Ribosomopathy-associated mutations cause proteotoxic stress that is alleviated by TOR inhibition

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Ribosomes are multicellular molecular machines that synthesize all of the proteins of living cells. Most of the genes that encode the protein components of ribosomes are therefore essential. A reduction in gene dosage is often viable albeit deleterious and is associated with human syndromes, which are collectively known as ribosomopathies1–3. The cell biological basis of these pathologies has remained unclear. Here, we model human ribosomopathies in Drosophila and find widespread apoptosis and cellular stress in the resulting animals. This is not caused by insufficient protein synthesis, as reasonably expected. Instead, ribosomal protein deficiency elicits proteotoxic stress, which we suggest is caused by the accumulation of misfolded proteins that overwhelm the protein degradation machinery. We find that dampening the integrated stress response4 or autophagy increases the harm inflicted by ribosomal protein deficiency, suggesting that these activities could be cytoprotective. Inhibition of TOR activity—which decreases ribosomal protein production, slows down protein synthesis and stimulates autophagy5—reduces proteotoxic stress in our ribosomopathy model. Interventions that stimulate autophagy, combined with measures of boosting protein quality control, could form the basis of a therapeutic strategy for this class of diseases.

Ribosomopathies encompass a wide range of syndromes. Common symptoms include a reduced number of blood cells, predisposition to cancer, skeletal abnormalities and growth retardation1–3. Neurological defects are also observed, although less frequently1–4. A subset of these symptoms, including growth retardation and mild autism, but not anaemia, was recently reported for a child carrying a de novo mutation in a gene encoding a component of the 40S subunit (RPS2367K5,6,7). We introduced this mutation into Drosophila and characterized the resulting phenotype. We used CRISPR–Cas9 to replace the third exon with a version encoding the R67K allele. This is not a peculiarity of this mutation, as R67K behaves like a classical Minute mutation. Thus, the patient's mutation is the starting point of this study, it is expected to trigger the same cell biological response as other ribosomopathy-associated mutations.

Cell competition involves short-range interactions that enable WT cells to trigger apoptosis in Minute+− cells8. However, we found that apoptotic figures were widespread in wholly heterozygous RPS2367K+−imaginal discs, much more so than in WT discs (Fig. 1b−f). This observation suggests that RPS2367K+−cells undergo apoptosis independently of any signal from WT cells. This is not a peculiarity of this mutation, as RPS3 and RPL14 heterozygotes have been reported to undergo widespread apoptosis, as assayed by TUNEL staining9. Adding to this evidence, we found that imaginal discs lacking one copy of RPS13, RPL5 or RPL14 had numerous Dcp1+−cells (Extended Data Fig. 1h−j). It is therefore clear that RP-deficient cells undergo apoptosis in a cell-competition-independent manner. To confirm that this effect is tissue intrinsic, we generated a conditional allele of RPS26 (Fig. 1g), a gene that is associated with Diamond–Blackfan anaemia10. Inactivation of one homologue in the posterior half of the discs, leaving the rest of the animal genetically WT, triggered apoptosis in only the heterozygous region (Fig. 1h,i), implying that a systemic signal is not involved.

The high rate of apoptosis in Minute heterozygotes suggests that these cells are intrinsically stressed. This is consistent with the widespread activation of JNK signalling that has been reported in RPS3+− and RPS15+−imaginal discs11, an observation that we confirmed in RPS2367K+−, RPS367K+−, RPL5+−, RPL14+− and RPS1367K+−imaginal discs (Extended Data Fig. 1f−j). Another characteristic of Minute-mutant cells is that they upregulate Xrp1, a protein that was recently shown to be required for cell competition12–22, and associated with the response to oxidative stress23 and the DNA damage response24. We found that the rate of apoptosis in RPS2367K heterozygous tissues was strongly reduced by knocking down Xrp1 (Extended Data Fig. 2). Therefore, Xrp1 is an essential component of the response to the cellular stress caused by RP deficiency, irrespective of cell competition. Overall, the above considerations and additional observations in other models of ribosomopathies13,24 show that apoptosis and cellular stress are characteristic responses to RP deficiency.
What could be the proximal cause of stress and apoptosis in RP-deficient tissue? Wing precursors undergo a ~1,000-fold increase in tissue size during larval development, and it is conceivable that a limited number of functional ribosomes might not suffice to meet the associated protein demand. Protein synthesis has indeed recently been shown to be depressed in RP-deficient cells. We therefore investigated whether this is also true in RP-deficient tissue. We generated imaginal discs in which one half was heterozygous mutant for RPS23, Wing imaginal discs—in which one half is heterozygous mutant for RPS23, as confirmed by the lack of mCherry expression. Note the increased number of apoptotic figures.

Fig. 1 | Apoptosis and depressed protein synthesis in RP-deficient tissue. a, Diagram of the RPS23\(^{R67K}\) allele, showing the 5’ and 3’ UTRs (grey), the coding exons (orange), attP (black), loxP (blue) and the Pax-mCherry selection cassette (red). b, c, Wing imaginal discs from WT (b) and RPS23\(^{R67K/+}\) (c) larvae labelled with anti-Nub antibodies (red) to highlight the pouch, and anti-Dcp1 antibodies (green) to mark apoptotic cells. d, Apoptotic coverage (percentage of surface area) in WT (black dots) and RPS23\(^{R67K/+}\) imaginal discs (red dots). e, f, Accumulation of pyknotic nuclei in a RPS23\(^{R67K}\) heterozygous disc (e) and high magnification view (f). g, Diagram of the conditional RPS26 allele (RPS26\(^{KD}\); the colour code is as described in a; the FRT sites used for gene inactivation are shown in green). h, Homozygous RPS26\(^{KD}\) imaginal disc (WT for RPS26 activity) showing the expression of the tubulin-mCherry cassette and low-level apoptosis (Dcp1 immunoreactivity). I, RPS26\(^{KD}\) imaginal disc expressing OPP under the control of hedgehog-GAL4. The posterior compartment is therefore heterozygous for the RPS26 mutation, as confirmed by the lack of mCherry expression. Note the increased number of apoptotic figures. j, k, Wing imaginal discs—in which one half is heterozygous mutant for RPS23\(^{R67K}\) (j, left) or RPS26\(^{KD}\) (k, right), and the other half is genetically WT (j, right; k, left)—were stained to visualize puromycilated peptides after incubation for 15 min in 1 μM of OPP. In both cases, incorporation was depressed in the mutant compartment. l, m, Dcp1 immunoreactivity in a WT wing imaginal disc expressing Rheb\(^{RNAi}\) in the anterior compartment (l) or an RPS23\(^{R67K/+}\) wing imaginal disc overexpressing Rheb in the anterior compartment (m). The anterior compartment is shown on the left, marked with anti-Ci antibodies (red). For d, data are mean±s.d. n=10 discs per genotype. Statistical analysis was performed using a two-tailed unpaired t-test. ***P<2.87×10^-7.

