeared after 2 h of thapsigargin treatment (Fig. 3a), and this induction of JNK phosphorylation was suppressed on knockdown of cdk5 or mekk1 (Fig. 3a), or when cells were treated with the CDK5 inhibitor, roscovitine (Fig. 3b). On the other hand, knockdown of other known mediators of the UPR, such as ire1, traf4, perk and atf6, had no discernible effects on JNK phosphorylation (Fig. 3a). Among other stress conditions examined, H2O2 treatment generated a similar outcome (Fig. 3c–f). The ability of H2O2 to induce JNK phosphorylation was significantly reduced in S2 cells pre-treated with double-stranded RNA (dsRNA) targeting cdk5 or mekk1. Whereas H2O2 treatment resulted in a more than sixfold increase in phospho-JNK levels in control cells, cdk5-knocked-down cells had on average only a twofold increase in phospho-JNK induction (Fig. 3d; n = 3, P = 0.018). Similarly, mekk1 knockdown reduced the extent of phospho-JNK induction in a statistically significant manner (Fig. 3f; n = 3, P = 0.0023).

Consistent with the results from S2 cells, Rh−1G47D mis-expressing imaginal discs showed signs of JNK signalling activation, as assessed through the puc–lacZ reporter (Fig. 3g–i). To determine whether JNK is required for ER-stress-induced apoptosis, we generated loss-of-function mosaic clones of the *Drosophila* JNK gene, *basket*. When Rh−1G47D was overexpressed in imaginal discs harbouring *basket−/−* clones, the number of apoptotic cells as assessed through TUNEL labelling was significantly reduced, with the remaining apoptotic cells primarily within the *basket*+ mosaic clones (Fig. 3j–l). We noticed that

**Figure 4** CDK5 phosphorylates MEKK1. (a) Conserved CDK5 consensus phosphorylation sites within MEKK1 of various *Drosophila* species. (b) CDK5 phosphorylates wild-type MEKK1 (MEKK1WT) on the Ser 1127 residue in vitro. Immunopurified MEKK1 was incubated with recombinant CDK5 and p35, and subsequently probed with an antibody against the phospho-Ser-1127 residue of MEKK1 (anti-p-MEKK1). Anti-Flag blots show total Flag-tagged MEKK1 levels (lower panel). (c) Phosphorylation at MEKK1 in transfected cells. The Flag-tagged MEKK1 was immunoprecipitated and probed with the anti-p-MEKK1 antibody. The average intensities of phospho-MEKK1 bands are shown in a histogram underneath the blot. Only CDK5-transfected cells show a statistically significant increase in the MEKK1 phosphorylation level after H2O2 treatment (n = 3, P = 0.0003). (d) Validation of the MEKK1 phosphorylation sites. HEK293T cells were transfected with CDK5, together with the indicated expression plasmids marked above the blot. Phospho-MEKK1 bands do not appear when a mutant MEKK1 plasmid lacking the putative phosphorylation sites is transfected (lanes 5 and 6). Anti-Flag blots show Flag-tagged MEKK1 levels (middle blot), and anti-HA bands show transfected CDK5 levels (lower blot). (e) MEKK1 phosphorylation bands disappear after phosphatase treatment (lane 4). Immunoprecipitated complexes were either untreated or treated with α-phosphatase (PP) before western blot analysis. (f) Co-immunoprecipitation of CDK5 and MEKK1. HEK293T cells were transfected with Flag-tagged wild-type MEKK1, together with HA-tagged wild-type CDK5. The protein complexes were immunoprecipitated using anti-Flag antibody and analysed by western blotting using anti-HA antibody. The interaction between wild-type CDK5 and wild-type MEKK1 was enhanced when cells were pre-treated with H2O2 (lane 2). WCL, whole-cell lysate; IP: immunoprecipitate; IB: immunoblot. Uncropped images of blots are shown in Supplementary Fig. S6.
Figure 5 The course of late-onset retinal degeneration of ninaE<sup>G69D</sup>+/+ flies is delayed on knockdown of CDK5, or in the mekk1<sup>l<sub>36</sub>-/—</sup> background. (a) Quantification of the degeneration process using the Rh1 > GFP fluorescence pattern. For each genotype, the percentage indicates the number of flies with intact ommatidial arrays as evidenced by the Rh1 > GFP pattern, from an average of eight independent crosses. Loss of mekk1 function delays the course of retinal degeneration of ninaE<sup>G69D</sup>/flies (n = 8, P = 0.0062). (b–e) Representative images of 20-day-old adult eye tangential sections. Genotypes are as indicated in the panels. Wild-type flies show clusters of seven rhabdomeres (stained as black circles) in a trapezoidal pattern within each ommatidia. Whereas this pattern is disrupted in the ninaE<sup>G69D</sup>+/retina, this degenerative phenotype is suppressed in ninaE<sup>G69D</sup>+/ flies with a mekk1<sup>l<sub>36</sub>-/—</sup> background. (f) The knockdown of CDK5 suppresses late-onset retinal degeneration of ninaE<sup>G69D</sup>+/+ flies (n = 5, P = 0.0004). IR, inverted repeat. (g,h) Representative images of 20-day-old adult retina downregulating CDK5 in the ninaE<sup>G69D</sup>+/+ background.

