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Gut-to-brain regulation of *Drosophila* aging through neuropeptide F, insulin, and juvenile hormone

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Affiliations are included on p. 10.

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Dietary restriction (DR) slows aging in many animals, while in some cases, the sensory signals from diet alone are sufficient to retard or accelerate lifespan. The digestive tract is a candidate location to sense nutrients, where neuropeptides secreted by enteroendocrine cells (EEC) produce systemic signals in response to food. Here, we measure how *Drosophila* neuropeptide F (NPF) is secreted into adult circulation by EEC and find that specific EEC differentially respond to dietary sugar and yeast. Female lifespan is increased when gut NPF is genetically depleted, and this manipulation is sufficient to blunt the longevity benefit conferred by DR. Depletion of NPF receptors at insulin-producing neurons of the brain also increases female lifespan, consistent with observations where loss of gut NPF decreases neuronal insulin secretion. The longevity conferred by repressing gut NPF and brain NPF receptors is reversed by treating adults with a juvenile hormone (JH) analog. JH is produced by the adult corpora allata, and inhibition of the insulin receptor at this tissue decreases JH titer and extends lifespan in both males and females, while this longevity is restored to wild type by treating adults with a JH analog. Overall, EEC of the gut modulate *Drosophila* aging through interorgan communication mediated by a gut–brain–corpora allata axis, and insulin produced in the brain impacts lifespan through its control of JH titer. These data suggest that we consider how human incretins and their analogs, which are used to treat obesity and diabetes, may impact aging.

incretin | aging | insulin | interorgan communication | juvenile hormone

Significance

Neuropeptide F (NPF) produced in the *Drosophila* gut is an insulin-regulatory hormone (incretin) that is secreted into adult circulation in response to feeding and diet. Suppression of gut NPF extends *Drosophila* longevity, as does knockdown of NPF receptors at the insulin-producing medial neurosecretory cells in the brain that control the titer of juvenile hormone (JH). Gut hormones and brain insulin regulate lifespan because they control JH titer, which itself is the master endocrine regulator of *Drosophila* aging. Gut NPF modulates *Drosophila* aging through the integration of nutrient sensing, insulin signaling, and JH. Given the role of incretin-mimetic drugs to treat diabetes and obesity, it may be time to consider how incretin analogs could impact human aging.

Drosophila provides insights into aging where we can readily manipulate genes, reproducibly measure lifespan and study specific tissues such as the gut. As with humans, the *Drosophila* gut is maintained with age through stem cells that proliferate and differentiate into enteroblasts, enterocytes (ECs), and enteroendocrine cells (EEC) (1). EEC produce and secrete neuropeptides that mediate digestion and communicate to other organs, including the brain (2). This gut–brain axis can play a central role in health, disease, and aging (3). Mammalian ghrelin produced in the gut induces appetite through its action upon the hypothalamus, while intestinal Peptide YY (PYY) is anorexigenic (4). Pancreatic insulin secretion is amplified by the gut-derived incretins glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) (5, 6). Importantly, analogs of GLP-1 are now used to treat diabetes and obesity (7, 8).

The EEC of the *Drosophila melanogaster* gut produce neuropeptides including allatostatins, Dh31, tachykinin, CCHamides, and Neuropeptide F (NPF) (9–12). Many of these neuropeptides are also produced in the brain (13, 14) but when derived just from the gut, they modulate intestinal stem cell (ISC) division, renal function, feeding, metabolism, and courtship (15–18). Among these, *Drosophila* NPF is an amidated PP-fold neuropeptide that resembles mammalian Neuropeptide Y (NPY) and Peptide YY (PYY) (19, 20). In addition, the *Drosophila* NPF receptor (NPFR) responds to mammalian NPY and PYY when expressed in *Xenopus* oocytes (21). NPFR in adult *Drosophila* are found in the ovary (22), fat body (23), *corpora cardiaca* (24), brain (25), and ventral nerve cord (26). Central to our current study, NPF secreted from the gut is an incretin that induces secretion of insulin from brain median neurosecretory cells (23, 24).

Insulin-like signaling has robust effects on *Drosophila* aging, as it does other animals (27). Reduced insulin signaling extends *Drosophila* lifespan when studies have repressed insulin production, mutated the insulin-like receptors or their substrates, or activated insulin-regulated transcription factors (28–30). Here, we hypothesize that gut NPF modulates aging by regulating insulin secretion from adult median neurosecretory cells of the brain, and that gut NPF mediates how dietary restriction (DR) extends lifespan. We test whether gut NPF impacts aging because it stimulates the corpora allata to produce juvenile hormone (JH). JH is a terpenoid lipid that controls insect development and adult

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The authors declare no competing interest.

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reproduction (31). Earlier work suggests that insulin signaling regulates JH, whereby JH in turn modulates lifespan (32). Here, we establish how gut NPF acts upon insulin signaling to regulate JH and thereby control aging.

We quantified how dietary yeast and sugar differentially influence the level of NPF secreted into the hemolymph by distinct gut EEC. Inhibition of gut NPF from open-type EEC blunted how high yeast diet reduced longevity, while inhibition of closed-type EEC extended lifespan independent of diet. Gut-derived NPF become localized at insulin-producing median neurosecretory cells within the brain, and lifespan was increased by blocking NPFR exclusively in these neurons. This inhibition impaired insulin secreted from the median neurosecretory cells, which limited JH released from the corpora allata. Our previous research demonstrated that lifespan conferred by mutation of *Drosophila* insulin receptors was reversed by treating adults with a JH analog (28). Now, we see that the insulin from the median neurosecretory cells regulates JH titer via insulin receptors upon the corpora allata, and JH is the ultimate endocrine factor by which reduced insulin extends lifespan in response to gut NPF. Gut NPF modulates *Drosophila* aging through the integration of nutrient sensing, insulin signaling, and JH titer.

Results

Closed-Type EEC Secrete NPF in Response to Feeding. Previous studies used the intensity of peptide labeled with antibodies within gut EEC to infer when these cells secrete NPF (23, 24, 33). High stain intensity implies retention of the hormones, but high intensity can also arise if neuropeptides are actively secreted yet produced at an even greater rate. We therefore developed a method to directly quantify secreted NPF in adult hemolymph. We adapted an approach where a biotin ligase BirA*G3-ER (UAS-BirA*G3) is expressed in targeted cells (34). Adult females with specific EEC drivers were fed biotin to label peptides produced in the endoplasmic reticulum of the target cells. Labeled neuropeptides from these cells could then be secreted into the hemolymph. We collected this hemolymph, captured the neuropeptides on streptavidin-coated plates, and quantified NPF with enzyme-linked immunosorbent assay (ELISA) (Fig. 1*A*).

NPF^{gut}-Gal4 (nSyb-Gal80; NPF-Gal4, see refs. 23, and 35) drives UAS-transgenes in closed-type EEC of the midgut R3 region (Fig. 1 *B*3 and *B*4) but not in NPF-producing neurons of the brain or in EEC of the midgut R2 (Fig. 1 *B*1 and *B*2 and *SI Appendix*, Fig. S1*A*). From the hemolymph of adult females, we detect gut NPF tagged with biotin by driving UAS-BirA*G3 with NPF^{gut}-Gal4, and this titer is reduced by simultaneously driving NPF-RNAi in the closed-type EEC (Fig. 1*C* and *SI Appendix*, Fig. S1*B*). Dietary yeast concentration (2% or 10%) did not affect the hemolymph NPF titer, although hemolymph NPF was reduced at *zeitgeber* 8 (ZT 8) relative to earlier in the day (ZT 5) (Fig. 1*D*). To assess how feeding itself impacts NPF from closed-type EEC, females were fasted for 24 h and then fed a full (sugar + yeast), sugar-only, or yeast-only diet. NPF in the hemolymph was equally elevated after refeeding across all diet types (Fig. 1*E*). These secretory responses were also observed with a calcium-dependent nuclear import reporter (CaLexA) (36, 37), in which GFP intensity, indicative of active secretion, was strong in EEC when flies were fed sugar or yeast (*SI Appendix*, Fig. S1*C*). Thus, closed-type EEC are not sensitive to the concentration of dietary yeast but secrete NPF when refed sugar or yeast, and this level declines some hours after feeding. NPF from the closed-type EEC may provide a satiety signal as suggested by Malita et al. (23) and by Gao et al. (33) where reduction of gut NPF *increases* adult feeding.