For b, c, e, f and h-m, scale bars, 50 μm. Genotypes for each figure panel are available in Supplementary Table 1. Source data are available online.
Expression of an RNA interference (RNAi) transgene against Rhei, a TOR activator, led to a decrease in OPP incorporation, whereas Rhei overexpression led to increase in OPP incorporation (Extended Data Fig. 3). Rhei knockdown in the anterior compartment was associated with a decrease in tissue size, as expected. However, this was not accompanied by increased apoptosis (Fig. 1I). By contrast, stimulation of TOR signalling by Rhei overexpression did not prevent, and even possibly enhanced, apoptosis in RP-deficient tissue (Fig. 1m). These results indicate that, although translation is depressed in RP-deficient cells, this is probably not the trigger of apoptosis in these cells. In fact, RPS23KO/KO cells do not seem to be limited in their ability to produce proteins since, upon inhibition of apoptosis, they gave rise to tumours, therefore generating biomass (Extended Data Fig. 4).

In the course of optimizing the conditions for OPP incorporation, we noticed that, at high doses and/or extended incorporation times (for example, 20 μM for 30 min), the OPP signal accumulated preferentially in RPS23KO/KO or RPS26KO/KO tissue compared with in the WT territory (Fig. 2a,b). This counterintuitive observation can be rationalized if one considers the fact that puromycin causes premature translation termination in a dose-dependent manner, with the resulting truncated polypeptides often failing to fold properly36. These defective ribosomal products are normally recognized and cleared by the ubiquitin–proteasome system and autophagy37,38. However, under conditions of impaired protein quality control response, the burden caused by OPP treatment becomes overwhelming and puromycylated defective ribosomal products accumulate in aggregates39. Interestingly, puromycylated polypeptides accumulated in large punctae after a high dose of OPP (20 μM for 30 min) and this was much more pronounced in the mutant compartment (RPS23KO/KO or RPS26KO/KO) compared with in the control tissue. This observation suggests that the disposal of misfolded proteins could be impaired in RP-deficient tissue.

To further test the ability of RP-deficient cells to face proteotoxic stress, we assessed how they handle an aggregation-prone protein. Huntington’s disease is characterized by polyglutamine expansion in the Huntingtin protein (HTT), which, over a threshold length (more than 36X Glu (36Q)), promotes the formation of oligomers and aggregates40. Shorter polyglutamine stretches are not considered to be pathogenic, but the behaviour of subpathological HTT25-Q truncations remains to be investigated. As expected, the fluorescence of HTT25-Q–cerulean in otherwise WT tissue was largely homogeneous, with only a few small clusters (Fig. 2c). However, in the RPS23KO/KO background, this protein accumulated in large punctae (Fig. 2d,e). These observations suggest that RP-deficient cells are less able than their WT counterparts to prevent the accumulation of aggregate-prone species such as truncated OPP-peptides or polyglutamine repeats. Expression of HTT53Q in an otherwise WT background leads to excess apoptosis (Extended Data Fig. 5), indicating that even WT cells cannot overcome a large number of repeats. We conclude that proteotoxic stress is sufficient to trigger apoptosis and RP-deficient cells are more readily overwhelmed by such stress compared with WT cells.

Hallmarks of proteotoxic stress include increased ubiquitination, which targets proteins for degradation, stimulation of autophagy and enlargement of lysosomes. Ubiquitination, assayed using an antibody, was higher in RPS23KO/KO compared with in control tissue (Fig. 2f,g). The p62 protein (also known as SQSTM1 or sequestosome-1), which is a ubiquitin-binding adapter that functions both as a receptor and cargo for selective autophagy37, also accumulated specifically in RPS23KO/KO (Fig. 2h,i) as well as in RPS26KO/KO cells (Extended Data Fig. 6a). A similar accumulation was observed in human cells (HEK293) that were engineered using CRISPR–Cas9 to carry this mutation (RPS23KO/KO/7; Fig. 2j–l). Correspondingly, the lysotracker-labelled acidic compartment, a proxy for lysosomes, was enlarged in the RPS23KO/KO territory (Fig. 2m), suggesting a high load on autophagy. To further assess autophagy, we took advantage of a dual-colour GFP–mCherry–Atg6a reporter41, which marks autophagosomes and autolysosomes by virtue of the differential sensitivity of GFP and mCherry to acidification (Extended Data Fig. 7a). Both markers were increased in RPS23KO/KO imaginal discs, suggesting altered progression through the autophagic pathway (Extended Data Fig. 7b,c). This observation combined with the accumulation of p62 punctae (Fig. 2h,i) indicates that, as a result of RP deficiency, ubiquitinated substrates exceeds the cell’s degradative capacity, a situation that probably causes proteotoxic stress. Proteotoxic stress, and the ensuing integrated stress response (ISR), is manifest by phosphorylation of the eukaryotic initiator factor 2 alpha (eIF2α). To investigate whether the ISR is activated in RP-deficient cells, we stained imaginal discs with antibodies against phosphorylated eIF2α (P-eIF2α). The signal was distinctly higher in RPS23KO/KO and RPS26KO/KO tissues than in the control half of the discs (Fig. 2n and Extended Data Fig. 6b). Thus, we conclude that RP-deficient cells experience proteotoxic stress and activate the ISR as a result.

Proteotoxic stress arises from the accumulation of misfolded proteins and/or the formation of protein aggregates. What could be the origin of such proteins in RP-deficient tissues? One can imagine that, in the absence of one functional protein component, dysfunctional ribosomes form. These could be less conducive to cotranslational folding and therefore generate an inordinate amount of unfolded proteins37. Alternatively, abnormal stoichiometry of ribosomal proteins could affect the translation of a subset of mRNAs and therefore upset the relative amounts of different proteins42, with excess proteins requiring additional degradation capacity. Another possibility is that partially functional ribosomes could be prone to making mistakes and therefore producing misfolded proteins. Translational fidelity has indeed been reported to be reduced in cultured RPS23KO/KO fibroblasts43. To determine whether this is also the case in RPS23KO/KO animals, we generated transgenic flies expressing a stop codon readthrough reporter inspired by the design of Grentzmann et al.44. This comprised DNA encoding firefly luciferase (Fluc) and nanoluciferase (Nluc) separated by a tetranucleotide

