Figures and figure supplements

Enhanced insulin signalling ameliorates C9orf72 hexanucleotide repeat expansion toxicity in *Drosophila*

Magda L Atilano *et al*

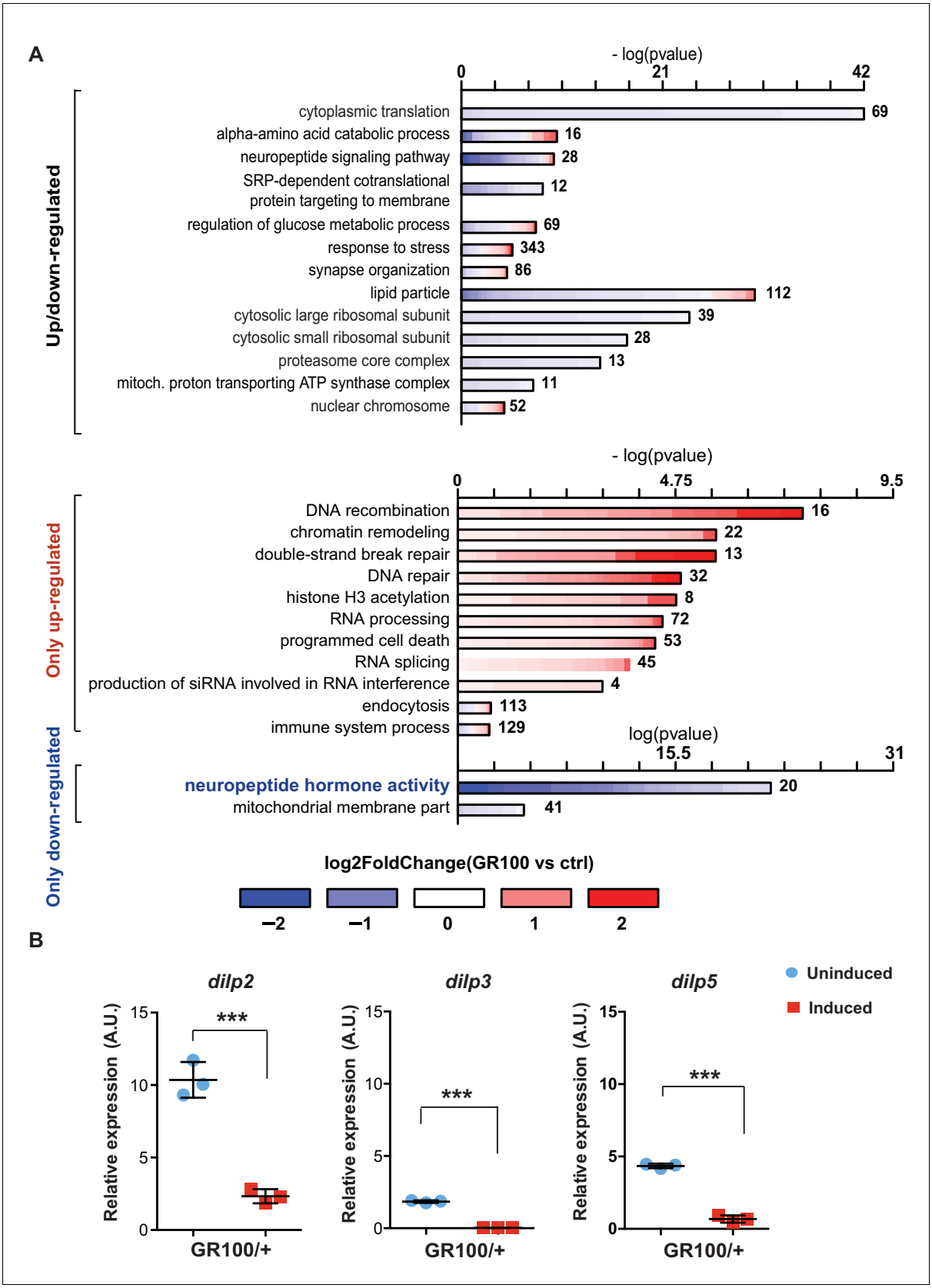

Figure 1. Insulin signalling is down-regulated in flies expressing *C9orf72* repeats. (**A**) Gene ontology enrichment of genes with altered expression when GR100 was expressed in neurons. In the top graph, bars represent enrichment of up- and down-regulated genes. In the bottom graph, upper bars

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represent only up-regulated genes, lower bars represent only down-regulated genes. Lengths of bars represent negative log-transformed, adjusted p-values for Fisher's exact enrichment test. Bar colour indicates log2-fold changes between GR100 and control per gene. Neuropeptide/hormone activity genes were down-regulated. (**B**) Quantitative RT-PCR analysis of dilp 2, 3, and 5 normalized against tubulin in fly heads expressing GR100 in neurons. Data was assessed by t-test and presented as mean ± SD, n = 3; dilp2: p=0.0004; dilp3: p<0.0001, dilp5: p<0.0001. Genotypes: (A) w; UAS-GR100/+; ElavGS/+ (GR100), w; +; ElavGS/+ (ctrl) and (B) w; UAS-GR100/+; ElavGS/+.