Open-Type EEC Secrete NPF in Response to Dietary Yeast Concentration. NPF is also seen in open-type EEC of the midgut R2 region. Open-type EEC (14) have an apical extension to the gut lumen (Fig. 2*A*1). The Tkg-Gal4 construct drives expression in NPF-positive open-type EEC of R2 (Fig. 2 *A*1 and *A*2), and in a few closed-type EEC in R3 (Fig. 2*A*3), but not in NPF-producing neurons of the brain (24). Unlike the dietary response observed in closed-type EEC, 10% dietary yeast increased NPF secretion to hemolymph from open-type EEC (Fig. 2*B*). Elevated secretion was apparent at ZT5 but not at ZT8. Likewise, CaLexA signal in open-type EEC was elevated in flies fed 10% yeast (Fig. 2 *C* and *D*). Notably, these secretory events occurred even while we saw high NPF staining intensity within these EEC (Fig. 2 *C* and *E*). Overall, high dietary protein promotes NPF secretion from open-type EEC (Fig. 2*F*). Consistent with previous observations from closed-type EEC (23, 33), feeding in adult females is increased when NPF is reduced in open-type cells (*SI Appendix*, Fig. S1*D*).

Lifespan Is Modulated by Gut NPF in Response to Dietary Protein. Yeast DR extends *Drosophila* longevity, and we now find that open-type EEC secrete NPF in response to high dietary yeast. We therefore determined whether limiting NPF from open-type EEC is required for diet restriction to extend lifespan. Knockdown of gut NPF with Tkg-Gal4 increased the lifespan of females upon high dietary yeast but not when adults were fed 2% yeast (Fig. 3 *A* and *B* and *SI Appendix*, Fig. S2*A*). Relative to wild type, depletion of gut NPF blunted the ability of DR to slow aging (genotype-by-diet interaction: $P < 0.0001$). In contrast, closed-type EEC secrete NPF in response to feeding but do not respond to the concentration of dietary yeast. Accordingly, knockdown of NPF with NPF^{gut}-Gal4 improved lifespan in females maintained on both high and low dietary yeast (Fig. 3 *C* and *D* and *SI Appendix*, Fig. S2*B*). NPF from closed-type EEC modulates aging independent of DR (genotype-by-diet: $P = 0.1519$).

Lifespan Is Modulated by NPFR at Insulin-Producing Cells of the Brain. Gut NPF secreted into hemolymph will act on tissues expressing the NPFR (23, 24, 35). To determine which tissue modulates lifespan in response to gut NPF, we measured female survival when NPFR were depleted in ovaries (38), fat body, *corpora cardiaca* (site of AKH synthesis), and brain median neurosecretory cells (site of insulin synthesis). Median lifespan was robustly extended (10 d, 21%), when NPFR were depleted in median neurosecretory cells (Fig. 4*A* and *SI Appendix*, Fig. S3*A*), while knockdown of NPF in other tissues increased or decreased median lifespan no more than two days (less than ±4%) (Fig. 4 *B*–*D* and *SI Appendix*, Table S1). Previous research found insulin secreted from median neurosecretory cells was reduced by knockdown of gut NPF or of median neurosecretory cells NPFR (24). We confirmed this observation (*SI Appendix*, Fig. S4) and further found NPF secreted from the gut can locate at brain median neurosecretory cells (*SI Appendix*, Fig. S5). Gut-secreted NPF may thus act directly upon median neurosecretory cells NPFR to modulate insulin secretion. Given that altered insulin signaling slows *Drosophila* aging (28–30), reduced gut NPF is expected to extend lifespan because this impairs insulin secretion from the median neurosecretory cells.

The NPF–Insulin Axis Regulates Aging Through Control of JH Titer. Mutation of the *Drosophila* insulin receptor (*dInr*) prolongs longevity and suppresses JH production in the adult corpora

