Proteotoxic stress is a driver of the loser status and cell competition

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Cell competition allows winner cells to eliminate less fit loser cells in tissues. In Minute cell competition, cells with a heterozygous mutation in ribosome genes, such as RpS3+/− cells, are eliminated by wild-type cells. How cells are primed as losers is partially understood and it has been proposed that reduced translation underpins the loser status of ribosome mutant, or Minute, cells. Here, using Drosophila, we show that reduced translation does not cause cell competition. Instead, we identify proteotoxic stress as the underlying cause of the loser status for Minute competition and competition induced by mahjong, an unrelated loser gene. RpS3+/− cells exhibit reduced autophagic and proteasomal flux, accumulate protein aggregates and can be rescued from competition by improving their proteostasis. Conversely, inducing proteotoxic stress is sufficient to turn otherwise wild-type cells into losers. Thus, we propose that tissues may preserve their health through a proteostasis-based mechanism of cell competition and cell selection.

Cell competition is a conserved mechanism that allows winner cells to eliminate viable but less fit loser cells in tissues1–3. This process acts as a mechanism of tissue quality control. By removing mis-specified or damaged cells, cell competition preserves tissue and organism health, potentially delaying ageing and disease onset4–6. Furthermore, an increasing body of evidence indicates that competitive interactions contribute to tissue colonization during cancer growth1.

The first form of competition discovered was Minute cell competition, wherein cells with a heterozygous mutation in ribosome genes are eliminated by neighbouring wild-type cells7. Over 80 genes make up the ribosome, and most display a dominant phenotype when mutated or lost, both in Drosophila and humans8,9. Based both on phenotypic dominance and on the high number of Minute genes, spontaneously occurring Minute cell competition is likely to be a frequent event relative to other types of cell competition. In addition, as ribosome genes are scattered across chromosomes, Minute cell competition may be frequent in diseases characterized by aneuploidy10, such as cancer, where deletions of large genomic regions often lead to single-copy loss of one or more ribosome genes11.

Despite its discovery over 40 years ago, our understanding of the mechanisms of Minute cell competition remains incomplete12. While several signals have been identified that act during cell competition13–19, the upstream signals priming cells as losers are mostly unknown20. It is, for instance, unclear how ribosome gene loss leads to the loser status12: Minute mutants exhibit a reduced translation rate17 and it has long been assumed that this drives the loser status17,18,21–24. However, the actual contribution of translation has not been investigated.

Here, we investigate how ribosome mutations lead to the loser status. We find that translation is not directly linked to the loser status in Minute competition. Instead, we find that ribosome gene mutations lead to defective autophagy and proteasome flux, accumulation of protein aggregates, and proteotoxic stress. These phenotypes are causative of the loser status. In addition, inducing proteotoxic stress through overexpression of aggregate-prone proteins phenocopies these protein catabolism defects and induces the loser status. Our work identifies proteotoxic stress as the leading cause of the Minute loser status and implicates cell competition in pathologies characterized by proteotoxic stress.

Results

Reduced protein synthesis does not confer the loser status. Minute cell competition is characterized by apoptotic elimination of Minute loser cells when they are in proximity to wild-type winner cells12–14. Thus, although Minute RpS3+/− cells display a modest increase in apoptosis compared with wild-type cells when they are in isolation (Fig. 1a,b and ref. 25), apoptosis is substantially elevated during competition in RpS3+/− cells that border wild-type cells12,13,27 (Fig. 1c,d). This region-specific induction of apoptosis at clone borders is a hallmark of certain types of cell competition, including Minute competition.

To investigate whether reduced translation triggers cell competition, we expressed a constitutively active form of the translational repressor 4E-BP (4E-BPΔA)28–30 in otherwise wild-type cells. In O-propargyl-puromycin (OPP) and l-azidohomoalanine (AHA) global translation assays, 4E-BPΔA expression induced a reduction in protein synthesis that was comparable to (Fig. 1e–g; OPP) or stronger than (Extended Data Fig. 1a–c; AHA) that seen in RpS3+/− cells. 4E-BPΔA expression resulted in little autonomous apoptosis (Fig. 1h). Furthermore, the frequency of dying cells was similar at 4E-BPΔA clone borders and clone centres (Fig. 1i,j). These data suggest that reducing rates of global protein synthesis alone, at levels equal to or greater than in RpS3+/− cells, is not sufficient to trigger cell competition, and indicate that additional properties induced by RpS3+/− mutations must also play a role.

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We have previously shown that RpS3\textsuperscript{−/−} cells and cells mutant in the loser gene and ubiquitin ligase *mahj*\textsuperscript{−/−} (*mahj*) share what we have termed the prospective loser status—a cellular state that predisposes cells to act as losers when confronted with wild-type winners\textsuperscript{26}. This state is characterized by activation of a range of stress response pathways, even in the absence of cell competition\textsuperscript{22}. For example, RpS3\textsuperscript{−/−} and mahj\textsuperscript{−/−} cells display chronic activation of c-Jun amino-terminal kinase (JNK) signalling\textsuperscript{13} and of the Nrf2-mediated oxidative stress response\textsuperscript{32}. Furthermore, Nrf2 activation is sufficient to induce the loser status in competition with wild-type cells\textsuperscript{22}. To determine whether a reduction in protein synthesis is sufficient to activate these pathways, we examined the levels of phospho-JNK and the activation of an Nrf2 reporter, green fluorescent protein (GFP)-driven by a glutathione S-transferase D1 promoter fragment (GstD1–GFP), in the absence of competition\textsuperscript{32}. As Minute cell competition does not occur across compartment boundaries, we were able to use compartment-specific transcriptional drivers to generate wing discs with two distinct but non-competing cell populations—one in the anterior compartment and one in the posterior compartment. Similar to RpS3\textsuperscript{−/−} cells, the levels of phospho-JNK were higher in wing disc cells expressing 4E-BPTa than in the wild-type compartment (Fig. 1j,k). However, GstD1–GFP levels were only minimally affected in 4E-BPTa cells (Fig. 1n). Thus, a reduction in protein synthesis can produce some aspects of the prospective loser status (JNK activation) but is insufficient to induce oxidative stress response activity or provoke cell competition.

Next, we asked whether reduced protein synthesis is necessary for *mahj*\textsuperscript{−/−} or RpS3\textsuperscript{−/−} cells to behave as losers. Knockdown of Mahj did not affect the protein translation rate (Extended Data Fig. 1d,e), indicating that translation inhibition does not play a role in priming *mahj*\textsuperscript{−/−} cells as losers. Next, we sought to boost rates of translation in RpS3\textsuperscript{−/−} cells and assess the resulting effect on the prospective loser status and on Minute competition. Growth arrest and DNA damage-inducible protein 34 (GADD34) can stimulate translation via dephosphorylation of the translation initiation factor eukaryotic initiation factor 2\alpha (elF2\alpha)\textsuperscript{−/−}. Indeed, GADD34 overexpression in RpS3\textsuperscript{−/−} cells caused a reduction in phospho-elF2\alpha (Extended Data Fig. 1lg) and a corresponding rescue of translation, as assessed by OPP incorporation (Fig. 1o,p). Surprisingly, GADD34-expressing RpS3\textsuperscript{−/−} cells displayed higher levels of the GstD1–GFP oxidative stress reporter (Extended Data Fig. 1h,i) and performed worse than RpS3\textsuperscript{−/−} cells in competition, with hardly any surviving at the point of dissection (Fig. 1q–s). Thus, in RpS3\textsuperscript{−/−} cells, translation inhibition seems to counter the loser status rather than contribute to it.