many apoptotic cells were found at the clonal boundaries. This property was also observed in mutant mosaic clones of dronc (Supplementary Fig. S4), which is an essential initiator caspase for apoptosis<sup>33,34</sup>. These observations support the idea that ER stress activates CDK5/MEKK1-mediated JNK signalling to cause caspase-dependent apoptosis.

Using a phosphorylation site prediction program (http://scansite.mit.edu), we detected two consensus CDK5 phosphorylation sites within the Drosophila melanogaster MEKK1 protein sequence, Thr 157 and Ser 1127. The putative phosphorylation sites within MEKK1 were conserved in other Drosophila species, indicative of its functional significance (Fig. 4a). To determine whether MEKK1 is in fact phosphorylated by CDK5, we generated antibodies directed against the putative phospho-residues (see Methods). Using one of these, an antibody directed towards the phosphorylated Ser-1127 residue, we were able to detect MEKK1 phosphorylation by CDK5 in vitro (Fig. 4b). We also detected phosphorylation of this residue in cultured HEK293T cells transfected with Flag-tagged MEKK1 (Fig. 4c). Notably, the intensity of the phospho-Ser-1127 band increased significantly in cells when CDK5 was co-transfected, and further enhanced when those cells were stressed with H<sub>2</sub>O<sub>2</sub> (Fig. 4c, lanes 3 and 4). On average, the degree of MEKK1 phosphorylation increased more than threefold after H<sub>2</sub>O<sub>2</sub> treatment (Fig. 4c, n = 3, P = 0.0003). We confirmed that this band corresponds to phospho-Ser-1127, as the signal did not appear when the MEKK1 Ser-1127 residue was mutated (Fig. 4d, lanes 5 and 6). Furthermore, the anti-phospho-MEKK1 failed to detect any band when the immunoprecipitate was treated with the λ-phosphatase (Fig. 4e). Moreover, the two proteins physically interacted, as evidenced by co-immunoprecipitation assays. Interestingly, the interaction was enhanced when the cells were pre-treated with H<sub>2</sub>O<sub>2</sub> (Fig. 4f). Taken together, these genetic and biochemical experiments support the idea that CDK5 and MEKK1 form a pathway to activate JNK signalling in response to ER stress.