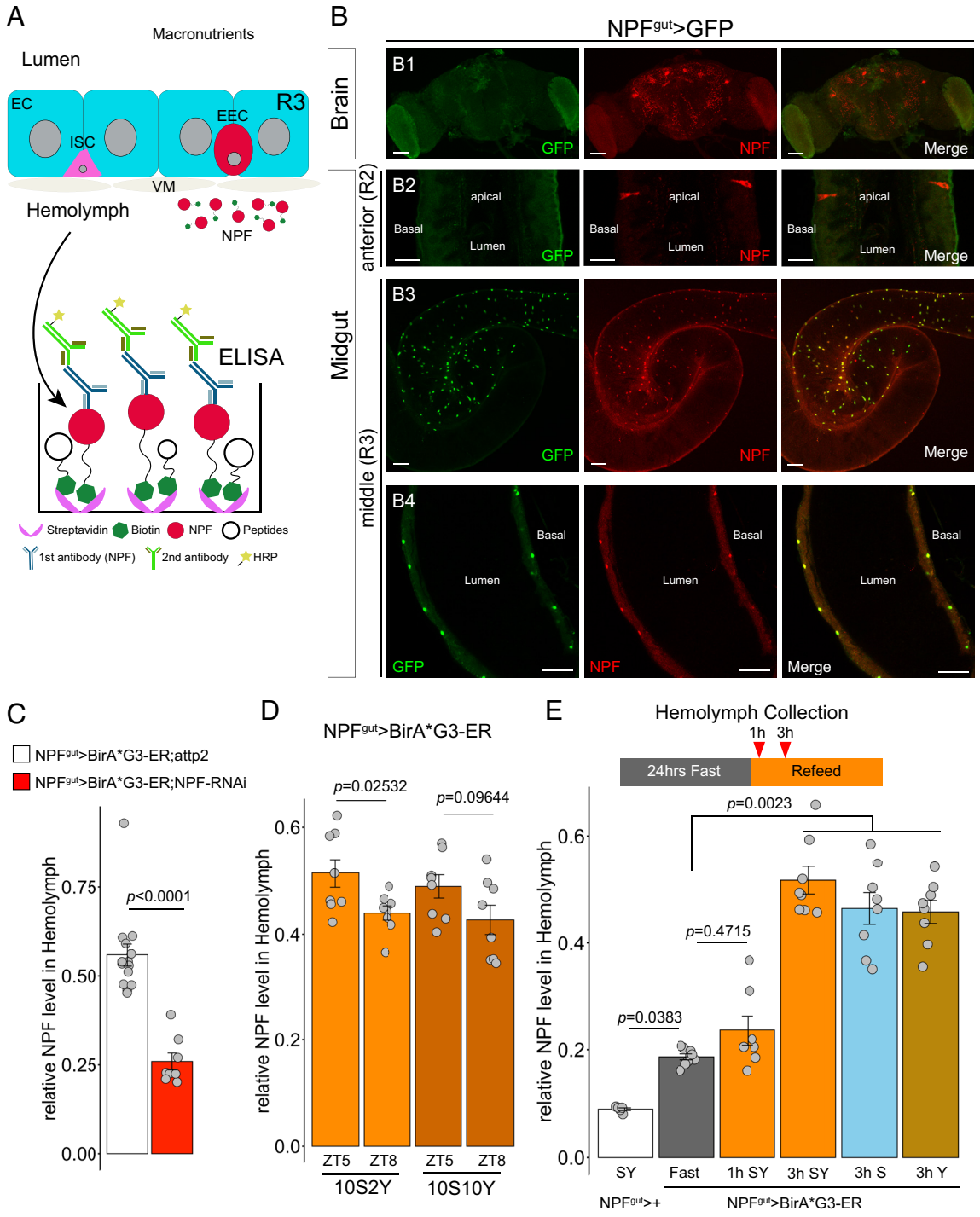

Fig. 1. Closed-type EEC secrete NPF in response to feeding. (*A*) Design for ELISA to measure NPF secreted into hemolymph. EC, EEC, ISC, and visceral muscle (VM). (*B*) Gal4 specific to R3 closed-type EEC (NPF^{gut}-Gal4: NPF-Gal4; nSyb-Gal80) drives UAS-GFP (green), colabeled with ab-NPF (red). GFP is absent from NPF-positive neurons in the brain (*B1*) and NPF-positive open-type EEC of the anterior midgut R2 (*B2*). GFP is expressed in most NPF-positive closed-type EEC of the middle midgut R3 (*B3* and *B4*). (Scale bars, *B1*—50 μm; *B2*—20 μm; *B3* and *B4*—50 μm.) (*C*) NPF in hemolymph measured by ELISA when gut NPF was knocked down in closed-type EEC (NPF^{gut} > BirA*G3-ER; NPF-RNAi) relative to NPF^{gut} > BirA*G3-ER; attp2 (*t* test). (*D*) NPF in hemolymph secreted from closed-type EEC from females maintained on low or high yeast diet at ZT 5 and ZT 8. (Two-way ANOVA with interaction: Diet-by-ZT, F = 0.075, *P* = 0.7861.) (*E*) NPF in hemolymph secreted from closed-type EEC from fasted females refed for 1 or 3 h upon different diets. (One-way Kruskal–Wallis ANOVA.)

allata, an endocrine gland innervated by median neurosecretory cells (28). This extended longevity of *dInr* mutants was restored to wild type by treating adults with the JH analog methoprene, and subsequent work showed that ablation of the adult corpora allata itself robustly extends lifespan (39). JH appears to be a master endocrine regulator of *Drosophila* aging. Accordingly, we determined if NPF regulates lifespan because the gut hormone controls JH titer.

We measured JH activity through its induction of Krüppel homolog 1 (*Kr-h1*), a target of the JH transcriptional complex Met/Gce (40). *Kr-h1* mRNA was reduced in females when gut NPF was depleted by RNAi (Fig. 5*A*) and when NPFR at the median neurosecretory cells were depleted (Fig. 5*B*). Furthermore, we quantified the level of the circulating epoxidated JH in females via LC–MS/MS. JH III and JH III bisepoxide (JHB_3) were reduced by depletion of NPFR at the median neurosecretory cells

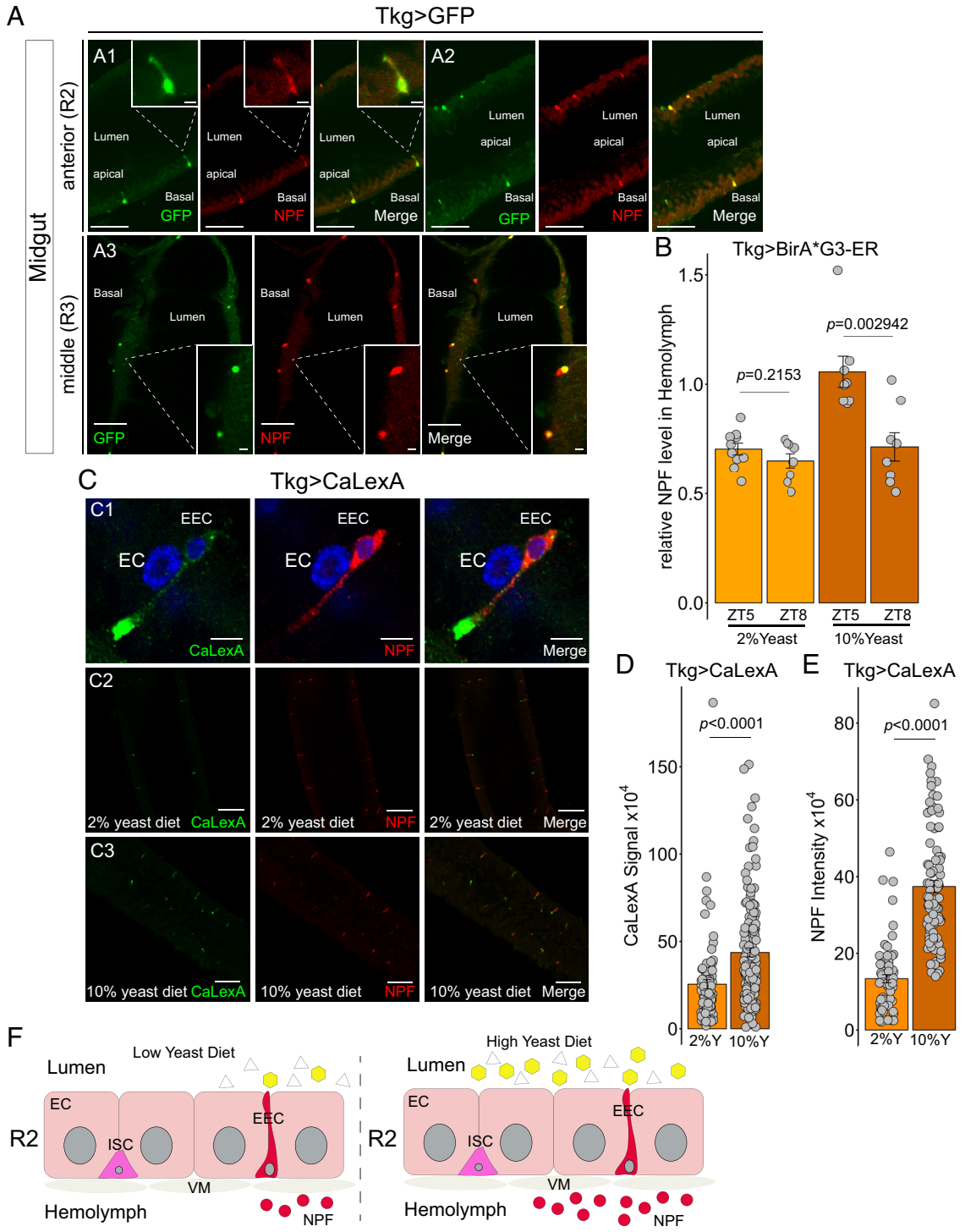

Fig. 2. Open-type EEC secrete NPF in response to dietary yeast concentration. (*A*) Tkg-Gal4 drives in open-type EEC that produce NPF, visualized from UAS-GFP (green) and anti-NPF (red) in anterior R2 midgut (*A1* and *A2*) and some closed-type EEC in the middle R3 midgut (*A3*). (*B*) Biotin tagged NPF (Tkg-Gal4 > BirA*G3-ER) in hemolymph from females maintained on low or high yeast diet, measured at ZT 5 and ZT 8. (Two-way ANOVA: Diet-by-ZT, F = 8.329, *P*= 0.0072.) (*C*) CaLexA activity (green) visualized at NPF-positive (red; nuclei: DAPI, blue) open-type EECs on low (C2) or high yeast (C3); quantified in (*D* and *E*) (Wilcoxon rank-sum tests). (Scale bars, *A*—50 μm (5 μm); C1—5 μm; C2 and C3—50 μm.) EC, EEC. (*F*) Summary for how dietary yeast impacts hemolymph NPF secreted from open-type EEC. EC, EEC, ISC, VM.