Fig. 2 | Hallmarks of proteotoxic stress in RP-deficient cells. a,b, Wing discs with one half RPS23KO/KO (a, left) or RPS26KO/KO (b, right), which were labelled to visualize puromycylated peptides after incubation for 30 min in 20 μM OPP. c,d, Wing primordia from WT (c) or RPS23KO/KO (d) larvae expressing HTT53Q–cerulean throughout the pouch (under the control of pdm2-GAL4). e, Quantification of HTT53Q punctae in RPS23KO/KO tissue (red dots, genotype as described in d; n = 7 discs). f,g, Anti-ubiquitin staining of a mosaic wing disc of which the anterior half is RPS26KO/KO (f). Increased puncta staining in the anterior compartment (left of the red dotted line) expressed as a ratio with the posterior control (g). n = 13 discs. h,i, Anti-p62 staining of the same genotype as described in (h) and quantified as described in (g). n = 9 discs. j,k, WT (j) or RPS26KO/KO (k) HEK293 cells were stained with anti-p62 antibodies (green) and 4,6-diamidino-2-phenylindole (DAPI; red). l, Coverage of p62 immunoreactivity in RPS23KO/KO (n = 13 images) relative to that in WT HEK293 cells (n = 11 images). m, Lysotracker staining in a wing disc with one half RPS23KO/KO, n, p-eIF2α immunoreactivity in a wing disc with one half RPS23KO/KO. For e, g, i and l, data are mean ± s.d. Statistical analysis was performed using two-tailed unpaired t-tests. **P = 5.81 × 10−8 (e), **P = 1.08 × 10−3 (g), ***P = 1.96 × 10−4 (i), ****P = 1.07 × 10−3 (l). For a–d, f, h, j, k, m and n, scale bars, 50 μm (a–h, m and n) and 10 μm (j and k). A list of the genotypes for each figure panel is provided in Supplementary Table 1. Source data are available online.
termination signal (UGAC, with UGA the termination triplet) and a flexible linker (Extended Data Fig. 8a). Thus, Nluc luminescence is expected only if an amino acid is wrongly incorporated at the stop codon and the ratio of Nluc to Fluc therefore provides an estimate of the rate of ribosomal mistakes. This reporter was validated in animals fed for 48h with G418, which causes stop codon

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**Figure Legends**

- **Figure 8a**: Images showing the effect of G418 on luminescence in wild-type (WT) and RPS23 R67K/+ mice.
- **Figure 8b**: Graphs illustrating the quantification of luminescence ratios in different genotypes.
- **Figure 8c**: Immunostaining images for ubiquitin expression in HEK293 cells with and without HTT25Q.
- **Figure 8d**: Quantification of p62 body area in HEK293 cells with and without RPS23 R67K/+.
- **Figure 8e**: Analysis of puncta area after treatment with OPP in WT and RPS23 R67K/+.
Ribosomal proteins comprise a large proportion of cellular proteins; in humans, they represent about 6% of the proteome by mass. In the absence of a single RP species, unassembled ribosomal proteins have a tendency to aggregate and need to be disposed of as they could lead to proteotoxic stress. To examine the possible contribution of orphan RPs to proteotoxic stress in RP-deficient tissues, we compared the proteome of RPS23<sup>67K/+</sup> relative to WT imaginal discs. On average, proteins of the small subunit (RPS, orange circles) are under-represented, while those of the large subunit (RPL, blue circles) are enriched in mutant discs. Differential effect of RP deficiency on the abundance of small-subunit (orange) and large-subunit (blue) proteins. As only half of the complement of functional RPS23 is produced in the RPS23<sup>67K</sup> heterozygotes, a large number of small-subunit proteins are orphaned and must be disposed of. By contrast, all of the large-subunit proteins produced by the cell can assemble in complete subunits. A list of the coloured proteins highlighted in a is provided in Supplementary Table 2.

As shown above, RP-deficient cells respond to proteotoxic stress by stimulating the phosphorylation of eIF2α. We now address whether this response is maladaptive or cytoprotective. To reduce the p-eIF2α pool, we overexpressed the conserved GADD34 protein, a substrate-selective regulatory subunit of the catalytic protein phosphatase 1 (PP1). As expected, GADD34 overexpression led to a decrease in p-eIF2α immunoreactivity in both WT and RPS23<sup>67K/+</sup> imaginal discs (Extended Data Fig. 9a–d). In otherwise WT animals, but not RPS23<sup>67K/+</sup> animals, this was accompanied by a relatively small, albeit significant, increase in tissue size (Extended Data Fig. 9e,f). Crucially, GADD34 overexpression increased apoptosis by about threefold in RPS23<sup>67K/+</sup> tissue despite having no or little impact on the rate of apoptosis in WT discs (Fig. 4a–d). Moreover, GADD34 overexpression led to an increase in the number of HTT<sup>210</sup> punctae in RPS23<sup>67K/+</sup> discs (Extended Data Fig. 9g–i). These observations suggest that the p-eIF2α contributes to limiting the proteotoxic stress caused by RP deficiency. We next investigated whether autophagy is also part of a cytoprotective process. Expression of an RNAi transgene against autophagy-related gene 1 (Atg1), which is required for autophagosome formation, had no detectable effect on the rate of apoptosis in otherwise WT tissue (Fig. 4e,h). However, expression of the same Atg1<sup>R86H</sup> transgene substantially enhanced the rate of apoptosis in RP-deficient cells (Fig. 4f–h). This implies that, conversely, stimulation of autophagy could dampen proteotoxic stress in RP-deficient cells. The contribution of the proteasome could not be unambiguously determined (Extended Data Fig. 10a–c) for lack of suitable genetic tools. Similarly, the relative importance of mechanisms involving heat-shock proteins, the unfolded protein response and/or dampening of translation also remains to be assessed.

Having established that p-eIF2α and autophagy limits the toxic effects of RP deficiency, we sought to experimentally emulate their downstream effects, with the hope of opening therapeutic avenues. One signalling pathway, that mediated by TOR, stands out since it...
regulates many of the relevant processes, including autophagy, translation and ribosome biogenesis. To test whether TOR inhibition could reduce proteotoxic stress and apoptosis in RP-deficient tissues, an RNAi transgene against Rheb was expressed in the anterior half of imaginal discs that were wholly heterozygous for $RPS23^{G67K}$ (Fig. 5a, compare Dcp1 immunoreactivity in the anterior half to that in the control $RPS23^{G67K/+}$ half). Crucially, p62 immunoreactivity was also reduced in the rescued compartment, suggesting that the improvement in cell survival resulted from a lessening of proteotoxic stress (Fig. 5b). As an alternative means of inhibiting TOR, we introduced rapamycin in larval food. A 48h feeding period led to a threefold reduction in the rate of apoptosis in $RPS23^{G67K/+}$ imaginal discs (Fig. 5c–c). Thus, reduction of TOR activity through two independent means alleviates the proteotoxic effect of RP deficiency. Inhibition of TOR by rapamycin could have multiple beneficial effects. By stimulating autophagy, it is expected to boost the removal of toxic species; by downregulating translation, it is predicted to decrease the burden on the protein-degradation machinery while at the same time allowing more time for cotranslational folding and, by dampening ribosome biogenesis, rapamycin would reduce the amount of orphaned ribosomal proteins. The relative importance of these processes remains to be assessed.