To determine whether this pro-apoptotic signalling pathway is also relevant to an age-dependent disease process, we turned to the Drosophila model for ADRP, where an endogenous mutant allele of the Rh-1 gene, ninaE<sup>G69D</sup>, causes the late-onset retinal degeneration phenotype associated with ER stress<sup>9,10</sup>. To track the course of retinal degeneration in live flies, we used the ninaE<sup>G69D</sup>+/ condition combined with a Rh-1 > GFP reporter<sup>35</sup>. Nearly 90% of ninaE<sup>G69D</sup>+/ flies lost the regular ommatidial array by day 28 after eclosion, indicative of age-related retinal degeneration. Those ninaE<sup>G69D</sup>+/ flies in a mekk1<sup>l<sub>36</sub>-/—</sup> background showed a delayed course of retinal degeneration, with only about half of the examined flies with disrupted Rh-1 > GFP patterns (Fig. 5a). Knockdown of cdk5 in the photoreceptors also delayed the course of retinal degeneration to a similar degree (Fig. 5f). This result was further validated through tangential sections of 20-day-old fly retina. Wild-type flies showed
regular ommatidial arrays (Fig. 5b), whereas the ninaED\textsuperscript{GFP} / + retina by this age showed disorganized ommatidia (Fig. 5c,g). This phenotype was largely rescued in the background of mekk1\textsuperscript{-/+} / - (Fig. 5d), or in cdk5-knockdown conditions (Fig. 5h).

These results indicate that the pro-apoptotic ER-stress response mediated by mekk1 and cdk5 is relevant to understanding age-related photoreceptor degeneration in ADRP. Moreover, our results indicate that CDK5/MEKK1/JNK forms a pathway that is independent of those UPR branches. Although it is unclear what lies upstream of CDK5 in our experimental system, we note that among the previously characterized CDK5-activating signals are ROS, calpains and Cam kinase II (refs 23, 25,36,37), which have also been associated with ER stress\textsuperscript{16,38}. Thus, it is possible to imagine a model where chronic proteotoxicity in the ER sends CDK5-activating signals to the cytoplasm, perhaps through ROS- or Ca\textsuperscript{2+}-mediated signalling. Once CDK5 is activated, it may send pro-apoptotic signals to the nucleus through the MEKK1/JNK pathway (Supplementary Fig. S5).

Many terminally differentiated cells without regenerative potential are known to acquire resistance to apoptosis during differentiation. In Drosophila, such apoptotic resistance can be attributed to the epigenetic silencing of major pro-apoptotic gene loci during development\textsuperscript{39}. This study showed that one of the consequences of stress-induced MEK1 signalling is to induce the expression of genes that are normally silenced through epigenetic mechanisms\textsuperscript{31}. On the basis of these observations, it is possible that terminally differentiated photoreceptors may have their pro-apoptotic loci in heterochromatin-like states, and the stress-induced CDK5/MEK1 pathway contributes to neurodegeneration by restoring those loci to an open chromatin state, an idea that needs to be investigated through future studies.

### METHODS

Methods and any associated references are available in the online version of the paper at www.nature.com/naturecellbiology

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**AUTHOR CONTRIBUTIONS**

M-J.K. and H.D.R. designed the experiments. J.C. carried out the EPgy2 screen. All authors read and edited the manuscript.

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The authors declare no competing financial interests.

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METHODS

Fly stocks and crosses. Genes were expressed in Drosophila eyes through the standard Gal4/UAS system.\(^6\) The specific genotypes for individual experiments are labelled in the figure legends. UAS-dtp1EGFP, UAS-Rh-\(^{1800}\) and UAS-Hrd1 (refs 3, 5) have been described previously. UAS-Rh-\(^{1800}\) was targeted for insertion into the 2R chromosome (5ID) using the phiC31 integration system. The transgenes were mis-expressed through the gnr–Gal80 driver. Mosaic loss-of-function clones were induced through the standard FLP/FRT system\(^7\). The loss-of-function mutant strain bsk\(^{1780}\) has been described previously\(^8\). The puc\(^{1800}\) allele was used to monitor JNK activation\(^9\). The D-mekki\(^{1800}\) allele and Flag-tagged Mekki complementary DNA (ref. 30) were used for cell culture and RNAi treatment.