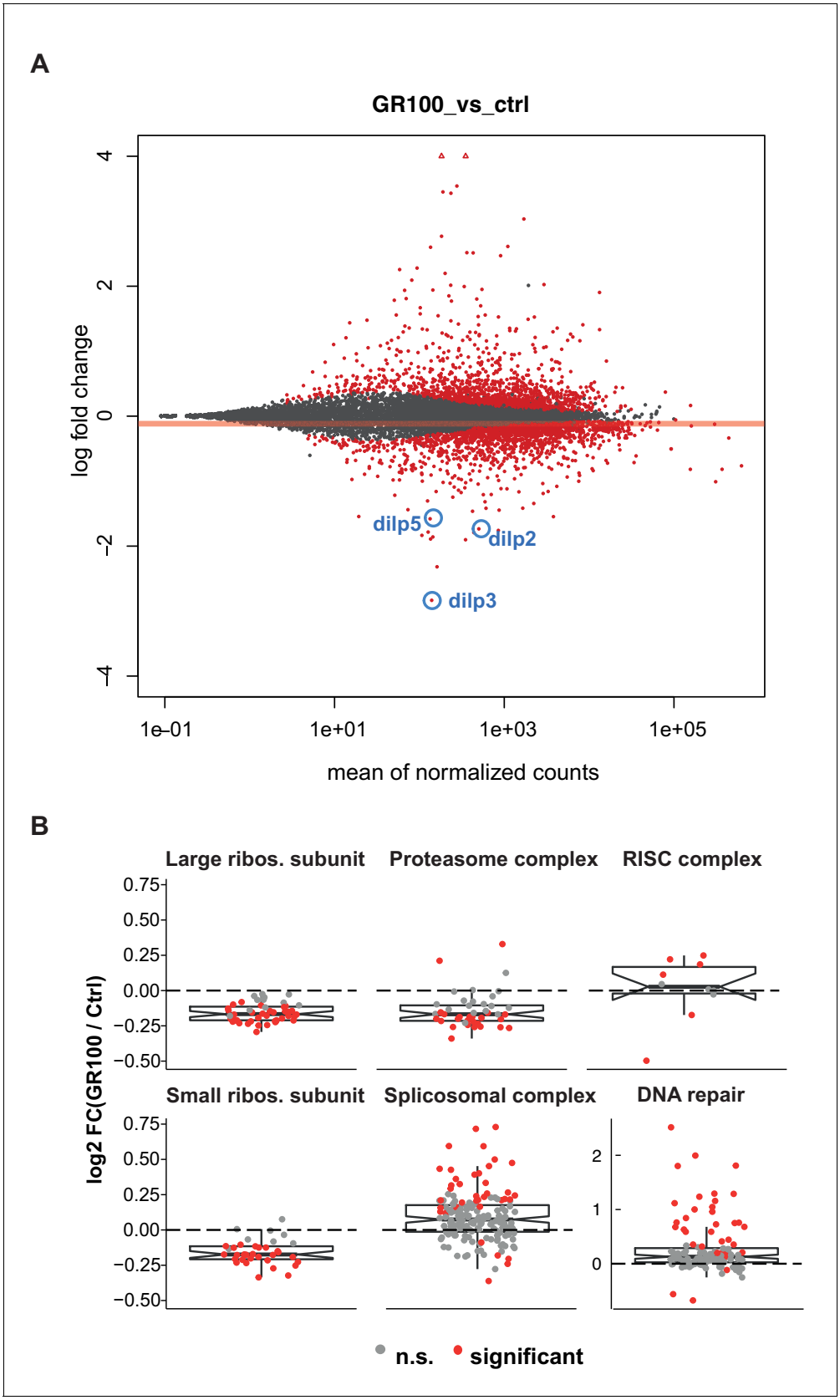

Figure 1—figure supplement 1. Expression of GR100 in fly neurons induces strong perturbation of the transcriptome. (**A**) MA-plot of DESeq2 output indicating genes that were significantly differentially expressed (red dots), non-significantly differentially expressed (grey circles), and significantly

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expressed outside the 4 to −4 log-fold change limit (red triangles) in GR100 over-expression flies relative to control flies. *Dilp2*, *3*, and *5* are annotated (blue circles). (**B**) Graphs display the relative expression of all genes associated with the identified GO-term categories with altered expression. Log2-fold change of the genes in GO categories: DNA repair, proteasome complex, spliceosome complex, RISC complex, and small and large ribosomal subunits. (**A and B**) Genotypes: w; UAS-GR100/+; ElavGS/+ (GR100) and w; +; ElavGS/+ (ctrl).

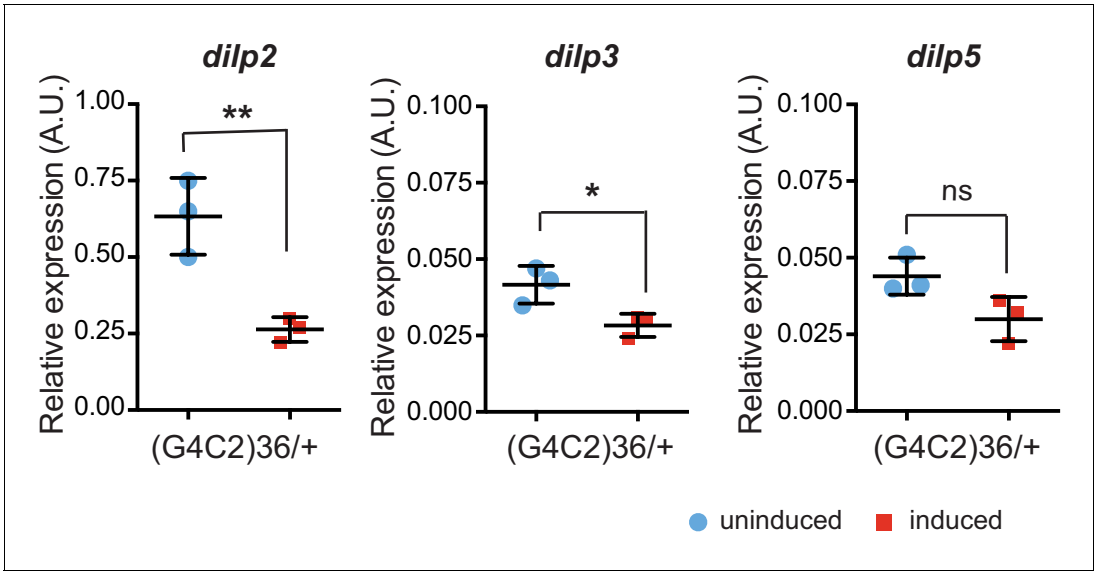

Figure 1—figure supplement 2. Insulin-like peptides 2, 3, and 5 are down-regulated in flies expressing (G4C2)36. Quantitative RT-PCR analysis of dilp 2, 3, and 5 normalized against tubulin in fly heads expressing (G4C2)36 in neurons. Blue circles show data obtained from uninduced flies, red squares data obtained from induced flies. Data was assessed by t-test and presented as mean ± SD, n = 3, *p=0.017, **p=0.008, ns p=0.0620. Genotype: w; UAS-(G4C2)36 /+; ElavGS/+.