**Prospective losers display dependence on autophagy and defective autophagic flux.** To seek out an alternative cause of the prospective loser status, we turned to a known rescue of Minute competition: inhibition of JNK signalling. In addition to rescuing RpS3\textsuperscript{−/−} cells from competition, JNK inhibition partially reverses activation of the transcriptional signature associated with prospective losers\textsuperscript{26}. Furthermore, it reduces GstD1–GFP reporter activation in RpS3\textsuperscript{−/−} cells (Extended Data Fig. 2a). Thus, we compared the transcriptional profiles of RpS3\textsuperscript{−/−} wing discs with or without JNK signalling inhibition\textsuperscript{26} to identify pathways associated with JNK inhibition and with a rescue of the loser status. This revealed differential expression of genes involved in protein catabolism, the proteasome, autophagy and the unfolded protein response (Supplementary Table 1). These pathways have all been implicated in Nrf2 regulation\textsuperscript{14,33}, supporting a potential role in cell competition.

To examine the role of autophagy in RpS3\textsuperscript{−/−} cells, we obtained wing discs from larvae carrying heterozygous mutations for both RpS3 and one of several autophagy-related genes: *p62* (ref\textsuperscript{2}P in *Drosophila*), *atg8* or *atg13* (ref. \textsuperscript{34}). We found that all three autophagy mutations caused a cell-autonomous increase in apoptotic events in an RpS3\textsuperscript{−/−} background, compared with RpS3\textsuperscript{−/−} or autophagy mutations alone (Fig. 2a,b and Extended Data Fig. 2b–d). Heterozygous mutations in another ribosome loser mutation, *Rpl27A*, also caused increased apoptosis in combination with heterozygous mutations in the autophagy gene *p62* (Extended Data Fig. 2e,f). Thus, Minute cells are acutely reliant on autophagy. However, autophagy inhibition did not impact the competitive status of RpS3\textsuperscript{−/−} cells, as knockdown of autophagy genes *atg1* or *atg9* by RNA interference (RNAi) did not affect clone coverage or competition-induced cell death in competing RpS3\textsuperscript{−/−} cells (except for a mild increase in competitive death in the case of *atg1* RNAi; Extended Data Fig. 2g–i). This contrasts with data from Nagata et al.,\textsuperscript{35} who have instead shown that inhibiting autophagy rescues *Minute* cells from competition. Non-competing RpS3\textsuperscript{−/−} cells also appeared to have more atg8\textsuperscript{+} foci (Fig. 2c) and had more p62\textsuperscript{+} foci (Fig. 2d,e) than wild-type cells.

Cells with reduced function of the loser gene and ubiquitin ligase *mahj* share with RpS3\textsuperscript{−/−} cells a cell-autonomous signature of hundreds of differentially expressed genes relative to wild-type cells, as well as a cell-autonomous activation of the oxidative stress response\textsuperscript{20}. This suggests that mutations in *mahj* and RpS3 lead to cell competition using a convergent mechanism\textsuperscript{22}. Thus, we examined the autophagic state in *mahj*\textsuperscript{−/−} cells. *mahj*\textsuperscript{−/−} homozygous clones in a background of *mahj*\textsuperscript{−/−} and wild-type cells also accumulated p62\textsuperscript{+} foci (Fig. 2f), whereas 4E-BP\textsuperscript{3A} had no effect on the number of p62\textsuperscript{+} foci (Fig. 2g). Thus, deregulated autophagy is associated with the prospective loser status of two functionally unrelated mutants, and this is not a consequence of reduced protein synthesis.

Accumulation of Atg8\textsuperscript{+} and p62\textsuperscript{+} autophagosomes can reflect either decreased or increased autophagic flux\textsuperscript{37}. To measure...
autophagic flux in prospective losers, we designed the reporter ReFlux, which measures the rate of p62 degradation\textsuperscript{37,38}. p62 is both an autophagy adaptor and an autophagy cargo that is degraded upon autophagosome degradation by the lysosome\textsuperscript{37}. Thus, measuring the rate of p62 degradation provides a direct measure of autophagic flux\textsuperscript{37}. In ReFlux, p62 is fused to GFP and driven by a heat-shock (hs) promoter for pulse-chase expression\textsuperscript{39} (Fig. 2h). As a control, we confirmed that ReFlux reports reduced autophagic flux upon depletion of the autophagy gene \textit{atg1} (Extended Data Fig. 3a–c). Then, we expressed ReFlux across wing discs containing \textit{RpS3}\textsuperscript{+/−} anterior and wild-type posterior compartments. We found that \textit{RpS3}\textsuperscript{+/−} and wild-type cells show similar GFP–p62 ReFlux signal intensity immediately following pulse expression. However, after a chase period, GFP–p62 ReFlux signal perdures in \textit{RpS3}\textsuperscript{+/−} cells compared with wild-type cells, indicating reduced autophagic flux (Fig. 2i–k). A reduced autophagic flux was also seen in competing \textit{RpS3}\textsuperscript{+/−} cells relative to competing wild-type cells (Extended Data Fig. 3d–f). Treatment with the autophagy inhibitor chloroquine led to persistence of the GFP–p62 ReFlux signal, confirming that GFP–p62 ReFlux loss is
Fig. 2 | Prospective losers display defective autophagic flux. a–b, Apoptotic cell death, as detected by anti-cleaved caspase-3 reactivity (green), in the wing discs of a p62+/− heterozygote (a; left), an RpS3+/− heterozygote (a; middle) and a p62+/− RpS3+/− transheterozygote (a; right), and corresponding quantification (n=10, 7 and 11, respectively; two-sided Mann–Whitney U-test without P adjustment for multiple comparisons) (b). c, Staining of autophagosomes and autolysosomes, as detected by atg8–GFP–mCherry expression (red) in the posterior compartment of wild-type (left) or RpS3+/− (right) wing discs. DAPI, 4′,6-diamidino-2-phenylindole. d–e, Immunostaining for p62 in wing discs harbouring RpS3+/− anterior cells and wild-type posterior cells (d) and corresponding fluorescence intensity quantification (n=9; two-sided paired t-test) (e). f, Immunostaining of p62 in a wing disc with mahj−/− clones (GFP−) induced in a mahj+/− heterozygous background (1xGFP). Wild-type twin spots are 2xGFP. g, Immunostaining for p62 in wing discs harbouring wild-type anterior cells and 4E-BPTa-expressing posterior cells (labelled by the absence of Ci; magenta). h, Schematic of ReFlux. The autophagy cargo p62 is fused to GFP and driven by a hs promoter for pulse-chase expression. i–k, GFP–p62 ReFlux signal (green) in wing discs harbouring RpS3+/− anterior cells (dsRed+) and wild-type posterior cells (dsRed−) immediately after heat shock (i) and 3 h later (j), and corresponding signal quantifications (n=7 and 8, respectively; two-sided Student’s t-test) (k). l–n, GFP–p62 ReFlux signal (green) in wing discs expressing mahj RNAi in the posterior compartment (RFP+) immediately after heat shock (l) or 3 h later (m), and corresponding signal quantifications (n=8 and 7, respectively; two-sided Student’s t-test) (n). RFP, red fluorescent protein. Scale bars, 50 µm. For all of the quantifications, the horizontal line represents the mean and the whiskers indicate 95% confidence intervals. All n values refer to the number of individual wing discs.
Fig. 3 | Autophagy impairment does not confer the loser status. a, b, Apoptosis detection by cleaved caspase-3 staining (red) in wing discs with mosaic expression of atg1 RNAi (GFP+ cells) (a) and corresponding quantifications (n = 9; two-sided Wilcoxon signed-rank test) (b). Cell death is classed as border death or centre death, as described in Fig. 1. c, p62 staining in wing discs of the same genotype as in a, d, p62 staining (left) and GstD1–GFP signal (right) in wing discs harbouring atg1 RNAi-expressing posterior cells and wild-type anterior cells. e–h, p62 staining (e) and apoptosis detection by cleaved caspase-3 staining (red) (f) in wing discs with atg13−/− clones (GFP+) induced in an atg13+/− heterozygous background (1×GFP), and corresponding cell death (g; n = 12; two-sided Wilcoxon signed-rank test) and clone size (h; n = 95 and 105, respectively; two-sided Mann–Whitney U-test) quantifications for atg13−/− clones and wild-type atg13+/− twin spots (2×GFP). Each data point on the graph in h represents one clone. The horizontal line represents the median and the whiskers indicate 95% confidence interval. i–k, Wing discs harbouring GFP+ clones expressing atg9 RNAi (j) or atg9 RNAi and 4E-BPTA (k) and stained for cleaved DCP1 (red), and corresponding cell death quantification in clone centres (cent.) versus borders (bord.) (n = 11 and 14, respectively; two-sided Wilcoxon signed-rank test) (i). Scale bars, 50 µm. For all of the quantifications provided other than in h, the horizontal line represents the mean and the whiskers indicate 95% confidence intervals. All n values refer to the number of individual wing discs, except in h, where n values refer to the number of individual twin-spot clones.
due to autophagic degradation (Extended Data Fig. 3g). ReFlux was eventually cleared from the RpS3+/− compartment (Extended Data Fig. 3h), indicating that autophagic degradation is delayed but not blocked. Knockdown of Mahj also reduced autophagic flux (Fig. 2l–n). Overexpression of 4E-BP1 also reduced autophagic flux, albeit with a substantially smaller effect size than RpS3+/−-mutations (Extended Data Fig. 3i–k).