(Fig. 5*C*). To confirm that the loss of JH slows aging when we deplete gut NPF or median neurosecretory cells NPFR, these females were treated with the JH analog methoprene. Methoprene reduced the survival of both genotypes to that of methoprene treated wild type controls (Fig. 5 *D* and *E* and *SI Appendix*, Fig. S6). Gut NPF ultimately modulates aging through the action of JH.

JH is synthesized in the adult corpora allata. NPF from the gut could potentially modulate the corpora allata directly, however the corpora allata is not reported to contain NPFR (41), and NPFR-RNAi driven in the corpora allata does not reduce systemic *Kr-h1* mRNA or the titer of JH (*SI Appendix*, Fig. S7*A*). In contrast, the corpora allata express insulin receptors (*dInr*) (42). To determine whether insulin peptides from the median neurosecretory cells

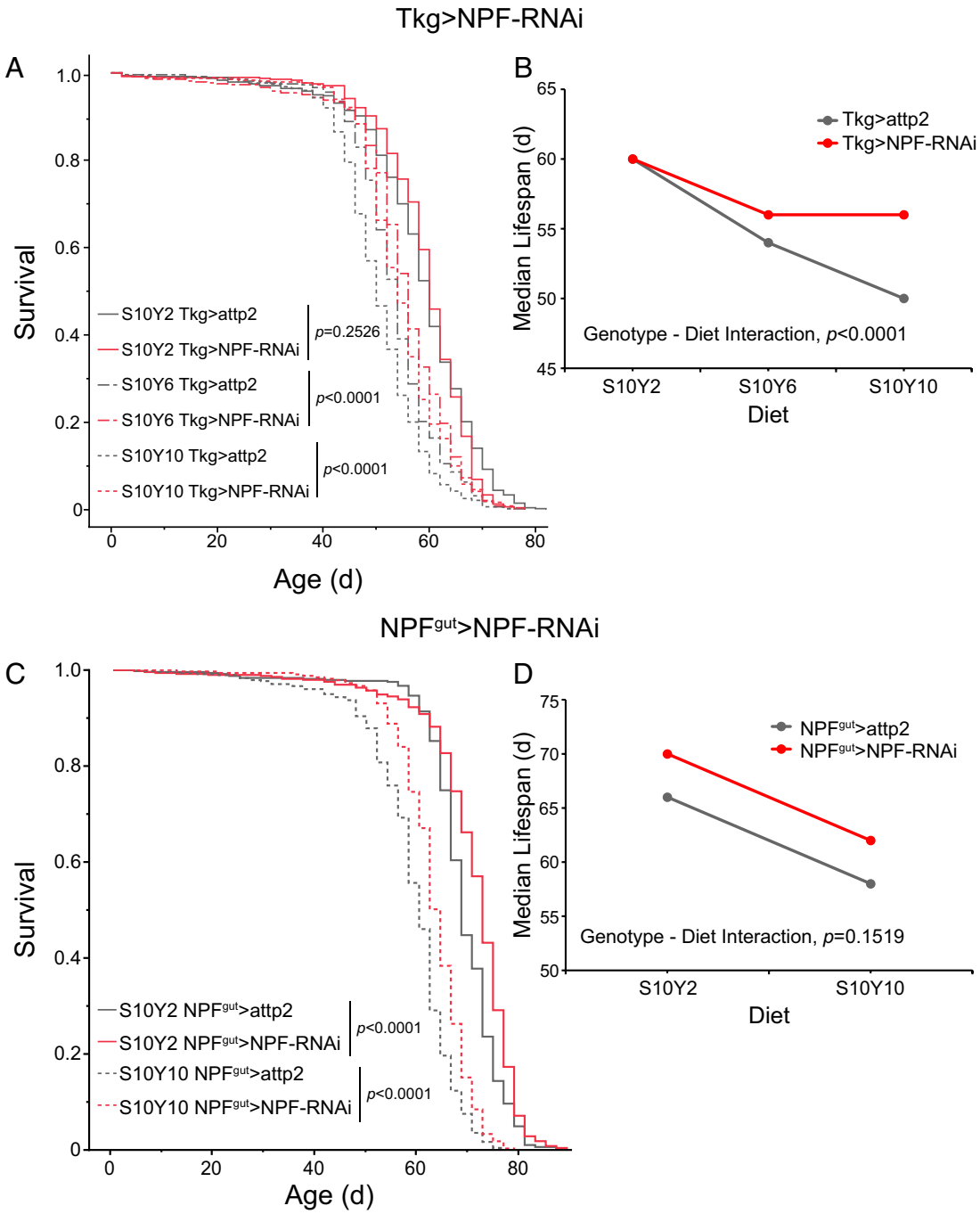

Fig. 3. Depletion of gut NPF slows aging. (*A*) Survival of adult females maintained on diets with 10% sugar (S10) and yeast at either 2% (Y2) or 10% (Y10), while expressing NPF-RNAi in open-type EEC (Tkg-Gal4) relative to wild type. Pairwise survival analyses by log-rank tests. (*B*) Reaction plots of median lifespan of panel *A* cohorts. We observe significant genotype-by-diet interaction inferred from proportional hazard analysis: Genotype, $P < 0.0001$; Diet, $P < 0.0001$; Genotype-by-Diet, $P < 0.0001$. (*C*) Survival was improved on both low and high yeast diet in females where NPF was depleted from closed-type EEC (NPF^{gut}-Gal4). (*D*) Reaction plots of median lifespan of panel *C* cohorts. Knockdown of NPF equally increases lifespan on 2% and 10% yeast diet (Proportional hazard analysis: Genotype, $P < 0.0001$; Diet, $P < 0.0001$; Genotype-by-diet, $P = 0.1519$). Further survival analysis statistics in *SI Appendix*, Table S1.

directly regulate JH, we inhibited insulin receptors in the corpora allata by expressing a receptor dominant negative construct (Aug-21 > $dInr^{DN}$). This reduced systemic *Kr-h1* mRNA (Fig. 5*F*) and robustly increased female lifespan (Fig. 5*G* and *SI Appendix*, Fig. S7*B*). Methoprene treatment reduced the survival benefit of the Aug-21 > $dInr^{DN}$ adults (Fig. 5*G*). Potentially, this rescue could arise if methoprene induces insulin synthesis, but the JH analog did not elevate *Drosophila insulin-like peptide 2* (*dilp2*) mRNA (*SI Appendix*, Fig. S7*C*). JH itself appears to regulate longevity downstream of insulin from the median neurosecretory cells.