Our research suggests that unresolved proteotoxic stress, and not insufficient protein production, could drive the pathology of ribosomopathies. Independent evidence for proteotoxic stress in

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**Fig. 4** The ISR and autophagy limit tissue damage caused by RP deficiency. a. The relatively low number of apoptotic figures (Dcp1, green) in imaginal discs overexpressing GADD34 throughout the wing pouch (under the control of $nubbin$-GAL4). b. c. The rate of apoptosis in heterozygous for $RPS23^{G67K}$ is enhanced considerably with GADD34 overexpression (c) versus without GADD34 overexpression (b). d. Quantification of apoptosis coverage (Fig. 1d) in the three genotypes shown in a-c. e. The relatively low number of apoptotic figures (Dcp1, green) in imaginal discs expressing an Atg1 RNAi transgene throughout the wing pouch (under the control of $rotund$-GAL4). f-g. The rate of apoptosis in heterozygous for $RPS23^{G67K}$ is enhanced considerably after Atg1 RNAi expression (g) versus without Atg1 RNAi expression (f). h. Quantification of apoptosis coverage in the three genotypes shown in e-g. For d, from left to right, $n=10$, $n=7$ and $n=10$ discs. For h, from left to right, $n=21$, $n=22$ and $n=25$ discs. For d and h, data are mean ± s.d. Statistical analysis was performed using two-tailed unpaired t-tests. ***$P=9.43\times 10^{-7}$ (d, left), ***$P=5.36\times 10^{-7}$ (d, right) ***$P=2.52\times 10^{-12}$ (h, left), ***$P=1.35\times 10^{-12}$ (h, right). For a-c and e-g, scale bars, 50 µm. A list of genotypes for each figure panel is provided in Supplementary Table 1. Source data are available online.
RP-deficient cells is described in the accompanying paper, with attention to its relevance to cell competition. We found that, upon RP deficiency, proteotoxic stress triggers eIF2α phosphorylation, which is known to slow down translation. Thus, although the reduction in protein synthesis observed in RP-deficient tissues could be attributed to limited biosynthetic capacity, our findings suggest that it could equally follow from the response to proteotoxic stress. In any case, we have shown that p-eIF2α and autophagy

Fig. 5 | Inhibition of TOR signalling reduces the accumulation of aggregates and apoptosis in RP-deficient cells. a, Wholly heterozygous RPS23\textsuperscript{R67K/+} disc expressing Rheb\textsuperscript{RNAi} in the anterior compartment (under the control of ci-GAL4), at the left of the red dotted line. The top panel shows anti-Ci to mark the domain of transgene expression and anti-Dcp1 to highlight apoptotic figures. Only Dcp1 is shown in the bottom panel. The preparation was stained with anti-Ci antibodies to mark the domain of expression and anti-Dcp1 antibodies to mark the apoptotic figures. b, Imaginal discs of the same genotype stained with anti-p62 antibodies (top), and a higher-magnification image (bottom). c, d, Discs from RPS23\textsuperscript{R67K/+} larvae that were fed for 48 h with vehicle (c) or rapamycin (d). Top panels show Dcp1 and DAPI, while only Dcp1 is shown at the bottom. e, The percentage of apoptotic coverage in RPS23\textsuperscript{R67K/+} imaginal discs from larvae fed with vehicle (black dots) or rapamycin (red dots). n = 29 discs for each condition. Data are mean ± s.d. Statistical analysis was performed using a two-tailed unpaired t-test. ***P = 1.41×10⁻¹¹. For a–d, scale bars, 50 μm. A list of genotypes for each figure panel is provided in Supplementary Table 1. Source data are available online.
are cytoprotective but insufficiently so. It is conceivable that cells cannot enhance this response further without triggering unacceptable side effects. We found that, to some extent, proteotoxic stress can be alleviated by TOR inhibition, an intervention that has been considered for proteinopathies, including Alzheimer’s disease, Parkinson’s disease, amyotrophic lateral sclerosis and Huntington’s disease. Although promising in our assays, rapamycin treatment should be seen only as a proof of principle intervention, as it is probably not appropriate for children or in tissues of high biosynthetic demand in which TOR activity is particularly important. Indeed, in fast-growing Drosophila larvae, we have not been able to identify a rapamycin regimen that completely suppresses proteotoxic stress without impairing growth. There is therefore a need to specifically target the downstream activities of TOR that are most likely to alleviate proteotoxic stress, such as autophagy. In this respect, it is of interest that a small-molecule inducer of autophagy enhanced erythropoiesis in models of Diamond–Blackfan anemia. This could be combined with additional means of reducing proteotoxic burden, for example, with drugs that favour protein folding or stimulate the clearance of misfolded proteins.

Online content
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Methods

Drosophila stocks. A list of all of the genotypes analysed in this study is provided in Supplementary Table 1. The following stocks were obtained from the Bloomington Drosophila Stock Center (BDSC), the Vienna Drosophila Resource Center (VDRC) or are described in Flybase: ci-GAL4 (FlyBase, FBrf007651), hh-GAL4 (FlyBase, FBt0012787), ru-GAL4 (BDSC, 7405), tub-GAL4 (BDSC, 5138), pbn2-GAL4 (BDSC, 49828), nab-GAL4 (FlyBase, FBr0105342), FRT24D (BDSC, 1802), FRT24D ubi-GFP (BDSC, 5626), TRE-GFP (BDSC, 5910), UAS-GFp (BDSC, 35786), UAS-FLP (FlyBase, FBr0018460), UAS-HTT252q-Cerulean (BDSC, 53460), UAS-HTW (BDSC, 56772), UAS-GFP-mCherry-Ayg8a (BDSC, 37749), UAS-Rhod (BDSC, 9688), UAS-GADD34 (BDSC, 76250), UAS-Atg1RNAi (BDSC, 44034), UAS-RhebRNAi (BDSC, 33966), UAS-Xrp1RNAi (BDSC, 34521), UAS-Rpn2RNAi (VDRC, 160457), UAS-Rfp68zRNAi (VDRC, 49244), RPL5RNAi (BDSC, 25907), RPL14RNAi (BDSC, 2247), RPS13RNAi (BDSC, 2246), Dpy-3(3.Gen41TA (BDSC, 4519).