Defective autophagy does not cause the loser status. Defective autophagy has been associated with the loser status in mouse embryonic stem cells40. Having observed reduced autophagic flux in both RpS3+/− and mahj+/− prospective losers, we next investigated whether reduced autophagy is sufficient to induce the loser status in these epithelia. Clones of cells expressing atg1 RNAi within wild-type imaginal discs did not show cell death enrichment at the clone borders (Fig. 3a,b), even though they accumulated p62 foci (Fig. 3c), indicative of impaired autophagy. atg1-depleted cells also failed to activate the oxidative stress response in a non-competitive context (Fig. 3d, right), despite confirmation of autophagy impairment from p62 accumulation (Fig. 3d, left). Similarly, inhibiting autophagy in clones by mutating atg13 caused accumulation of p62 foci (Fig. 3c) but did not result in cell competition with wild-type cells, as neither cell death nor clonal disadvantage were observed (Fig. 3f–h). Therefore, reduced autophagic flux is observed in RpS3+/− cells both in the absence of and during competition, but is not sufficient to cause cell competition.

As reduced protein synthesis and autophagy flux are observed in RpS3+/− losers but neither is sufficient to confer the loser status, we asked whether they might do so in concert. However, co-expressing atg9 RNAi and 4E-BP1 in clones of cells in a wild-type wing disc did not result in border cell death, indicating that reduced protein synthesis and defective autophagy together are not sufficient to induce the competitive elimination of losers (Fig. 3i–k).

Prospective losers have defective proteasome flux. Proteasome genes were also differentially expressed in RpS3+/− cells upon JNK signalling inhibition (Supplementary Table 1), prompting us to investigate the role of the proteasome in Minute cells. Heterozygosity of a proteasomal core subunit gene caused increased apoptosis in RpS3+/− cells and in Rpl27A−/− cells (Extended Data Fig. 4a–d). Similarly, feeding flies the proteasome inhibitor bortezomib increased the number of dying cells in wild-type imaginal discs. Clones of cells expressing 4E-BP TA also reduced autophagic flux, albeit with a substantially smaller effect size than RpS3+/−+/- and RpS3+/−−/− wing disc cells (Extended Data Fig. 4k). We reasoned that RpS3+/− mutations could lead to a stoichiometric imbalance in ribosome proteins, which could turn cause proteotoxic stress and overload the proteasome and autophagy machineries. To test this, we measured relative levels of ribosomal proteins, by tandem mass tag (TMT) spectrometry of RpS3+/− and wild-type wing discs. TMT successfully identified 78 ribosomal proteins of the 93 reported on FlyBase (of the missing 15, eight are not expected to be expressed in wing discs). This showed that the RpS3+/− mutation causes a log[fold change] reduction in RpS3 protein of 0.291 relative to wild-type levels. Interestingly, a reduction was observed for all small ribosome subunit proteins detected (Fig. 4), indicating coordinated regulation, but this was not seen for components of the large subunit, whose levels were, with few exceptions, equal to or higher than in wild-type cells (Fig. 4). Thus, at steady state, RpS3+/− cells have a stoichiometric excess of ribosome proteins from the large subunit relative to small subunit ribosome proteins. This could contribute to proteasome and autophagy overload.

When they are not efficiently cleared by degradation, ribosome proteins can form protein aggregates46,47. To test this, we used ProteoStat, a dye that fluoresces upon intercalation with protein aggregate-associated quaternary structures. Indeed, ProteoStat staining detected accumulation of protein aggregates in RpS3+/− cells relative to wild-type cells, in the absence of cell competition (Fig. 4k). Protein aggregates are often ubiquitin positive48,49, and immunostaining with the FK2 antibody, which detects mono- and poly-ubiquitin conjugates, revealed that RpS3+/− cells, but not RpS3−/− cells, express these aggregates (Fig. 4k).

**Fig. 4 | Prospective losers display proteotoxic stress.** a, b, Apoptosis detection by cleaved caspase-3 staining (red) in wild-type (a) or RpS3+/− (b) wing discs fed dimethyl sulfoxide (DMSO) or 10 μM bortezomib (BORT), as indicated. c, Quantification of dying cell numbers within the pouch region of wing discs under the conditions indicated in a and b (n = 8, 8, 7 and 5, respectively; two-sided Mann–Whitney U-test without P adjustment for multiple comparisons). CT, control. d, Schematic of ProteoFLUX. Fusion of GFP with the proteasome degradation signal CL1 is driven by a hs promoter for pulse-chase expression. e, f, ProteoFLUX CL1–GFP signal (green) in wing discs expressing RNAi against the proteasomal subunit Rpt6 specifically in posterior cells, immediately after heat shock or 2 h later, as indicated (e), and corresponding signal quantifications (n = 3 and 11, respectively; two-sided Mann–Whitney U-test) (f). g, i, ProteoFLUX CL1–GFP signal (green) in wing discs harbouring RpS3+/− anterior cells (dsRed+) and wild-type posterior cells (dsRed−) immediately after heat shock (g) or 2 h later (i), and corresponding signal quantifications (n = 7 and 7, respectively; two-sided Student’s t-test) (i). j, Abundance of ribosomal subunit proteins in RpS3+/− wing discs relative to wild-type wing discs by TMT mass spectrometry. The bars indicate average log[fold change] values across two independent biological replicates. k, ProteoStat protein aggregate staining (green) in wing discs harbouring RpS3+/− anterior cells and wild-type posterior cells. l, FK2 anti-conjugated ubiquitin (green) and anti-p62 (red) staining in a wing disc harbouring a RpS3+/− anterior compartment and a wild-type posterior compartment, as indicated. Yellow boxes mark inset locations. Scale bars, 50 μm. For all of the quantifications provided, the horizontal line represents the mean and the whiskers indicate 95% confidence intervals. All n values refer to the number of individual wing discs.
wild-type cells, accumulate large, ubiquitin-positive foci in the cytoplasm (Fig. 4l). Many of these foci were also positive for the autophagy adapter/cargo p62 (Fig. 4l), which is often recruited to cytosolic protein aggregates49. Furthermore, phospho-eIF2α, a marker of proteotoxic stress and of the integrated stress response33, was upregulated in RpS3Δ/− cells, both under homotypic conditions (Extended Data Fig. 4l,m) and during cell competition (Extended Data Fig. 4n,o). Collectively, RpS3Δ/− cells show reduced autophagy flux, reduced proteasome flux, accumulation of ubiquitinated protein aggregates, and markers of proteotoxic stress.