Cell culture and RNAi treatment. Drosophila Schneider S2 cells were cultured in DME supplemented with 25 mM glucose, 20% FCS and 55 mM β-mercaptoethanol. CDK5 knockdown was achieved using Mission short hairpin RNA (shRNA)-encoding lentiviruses directed to mouse CDK5 mRNA (Sigma-Aldrich; GenBank/EMBL/DDBJ accession no. NM_007668.3), following the manufacturer’s protocols. Knockdown clone CDK5 was targeted with shRNA TRCN0000278085. After transduction and selection with puromycin at 1.8 μg/ml for 7 d, the cells were exposed to 5 mg/ml\(^2\) of tunicamycin for 18 h and fixed and stained with TUNEL labelling kit (ApopTag Red In Situ Apoptosis Detection Kit, Millipore).

In vitro kinase assay. Histidine-tagged CDK5 and p35 were expressed in Escherichia coli (BL21 strain) and purified using a Ni-NTA His-Bind Superflow kit (Novagen). Flag-tagged Mekki was immunopurified using anti-Flag antibody and protein G-Sepharose beads (Roche). Immunopurified wild-type Mekki was incubated with CDK5/p35 in kinase assay buffer (20 mM HEPES, 5 mM EGTA, 7.5 mM MgCl\(_2\), 1 mM dithiothreitol, protease inhibitor and phosphatase inhibitor (Roche) containing 50 μM ATP) for 60 min at 30 °C. The phospho-ylated proteins were subjected to SDS–PAGE and blotted with anti-phospho-MEK1\(^{1187}\) antibody.

Analysis of retinal degeneration. Flies with the relevant genotypes were crossed into the cn, br-/- background to eliminate eye pigments, which may otherwise affect the course of retinal degeneration. These flies were selected and reared in vials (30–50 flies in each vial), in permanent light at 25 °C. The vials were changed frequently to avoid mixing the flies with eventual progeny. The quantification of pseudopupils was carried out on a pad under blue fluorescent light after anaesthetizing the flies with CO\(_2\). Cross-sectioning was carried out as described previously\(^10\) and toluidine blue was used as a dye to increase the contrast.

Scanning electron microscopy. Standard procedures were used for sample preparation, including fixation in 2% glutaraldehyde, dehydration and drying in HMDS solvent. Gold–paladium was used to coat adult flies and images were taken at ×180 and ×1,000 magnification.

Statistics. Statistical significance was applied through unpaired Student’s \(t\)-test analyses. Error bars represent s.e.m. throughout.

Immunoprecipitation. Cells were lysed in a lysis buffer (50 mM Tris–Cl (pH 8.0), 150 mM NaCl, digitonin and 1% Triton X-100) for 20 min, and centrifuged at 16, 100 g. The resulting supernatant was used for subsequent immunoprecipitation. Immunoprecipitation was carried out with anti-Flag (Sigma) and protein-G-coupled Sepharose beads (Roche). Beads were washed in low-ionic-strength buffer (50 mM Tris–Cl (pH 8.0), 100 mM NaCl and 1% Triton X-100) three times, and boiled in Laemmli’s sample buffer for standard western blots assays.

Cell culture and RNAi treatment. Drosophila Schneider S2 cells were cultured following the vendor protocols (Invitrogen). A standard protocol was followed for dsRNA treatment of cultured cells\(^4\). In brief, 10\(^2\) cells were split into 6-well dishes 1 day before treatment with dsRNA. The next day, 20 μg of mekki dsRNA or cdk5 dsRNA was added to each well, which was followed by another boost of 20 μg dsRNA at day 4. The cells were split at day 6 and treated with thapsigargin or H\(_2\)O\(_2\) at day 7 to detect MAPK activation. The mekki dsRNA consisted of a 503-nucleotide region (Ambion Inc: DRSC16302) described by the Drosophila RNAi Screening Center (http://www.flyrnai.org). The following primers were used to amplify this sequence from a S2 cell cDNA library: ‘R’ primer, 5’-GAGACCGTCTCAGGCCA-3’; ‘S’ primer, 5’-CTGTGAGCAGCATTGTG-3’. This amplicon has no predicted off-targets.