The NPF–JH Axis Differs in Males. Systems that modulate aging can differ among the sexes. We therefore compared how gut NPF affects male lifespan relative to its function in females. Knockdown of NPF in gut EEC by Tkg-Gal4 did not extend the lifespan of males on 2% yeast diet (*SI Appendix*, Fig. S8*A*), unlike the benefit seen in females. Likewise, this gut NPF knockdown in males did not appreciably reduce *Kr-h1* mRNA (*SI Appendix*, Fig. S8*A*), indicating it did not affect JH signaling. Knockdown of the NPFR within male median neurosecretory cells reduced lifespan and increased *Kr-h1* mRNA (*SI Appendix*, Fig. S8*B*), opposite

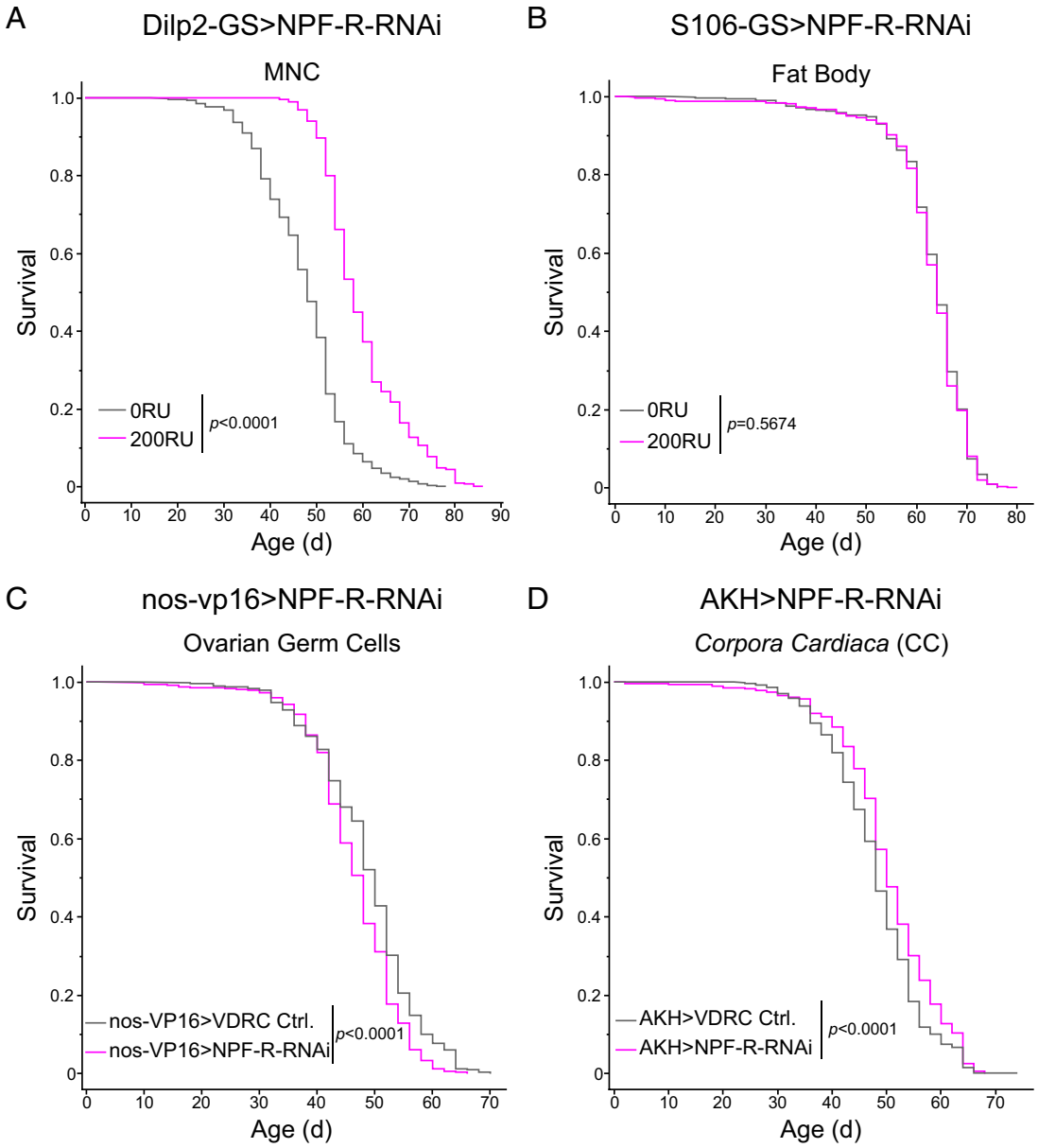

Fig. 4. Knockdown of NPFR in the MNC extends lifespan. (*A*) Female survival was robustly increased by *dilp2*-GS > UAS-*NPFR*-RNAi activated by RU486 relative to the same genotype without RU (log rank test $P < 0.001$). (*B–D*) Survival was minimally affected when NPF was depleted (UAS-NPF-RNAi) in (*B*) fat body; (*C*) germline stem cells; (*D*) *Corpora cardiaca*. Further survival statistics in *SI Appendix*, Table S1.

of what we observed with females. But consistent with females, blocking dInr in the corpora allata (Aug-21 > $dInr^{DN}$) extended male lifespan and reduced JH-associated signaling (*SI Appendix*, Fig. S8*C*). Thus, JH signaling correlates with lifespan in both sexes, as previously seen when ablation of the corpora allata increased longevity for males and females (39). However, the way NPF modulates lifespan and JH differs among the sexes. Detailed analysis is required to unravel the NPF network in males.

Discussion

Multiple Pathways Modulate JH Synthesis. JHs are central regulators of insect development and life history where the biosynthesis of JH is concurrently influenced by internal and environmental factors (43). To regulate the rate of JH synthesis in response to this complexity, the corpora allata expresses receptors for many regulatory factors (44). Ecdysis triggering hormone synthesized by tracheal Inka cells stimulate JH biosynthesis. Likewise, in many insects although not in *Drosophila*, Allatotropin derived from neurosecretory cells of the brain stimulate JH synthesis in the corpora allata. Allatostatic molecules that inhibit JH synthesis include Allatostatin C and Diuretic Hormone 31. While the pathways of these regulators in response to developmental stage, mating, circadian rhythms, and temperature have been recently described (45–49), little is known about how nutrients are sensed in the gut to affect corpora allata activity and in turn how this affects lifespan.

Gut NPF Controls Longevity in Response to Diet. EEC are scattered throughout the epithelium of the *Drosophila* gastrointestinal tract. Here, we find that limiting dietary yeast, which is associated with extended lifespan (50, 51), reduces the secretion of NPF from open-type EEC into the hemolymph. Previous work with *Drosophila* showed that survival was decreased when the nonspecific secretion from all NPF-positive cells was increased (52). Here, we show that specific knockdown of gut NPF is sufficient to extend adult