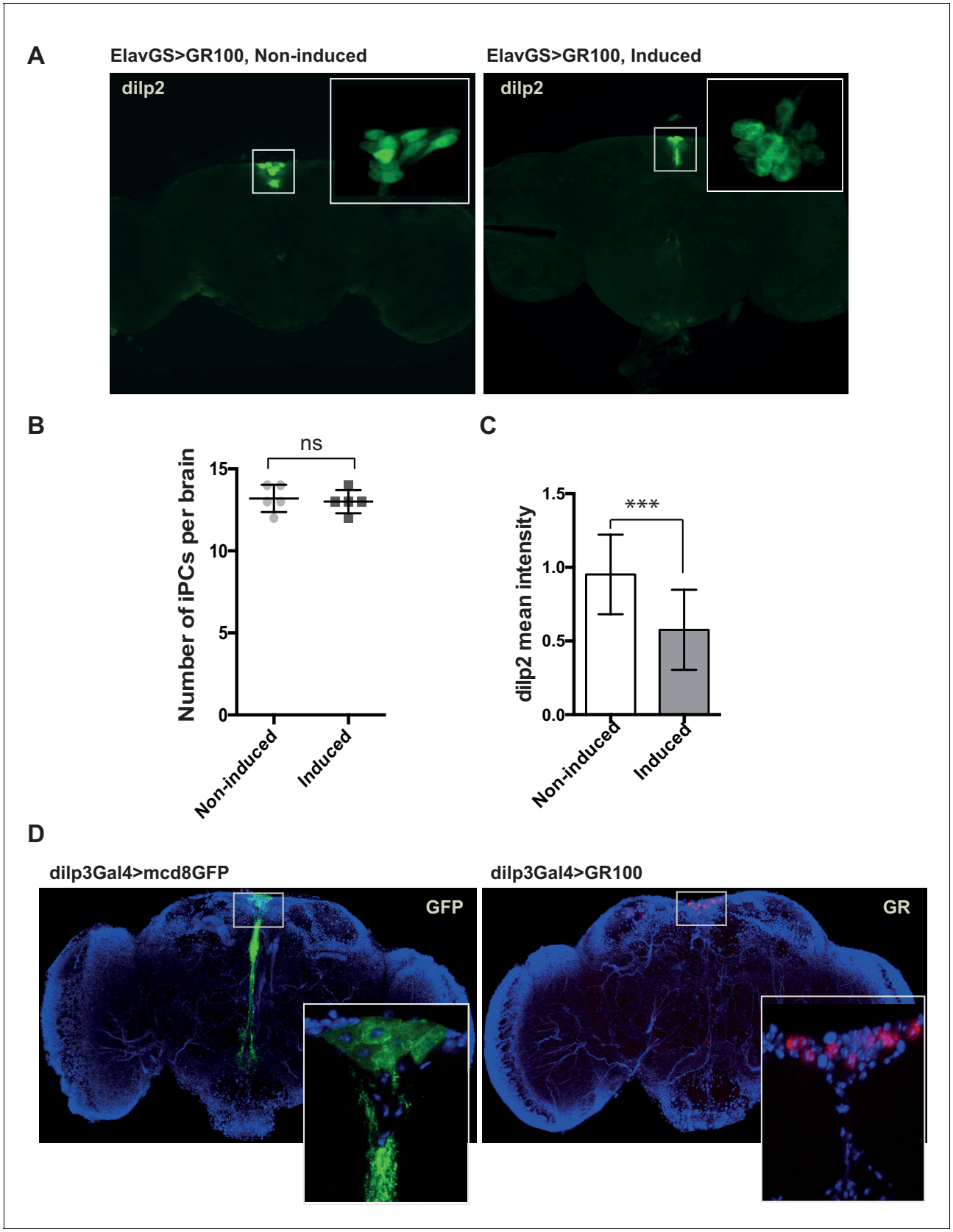

Figure 1—figure supplement 3. Expression of GR100 in fly neurons does not induce loss of IPCs. (**A**) Representative confocal images of dilp2 immunostaining (green) in the IPCs of adult female flies expressing neuronal GR100 (induced) or not (non-induced). (**B**) Quantified IPC number present

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in brains of non-induced and induced flies expressing GR100. No significant difference (p=0.694) was found. Data was assessed by t-test and presented as mean ± SD, n = 5. (**C**) Quantified dilp2 mean immunofluorescence intensities in IPCs. Flies expressing GR100 had significantly reduced dilp2 levels (Mann-Witney test, ***p<0.0001). Data are presented as mean ± SD, n = 24. (**D**) IPC immunostaining with anti-GR (red) and anti-GFP (green) antibodies. Local expression of poly-GR repeats in IPCs using dilp3-Gal4 driver showed that the IPCs were still able to produce GR100 (red). Flies expressing GFP in IPCs were used as control. Genotypes (**A**), (**B**), and (**C**) w; UAS-GR100/+; ElavGS/+. (**D**) w; UAS-GR100 /dilp3-Gal4 and w; UAS-mcd8-GFP /dilp3-Gal4.