**Figure 4:**
- **a** shows Caspase-3 activity in DMSO and 10 μM BORT treated WT samples.
- **b** displays similar effects in RpS3Δ/− samples.
- **c** illustrates Caspase+ cell counts with statistical significance.
- **d** illustrates heat shock inducible promoter CL1-GFP and heat shock inducible promoter CL1–GFP signal intensity over time.
- **e** shows CL1–GFP signal intensity over time.
- **f** depicts CL1–GFP signal intensity over time with statistical significance.
- **g** and **h** demonstrate RpS3Δ/− and WT samples with DAPI/dsRed and CL1–GFP signals.
- **i** compares CL1–GFP signal intensity over time with statistical significance.
- **j** compares the expression of small and large subunit proteins.
- **k** and **l** show RpS3Δ/− and WT samples with DAPI, ProteoStat, and FK2 signals.

**Legend:**
- CL1-GFP: Heat shock inducible promoter
- CL1–GFP: Heat shock inducible promoter
- DAPI/dsRed: Staining for DNA and dsRed
- CL1–GFP signal intensity: Measured over time
- NS: Not significant
- P: Statistical significance
- Fold change: Logarithmic scale
Improving proteostasis in *Rps3*⁻/⁻ cells rescues their loser status. Proteotoxic stress can induce Nrf2 activation⁵⁰ and this in turn is linked to the loser status⁵⁹, suggesting a link between proteotoxic stress and the prospective loser status. Consistent with this, inhibiting the proteasome with bortezomib was sufficient to elevate GstD1–GFP signal in non-competing wild-type and *Rps3*⁻/⁻ wing disc cells (Extended Data Fig. 5a–c). We therefore asked whether alleviating proteotoxic stress would rescue loser cells from competition. Rapamycin inhibits target of rapamycin signalling and promotes proteostasis via multiple mechanisms, including inhibiting translation and activating autophagy and proteasome functions⁵¹,⁵². We found that rapamycin feeding reduced the frequency of competition-induced apoptosis in *Rps3*⁺/⁻ cells bordering wild-type cells (Fig. 5a–c). Rapamycin feeding also reduced the
cell-autonomous activation of the oxidative stress reporter Gsd1–GFP in RpS3<sup>−/−</sup> cells (Fig. 5d,e). As rapamycin was fed systemically, the observed rescue of competition-induced cell death could in part arise from the effects of rapamycin on wild-type cells. We therefore sought to improve proteostasis specifically in RpS3<sup>−/−</sup> cells. To this end, we overexpressed, in RpS3<sup>−/−</sup> cells, the transcription factor FOXO, which is inhibited by target of rapamycin signalling<sup>53,54</sup> and promotes both autophagy and proteasome functions<sup>54</sup>. FOXO overexpression reduced the number of p62<sup>+</sup> aggregates (Fig. 5f), increased protein synthesis (Fig. 5g,h) and reduced mildly the levels of phospho-eIF2α (Fig. 5i,j) in RpS3<sup>−/−</sup> cells, indicating overall improved proteostasis. Strikingly, FOXO overexpression in RpS3<sup>−/−</sup> cells abolished competition-induced cell death, as very few apoptotic bodies could be detected in competition with wild-type cells (Fig. 5k–m). These data indicate that reducing proteotoxic stress inhibits the competitive elimination of RpS3<sup>−/−</sup> cells.

Proteotoxic stress is sufficient to cause the loser status. We considered that protein aggregation and proteotoxic stress could be sufficient to cause the loser status in competitive contexts. To test this...
hypothesis, we ectopically expressed the human aggregate-prone polyQ protein ataxin-3 (SCA3/MJDQ78), which is responsible for the human neurodegenerative disorder Machado–Joseph disease and has been used in Drosophila to model this neurodegenerative condition. MJDQ78 expression was sufficient to recapitulate many features shared by RpS3Δ/Δ and mahjΔ/Δ prospective losers; namely, upregulation of GstD1–GFP (Fig. 6a,b), reduced autophagic flux (Fig. 6c) and accumulation of p62+ structures (Fig. 6d,e). MJDQ78, however, did not perceptibly impact on rates of translation, as measured by OPP incorporation (Fig. 6f,g). Importantly, clones overexpressing MJDQ78 in wild-type wing discs showed a local induction of apoptosis, specifically at their borders with wild-type cells (Fig. 6h,i), and grew poorly relatively to wild-type clones (Fig. 6j–l), indicating that these cells are eliminated by cell competition. This was specifically induced by proteotoxic stress, as clones expressing the wild-type version of Ataxin-3 (MJDQ27) did not show induction of border death (Extended Data Fig. 5d–f).

Thus, proteotoxic stress is sufficient to turn otherwise wild-type cells into losers (Fig. 6m).

Discussion

Our work shows that single-copy loss of ribosome genes leads to major defects in cellular protoxosis, as also shown in the accompanying paper by Recasens-Alvarez et al. Heterozygosity of ribosome genes in humans leads to genetic disorders collectively known as ribosomopathies, which are characterized by severe malformations and pathologies. The mechanisms through which ribosomal mutations lead to these defects are only partially understood. Our work suggests that proteotoxic stress may be an underlying cause for some such defects and that they might be improved by drugs that promote proteostasis, such as the Food and Drug Administration–approved compound rapamycin that we used in this study.

Our work shows that proteotoxic stress is sufficient to confer the loser status. This finding broadens the scope of cell competition and suggests it may be an active mechanism in physiological and pathological contexts characterized by proteotoxic stress. This may help to explain the competitive elimination of neurons in Drosophila models of neurodegenerative diseases. It may be especially relevant to cancer, where proteotoxic stress is often observed. Our findings suggest that cancer cells might represent concealed losers that have escaped proteotoxic stress-induced cell competition through masking mutations. Understanding how Minute mutations and proteotoxic stress lead to cell competition may help to unmask the loser status in cancer cells in ways that could be exploited therapeutically.

Healthy proteostasis is a driver of organism fitness and contributes to organism longevity, whereas impaired proteostasis is associated with ageing and age-related pathologies. We propose that tissues preserve their health and youth through a proteostasis-based mechanism of cell elimination. By measuring cell fitness on the basis of proteostasis and converting it into the loser status through the activation of the oxidative stress response, proteostasis-based cell competition could act as a general mechanism of cell selection in adult homeostasis. How proteotoxic stress induces the loser status remains to be established.

Online content

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Methods

Fly husbandry. Fly lines were maintained at 25°C on a flour-based food supplemented with yeast. Our standard recipe contains 7.5 g/l agar powder, 50 g/l baker’s yeast, 35 g/l glucose, 35 g/l wheat flour, 2.5% nipagin, 0.4% propionic acid and 1.0% penicillin/streptomycin. For some chemical feeding experiments, drugs were diluted in Nutri-Fly GF food (Scientific Laboratory Supplies) made to manufacturer’s instructions. Eggs were collected for 24 h and wing discs were dissected from wandering third-instar larvae. For each dataset, including across different vials and genotypes, egg collections, heat shocks and harvesting of wandering larvae, wing discs for dissections were done in parallel. All of the Drosophila strains used in this study are listed in Supplementary Table 2 and the genotypes for all experimental crosses are provided in Supplementary Table 3.