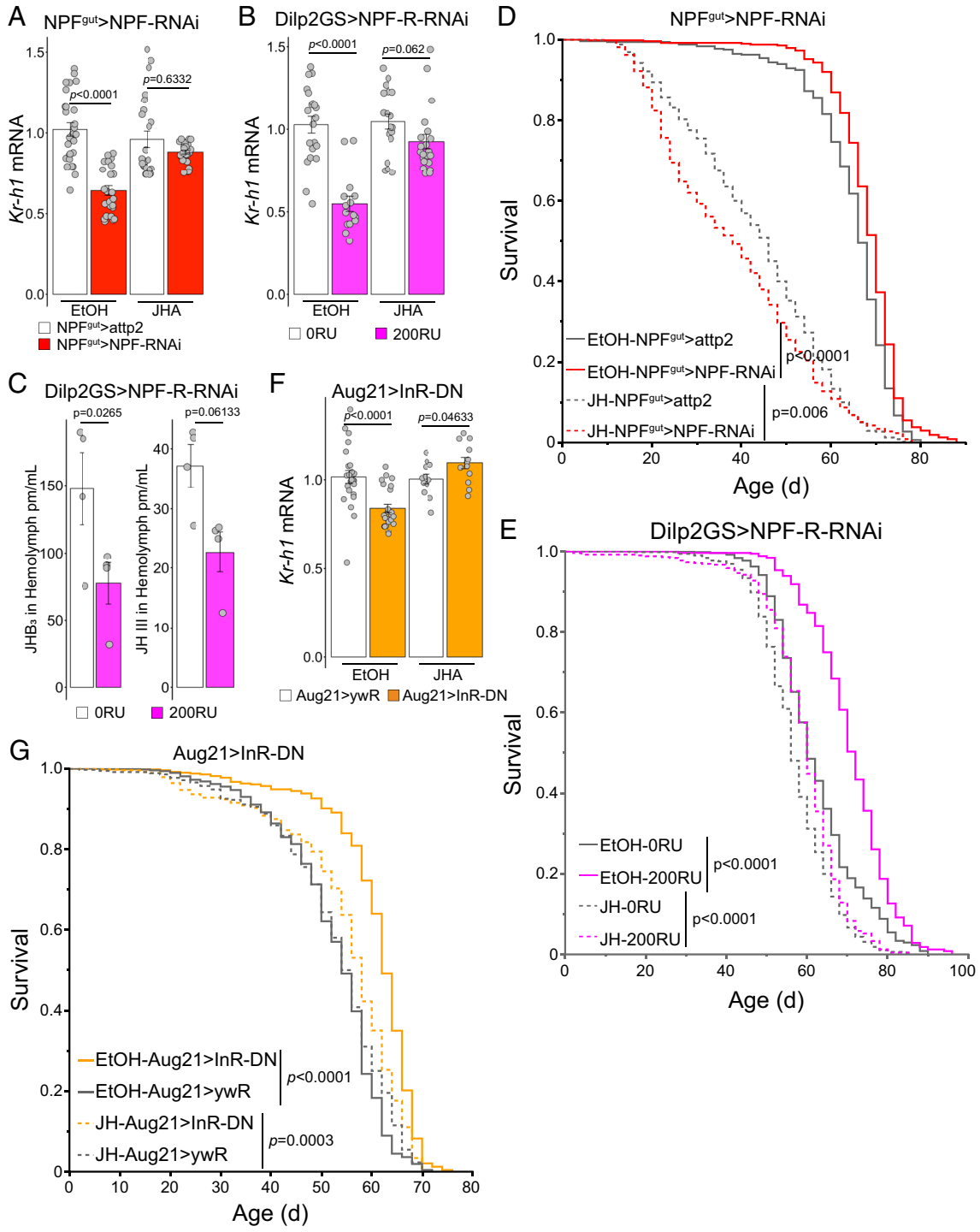

Fig. 5. JH modulates longevity conferred by gut NPF. (*A*) Knockdown of gut NPF (NPF^{gut} > NPF-RNAi) reduced expression of *Kr-h1*, where treatment with JHA restored *Kr-h1* mRNA (Two-way ANOVA: Genotype, F = 39.204, *P* < 0.0001; Treatment, F = 6.482, *P* = 0.0125; Genotype*Treatment, F = 17.917, *P* < 0.0001.) (*B*) Knockdown of NPFR in MNC (Dilp2GS > NPFR-RNAi) activated by RU reduced *Kr-h1* mRNA, where treatment with JHA restored *Kr-h1* mRNA to the level of wild type (Two-way ANOVA: RU, F = 32.93, *P* < 0.0001; Treatment, F = 15.82, *P* < 0.0001; RU*Treatment, F = 14.62, *P* = 0.0003.) (*C*) JHB_3 and JH III in hemolymph is reduced by RU-induced knockdown of NPFR in MNC (Dilp2GS > NPFR-RNAi). (*D*) Knockdown of NPF in the gut extends lifespan (NPF^{gut} > NPF-RNAi), where JHA reduces lifespan and eliminates the advantage conferred by NPF knockdown (Two-way Cox-proportional hazard analysis, treatment-by-genotype interaction, *P* = 0.0002). (*E*) Knockdown of NPFR in the MNC extends lifespan (Dilp2GS > NPFR-RNAi), where JHA rescues lifespan to the level of wild type (Two-way Cox-proportional hazard analysis, treatment-by-RU interaction, *P* < 0.0001). (*F*) Inhibition of *dInr* the dominant-negative *dInr^{DN}* expressed in the corpora allata (Aug21 > dInr^{DN}) reduced systemic *Kr-h1* mRNA, while treatment with JHA restored *Kr-h1* mRNA to wild type (Two-way ANOVA: Genotype, F = 7.278, *P* = 0.0088; Treatment, F = 12.766, *P* = 0.0007; Genotype-by-Treatment, F = 15.416, *P* = 0.0002.) (*G*) Inhibition of insulin receptors in the corpora allata extended lifespan, while treatment with JHA rescued this survival toward that of control genotype with and without JHA (Two-way Cox-proportional hazard analysis, treatment-by-RU interaction, *P* < 0.0001). Further survival statistics in *SI Appendix*, Table S1.

Drosophila survival. Notably, NPF from open-type EEC of the midgut R2 region modulates how dietary (yeast) restriction slows aging. NPF is also secreted from closed-type EEC, but blunting NPF from these cells extends lifespan equally on low- and high-protein diets. Overall, gut NPF can regulate fly aging in response to dietary protein but also independently of diet quality.