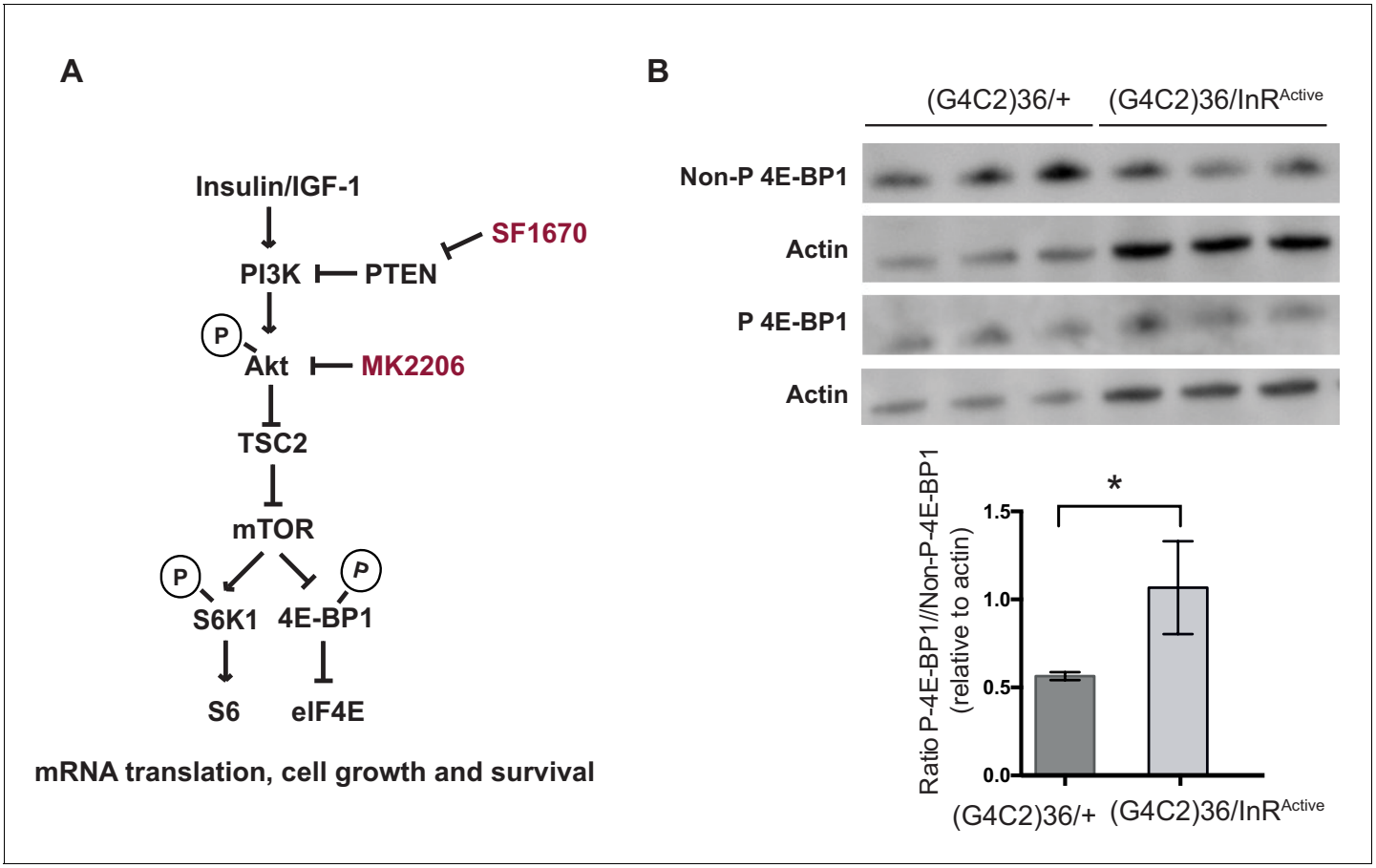

Figure 1—figure supplement 4. Insulin pathway activity is down-regulated in flies expressing (G4C2)36. (**A**) Insulin signalling pathway. Insulin binds to insulin receptor, activating insulin receptor substrates and subsequently PI3K-Akt-mTOR pathway. SF1670 is a PTEN inhibitor and MK2206 is a pan-Akt inhibitor. (**B**) Western blot analysis of the phosphorylated and non-phosphorylated 4E-BP1. Flies expressing (G4C2)36 had a significantly decreased ratio of phosphorylated 4E-BP1 to the non-phosphorylated form compared with flies expressing both (G4C2)36 and InR^{Active} (*p=0.03, unpaired t-test). Data are presented as mean ± SD, n = 3. Genotype (**B**) w; UAS-(G4C2)36/+; ElavGS/+ and w; UAS-(G4C2)36 /UAS-InR^{Active}; ElavGS/+.

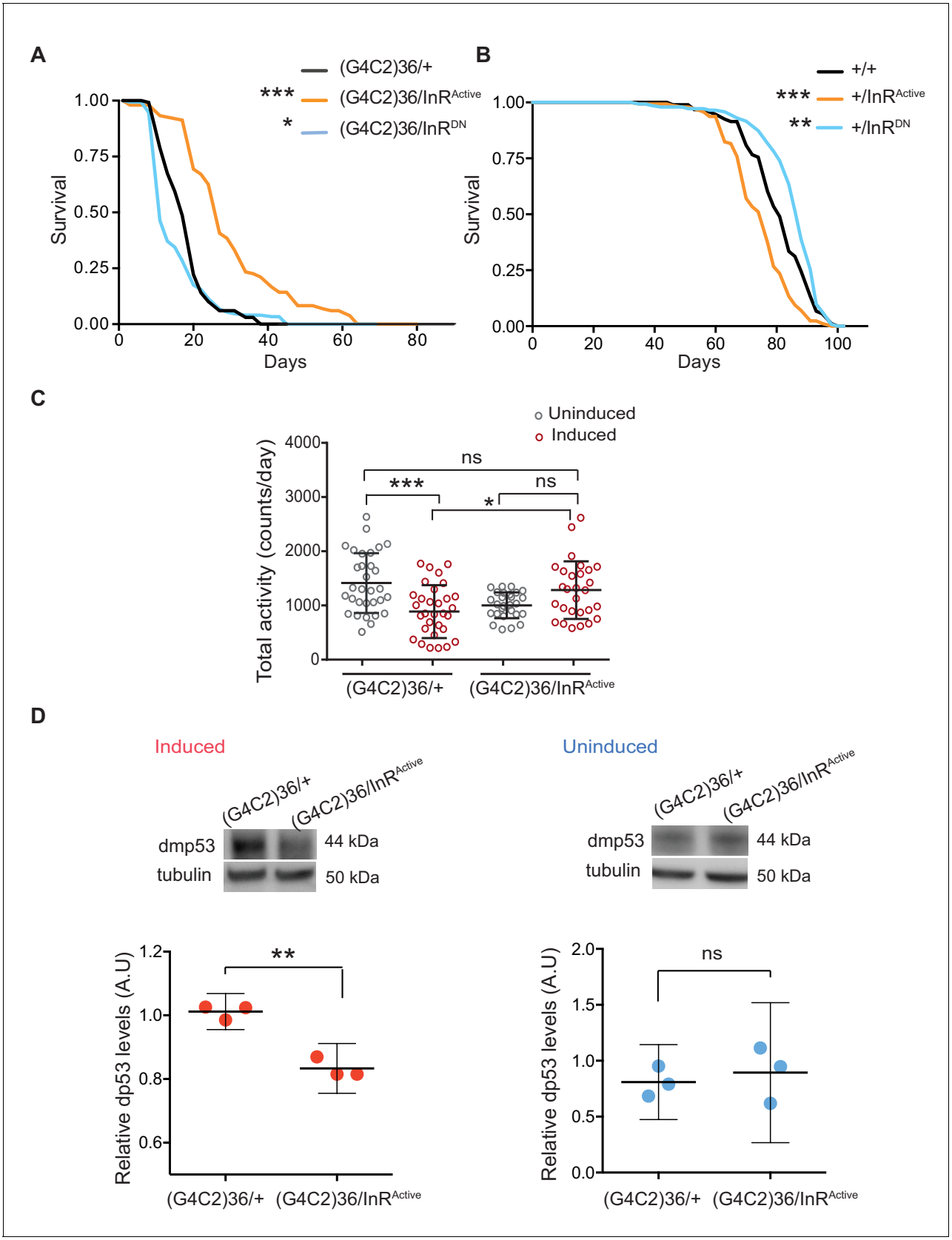

Figure 2. Activation of insulin signalling reduces G4C2 repeat toxicity in vivo. (**A**) Lifespan of flies (n = 150) expressing (G4C2)36 or co-expressing InR constructs (InR^{Active}, InR^{DN}) in neurons. Lifespan was significantly extended in (G4C2)36 disease flies co-expressing InR^{Active} compared with (G4C2)36

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expressing flies (***p=2.8×10^{-21} – log-rank test) and decreased when co-expressed with InR^{DN} (*p=0.027). (**B**) Lifespan of wild-type flies (n = 150) expressing InR^{Active} or InR^{DN} in neurons. Lifespan was significantly reduced in flies expressing InR^{Active} compared with control flies (***p=2.64×10^{-6} – log-rank test) and increased in flies expressing InR^{DN} (**p=0.0035). (**C**) Total activity of flies expressing (G4C2)36 in neurons was significantly reduced compared with uninduced control flies (***p=0.0003). (G4C2)36 flies co-expressing InR^{Active} showed increased activity (*p=0.018) compared with flies expressing (G4C2)36 alone (two-away ANOVA followed by Holm-Sidak's comparison test). Data are presented as mean with SD (n = 30 per genotype). (**D**) Flies expressing (G4C2)36 alone had significantly increased levels of p53 compared with flies expressing InR^{active} (**p=0.0014, t-test). Data are presented as mean ±95% confidence intervals, n = 3. Genotypes (**A**) w; UAS-(G4C2)36/+; ElavGS/+, w; UAS-(G4C2)36/UAS-InR^{Active}; ElavGS/+, w; UAS-(G4C2)36/UAS-InR^{DN}; ElavGS/+. (**B**) w; ElavGS/+, w; +/UAS-InR^{Active}; ElavGS/+, w; +/UAS-InR^{DN}; ElavGS/+. (**C and D**) w; UAS-(G4C2)36/+; ElavGS/+, w; UAS-(G4C2)36/UAS-InR^{Active}; ElavGS/+.