Immunostaining. Wing discs were dissected in phosphate-buffered saline (PBS) before fixation in 4% formaldehyde/PBS solution for 20 min at room temperature. Dissected hemi-larvae were subsequently washed three times in PBS (30 s each) before permeabilization in PBS containing 0.25% Triton X-100 (PBS-T). Samples were next incubated in blocking buffer (PBS-T supplemented with 4% foetal calf serum) for 30 min at room temperature. Primary antibodies were diluted in blocking buffer and incubated overnight at 4°C. Samples were washed three times in PBS-T (10 min each) before incubation in secondary antibody (diluted in blocking buffer) for 1 h at room temperature. The secondary antibodies were conjugated with Alexa Fluor 488, Alexa Fluor 555 or Alexa Fluor 633 dyes (Molecular Probes). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (0.5 µg/ml) in PBS for 5 min. After 5 min wash in PBS-T, wing discs were mounted in VECTASHIELD (Vector Laboratories) on a boric acid glass slide (number 1.5; VWR International). For anti-FK2 staining, the blocking buffer was substituted with a 3% bovine serum albumin in PBS solution. Details and sources of all antibodies are provided in Supplementary Table 2. For the immunoblotting experiments, the primary antibodies used were as follows: 1:1000 in PBS-T for anti-p–JNK, 1:1000 in 1% anti–Ci, 1:2000 for anti–Ref(2)BP1-3, 1:25000 for anti–cleaved caspase-3, 1:2500 for anti–DCPI, 1:500 for anti–p–eIF2α and 1:5000 for anti-FK2.

Clonal analysis. Mosaic wing discs were generated using the FLP/FRT system, employing hs–FLP or en–Gal4–UAS–FLP transgenic strains. For clone induction, heat shocks were carried out 2–4 d after egg laying (depending on the experiment) in a 37°C water bath before returning the flies to a 25°C incubator or, for experiments employing a temperature-sensitive Gal80 (Gal80TS), to a water bath set at 29°C. Dissected hemi-larvae were subsequently washed three times in PBS before separating wing discs from the larval body and mounting in PBS under our standard cover slips. Wing discs were imaged immediately. Details of the reagents are provided in Supplementary Table 2.

Identification of proteostasis genes. The full list of genes differentially expressed in Rp35−/− cells ± expression of the JNK inhibitor puc was reported previously. To identify differentially expressed proteostasis genes from this list, we selected genes associated with the following Gene Ontology terms: autophagy; response to unfolded proteins; proteasome complex; and proteasome catalytic process.

ReFlux and ProteoFlux assays. ReFlux and ProteoFlux assays were carried out as pulse-chase experiments. Third-instar wandering larvae were heat shocked for 40–45 min to induce a pulse of GFP–p62 or CL1–GFP, respectively. Larvae were incubated at 25°C for the indicated times to chase protein levels before dissection.

ProteoStat assay. For the ProteoStat Protein Aggregation assay, larvae were dissected and inverted in PBS before transfer to a 1.5-ml Eppendorf tube containing 4% formaldehyde diluted in 1x ProteoStat assay buffer (PAB). The samples were subsequently permeabilized in 0.5% Triton X-100 and 3 mM ethylenediaminetetraacetic acid (pH 8.0) diluted in 1x PAB, before staining with ProteoStat detection reagent diluted 1:1 in 20,000 together with Hoechst 33342 at 1 µg/ml in PAB. Hemi-larvae were subsequently washed three times in PBS before separating wing discs from the larval body and mounting in PBS under our standard cover slips. Wing discs were imaged immediately. Details of the reagents are provided in Supplementary Table 2.

Transmission electron microscopy. Larvae were washed and dissected in Schneider’s Insect Medium, and imaginal wing discs were dissected out and subjected to high-pressure freezing in a 20% bovine serum albumin solution followed by an osmium tetroxide freeze substitution and acetone embedding. The resulting blocks were sectioned onto grids using an ultramicrotome and stained with uranyl acetate and lead citrate. Sections were then imaged on a Tecnai 12 transmission electron microscope.

Chemical feeding. For bortezomib feeding, eggs were collected for 24 h and larvae were grown on normal food for 72 h before being floated in a 20% sucrose solution. Floating larvae were thoroughly washed with PBS before transferring to Nutri-Fly GF premixed food containing 10 µM bortezomib or the equivalent volume of dimethyl sulfoxide (as a carrier control). Larvae were grown until they were at third-instar wandering stages. For rapamycin feeding, 4 µM rapamycin was diluted in ice-cold glucose-based food and floating larvae were maintained in water (or equivalent carrier control of ethanol) until the wandering stage. For chloroquine incubation, dissected larvae were incubated in 50 µM chloroquine diluted in normal Schneider’s medium (or the equivalent volume of water as a carrier control) for 3 h at 25°C, before washing in PBS. Details of the reagents are provided in Supplementary Table 2.

Proteomics. Third-instar larvae raised on normal food were dissected in ice-cold PBS containing 1x PhosSTOP phosphatase inhibitor and 1x HalT Protease Inhibitor cocktail. Wing discs were then centrifuged in an Eppendorf tube containing 30 µl PBS/inhibitor cocktail 30 s at 6,000 RCF for 30 s. Following 30 s resuspended in ice-cold RIPA lysis buffer. Lysed samples were centrifuged at 12,500 RCF at 4°C for 10 min. Aliquots of 50 µg of each sample were digested with trypsin (1.25 µg trypsin; 37°C, overnight) and labelled with TMT ten-plex reagents according to the manufacturer’s protocol (Thermo Fisher Scientific) before samples were pooled. A total of 40 µg of the pooled sample was desalted using a Sep-Pak cartridge according to the manufacturer’s instructions (Waters). Eluate from the Sep-Pak cartridge was evaporated to dryness and resuspended in buffer A (20 mM ammonium hydroxide (pH 10)) before fractionation by high-pH reversed-phase chromatography using an UltiMate 3000 liquid chromatography system (Thermo Fisher Scientific). In brief, the sample was loaded onto an XBridge BEH C18 Column (150 A, 3.5 µm; 2.1 mm x 150 mm; Waters) in buffer A and peptides were eluted with an increasing gradient of buffer B (20 mM ammonium hydroxide in acetonitrile (pH 10)) from 0–95% over 60 min. The resulting fractions were evaporated to dryness and resuspended in 1% formic acid before analysis by nanoscale liquid chromatography coupled to tandem mass spectrometry using an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific).

High-pH reversed-phase fractions were further fractionated using an UltiMate 3000 nanoscale liquid chromatography system in line with an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific). All spectra were acquired using an Orbitrap Fusion Lumos mass spectrometer controlled by Xcalibur 3.0 software (Thermo Fisher Scientific) and operated in data-dependent acquisition mode using an SPS-MS3 workflow. FTMS2 spectra were collected at a resolution of 120,000 with an automatic gain control (AGC) target of 400,000 and a maximum injection time of 100 ms. Precursors were filtered with an intensity threshold of 5,000, according to the charge state (to include charge states 2–7) and with monoisotopic peak determination set to peptide. Previously interrogated 120,000 FTMS2 spectra were excluded from the analysis. Peptide precursors were isolated with a quadrupole isolation window of 0.7 m/z. FTMS2 spectra were collected with an AGC target of 10,000, a maximum injection time of 70 ms and a collision-induced dissociation energy of 35%.