The cdk5 dsRNA consisted of a 351-nucleotide region (Ambion Inc: DRSC07359). The following primers were used to amplify this sequence from an embryo cDNA library: ‘R’ primer, 5’-GATCGGATCTGAAACC-3’; ‘S’ primer, 5’-GGAAGCAGCAGTGATC-3’. This amplicon has no predicted off-targets.

Mint cells were cultured in DME supplemented with 25 mM glucose, 20% FCS and 55 mM β-mercaptoethanol. CDK5 knockdown was achieved using Mission short hairpin RNA (shRNA)-encoding lentiviruses directed to mouse CDK5 mRNA (Sigma-Aldrich; GenBank/EMBL/DDBJ accession no. NM_007668.3), following the manufacturer’s protocols. Knockdown clone CDK5 was targeted with shRNA TRCN0000278085. After transduction and selection with puromycin at 1.8 μg/ml for 7 d, the cells were exposed to 5 mg/ml\(^2\) of tunicamycin for 18 h and fixed and stained with TUNEL labelling kit (ApopTag Red In Situ Apoptosis Detection Kit, Millipore).

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Figure S1 Establishment of a Drosophila eye based assay system to study ER stress response. Indicated genes were misexpressed in the developing eyes using the gmr-Gal4 driver. (A-C) Scanning EM image of external adult eyes. (A) Wild type fly eye. Expression of Rh-1<sup>G69D</sup> in larval eye discs generates small adult eyes with abnormally smooth surface (B), which is suppressed when hrd1 is co-expressed (C). (D, E) Eye imaginal discs expressing the ER stress reporter XBP1-EGFP alone (D), or together with Rh-1<sup>G69D</sup> (E). (F) DTT-induced Unfolded Protein Response assessed from eye disc extracts. Left panel: An anti-ATF4 western blot with control disc extracts (lane 1) or with those exposed to DTT (lane 2). Anti-β-tubulin blot is shown as a loading control (lower panel). Right panel: DTT treatment induces other UPR responses. Eye imaginal discs were treated with DTT for 6 h and levels of XBP1 mRNA splicing were determined by RT-PCR. Spliced and unspliced PCR fragments are indicated as Xbp1<sub>S</sub> and Xbp1<sub>U</sub>. The Hsc3 gel shows semi-quantitative RT-PCR of BiP/Hsc3 upon DTT treatment. CG16974 is used as a loading control (bottom). (G-I) Eye imaginal discs labeled with anti-ATF4 antibody (red) shows ATF4 induction upon Rh-1<sup>G69D</sup> misexpression (H), but not in control discs (G). Such ATF4 induction was suppressed when hrd1 was co-expressed (I). (J-L) Superoxide indicator DHE (red) shows evidence of ROS generation in response to Rh-1<sup>G69D</sup> overexpression (K), but not in control eye discs (J). ROS accumulation was reduced by Hrd1 overexpression. Scale bars represent 100 μm (A, D). Genotype: canton S (A), gmr-Gal4, UAS-Rh-1<sup>G69D</sup>/UAS-lacZ (B, H, K), gmr-Gal4, UAS-Rh-1<sup>G69D</sup>/UAS-hrd1 (C, I, L), gmr-Gal4/+;UAS-xbp1-EGFP/+ (E), yw (G), gmr-Gal4/+ (J).
Figure S2 Cdk5 knockdown in mouse Min6 cells protect against tunicamycin treatment-induced apoptosis. Levels of apoptosis in Min6 cells were assessed by TUNEL labeling. Shown are representative images of Min6 cell lines stably expressing control shRNA (Scr: scrambled), or shRNA-Cdk5 that targets the mouse Cdk5 gene (TRCN0000278085). These cells were either exposed to 5 mg/ml of Tunicamycin for 18 hours, or not treated (labeled as NT). Scale bars present 50 μM.