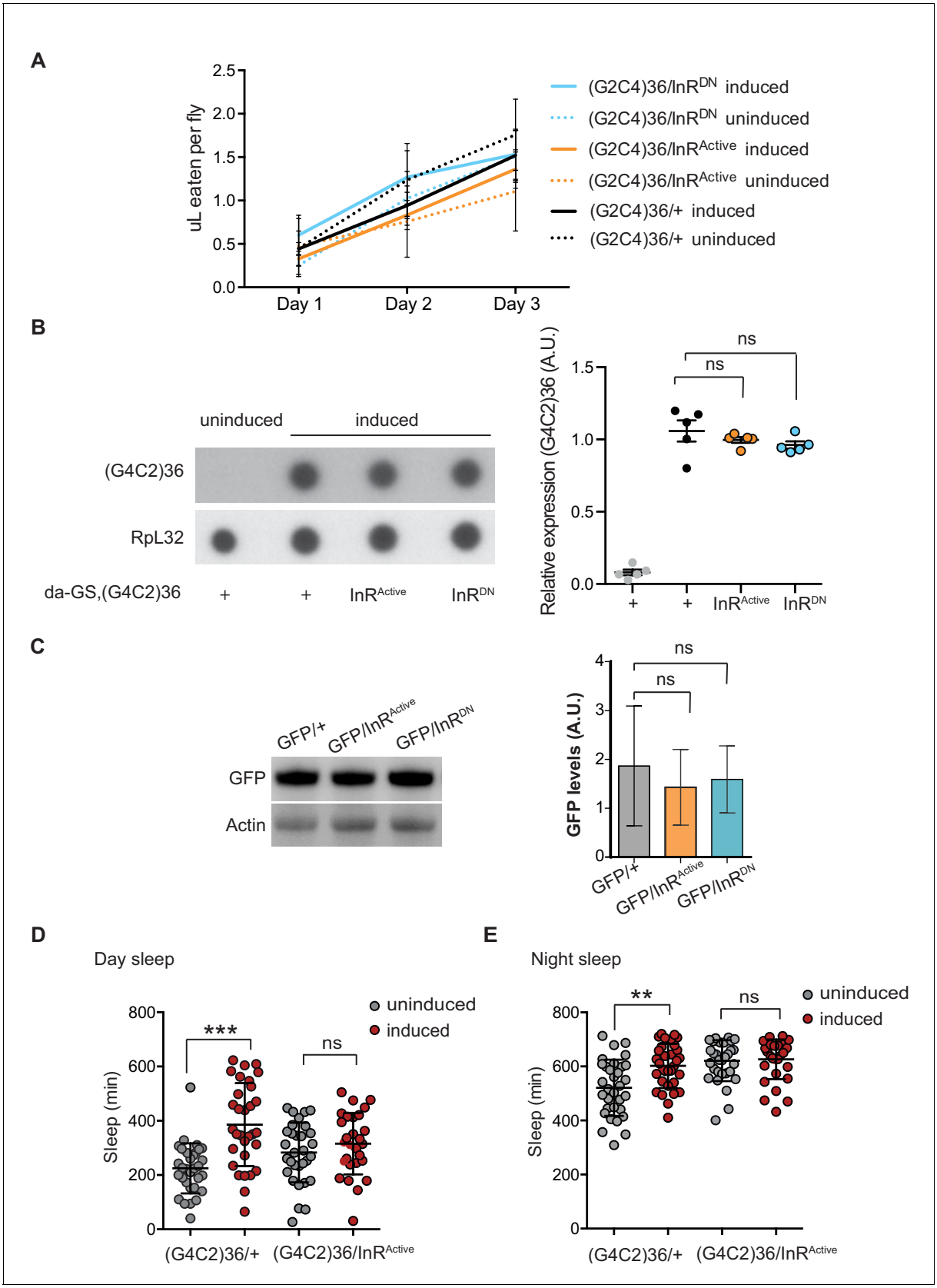

Figure 2—figure supplement 1. Expression of InR construct does not affect fly feeding or ElavGS expression system. (**A**) There were no significant differences in food intake between flies expressing (G4C2 alone or co-expressing InR constructs, or between flies induced or uninduced with RU486,

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one-way ANOVA, followed by Tukey's multiple comparisons test). Data is presented as mean with standard errors of the mean. (**B**) Dot blot analysis of (G4C2)36 transcript levels in flies ubiquitously expressing (G4C2)36 repeats alone or co-expressing InR^{Active} and InR^{DN} transgenes showed no significant differences across the genotypes (one-way ANOVA, followed by Dunnett's multiple comparisons test). (**C**) GFP expression levels in UAS-GFP; ElavGS flies co-expressing InR^{Active} or InR^{DN} determined by western blot analysis. There were no significant differences (p=0.4) in GFP levels between genotypes (one-way ANOVA, followed by Tukey's multiple comparisons test). Data is presented as mean with ±95% confidence intervals, n = 3. (**D**) Expression of (G4C2)36 in neurons increased day sleep of flies (***p<0.0001, two-away ANOVA followed by Holm-Sidak's comparison test), while this was abolished when (G4C2)36 was co-expressed with InR^{Active} (ns; p=0.87). Data are mean ± SD (n = 32 per genotype). (**E**) Expression of (G4C2)36 in neurons increased night sleep of flies (**p=0.0007, two-away ANOVA followed by Holm-Sidak's comparison test), while co-expression of InR^{Active} abolished this phenotype (ns; p=0.81). Data are mean ± SD (n = 32 per genotype). Genotype (**A**) w; UAS-(G4C2)36/+; ElavGS/+, w; UAS-(G4C2)36/UAS-InR^{Active}; ElavGS/+ and w; UAS-(G4C2)36 /UAS-InR^{DN}; ElavGS/+. (**B**) w; da-GS, UAS-(G4C2)36/+, w; da-GS, UAS-(G4C2)36/UAS-InR^{Active} and w; da-GS, UAS-(G4C2)36/UAS-InR^{DN}. (**C**) w; UAS-GFP/+; ElavGS/+, w; UAS-GFP/UAS-InR^{Active}; ElavGS/+ and w; UAS-GFP/UAS-InR^{DN}; ElavGS/+. (**D and E**) w; UAS-(G4C2)36/+; ElavGS/+, w; UAS-(G4C2)36/UAS-InR^{Active}; ElavGS/+.