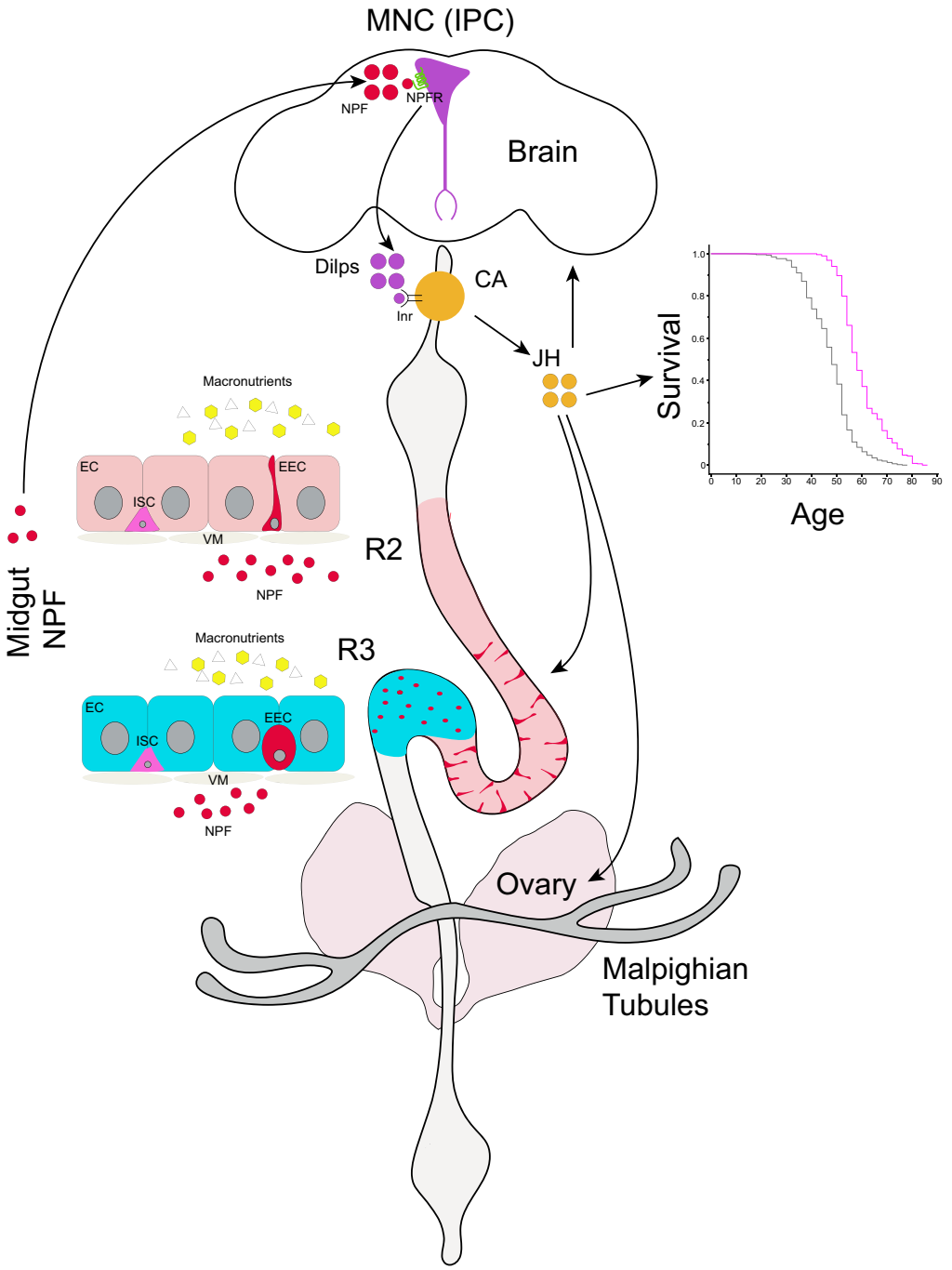

Fig. 6. Model of interorgan communication between gut–brain–corpora allata in *D. melanogaster*. The *Drosophila* midgut contains EC cells, ISC, and EEC. Dietary macronutrients (yeast and sugar) induce EEC to secrete NPF that crosses the ventral muscles (VM) into the hemolymph. Open-type EEC of gut region R2 secrete NPF in response to the concentration of dietary yeast. Closed-type EEC of gut region R3 secrete NPF in response to feeding. Gut NPF enters the brain where it can localize at median neurosecretory cells (MNC) that produce insulin (insulin producing cells, IPC). NPFR at the MNC will be activated by the gut-produced NPF and stimulate these neurons to secrete *Drosophila* insulin-like peptides (Dilps). Dilps stimulate insulin-like receptors (Inr) at the corpora allata (CA), which induces the synthesis and release of JH. JH is highly pleiotropic, acting at many tissues to affect a range of adult phenotypes including behavior (brain), gut remodeling, egg production (ovary), and adult survival.

An NPF–Insulin–JH Axis Controls Aging. Gut NPF secreted into the hemolymph may act on several tissues. Yoshinari (24) previously showed that knockdown of gut NFP reduces insulin secreted from median neurosecretory cells of the brain. Here, we biotinylated neuropeptides produced in gut EEC and tracked secreted NPF to the median neurosecretory cells. Lifespan was increased when NPFR at the median neurosecretory cells were depleted. This receptor knockdown reduced insulin peptide mRNA, as seen in Yoshinari (24). Many studies suggest altered insulin/IGF signaling nonautonomously slows aging, but the underlying mechanisms are poorly understood. We previously suggested insulin signaling affects *Drosophila* aging through control of JH titer (30, 53), and now show that gut NPF and NPFR at the median neurosecretory cells regulate aging because they control JH titer in response to insulin-like peptide.

Our data demonstrate that aging is modulated by interorgan communication between the gut, the brain, and the corpora allata and that insulin signaling affects aging because *Drosophila* insulin is an allatotropic hormone, increasing JH biosynthesis. This outcome suggests a model that coalesces previous observations (Fig. 6). The adult corpora allata produces JH, which modulates a range of adult traits including egg production (46, 54), behavior (48), intestinal remodeling (55), and lifespan (39). The synthesis of JH by the corpora allata is regulated through multiple allatostatic and allatotropic factors (41, 42, 44). Although we have long known that altered insulin likewise mediates JH production in *Drosophila* (28, 53), here we show this control is achieved directly at the corpora allata through insulin receptors that respond to insulin produced by median neurosecretory cells of the brain. Insulin from the median neurosecretory cells is regulated by many

inputs (serotonin, octopamine, GABA, short NPF (sNPF), corazonin, and tachykinin-related peptide) (56), including by gut NPF as first reported by Yoshinari (24). Each therefore has the potential to impact adult JH production. Here, we find that NPF produced by the gut can regulate JH produced in the corpora allata, where this connection is relayed by brain median neurosecretory cells.

A Potentially Conserved Gut–Brain–Endocrine Axis Controls Aging. How does JH ultimately modulate the aging process? JH is transiently elevated in newly eclosed adult *Drosophila* (54) where it controls the final development of the adult gut, fat body, and gonads (55). Adult JH likewise promotes ISC division during gut regeneration (57). JH is repressed during adult *Drosophila* reproductive diapause, a state demonstrated to slow aging (58). To further understand how JH affects *Drosophila* aging, Yamamoto et al. (39) described transcriptional profiles from adults where ablation of the corpora allata extended lifespan. Slow aging was associated with an increase in the amino acid storage protein larval serum protein 1 (LSP1), fatty acid binding protein 1 (FBP1), and the odorant binding protein Obp99b, as well as downregulation of proteases such as Jon25Bii. Most recently, Kim et al. (59) reported that a transient increase in JH titer in early adulthood disrupts protein homeostasis (proteostasis) and decreases lifespan.

Mammals have no direct analog of JH, but the insect corpora allata and mammalian hypothalamic-pituitary system may represent parallel endocrine architectures, which suggest growth hormone or thyroid hormone may modulate aging as we see with JH (60). The *Drosophila* incretin NPF resembles mammalian NPY, peptide YY (PYY), and pancreatic polypeptide (PP). Although NPY is a hypothalamic hormone, it is also produced in the mammalian gut where it influences gastrointestinal motility, electrolyte secretion, and immune function (61). Likewise, PYY is produced in small intestine enteroendocrine L-cells where it is coreleased with the incretin glucagon-like peptide 1 (GLP-1) (62). Both NPY and PYY are proposed to influence mammalian age associated diseases and longevity (63–65), while PYY is thought to act directly on Y1 receptors in the pancreas as well as in the central nervous system to inhibit insulin secretion (66, 67). Here, we find reduced gut NPF is sufficient to slow *Drosophila* aging. This result prompts us to ask whether secretion of gut neuropeptides from EEC might impact human aging, and whether treatment with analogs of gut incretin hormones may alter the aging trajectory independent of their immediate metabolic benefits.

Material and Methods

Fly Husbandry and Stocks. Stocks were reared on standard *Drosophila* medium containing 11% sugar, 2.5% yeast, 5.2% 0.8% agar (w/v in 100 mL water) with 0.2% Tegosept (methyl 4-hydroxybenzoate, Sigma), and maintained at 25 °C, 40% RH, and 12:12 light–dark cycle (LD). RU486 (mifepristone, Sigma) used to activate GeneSwitch-Gal4 was dissolved in ethanol to a concentration of 200 μM and added to cooked food.

Fly lines were backcrossed to *yw* background for at least eight generations. Stocks: NPF-Gal4 (BDSC#25682), Tkg-Gal4 (provided by N. Perrimon, HHMI), VP16-nos-Gal4 (BDSC#4937), Dilp2-GeneSwitch-Gal4 (68), S106-GeneSwitch-Gal4 (69), AKH-Gal4 (BDSC#25683), Aug21-Gal4 (BDSC#30137), nsyb-Gal80 (35) (provided by S. Pletcher, University of Michigan), UAS-NPF-RNAi (BDSC#27237), UAS-attp2 (BDSC#36303), UAS-InR^{DN} (BDSC#8253), UAS-NPF-R-RNAi (VDRC KK107663), UAS-VDRC-control (VDRC 60100), UAS-20xUAS-IVS-mCD8::GFP (BDSC#32194), UAS-CaLexA (BDSC#66542), and UAS-BirA*G3-ER-Myc (34) (provided by N. Perrimon, HHMI).