For FTMS3 analysis, the Orbitrap was operated at a resolution of 30,000 with an AGC target of 50,000 and a maximum injection time of 105 ms. Precursors were fragmented by high-energy collision dissociation at a normalized collision energy of 60% to ensure the maximum TMT reporter ion yield. Synchronous precursor selection was used to include up to five MS2 fragment ions in the FTMS3 scan. The raw data files were processed and quantified using Proteome Discoverer software version 2.1 (Thermo Fisher Scientific) and searched against the UniProt Drosophila melanogaster database (downloaded in March 2020; 41,311 entries) using the SEQUEST HT algorithm. The peptide precursor mass tolerance was set at 10 ppm and the tandem mass spectrometry tolerance was set at 0.6 Da. The resulting database search options were filtered using the SEQUEST HT algorithm. The peptide precursor mass tolerance was set at 10 ppm and the tandem mass spectrometry tolerance was set at 0.6 Da. Searches were performed with full trypptic digestion, and a maximum of two missed cleavages were allowed. The reverse database search option was enabled and all data were filtered to satisfy a false discovery rate of 5%. Ribosomal proteins were identified by cross-referencing the protein list against the ribosomal protein category in FlyBase using R statistical software. Average fold changes were obtained for ribosomal proteins that exhibited a consistent change in relative abundance across both biological replicates. Two biological replicates were performed.

Cloning and transgenes. To isolate genomic DNA, a single fly was homogenized in 50 µl extraction buffer containing 10 mM Tris HCl (pH 8.2), 2 mM ethylenediaminetetraacetic acid (pH 8.0), 0.1% Triton X-100 and 200 µg/ml protease K. Samples were then heated to 55°C for 30 min in a Thermoshaker with occasional vortexing, before increasing the temperature to 95°C for 15 min to inhibit protease activity. Samples were then cooled to 4°C and centrifuged at
5,000g for 5 min at 4°C. The supernatant was subsequently transferred to a fresh 0.5-ml Eppendorf tube and stored at 4°C. Alternatively, DNA was isolated from 10–15 flies using a Gentra Puregene Tissue Kit using the following protocol. Flies were homogenized using a motorized pestle in 200 μl cell lysis buffer and incubated at 65°C in a Thermoshaker for 15 min. Then, 1 μl RNAase A solution was added before incubation at 37°C for a further 15 min. A volume of 100 μl protein precipitation buffer was subsequently added and samples were thoroughly mixed and incubated on ice for 5 min. Samples were centrifuged for 10 min at 4°C at maximum speed before adding 300 μl isopropanol to the supernatant, mixing well and centrifuging for a further 15 min. The resulting pellet was washed twice with 70% ethanol before re-suspending in 50 μl DNase-free water.

For cloning of both the ReFLUX (hs–GFP–p62) and ProteoFLUX (hs–CL1–GFP) constructs, genomic DNA was isolated from 10–15 flies of the genotypes UAS–GFP–p62 or UAS–CL1–GFP, respectively. The resulting genomic DNA was used as a template for a PCR using primers designed to amplify constructs introduced in the common pUAST vector. To generate pCaSpør–hs–GFP–p62, three different pairs of primers were used to generate a PCR product that could be inserted into the pCR4-TOPO vector. The resulting pTOPO–GFP–p62, together with pCaSpør–hs, was digested with XbaI and NotI restriction enzymes (New England Biosciences) to produce a fragment containing GFP–p62 that could be ligated into the pCaSpør–hs backbone. For hs–CL1–GFP, a protocol using an In-Fusion HD Cloning Plus Kit was designed to infuse a PCR product containing the CL1–GFP sequencing into the pCaSper–hs backbone. For cloning of the act–RpS3–Gαδ construct, the In-Fusion HD Cloning Plus Kit (Clontech; 638909) was used to linearize an extant pCaSper2–act > CD2 > Gαδ vector, by digestion with the Acc65I restriction enzyme (New England Biolabs). Two PCR products from a plasmid encoding RpS3 together with Hsp70 terminator sequences, were then sequenced. The resulting plasmid was transformed into Stellar competent cells (Clontech; 636766).

Plasmids for all constructs were sent for injection into a w[118] line by Genetics Services, University of Cambridge or BestGene Drosophila embryo injection services. The exact primers used are provided in Supplementary Table 2.

**Image acquisition and processing.** Confocal images were acquired using Leica SP5 and SP8 confocal microscopes with a 40× 1.3 numerical aperture PL Apo Oil immersion objectives. All wing discs were imaged as z-stacks with each section corresponding to 0.5–1.0 μm. Images were subsequently analysed and processed using Fiji2 and Photoshop (Adobe Version CS6). Clonal areas were determined using a custom script built in Fiji. For cell death quantifications, caspase-3 or DCP1 sequences, were then infused. The resulting plasmid was transformed into Stellar competent cells (Clontech; 636766).

**Quantifications.** For immunofluorescence and fluorescent reporter microscopy-based assays, all measurements were derived from the pouch region of the wing disc. For cell death assays, death counts were normalized to the area of the wing pouch or to the specified region of the clones within the pouch. For all scatter dot plots, unless otherwise specified, the horizontal line represents the mean and the whiskers indicate 95% confidence intervals.

**Statistics and reproducibility.** All data used for statistical tests, along with the specific test used for each experiment, are shown in the source data. Statistical tests were performed using GraphPad Prism 7.0a and RStudio software. P values were determined using univariate statistics. We consider P > 0.05 to be not significant. Parametric tests were used in cases where assumptions of normality and equivalence of variance were met. Non-parametric tests were used otherwise. The parametric tests used were Student’s t-tests and paired t-tests for matched data. The non-parametric tests used were either Kolmogorov–Smirnov tests or Mann–Whitney U-tests, or Wilcoxon matched-pairs signed-rank tests for matched data. P value corrections for multiple comparisons were not considered due to the low number of comparisons. All statistical tests were two sided.

A minimum of three biological repeats were used for experiments comparing across separate wing discs. For matched experiments containing an internal control, a minimum of two biological repeats were performed. Functional validation of reagents and Drosophila stocks (for example, RNAi) was carried out at least once. All data points for all replications for specific quantifications are provided in the source data.

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

**Data availability**

The following publicly available databases were used in this study: FlyBase (https://flybase.org) and the UniProt D. melanogaster proteome (https://www.uniprot.org/proteomes/UP00000803). Source data are provided with this paper. All other data supporting the findings of this study are available upon reasonable request.

**Code availability**

The Fiji-based custom-made script can be made available to individuals upon reasonable request while we seek to publish it independent of this study.