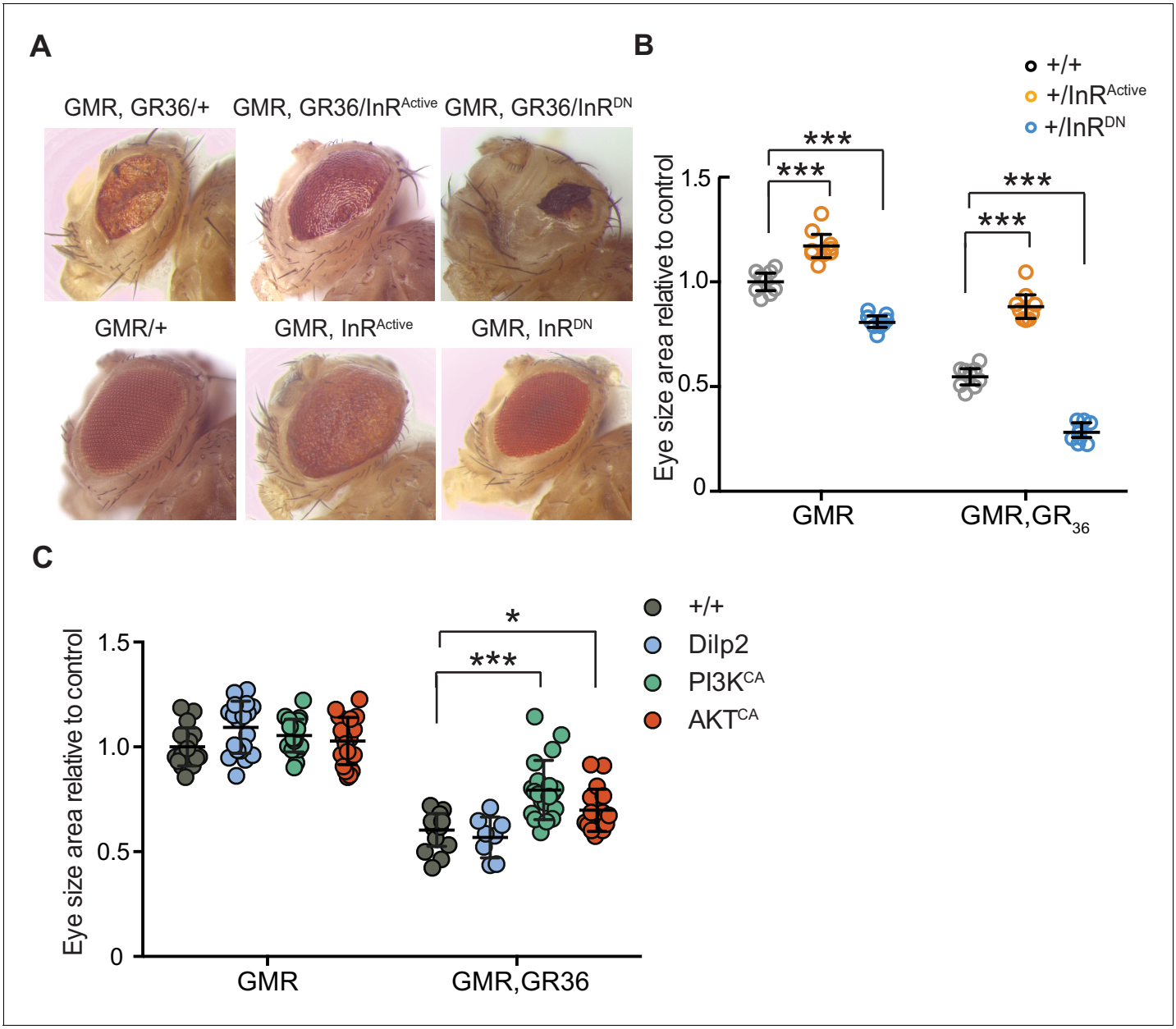

Figure 3. Activation of insulin signalling reduces poly-GR toxicity via InR/PI3K/Akt. (**A**) Stereomicroscopy images of representative 2-day-old adult *Drosophila* eyes expressing InR^{Active} or InR^{DN} using the GMR-GAL4 driver (bottom panel) or co-expressing both GR_{36} and InR constructs (top panel). (**B**) Eye size of flies (n = 10 per genotype) normalized to the mean of the control eye size. Expression of InR^{Active} in a wild-type background with GMR driver caused eye overgrowth, while InR^{DN} decreased eye size ($p<0.001$). Co-expression of the GR_{36} with InR^{DN} greatly decreased eye size (***$p<0.0001$), while with InR^{Active} substantially increased it (two-way ANOVA followed by Holm-Sidak's multiple comparison test). Two-way ANOVA showed a significant interaction between InR genotype and expression of the repeats ($p<0.0001$). Data is presented as mean ± 95% confidence intervals. (**C**) Eye size (n = 20) of 2-day-old adult *Drosophila* eyes expressing dilp2, $PI3K^{CA}$, or Akt^{CA} using the GMR-GAL4 driver. Co-expression of $PI3K^{CA}$ or Akt^{CA} with GR_{36} repeats yielded a partial rescue of the size of the eye (***$p<0.0001$ and *$p=0.036$ respectively, two-way ANOVA, followed by Holm-Sidak's multiple comparison test). Data are presented as mean ± SD. Genotypes: (**A and B**) w; GMR-Gal4/+, w; GMR-GAL4/UAS-InR^{Active}, w; GMR-GAL4/UAS-InR^{DN}, w; GMR-Gal4, UAS-GR_{36}/+, w; GMR-Gal4, UAS-GR_{36}/UAS-InR^{Active}, w; GMR-Gal4, UAS-GR_{36}/UAS-InR^{DN}. (**C**) w; GMR-Gal4, UAS-GR_{36}/+, w; GMR-Gal4, UAS-GR_{36}/+;UAS-dilp2/+, w/$PI3K^{CA}$; GMR-Gal4, UAS-GR_{36}/+, w; GMR-Gal4, UAS-GR_{36}/Akt^{CA}, w; GMR-Gal4/+, w; GMR-Gal4/+; UAS-dilp2/+, w/$PI3K^{CA}$; GMR-Gal4/+, w; GMR-Gal4/Akt^{CA}.