Figure S3 An EP screen for suppressors of the Rh-1<sup>WT</sup> overexpression eye phenotype. External adult eyes of a wild type canton S fly (A), control gmr-Gal4/ fly (B), Rh-1<sup>WT</sup> and lacZ co-expressing fly (C) and those misexpressing Rh-1<sup>WT</sup> together with genetic modifiers identified by EP screen (D-J). Notably, the EPgy2 element EY00710 is associated with the thread (diap1) locus (D), EY11980 is with hrd1 (G), and EY02276 is with mekk1 (J). Genotypes: Canton S (A), gmr-Gal4/+ (B), gmr-Gal4/ UAS-lacZ/UAS-Rh-1<sup>WT</sup>/ (C), gmr-Gal4/+;UAS-Rh-1<sup>WT</sup>/EY00710 (D), gmr- Gal4/+;UAS-Rh-1<sup>WT</sup>/EY06824 (E), gmr-Gal4/+;UAS-Rh-1<sup>WT</sup>/EY08547 (F), gmr-Gal4/+;UAS-Rh-1<sup>WT</sup>/EY11980 (G), gmr-Gal4/+;UAS-Rh-1<sup>WT</sup>/EY12388 (H), gmr-Gal4/+;UAS-Rh-1<sup>WT</sup>/EY12810 (I), gmr-Gal4/+;UAS-Rh-1<sup>WT</sup>/ EY02276 (J).
Figure S4 Higher magnification view of Rh-1\textsuperscript{G69D} triggered apoptosis in apoptosis-defective mosaic clones. Shown are magnified views of eye imaginal discs overexpressing Rh-1\textsuperscript{G69D}. Mutant clones defective of apoptosis are marked by the absence of GFP. Specifically, shown are discs with clones of basket \textsuperscript{--} (A-C), or dronc \textsuperscript{--} (D-F). The pattern is similar, with many TUNEL positive cells appearing at clonal boundaries. Some TUNEL positive cells appear to be within the mutant clones, and this may be an illusion due to the apoptotic cells being engulfment by neighboring (mutant) cells. Alternatively, it may be due to non-autonomous death signaling by caspases, implicated in other neurodegenerative disorders (Burguillos et al., Nature (2011) 472(7343):319-324). Genotype: gmr-Gal4, ey-flp/+;bsk\textsuperscript{170B}, FRT40, UAS-Rh-1\textsuperscript{G69D}/ubi-GFP, FRT40;+/+ (A-C), gmr-Gal4, ey-flp/+;UAS-Rh-1\textsuperscript{G69D}/; dronc\textsuperscript{129}, FRT80/ubi-GFP, FRT80 (D-F).
**Figure S5** A Model for Rh-1G69D-induced Apoptosis. Misfolded protein overload in the ER activates a pro-apoptotic pathway that consists of Cdk5, its regulatory subunit p35 (Cdk5alpha) and Mekk1. These genes are required for Jun N-terminal Kinase activation and cell death. Mekk1 is a MAP KKK that is phosphorylated by Cdk5. While this pathway is required for apoptosis, it does not affect the overall levels of misfolded protein load in the ER, as assessed by the activity of other Unfolded Protein Response reporters. This is in contrast to Hrd1, which reduces toxicity by reducing misfolded protein load in the ER through ubiquitin-mediated protein degradation.
Figure S6 Full scan
Table S1 List of Drosophila lines screened in this study. (Left top) The list of RNAi lines that target Drosophila phosphatases. (Left bottom) The list of RNAi lines that target Drosophila kinases. The names of the targeted genes are listed on the left column. These lines were from the Vienna Drosophila RNAi Center, and their stock numbers are listed on the right column. (Right) List of EPgy2 lines used in the gene overexpression screen. The lines with insertions on the 3R chromosome are listed on the left column, and those on the 3L chromosome are listed on the right.