Demography and Survival Analysis. Newly eclosed adults were transferred to fresh standard food in bottles to mate for 48 h at 25 °C, 40% RH, and 12:12 LD cycle. Forty-eight hours old female adults were sorted with light CO_2 and then pooled in 1L demography cages maintained at 25 °C, 40%RH and 12:12 LD cycle with an initial density of 125 adults per cage. Four cages were initiated per genotype. Food vials of demography cages contained 10% sugar, 5.2% cornmeal, 0.8% agar and 2% or 10 % yeast for 2%- or 10%-yeast diet, respectively. Food vials were changed daily for the first 45 d and every 2 d thereafter. Dead flies were removed from each cage and counted every 2 d. Treatment with JH analog (Methoprene; Sigma PESTANAL, racemic mixture) followed the methods of Yamamoto (39): 10 μL of 32.2 μM methoprene in ethanol was applied to a cotton bud fixed within each demography cage and replenished twice weekly.

Immunostaining and Imaging. Tissues of adult flies were dissected in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (PFA) in PBS at room temperature with gentle shaking for 45 min (gut) or 90 min (adult brain). After six washes with PBS+0.3% Triton-X, samples were blocked in PBS+0.3% Triton-X with 5% normal goat serum (NGS, Thermo Fisher) at room temperature for 2 h. After six further washes with PBS+0.3% Triton-X, samples were incubated in primary antibody dilutions in PBS+0.3% Triton-X with 5% NGS at 4 °C overnight. Primary antibodies included anti-NPF 1:1,000 (RayBiotech RB-19-0001), anti-GFP 1:2000 (Abcam ab13970), and anti-P-Glycoprotein (C219) 1:100 (Invitrogen MA1-26528). Washed samples (6X) were incubated in secondary antibody dilutions and additional staining reagents in PBS+0.3% Triton-X with 5% NGS at room temperature for 2 h. Secondary antibodies and reagents included Goat anti-Chicken IgY 488, 1:200 (Invitrogen A11039), Goat anti-Mouse IgG 488 (Invitrogen A11029), Goat anti-Rabbit IgG 555 (Invitrogen A21428), anti-Streptavidin 647 (Invitrogen S32357), and DAPI, 0.5 μg/mL (Sigma). Samples were mounted in 80% glycerol. Images were acquired with a Zeiss LSM 800 Confocal Microscope and analyzed using ImageJ software. Total fluorescence intensity of identified EEC in confocal sections was measured using the 3D Object counter of ImageJ.

Biotin-Containing Fly Food and *Drosophila* Culture. Biotin-containing food was prepared following Droujinine (34). 18 mM biotin stock was made by dissolving solid biotin (Sigma B4639) in water, adjusting the final pH to 7.2. Food with biotin at a final concentration of 100 μM was prepared, dried overnight, and stored at 4 °C. EEC driver lines (−Gal4) were crossed to UAS-BirA*G3-ER. Progeny were allowed to mate for 2 d at 25 °C on standard food without biotin. Females were switched to 29 °C for 2 d on standard food and then transferred to food with 100 μM biotin while at 29 °C for 6 d. Fresh food was provided daily.

Hemolymph Collection. Hemolymph was processed from biological replicates, each made from a pool of 20 flies. Flies were punctured at the thorax with a tungsten needle (Fine Science Tools) and transferred to a 0.5 mL tube where the bottom contained a 25-gauge hole. To collect hemolymph for ELISA, the 0.5 mL tube was inserted into 1.5 mL low-protein binding tube and the two-tube assembly centrifuged at 9,000 rpm for 5 min at 4 °C. The supernatant was kept on ice and biotinylated NPF was pulled down on 96-well streptavidin-coated plates and quantified by ELISA (see below). To collect hemolymph for JH measurement, the 0.5 mL tube with flies was inserted into a siliconized glass vial (Fisher Scientific 03-395G), with the assembly centrifuged at 9,000 rpm for 5 min at 4 °C. The supernatant was kept on ice and hemolymph JH was extracted and measured by LC–MS/MS (see below).

Protein Lysate Preparation. For lysis buffer, RIPA (Pierce) was supplemented with PMSF (Sigma) PhosSTOP (Roch) and cOmplete EDTA-free Tablet (Roch). For each biological replicate, 10 dissected brains were placed in lysis buffer with beads and homogenized with a TissueLyser (Retsch) in three cycles of 2 min. Samples were centrifuged for 15 min at 4 °C. The collected supernatant was assayed for total protein concentrations (BCA protein assay kit, Pierce) and biotinylated gut-NPF was pulled down with streptavidin-coated plated and quantified by ELISA (see below).

Streptavidin Plate Pulldown and ELISA. For each biological replicate of hemolymph or brain proteins, 1 μL of hemolymph or 2 μg of protein lysate was diluted into 200 μL of PBS + 0.05% Tween20. Then, 100 μL of samples was pipetted into each well of a 96-well streptavidin-coated plate (Thermo Fischer 15125) and incubated on a shaker at 4 °C overnight. Incubated plates were washed six times with PBS + 0.05% Tween20 and then exposed to anti-NPF antibody (1:1,000) for 3 h. After six washes, samples were incubated with HRP-conjugated secondary

antibody at 1:5,000 (Jackson Immuno Research 111-035-144) for 2 h. After six final washes, samples were treated with TMB solution (Cell signaling 7004) for 30 min until terminated with Stop solution (Cell signaling 7002). Absorbance was recorded at 450 nm using a SpectraMax M5 (Molecular Devices LLC).

JH Extraction and Measurement. First, 5 μL hemolymph of each biological replicate was transferred into prechilled siliconized glass vials (Fisher Scientific 03–395G) on ice with a siliconized glass pipette and supplemented with 150 μL of chilled PBS from the same tip. Then, 10 μL of 6.25 pg/μL JH III–D3 (Toronto Research Chemicals, #E589402) in acetonitrile was added into each sample along with 600 μL of hexane. Samples were vortexed for 1 min and centrifuged for 5 min at 4,500 rpm. Then, 500 μL of the organic phase was transferred to a fresh silanized glass vial. JH titers were measured using LC–MS/MS as described in Ramirez et al. (70).

qRT-PCR. Total mRNA was extracted from five biological replicates of eight females (once-mated, aged 9 d) for each genotype using Trizol reagent (Invitrogen 15596018) and Direct-zol RNA Miniprep Plus Kits (R2072). RNA yield was determined with a NanoDrop ND-1000 Spectrophotometer. cDNA was synthesized using iScript (Bio-Rad 1708890), and RT-qPCR performed by QuantStudio 3 (Applied Biosystems) with SYBR Green PCR Master Mix (Applied Biosystems 4309155). Relative mRNA abundance of each gene was normalized to *rp49*; see *SI Appendix*, Table S2 for primer sequences.

Statistical Analysis. Data are presented as mean ± SEM from independent biological replicates. For data confirmed to be normally distributed, comparisons were based on Student's *t* tests or one-way ANOVA with post hoc multiple-testing, and Wilcoxon rank-sum or one-way Kruskal–Wallis ANOVA were used to evaluate nonnormally distributed data. Two-way ANOVA was performed to determine the interaction between JHA treatment and manipulated genes, or diets and ZT. Survival analysis was conducted in JMP Pro 16 software with combined data from replicate cages. Log-Rank tests provided pairwise comparison of survival distributions. Proportional hazard survival analysis was used to infer whether lifespan was affected by the interaction between JHA treatment and manipulated genes.

Data, Materials, and Software Availability. Electronic repository data have been deposited in Brown Digital Repository (https://doi.org/10.26300/5909-pk25) (71).

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