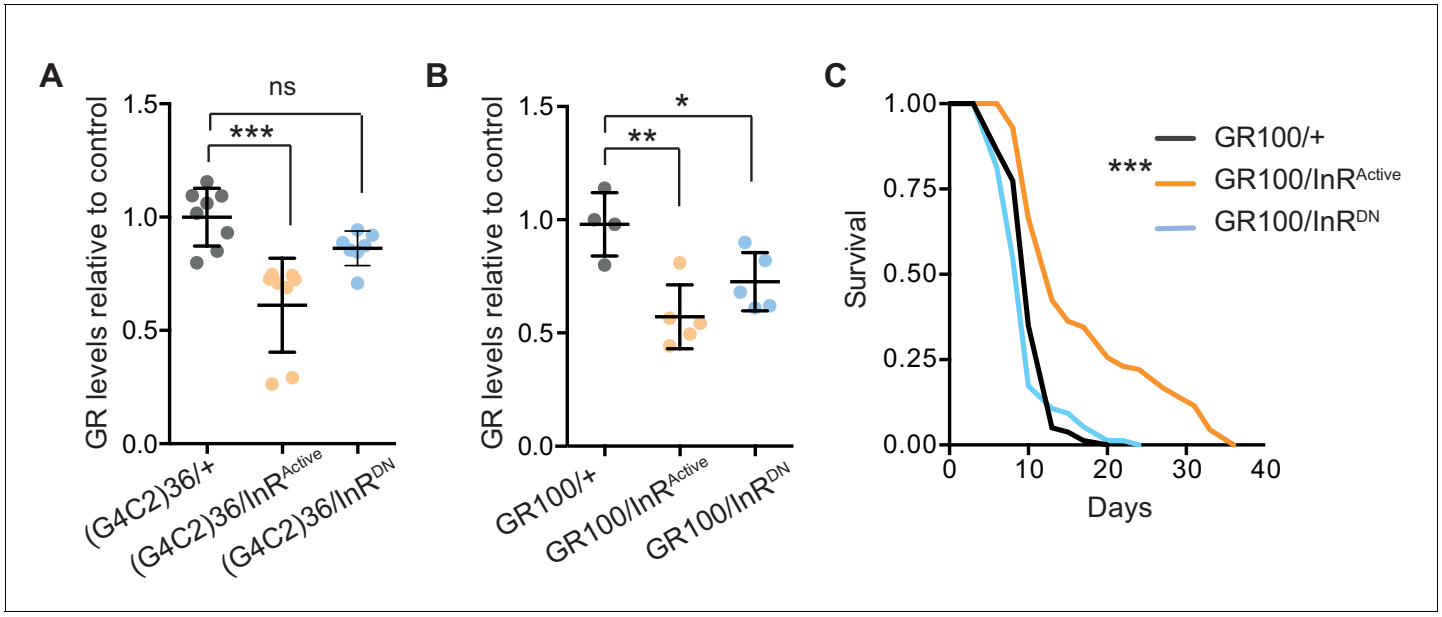

Figure 4. Activation of insulin signalling reduces poly-GR levels in flies. (**A**) GR dipeptide levels determined by Meso Scale Discovery (MSD) immunoassay were reduced in (G4C2)36 flies expressing InR^{Active} compared to (G4C2)36 alone flies (***p=0.0001, one-way ANOVA, followed by Tukey's multiple comparisons test). Levels of GR were normalized to the mean GR levels of control (G4C2)36 flies. Data is presented as mean ± SD, n = 8. (**B**) Expression of poly-GR determined by MSD immunoassay was also reduced in flies expressing both GR100 and InR^{Active} compared to flies expressing GR100 alone (**p=0.0025, one-way ANOVA followed by Tukey's multiple comparison test). Co-expression of InR^{DN} slightly reduced poly-GR levels (*p=0.044). Levels of GR were normalized to the mean GR levels of control (G4C2)36. Data are presented as mean ± SD, n = 5. (**C**) Lifespan was significantly extended in flies (expressing ATG driven GR100 with over-expression of InR^{Active} compared to flies only expressing GR100; ***$p=1.62\times10^{-11}$ – log rank test). Genotypes (**A**) w; UAS-(G4C2)36/+; ElavGS/+, w; UAS-(G4C2)36/UAS-InR^{Active}; ElavGS/+, w; UAS-(G4C2)36/UAS-InR^{DN}; ElavGS/+. (**B**, **C**) w; UAS-GR100/+; ElavGS/+, w; UAS-GR100/UAS-InR^{Active}; ElavGS/+, w; UAS-GR100/UAS-InR^{DN}; ElavGS/+.

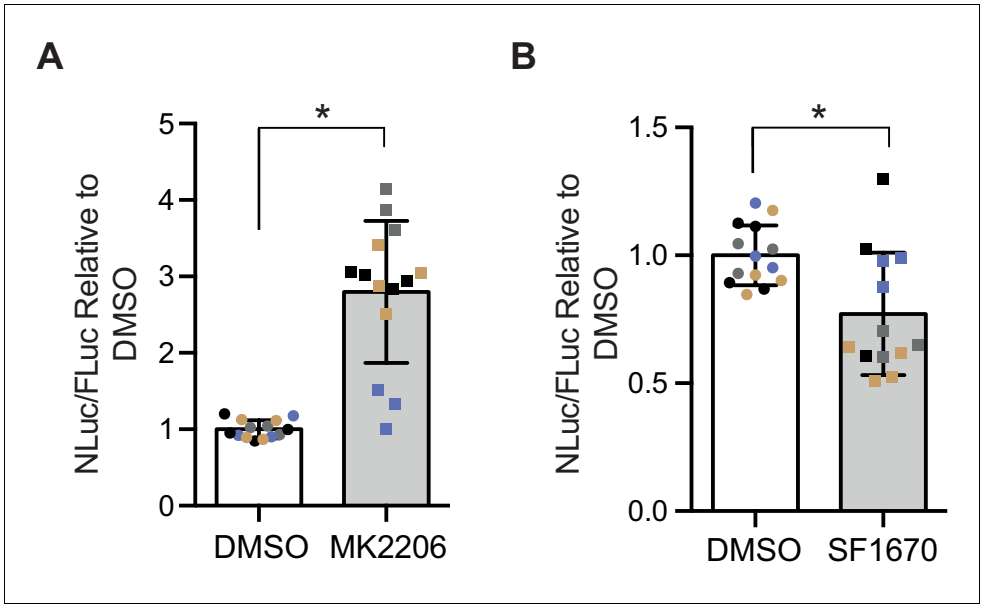

Figure 5. Poly-GR levels are increased by Akt inhibition and decreased by PTEN inhibition in mammalian cells. Poly-GR levels were measured using a NLuc reporter assay following a 48 hr treatment with either 1 μM MK2206 (AKT inhibitor) or SF1670 (PTEN inhibitor). (**A**) MK2206 significantly increases poly-GR levels (*p=0.0168). (**B**) SF1670 significantly decreases poly-GR levels (*p=0.0401). Each NLuc reading was normalized to FLuc for each well and further normalized to DMSO control treatment. Data given as mean ± SD of 4 biological replicates with 3–4 technical replicates per biological replicate. Data analyzed via two-tailed, unpaired Student's t-test on the mean of each biological repeat.