Genetic engineering of flies and HEK293 cells. The RPS25lacZ allele was generated using CRISPR–Cas9-mediated homology-directed repair. Suitable 5' and 3' homology arms, as well as the fragment carrying the R67K substitution, were amplified from pSGDlucV3.0 (Addgene, 119760) and Nluc from pUAS-NanoLuc (Thermo Fisher Scientific, A43628). The Fluc coding sequence was deleted in the first step along with a cas9-cherry fragment. This was achieved by cloning the RPS26 region and tubulin-mCherry between the two FRT sites of the pR26-Pax-mCherry vector, which enabled F3-mediated integration into the attP site of RPS26-cherry (ref. 37). In the resulting strain, RPS26 could be inactivated in a spatially restricted domain (marked by the loss of mCherry expression) with FLP recombination.

A stop-codon readthrough reporter was generated by cloning Fluc and Nluc into a pUAS-attP vector (pFRC81, Addgene, 36432). The Fluc coding sequence was amplified from pSGDlucV3.0 (Addgene, 119760) and Nluc from pUAS-NanoLuc (Addgene, 84139) and cotransfected with single-stranded oligodeoxynucleotides (ssODNs) containing the R67K mutation and the pSpCas9(BB)-2A-Puro vector (PX459, Addgene, 48139) and cotransfected with Flp recombinase.

To generate RP heterozygous mutant wing disc compartments. After introducing an FRT site proximal to the RPS23 promoter, the anterior compartment remained genetically WT, we took advantage of the fact that the RPS26 allele is conditional. A FLP transgene was expressed in the posterior half to excise the second exon, along with the tubulin-mCherry cassette, from RPS26-GFP, while leaving the WT homologous chromosome unchanged. This genetic manipulation therefore generated a heterozygous posterior compartment (RPS26+/−) marked by the loss of mCherry fluorescence and a control (WT) anterior compartment.

Developmental timing. Developmental timing was determined in both WT and RPS23lacZ animals with four replicates each. Adult flies were allowed to lay eggs for a period of 4 h. Freshly hatched first instar larvae (L1) were collected and transferred to vials and new pupae were scored every 4 h. The datasets of all of the replicates were pooled, and the corresponding mean ± s.d. values were calculated for each timepoint using Microsoft Excel. Results were represented in a cumulative percentage distribution plot using GraphPad Prism 8.

Translational fidelity assay and quantification. Expression of UAS-Fluc-stop-Nluc was driven ubiquitously by the tubulin-GAL4 driver. Fluc and Nluc luminescence was measured using the Nano-Glo Dual-Luciferase Reporter Assay System (NanoDLR, Promega, N1610). Positive controls and experimental animals were processed and assayed in parallel. Larvae were collected, washed in PBS and three larvae per tube were homogenized with a pestle in 300 µl of 1x passive lysis buffer (Promega, E1941). The tubes were then placed in an orbital rotator for 10 min at 4 °C to complete lysis. Tubes were centrifuged in a benchtop centrifuge for 2 min at 17,000 × g for 3 rain and the top fatty layer was discarded. For the Fluc reaction, 80 µl of lysate was transferred into a new tube and mixed with 80 µl of One-Glo EX reaction mix. Fluc relative luminescence units (RLU) were measured immediately with the GloMax 20/20 single-tube luminometer (Promega, E5311). To assay for Nluc luminescence, 80 µl of the NanoDLR Stop & Glo reaction mix was added and the tubes were kept in the dark for 10 min to fully quench Fluc activity. Nluc luminescence was then measured using the GloMax 20/20 luminometer. The ratio of Nluc/Fluc RLU served as an estimate of stop codon readthrough. Ratios were normalized to the average of that in WT control larvae to represent the fold change. To validate the reporter assay, three genetic markers were tested. In each case, two tubes containing 10 µl of food supplemented with 300 µg/ml of G418 (Sigma–Aldrich, G8168) were allowed to feed during 48 h before being processed for luciferase activity as described above.

Rapamycin treatment. Recently hatched L1 larvae were grown into 50 mm Petri dishes with normal food. When larvae reached the L3 stage, they were transferred into new dishes containing 10 ml of food supplemented with 40 µM of rapamycin (Sigma–Aldrich, 37094) or vehicle and allowed to feed during 48 h before being processed for immunostaining.

Mammalian cell culture. WT and RPS25lacZ/+. HEK293 cells were cultured in complete Dulbecco’s modified Eagle’s medium (Thermo Fisher Scientific, 31966021) supplemented with 10% (v/v) fetal bovine serum (Thermo Fisher Scientific, A3160402) and 1% (v/v) penicillin–streptomycin (Thermo Fisher Scientific, 15140122) at 37 °C in a humidified chamber under an atmosphere of 5% CO2.

Antibodies and dyes. The following primary antibodies were used: rat anti-Ci (1:50, DSHB, 2A1), mouse anti-Nubi (1:50, DSHB, D24), mouse anti-Wg (1:500, DSHB, 4D4), rabbit anti-Dcp1 (1:500, CST, 9578), rabbit anti-P-Elf2α (1:100, clone D98G, CST, 3398), mouse anti-abiquitin (1:10,000, clone FK2, Merck, 04-263), rabbit anti-Drp1 (1:600, gift from T. Rusten), mouse anti-human p62 (1:300, clone 2C11, Novus Biologicals, H0000887-M01). Alexa Fluor and Alexa Fluor Plus secondary antibodies were raised in goat were obtained from Thermo Fisher Scientific and used at a dilution of 1:1000 (A32731, A32732, A32723, A-21247). DAPI was used at final concentration of 0.1 µg/ml (Sigma–Aldrich, D9542).

Immunostaining protocol. Larvae were inverted in ice-cold PBS, fixed in 4% methanol-free formaldehyde (Pierce, Thermo Fisher Scientific, 28096) for 30 min and then rinsed in PBS. Inverted larvae were permeabilized in PBS+0.1% Triton X-100 (PBTA 0.1%) for 30 min and blocked for 1 h in 0.1% PBS with 2% normal goat serum (Thermo Fisher Scientific, 01-6201) before incubation in a solution of primary antibodies diluted in PBTA 0.1% (without blocking agents or carrier proteins) overnight at 4 °C with gentle rotation. After incubation with primary antibodies, samples were rinsed three times for 15 min in PBTA 0.1% and incubated with the samples at room temperature for 1.5 h in an orbital rotator. After secondary incubation, the samples were rinsed three times for 15 min in PBTA 0.1% before being washed with PBS. Wing discs were dissected out of the inverted larvae and mounted in glycerol-PBS mounting medium (40 ml glycerol + 5 ml 10X PBS + 4.5 µl N-propyl-gallate (50% (m/v) in ethanol)).