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**Author contributions**

E.P. led the project. All authors conceived of the experiments. M.P.D., M.E.B., I.K. and R.P.L. performed and analysed the experiments. M.P.D., M.E.B., P.F.L. and E.P. wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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Extended Data Fig. 1 | Protein synthesis and its regulation in Rps3<sup>−/−</sup> cells. (a-c) AHA (grey) protein synthesis assay in wing discs harboring either Rps3<sup>−/−</sup> clones (GFP-positive) (a) or clones overexpressing 4EBP<sup>TA</sup> (GFP-positive) (b), and corresponding quantification (n = 7 and 7, respectively, two-sided paired t-test without p-adjustment for multiple comparisons) (c). (d-e) OPP (green) protein synthesis assay in a wing disc expressing mahj-RNAi in the P compartment (positively labelled with RFP) (d) and corresponding quantification (n = 10, two-sided Wilcoxon signed-rank test) (e). (f-g) An Rps3<sup>−/−</sup> wing disc expressing GADD34 in the P compartment and labelled with phospho-eIF2α (red) (f), and corresponding quantification (n = 10, two-sided paired t-test) (g). (h-i) GST-GFP reporter (green) activation in an Rps3<sup>−/−</sup> wing disc expressing GADD34 in the P compartment (h), and corresponding quantification (n = 10, two-sided paired t-test) (i). For all micrographs, scale bars correspond to 50 µm. For all quantifications provided, the horizontal line represents the mean and whiskers indicate 95% confidence intervals. All n numbers refer to the number of individual wing discs.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | The role of autophagy in prospective losers. (a) GstD1-GFP signal (green) in a RpS3+/− wing disc expressing Puc in P cells (labelled by the absence of Ci, magenta). (b–c) Apoptotic cell death, as detected by anti-cleaved Caspase-3 reactivity (red), in wing discs of an atg8+/− heterozygote (b, left), RpS3+/− heterozygote (b, middle), or atg8+/−, RpS3+/− transheterozygote (b, right) and corresponding quantification (n = 9, 8, and 9 respectively, two-sided two sample Kolmgorov-Smirnov test without p-adjustment for multiple comparisons) (c). (d) Apoptotic cell death, as detected by anti-cleaved Caspase-3 reactivity (red), in wing discs of an atg13+/− heterozygote (d, left), RpS3+/− heterozygote (d, middle), or atg13+/−, RpS3+/− transheterozygote (d, right). (e–f) Apoptotic cell death, as detected by anti-cleaved dcp1 antibody staining (red), in wing discs of a p62+/− heterozygote (f, left), RpL27A+/− heterozygote (f, middle), or RpL27A+/−, p62+/− transheterozygote (f, right) and corresponding quantification (n = 10, 10, and 12 respectively, two-sided Mann-Whitney U test without p-adjustment for multiple comparisons) (e). (g–i) Wing discs harboring RpS3+/− clones (GFP-positive) (g, left), RpS3+/− clones expressing atg1-RNAi (GFP-positive) (h, middle), or RpS3+/− clones expressing atg9-RNAi (GFP-positive) (h, right) stained with cleaved-dcp1 (red) and corresponding quantification of border cell death (n = 16, 12, and 9 respectively, two-sided Mann-Whitney U test without p-adjustment for multiple comparisons) (g) and clone coverage (n = 16, 12, and 9 respectively, two-sided student’s t-test without p-adjustment for multiple comparisons) (i). For all micrographs, scale bars correspond to 50 µm. For all quantifications provided, the horizontal line represents the mean and whiskers indicate 95% confidence intervals. All n numbers refer to the number of individual wing discs.
Extended Data Fig. 3 | Autophagy flux in ribosome mutants and upon translation inhibition. (a–c) GFP-p62 ReFlux signal (green) in wing discs expressing RNAi against the autophagy gene atg1 specifically in P cells (labelled by the absence of Ci, magenta), immediately after heat shock (a) or three hours later (b), and corresponding signal quantifications (n = 7 and 6 respectively, two-sided two sample Kolmgorov-Smirnov test) (c). (d–f) GFP-p62 ReFlux signal (green) in a wing disc harboring RpS3+/− clones (dsRed-positive) three hours after heat-shock (d) and corresponding quantification of GFP-p62 signal intensity (e) and number of GFP-p62 foci per area (f) (for both measurements, n = 5, two-sided paired t-test). (g) GFP-p62 ReFlux signal (green) in wing discs harboring RpS3+/− A cells and wild-type P cells, three hours after heat-shock, with or without addition of chloroquine, as indicated. (h) GFP-p62 ReFlux signal (green) in wing discs harboring RpS3+/− A cells (dsRed-positive) and wild-type P cells (dsRed-negative) twenty-four hours after heat-shock. (i–k) GFP-p62 ReFlux signal (green) in wing discs harboring wild-type A cells and 4E-BPα-expressing P cells (labelled by the absence of Ci, magenta), immediately after heat shock (i) or three hours later (j), and corresponding signal quantifications relative to wing discs containing an RpS3+/− A compartment and wildtype P compartment (images not shown) (n = 9 and 8 for 0 and 3 hour 4E-BPα, and n = 7 and 8 for 0 and 3 hour RpS3+/−, respectively; two-sided two-sample Kolmgorov-Smirnov test without p-adjustment for multiple comparisons) (k). For all micrographs, scale bars correspond to 50 μm. For all quantifications provided, the horizontal line represents the mean and whiskers indicate 95% confidence intervals. All n numbers refer to the number of individual wing discs.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Proteasome defects are linked to the prospective loser status but not to translation inhibition. (a–b) Apoptosis as detected by anti-cleaved caspase-3 reactivity (green), in Prosβ2+/− (a, left), RpS3+/− (a, middle), or Prosβ2+/−, RpS3+/− transheterozygote (a, right) wing discs and corresponding quantification (n = 10, 10, and 10 respectively, two-sided two sample Kolmogorov-Smirnov test without p-adjustment for multiple comparisons) (b). (c–d) Apoptotic cell death as detected by cleaved-dcp1 (red) in Prosβ2+/− (c, left), a RpL27A+/− (c, middle), or a RpL27A+/−, prosβ2+/− transheterozygote (c, right) wing discs, and corresponding quantification (n = 8, 13, and 10 respectively, two-sided Mann-Whitney U test without p-adjustment for multiple comparisons) (d). (e–g) ProteoFLUX CL1-GFP signal (green) in wing discs expressing mahj-RNAi in the P compartment (RFP-positive), immediately after heat shock (e) or two hours later (f) and corresponding signal quantifications (n = 9 and 7 respectively, two-sided two sample Kolmogorov-Smirnov test) (g). (h–j) ProteoFLUX CL1-GFP signal (green) in wing discs harboring wild-type A cells and 4E-BPα-expressing P cells (labelled by the absence of Ci, magenta), immediately after heat shock (h) or two hours later (i), and corresponding signal quantifications relative to wing discs containing an RpS3+/− A compartment and wildtype P compartment (images not shown) (n = 9 and 10 for 0 and 2 hour 4E-BPα, and n = 7 and 7 for 0 and 2 hour RpS3+/−, respectively; two-sided two-sample Kolmogorov-Smirnov test without p-adjustment for multiple comparisons) (j). (k) Transmission Electron microscopy images of a wing disc with wildtype P (left panel) and RpS3+/− A compartments (right panel). Red arrows indicate phago-lysosomal structures containing ribosomes. The scale bar is 500 nm. (l–m) Phospho-eIF2α staining (red) in wing discs harboring RpS3+/− A cells (GFP-positive) and wild-type P cells (GFP-negative) (l) and corresponding signal quantifications (n = 6, two-sided Wilcoxon ranked-sum test) (m). (n–o) A wing disc harboring RpS3+/− clones (GFP-positive) and stained for phospho-eIF2α (red) (n) and corresponding signal quantification (n = 9, two-sided paired t-test) (o). For all micrographs other than those in (k), scale bars correspond to 50 μm. For all quantifications, the horizontal line represents the mean and whiskers indicate 95% confidence intervals. All n numbers refer to the number of individual wing discs.
Extended Data Fig. 5 | Proteostasis and the oxidative stress response. (a–c) GstD1-GFP signal (green) in wild type (a) or RpS3−/− wing discs (b) fed DMSO control or 10μM bortezomib, as indicated, and corresponding quantification (n=7, 8, 12, and 12, two-sided Mann-Whitney U test without p-adjustment for multiple comparisons) (c). (d–f) Wing discs harboring GFP-positive clones expressing MJDQ27 (d) or MJDQ78 (e) and stained with cleaved-dcp1 (red) and corresponding quantification of cell death (n=17 and 15 respectively, two-sided Wilcoxon signed-rank test without p-adjustment for multiple comparisons) (f). For all micrographs, scale bars correspond to 50μm. For all quantifications provided, the horizontal line represents the mean and whiskers indicate 95% confidence intervals. All n numbers refer to the number of individual wing discs.
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted. Give P values as exact values whenever possible.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

| Data collection | Image acquisition was performed using Leica SP5 and SP8 confocal microscopes and their corresponding image acquisition softwares. |
|-----------------|-------------------------------------------------------------------------------------------------------------------------|
| Data analysis   | Images were analysed and processed using Fiji2 (version 1.53g). For some experiments we used a Fiji-based custom-made script, which can be made available to individuals upon reasonable request, while we seek to publish it independently of this study. Statistical analyses were performed using GraphPad Prism 7.0a and RStudio Statistical Software vs3.6.3. Proteome data were analysed using Proteome Discoverer 2.1 and the Drosophila melanogaster database. |

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

All source numerical data are provided in the Statistics Source Data table. All other data supporting the findings of this study are available upon reasonable request. The following publicly available databases were used in this study: Flybase (https://flybase.org), Uniprot D. melanogaster proteome (https://www.uniprot.org/proteomes/UP0000000803).
Field-specific reporting

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- Life sciences  □ Behavioural & social sciences  □ Ecological, evolutionary & environmental sciences

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

**Sample size**

We aimed to obtain around 10 wing discs per sample and no experiment had fewer than five wing discs. The sample size of 10 wing discs provides an optimal compromise between good statistical power and cost of the experiment. For those experiments with lower sample size this was deemed sufficient because they had internal control within the same wing discs, i.e., anterior compartment was control and posterior compartment was experimental condition. This minimized biological or experimetal variation between the two sample types and eliminated confounding factors.