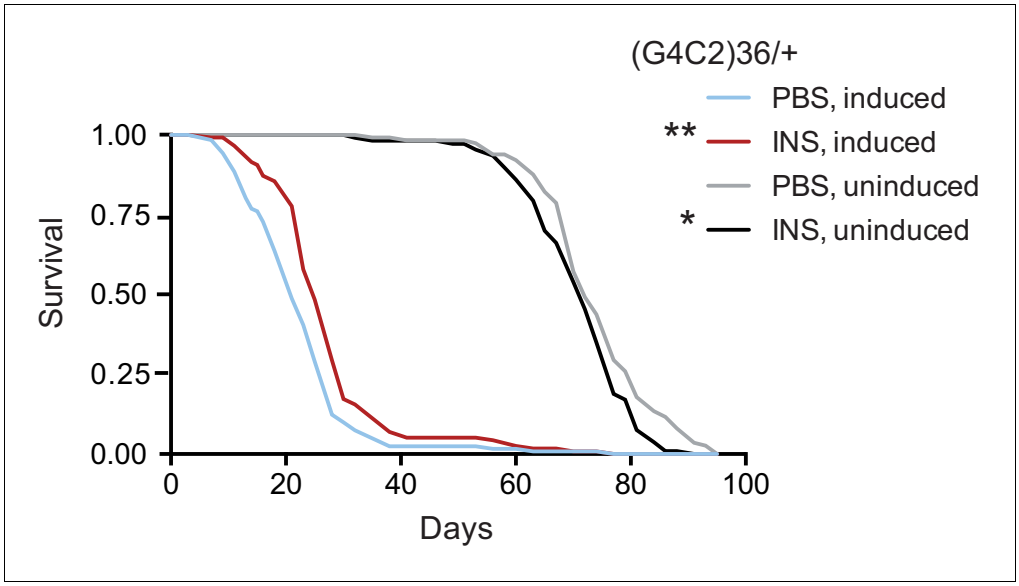

Figure 6. Systemic injection of insulin rescues (G4C2)36 toxicity in *Drosophila*. Injection of 0.03 mg/ml insulin (INS) significantly extended lifespan of flies (n = 120) expressing (G4C2)36 when compared with flies injected with PBS (**p=0.00034, log-rank test), while it slightly shortened lifespan in non-induced flies (*p=0.043). Genotype: w; UAS-(G4C2)36 /+; ElavGS/+.

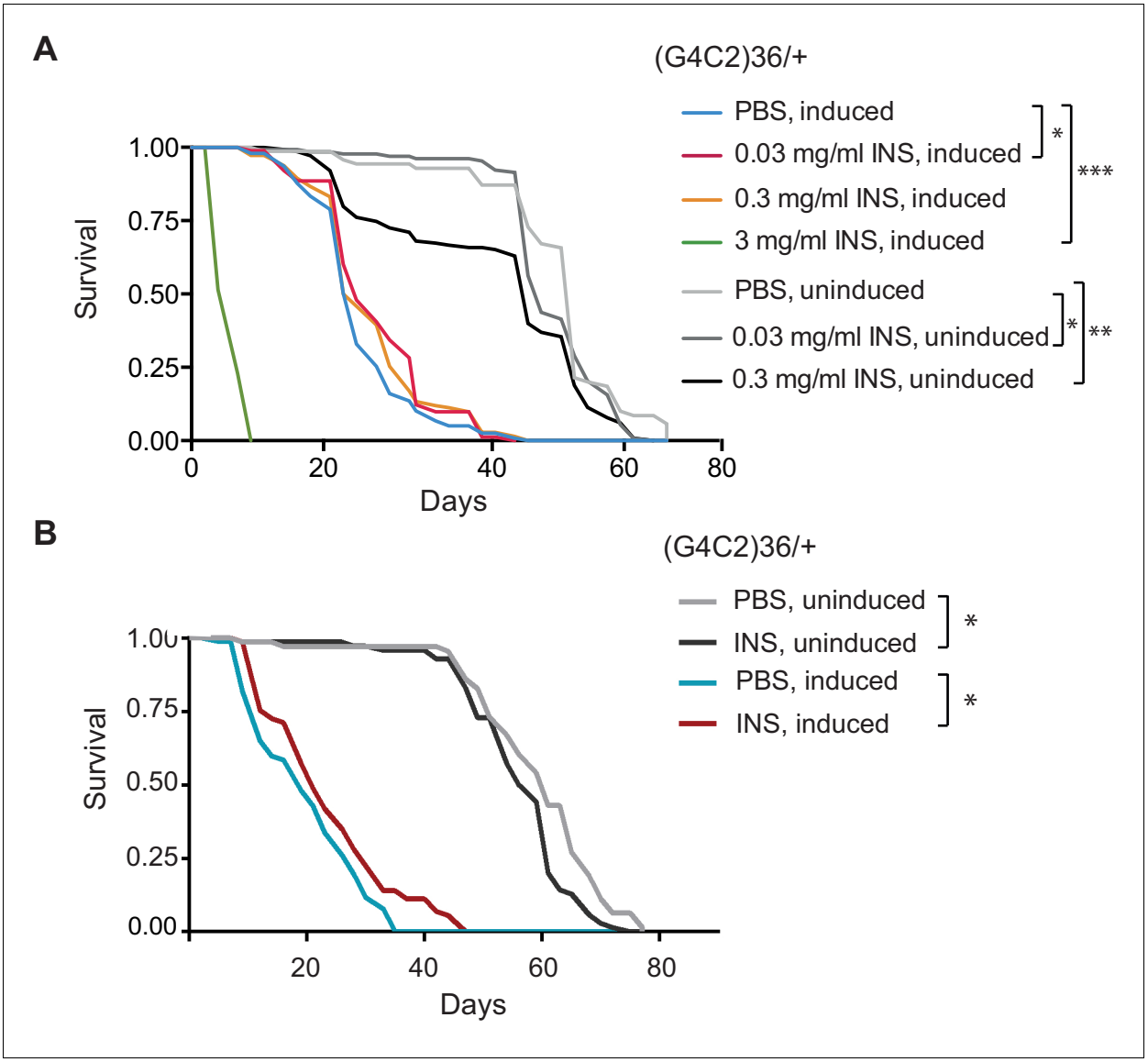

Figure 6—figure supplement 1. Systemic injection of insulin reduces (G4C2)36 toxicity in *Drosophila*. (**A**) Injection of different concentrations of insulin 0.03, 0.3, and 3 mg/ml into (G4C2)36 flies haemolymph (n = 120). Injection of 3 mg/ml insulin into (G4C2)36-induced flies greatly shortened their lifespan ($p<0.0001$, log-rank test), while 0.3 mg/ml had no significant effect ($p=0.083$), and 0.03 mg/ml modestly increased lifespan ($p=0.038$, log-rank test, PBS induced vs. 0.03 mg/ml INS induced). In uninduced (G4C2)36 flies, injection of insulin at 0.3 or 0.03 mg/ml shortened lifespan ($p<0.0001$, log-rank test, PBS uninduced flies vs. 0.3 mg/ml INS uninduced; $p=0.039$, log-rank test, PBS uninduced flies vs 0.03 mg/ml INS uninduced). (**B**) Injection of insulin (INS) at 0.03 mg/ml significantly extended lifespan of flies (n = 80) expressing (G4C2)36 when compared with flies injected with PBS (*$p=0.037$, log-rank test) in induced flies, while it shortened lifespan in non-induced flies (*$p=0.007$). Genotype (A, B) w; UAS-(G4C2)36/+; ElavGS/+.