Lysotracker staining. Larvae were inverted in prewarmed PBS at 35 °C and transferred into a 1.5 ml tube containing prewarmed PBS. The buffer was then replaced with 1 µM Lysotracker Red DND-99 (Thermo Fisher Scientific, L7528) in PBS and the tubes were placed in an orbital rotator for 10 min at room temperature. The larvae were then rinsed three times for 1 min in PBS to remove residual dye and fixed in 4% methanol-free formaldehyde. After fixation, larvae were rinsed in PBS and the discs were mounted in mounting medium.

Puromycin incorporation assay. Puromycin incorporation was assayed using the Click-it Plus OPP Alexa Fluor Protein Synthesis Assay Kit (Thermo Fisher Scientific, C10458 and C10457) according to manufacturer’s instructions with
minor modifications, as described below. Larvae were inverted in prewarmed Schneider’s Drosophila Medium at 25°C (Gibco, Thermo Fisher Scientific, 21702024) and transferred into a 1.5 ml tube with prewarmed Schneider’s medium. The medium was then replaced with either 1 µM OPP in Schneider’s medium for 15 min or 20 µM OPP for 30 min with gentle rotation at room temperature. After OPP incorporation, inverted larvae were rinsed in PBS, fixed in 4% methanol-free formaldehyde and rinsed again in PBS before a permeabilization and blocking step. The Click-iT reaction mix was prepared according to the manufacturer’s instructions and larvae were incubated for 30 min in the dark at room temperature. The samples were finally rinsed in reaction rinse buffer and stained with DAPI. Discs were dissected out and mounted in mounting medium.

Image acquisition and processing. Samples were imaged using a Leica TCS SP5 confocal microscope and confocal stacks (1 µm step size) were processed and analysed using Fiji (Image), National Institutes of Health). Images were assembled into figures using Adobe Photoshop CC 2018, 19.0 release. Cartoons were drawn using Adobe Illustrator CC 2018, 22.0.1 release.

Quantification of apoptosis in imaginal discs. Apoptosis was quantified in the wing pouch, marked by the fluorescence of HTTω−25Q aggregates was quantified in the wing pouch, marked by the fluorescence of HTTω−25Q−cerulean, which was expressed under the control of the pouch-specific driver pdm2-GAL4. Confocal planes covering all fluorescent signal along the apico–basal axis of wing discs were maximum-projected using Fiji. The pouch was outlined, and its area was measured. To segment the apoptotic cells, a threshold excluding the background was applied to generate a binary mask. The resulting regions of interest (ROIs) were used to determine the area covered by apoptotic cells. Apoptosis coverage was calculated as the percentage of the total wing pouch area occupied by apoptotic cells. Graphical representation of all data points was generated as scatter plots using GraphPad Prism 8. For statistical analysis, two-tailed unpaired t-tests were performed using Microsoft Excel. **P < 0.001.

Quantification of HTT aggregates in imaginal discs. The formation of HTTω−25Q aggregates was quantified in the wing pouch, marked by the fluorescence of HTTω−25Q−cerulean, which was expressed under the control of the pouch-specific driver pdm2-GAL4. Confocal planes covering all fluorescent signal along the apico–basal axis of wing discs were maximum-projected using Fiji. The pouch was outlined, and its area was measured. To segment HTTω−25Q aggregates, a threshold excluding the background was applied to generate a binary mask and the area covered by aggregates was calculated as the ratio of aggregate area/wing pouch area. The ratios were normalized to the WT control and represented as fold change. All data points are represented as scatter plots using GraphPad Prism 8. For statistical analysis, two-tailed unpaired t-tests were performed using Microsoft Excel. ***P < 0.001.

Quantification of p62 bodies in HEK293 cells. The presence of p62 bodies was quantified in WT and in RPS26ΔheK293 HEK293 cells with anti-p62 staining. Control and experimental cells were fixed with 30% cold methanol and immunostained in parallel, and images were acquired under identical confocal settings. To segment p62 puncta, confocal planes covering all fluorescent signal along the apico–basal axis were maximum-projected using Fiji. The background was then subtracted using a 10-pixel radius rolling ball and identical thresholds were subsequently applied to each image to cover the p62 bodies. Images were then converted into binary masks, the segmented particles were selected as ROIs, and the total area that was covered by p62 bodies was measured. For each image, the ratio of p62 body area/number of nuclei was calculated and normalized to the average of the WT control. The p62 coverage was represented as the fold change in scatter plots using GraphPad Prism 8. For statistical analysis, two-tailed unpaired t-tests were performed using Microsoft Excel. **P < 0.01.

Quantification of ubiquitin and p62 punctae in wing imaginal discs. The accumulation of ubiquitin and p62 was quantified in imaginal discs composed of one half heterozygous for the RPS26ΔheK293 allele and the other half genetically WT (therefore serving as an internal control). To segment ubiquitin and p62 punctae in wing discs (Fig. 2f–i), confocal planes covering all fluorescence signal along the apico–basal axis were maximum-projected and the same threshold was subsequently applied to all of the images. Images were then converted into binary masks, the segmented particles were selected as ROIs, and the area covered by ubiquitin or p62 was calculated relative to the area of the mutant or WT compartment. The ubiquitin and p62 data were represented in scatterplots as the fold change in the mutant compartment versus the WT compartment. For statistical analysis, two-tailed unpaired t-tests were performed using Microsoft Excel. **P < 0.01, ***P < 0.001.

Proteomic analysis. Fifty imaginal discs were dissected and lysed in 40 µl of extraction buffer containing 1% Triton X-100, 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2X Halt protease/phosphatase inhibitors (Thermo Fisher Scientific, 78441), 5 mM EDTA, 1 mM dithiothreitol. Proteins were reduced and alkylated using dithiothreitol and iodoacetamide. The sample volumes were then adjusted to 100 µl using LC–MS-grade water and processed for acetone precipitation to remove detergents. Air-dried acetone-precipitated pellets were resuspended 25 µl of 100 mM HEPES, 1 M Guanidinium pH 18 in an ultrasonics bath. Endoprotease Lys-C was added at a ratio of 1:50 (enzyme:protein) and incubated at 37°C for 3h. The sample was then diluted twofold with MilliQ water and trypsin was added at a ratio of 1:50 (enzyme:protein) and incubated overnight at 37°C. The digests were acidified using TFA and prepared for mass spectrometry analysis using an EksigentOne liquid chromatography system coupled to an Orbitrap Fusion Lumos Mass Spectrometer run in data-dependent acquisition mode. Raw data were then processed using MaxQuant v.1.6.12.0 specifying IBAQ quantification with further downstream statistical analysis in Perseus v.1.4.0.2.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data supporting the findings of this study are available within the paper and its Supplementary Information. The mass spectrometry dataset is available at ProteomeXchange under the identifier PXD023021. Accession numbers and names for the proteins identified by mass spectrometry are available at UniProt (https://www.uniprot.org/) and FlyBase (http://flybase.org/). Source data are provided with this paper.