**Data exclusions**

No data were excluded from the analysis.

**Replication**

A minimum of three biological repeats were used for experiments comparing across separate wing discs. For matched experiments containing an internal control, a minimum of two biological repeats were performed. Functional validation of reagents and Drosophila stocks (e.g., RNAi) was carried out at least once. In all experiments where internal controls worked and chemicals were confirmed to be biologically active reproducibility was 100%.

**Randomization**

In all experiments where comparing across conditions (as opposed to having an internal reference), flies and larvae were allocated to treatment or control group randomly. In experiments with internal reference this was not applicable as both treatment and control were within the same sample.

**Blinding**

Experiments comparing GST-GFP levels upon drug treatments or comparing cell death across different homotypic genotypes were carried out in a blinded fashion. For all other experiments investigators were not blinded, as the experimental design or the presence of identifying traits in the samples did not make it possible. Experiments were designed so as to avoid co-variables introduced by balanced covariates. Specifically, all final experimental genotypes did not contain balance chromosomes (see genotypes table), as we found that those can modify phenotypes.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|---------------------------------|---------|
| n/a                             | Involved in the study |
| - Antigens                      | □ ChIP-seq |
| - Eukaryotic cell lines         | □ Flow cytometry |
| - Palaeontology                 | □ MRI-based neuromaging |
| - Animals and other organisms   |         |
| - Human research participants   |         |
| - Clinical data                 |         |

**Antibodies**

| Antibodies used | Rabbit anti-pJNK pThrP (used 1:500) Promega Cat#7933B |
|-----------------|---------------------------------------------------------|
|                 | Rat anti-CI (used 1:1000) DSHB Cat#2A1                 |
|                 | Rabbit anti-Ref(2)P (used 1:2000) Tor Erik Rusten Lab |
|                 | Rabbit anti-cleaved Caspase-3 (used 1:25000) Abcam Cat#13847 |
|                 | Rabbit anti-Omp1 (used 1:25000) Cell signalling Cat#95765 |
|                 | Rabbit anti-p-eIF2 (used 1:5000) Cell signalling Cat#33587 |
|                 | Mouse anti-Fg (used 1:5000) Merck Cat#34-263            |

**Validation**

Rabbit anti-pJNK pThrP has been validated to detect pJNK protein independently by several groups. Our own group has shown that it detects p-JNK upon JNK overexpression and that it shows reduction in signal upon expression of a JNK phosphatase, unpublished data, Piddri lab.)
Rat anti-CI shown to stain the anterior compartment by IF in Aza-Bianc et al. 1997, DOI: http://doi.org/10.1016/S0092-8674(00)0292-5.

Rabbit anti-Ref(2)P shown to work in IF by Tor Eric Rusten in multiple papers (e.g. Katherder et al. 2017, DOI: 10.1038/nature20815), furthermore, inhibition of autophagy leads to p62 accumulation in our hands (see figure 3C and E).

Rabbit anti-cleaved Caspase 3. Used in several cell competition papers by several groups to detect apoptosis in Drosophila wing discs. See e.g. Kurcinski et al., Nat Comm 2017.

Rabbit anti-Dsp1. Validated in S2 cells by manufacturer (see product page on manufacturer’s website). We have also tested this antibody by RNAi knockdown of genes essential for competition-mediated apoptosis (e.g. Drone and 3rp1) in competing wing discs and observed that the signal declines (unpublished data, Piddini Lab).

Rabbit anti-a-elf2alpha. Validated for western blot by manufacturer. In our hands, for immunofluorescence, we see that overexpression of Gadd34, an elf2alpha phosphatase, leads to a decrease in signal intensity (see Extended Data figure 1D). We have also seen that an RNAi against Gadd34 leads to an increase in signal (unpublished data, Piddini Lab).

Mouse anti-FG2. This antibody has been shown to stain mono- and poly-ubiquitylated proteins by western blot (see manufacturer's product page). For IF, data in the literature are consistent with this. For instance, proteasome mutation yields an increase in FK2 staining (Lee et al. 2016, DOI: 10.1038/cd3.2016.43) and an increase in signal is also observed upon mutation of UbA4, the sole ubiquitin activating enzyme in Drosophila (Lee et al. 2006, DOI: 10.1242/dev.01126B).

**Animals and other organisms**

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

| Laboratory animals | We used Drosophila melanogaster third instar larvae, of the genotypes indicated, selected at wandering stage. We dissected females for the following experiments (including where relevant for the matched control): MIBQ28 expression experiments, Atg RNAi in Minute competition experiment, UAS-GADD34 in Minute competition experiment, and for the p62/prox32/Ref(2)P synthetic lethality studies. In addition in some experiments genotype segregated with a specific gender for X chromosome-linked mutations or transgenes. Gender was random, for all other experiments.

| List of strains used: |
| Drosophila ap53[PlacQ2] |
| Drosophila re53* |
| Drosophila rl27A[1] |
| Drosophila hs-FLP; FRT82B y [1], W[1]1118 |
| en-Ga4, UAS-FLP; FRT82B |
| Mh-Ga4/TM6B |
| Drosophila FRT42D, ubi-GFP/Cyo FRT82B, Fpc3[PlacQ2], hs-Ga4 |
| Drosophila hs-FLP, UAS-CG38-GFP; FRT82B, Bbo3[PlacQ2], act>Bbo3>Ga4/TM6b |
| Drosophila tub>Gal80TS |
| Drosophila UAS-GFP-attB-mCherry |
| Drosophila FRT42D mah |
| Drosophila UAS-puc |
| Drosophila UAS-4E-BP7A |
| Drosophila w+/w; tub>CD2>Gal4, UAS-GFP; tub-Gal80TS |
| Drosophila hs-FLP122; act>CD2>Gal4, UAS-GFP/TM6b |
| Drosophila (UAS-GADD34 |
| Drosophila UAS-uF0XO |
| Drosophila Pros22EP3067/TM6b |
| Drosophila UASp-GFP-mCherry-Atg8a |
| Drosophila hs-flp; FRT823, Atg1-RNAi |
| Drosophila UAS-Atg1 RNAi |
| Drosophila UAS-Atg9-RNAi |
| Drosophila UAS-p62-RNAi |
| Drosophila UAS-Rpr6 RNAi |
| Drosophila Atg8aG57569/FM7c |
| Drosophila Ref(2)P>Q2>Qyo |
| Drosophila UAS-mah RNAi |
| Drosophila GstD1-GFP |
| Drosophila hs-CL1-GFP (ProtopLEX) |
| Drosophila hs-p62-FF(reflex) |
| Drosophila UAS-Hsp/MiD-Q27 |
| Drosophila UAS-Hsp/MiD-Q28 |

| Wild animals | The study did not involve wild animals. |
| Field-collected samples | The study did not involve samples collected from the field. |
| Ethics oversight | The use of Drosophila does not require ethical approval. |

Note that full information on the approval of the study protocol must also be provided in the manuscript.