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Author contributions

This project was conceived by C.R.-A., H.N. and J.-P.V.; H.N. created the RPS26ΔheK293 strain, as well as the RPS26ΔheK293 strain, which was used as the starting point for generating RPS26ΔheK293 by C.A. and C.R.-A.; H.N. also performed the developmental timing measurements. D.J.H generated the RPS26ΔheK293 cells. C.A. and C.R.-A. generated the translation fidelity reporter. I.K. and A.P.S. generated and analysed the mass spectrometry data. The manuscript was written by C.R.-A. and J.-P.V.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to C.R.-A. or J.-P.V.

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Extended Data Fig. 1 | Phenotypes of Minute heterozygotes. (a, b) Scutellar region of control and RPS23^{R67K/+} flies showing the short-bristle phenotype that characterises Minute heterozygotes. c, Cumulative distribution of pupariation time for control (n = 143 larvae) and RPS23^{R67K/+} (n = 134 larvae). Error bars represent standard deviation. (d) Control mosaic imaginal disc harbouring wildtype clones (2X GFP) and their wild type twin clones (absence of GFP), induced by heat shock-mediated expression of FLP (hs-FLP). Note the low number of Dcp1-positive cells (red and grey). e, Mosaic imaginal discs harbouring wild type clones (2X GFP) in a RPS23^{R67K/+} background (1X GFP), also induced with hs-FLP. Here the twin clones (RPS23^{R67K/R67K}) are rapidly eliminated and the wild type cells outcompete the RPS23^{R67K/+} cells, which undergo a high rate of apoptosis (Dcp1, red and grey). (f, g) JNK signalling (indicated by expression of the TRE-GFP reporter and apoptosis (Dcp1) in RPS23^{R67K/+} are fully suppressed by a wild type copy of RPS23 from a genomic duplication (g). (h–j) JNK signalling and apoptosis in a panel of heterozygous Minute mutants (RPL5, RPL14, and RPS13). Scale bars represent 50 µm. Genotypes for each figure panel are available in Supplementary Table 1. Source data is available for this figure.
Extended Data Fig. 2 | Xrp1 is required for activation of apoptosis in RPS23R67K+/+. a, Imaginal disc of a RPS23R67K/+ larva expressing an RNAi transgene against Xrp1 in the anterior compartment (marked with anti-Ci). The number of Dcp1-positive cells is lower in the anterior than in the posterior compartment where Xrp1 activity is unaffected. Scale bars represent 50 µm. Genotypes for each figure panel are available in Supplementary Table 1. Experiments were repeated independently three times with similar results.
Extended Data Fig. 3 | Manipulation of Rheb activity affects OPP incorporation in wild type and RPS23R67K/+ imaginal discs. (a–d) Wing imaginal discs (wild type and RPS23R67K/+; as indicated) overexpressing Rheb or RhebRNAi in the anterior compartment (left hand side of the disc). The discs were explanted and incubated for a 15 min in 1 µM OPP before staining for puromycilated peptides (grey scale). Rheb overexpression stimulated OPP incorporation in both genotypes, while RhebRNAi had the opposite effect. Scale bars represent 50 µm. Genotypes for each figure panel are available in Supplementary Table 1. Experiments were repeated independently three times with similar results.
Extended Data Fig. 4 | RPS23R67K/+ imaginal discs develop tumours upon inhibition of apoptosis. (a,b) Wing disc from a RPS23R67K/+ larva expressing P35, a baculovirus-derived inhibitor of effector caspases in the pouch (under the control of rotund-GAL4). Note the overgrowth characterised by epithelial folds and ectopic Wingless expression (green), shown in grey scale at higher magnification in b. Formation of these tumours shows that RP-deficient cells are not inherently incapable of growth. Tumour formation may be relevant to the increase cancer risk associated with human ribosomopathies as well as to the observation that ribosomal protein genes are frequently deleted in human cancers, often in concert with the loss of TP53. Scale bars represent 50 µm. Genotypes for each figure panel are available in Supplementary Table 1. Experiments were repeated independently three times with similar results.
Extended Data Fig. 5 A toxic form of Huntington (HTT96Q) triggers apoptosis. (a,b) Expression of HTT96Q throughout the pouch (with rotund-gal4) triggers an increased rate of apoptosis relative to that seen with GFP expression, which is expected to be innocuous. c. Quantification of Dcp1 coverage in the two genotypes shown in panels a and b (n = 5 discs per genotype). Error bars denote standard deviation. For statistical analysis, a two-tailed unpaired t-test was carried out. **P = 4.11E-03. Scale bars represent 50 µm. Genotypes for each figure panel are available in Supplementary Table 1. Source data is available for this figure.
Extended Data Fig. 6 | Accumulation of p62 and P-eIF2α in RPS26KO/+.

(a, b) RPS26<sup>lox</sup> was inactivated (and tubulin-mCherry deleted) by crossing to hedgehog-GAL4, UAS-FLP. In the resulting RPS26<sup>ko</sup> posterior compartment, immunoreactivity against p62 and P-eIF2α was higher than in the control anterior compartment. Scale bars represent 50 µm. Genotypes for each figure panel are available in Supplementary Table 1. Experiments were repeated independently three times with similar results.
Extended Data Fig. 7 | RP deficiency alters the activity of an autophagy reporter. a, Cartoon showing progression through autophagy as monitored by the GFP-mCherry-Atg8a reporter. Yellow indicates the simultaneous presence of GFP and mCherry in the phagophore and autophagosome. Autolysosomes only retain the red colour because of the drop in pH, which quenches GFP fluorescence. (b, c) Fluorescence from GFP-mCherry-Atg8a, expressed with tubulin-GAL4 in wild type or RPS23R67K/+ . Single fluorescence channels are also shown in grey. Scale bars represent 50 μm. Genotypes for each figure panel are available in Supplementary Table 1. Experiments were repeated independently three times with similar results.
Extended Data Fig. 8 | Translation fidelity is unaffected in RPS23R67K/+.

a, The stop codon readthrough reporter comprises 10X Upstream Activator Sequences (UAS), which confers GAL4 responsiveness, the 5’ UTR from Syn21, the coding region of Firefly luciferase (Fluc), a STOP codon (UGAC), a flexible linker, the coding region of Nanoluciferase (Nluc), and the 3’UTR from p10.

b, Quantification of the Nluc/Fluc ratio, measured from whole larvae lysates and normalised to that in control larvae. Statistical analysis: 4 replicates for each condition. Error bars denote standard deviation. A two-tailed unpaired t-test was carried out. P>0.05, no significant increase was seen in RPS23R67K/+ larvae. Genotypes for each figure panel are available in Supplementary Table 1. Source data is available for this figure.
Extended Data Fig. 9 | Validating the effect of GADD34 overexpression. a, P-eIF2α immunoreactivity in a wild type imaginal disc. b, This is reduced by GADD34 overexpression (GADD34OE) driven by nubbin-GAL4. (c) P-eIF2α immunoreactivity is similarly decreased in RPS23R67K/+ larvae overexpressing GADD34. d, Schematic representation of the domain where GADD34 was overexpressed. (e,f) GADD34 overexpression causes a mild but significant increase in wing size in otherwise wild type flies but not in RPS23R67K heterozygotes. Note that the wing of RPS23R67K heterozygotes is smaller than that of wild type. (g,i) GADD34 overexpression exacerbates the formation of HTT25Q punctae in RPS23R67K heterozygotes. Statistics: error bars denote standard deviation. In f, n = 12 adult wings for each genotype. In i, from left to right, n = 10 and 8 discs. A two-tailed unpaired t-test was carried out. P-values in f, from top to bottom: 2.48E-01, 1.31E-07 and 7.09E-06. P-value in i, 9.65E-04. Scale bars represent 50 µm. Genotypes for each figure panel are available in Supplementary Table 1. Source data is available for this.
Extended Data Fig. 10 | Effect of proteasome inhibition on the rate of apoptosis in RP-deficient tissues. a, Extent of apoptosis (coverage of Dcp1 immuno-reactivity) in the pouch of discs of genotypes indicated. Rpt6RNAi denotes rotund-gal4-driven expression of a Rpt6RNAi transgene. This particular Rpt6RNAi transgene had only a minor effect on apoptosis in wildtype tissue. Expression of this RNAi transgene did not enhance the rate of apoptosis in RPS23R67K heterozygotes. b, The effect of stronger proteasome knockdown (with Rpn2RNAi) on the rate of apoptosis in RP-deficient tissue could not be assessed because it triggered extensive apoptosis in otherwise wild type imaginal discs. Statistics: error bars denote standard deviation. n = 9 discs for each genotype. A two-tailed unpaired t-test was carried out. P > 0.05, no significant difference was seen. Genotypes for each figure panel are available in Supplementary Table 1. Source data is available for this figure.
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Software and code

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Data collection
- The Leica LAS AF software Leica LAS (v.2.7.3.9723) was used to control the Leica SP5 microscope and to acquire confocal images.

Data analysis
- Fiji - image analysis and quantification
- Microsoft Excel (Office 365) - data tables and statistical analysis
- Adobe Photoshop CC 2018, 19.0 release - figure assembly
- Adobe Illustrator CC 2018, 27.0.1 release - drawing cartoons and diagrams
- MaxQuant v.1.6.12.0 and Perseus 1.4.0.2 - specifying (iBAQ) quantification with further downstream statistical analysis
- Prism (v. 8.0.0 (131)) - data representation in plots

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**Sample size**
Sample sizes were chosen on the basis of published literature about the methods used and in order to perform statistical analyses. PMID: 27893747, 23878226

**Data exclusions**
Imaginal discs with stainings of poor quality or with high background signal that impeded analysis were not included. Imaginal discs that had been broken or its normal shape distorted during the immunostaining protocol or sample mounting were also not included.

**Replication**
Experiments were performed in at least three independent biological replicates and all attempts at replication were successful.

**Randomization**
Randomization was not relevant to this study. All the prepared samples were analysed.

**Blinding**
Blinding was not used for this study because for each experiment, image analysis used the same threshold for experimental and control samples.

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| Clinical data | |

**Antibodies**

| Antibodies used | Validation |
|-----------------|------------|
| rat anti-C (1:50, DSHB #2A1), mouse anti-Nub (1:50, DSHB #2D4), mouse anti-Wg (1:500, DSHB #4D4), rabbit anti-Dcr1 (1:500, CST #9578), rabbit anti-P-ef2ko (1:100, clone D9GB, CST #3398), mouse anti-Ubiquitin (1:10000, clone FK2, Merck #04-263), rabbit anti-Drosophila p62 (1:2000, kind gift from Tor Erik Rusten, Nezis et al., Journal of Cell Biology 2008), mouse anti-human p62 (1:300, clone 2C11, Novus Biologicals H00008878-M01). Alexa Fluor and Alexa Fluor Plus secondary antibodies raised in goat were obtained from ThermoFisher and used at 1:1000 dilution (MA32731, MA32752, MA32729, MA32777, MA-21247). |

| | Previous validations and citations can be found by using the RRID numbers. (DSHB Cat# 2A1, RRID:AB_2109711), (DSHB Cat# Nub 2D4, RRID:AB_2722119), (DSHB Cat# 4D4, RRID:AB_528512), (Cell Signaling Technology Cat# 9578, RRID:AB_2721060), (Novus Cat# H00008878-M01, RRID:AB_548364). Drosophila p62 antibodies were generated, validated and provided by Dr. Tor Erik Rusten (Nezis, I. P. et al. Ref[2J], the Drosophila melanogaster homologue of mammalian p62, is required for the formation of protein aggregates in adult brain. J. Cell Biol. 180, 1065–1071 (2008). The rabbit anti-P-ef2ko (Cell Signalling Technology Cat# 3398, RRID:AB_AB_2006481) reacts with Drosophila P-ef2ko according to the antibody's manufacturer. https://www.cellsignal.co.uk/products/primary-antibodies/phospho-ef2a-ser51-d9g88-xp-rabbit-mab/3398. This antibody is further validated in Extended data figure 9b and 9c by overexpressing the P-ef2ko phosphatase GADD34, which causes a decrease in the signal detected by the P-ef2ko antibody. The mouse anti-Ubiquitin antibody, clone FK2 (Millipore Cat# 04-263, RRID:AB_612093) recognizes mono and polyubiquitylated proteins but not free ubiquitin in a wide range of species including Drosophila according to the manufacturer's information https://www.merckm Millipore.com/GB/en/product/Anti-Ubiquitin/ubiquitinated-Proteins-antibody-clone-FK2-MM-1F-04-263. It has been validated previously in Drosophila PMID: 2432251, 28510997, 28806139. https://antibodyregistry.org/search.php?q=AB_612093 |
## Eukaryotic cell lines

Policy information about [cell lines](#)

| **Cell line source(s)** | HEK-293 (ATCC CRL-1573) wild type and engineered to carry the RPS23[R67K] mutation |
|-------------------------|----------------------------------------------------------------------------------|
| **Authentication**      | Authentication was not performed for this study.                               |
| **Mycoplasma contamination** | The cells tested negative for mycoplasma contamination. |
| **Commonly misidentified lines (See ICLAC register)** | No commonly misidentified cell lines were used in this study |