PNAS RESEARCH ARTICLE | GENETICS OPEN ACCESS

Trametinib ameliorates aging-associated gut pathology in *Drosophila* females by reducing Pol III activity in intestinal stem cells

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Edited by Lynn Riddiford, University of Washington, Friday Harbor, WA; received July 12, 2023; accepted November 17, 2023

Pharmacological therapies are promising interventions to slow down aging and reduce multimorbidity in the elderly. Studies in animal models are the first step toward translation of candidate molecules into human therapies, as they aim to elucidate the molecular pathways, cellular mechanisms, and tissue pathologies involved in the anti-aging effects. Trametinib, an allosteric inhibitor of MEK within the Ras/MAPK (Ras/Mitogen-Activated Protein Kinase) pathway and currently used as an anti-cancer treatment, emerged as a geroprotector candidate because it extended lifespan in the fruit fly *Drosophila melanogaster*. Here, we confirm that trametinib consistently and robustly extends female lifespan, and reduces intestinal stem cell (ISC) proliferation, tumor formation, tissue dysplasia, and barrier disruption in guts in aged flies. In contrast, pro-longevity effects of trametinib are weak and inconsistent in males, and it does not influence gut homeostasis. Inhibition of the Ras/MAPK pathway specifically in ISCs is sufficient to partially recapitulate the effects of trametinib. Moreover, in ISCs, trametinib decreases the activity of the RNA polymerase III (Pol III), a conserved enzyme synthesizing transfer RNAs and other short, non-coding RNAs, and whose inhibition also extends lifespan and reduces gut pathology. Finally, we show that the pro-longevity effect of trametinib in ISCs is partially mediated by Maf1, a repressor of Pol III, suggesting a life-limiting Ras/MAPK-Maf1-Pol III axis in these cells. The mechanism of action described in this work paves the way for further studies on the anti-aging effects of trametinib in mammals and shows its potential for clinical application in humans.

Trametinib | aging | *Drosophila* | gut pathology | Pol III

Average life expectancy in humans has doubled during the last 100 y, currently surpassing 83 y in wealthy countries such as Switzerland, Australia, and Japan (1). However, healthy lifespan, or "healthspan," is not increasing at the same rate. This has resulted in an increasing prevalence of age-related diseases, such as cardiovascular dysfunctions, cancers, and neurodegenerative disorders, associated with an escalating economic burden and pressure on healthcare services (2, 3). Some pharmacological agents already used in the clinic, such as the mammalian target of rapamycin (mTOR)-inhibitor rapamycin, can counteract aging-related phenotypes and diseases in animal models. Repurposing of these and other drugs as potential geroprotective treatments is hence being proposed to compress the period of morbidity in older people (4). Evidence from animal models, including the tissues and pathologies improved by these drugs, the molecular pathways involved, and sexually dimorphic responses and side-effects are needed to accelerate the transition of these treatments to human clinical trials.

Trametinib is an anti-cancer drug currently used for the treatment of metastatic melanoma, anaplastic thyroid cancer, and non-small cell lung cancer and has been described as a potential anti-aging drug based on data from the fruit fly *Drosophila melanogaster* (5, 6). It is an allosteric inhibitor of MEK, the Mitogen-Activated Protein Kinase (MAPK) of the Extracellular-Signal-Regulated Kinase (ERK), part of the Ras/MAPK pathway, a highly conserved signaling cascade of kinases controlling cell survival, proliferation, growth, and differentiation (7, 8). The Ras/MAPK pathway can be activated by different receptor tyrosine kinases present at the plasma membrane of the cell, including insulin receptor and epidermal growth factor receptor. This results in the activation of Ras small nucleotide guanosine triphosphate hydrolases (Ras GTPases) and the downstream phosphorylation cascade composed of Raf, MEK, and ERK kinases (9–11). Once phosphorylated, ERK can modify a wide range of cytoplasmic and cytoskeletal protein substrates, as well as several nuclear transcription factors, and hence activate cell division, differentiation, survival, and growth (12).

Ras/MAPK pathway hyperactivation leads to uncontrolled cell proliferation and is one of the best-described mechanisms leading to tumor formation (13). On the other hand, direct inhibition of Ras orthologs and other components of the Ras/MAPK pathway

Significance

Human life expectancy has increased markedly during the last decades thanks to advances in medicine, hygiene, and nutrition, among other factors. However, this demographic change brings an increased prevalence of age-related diseases, such as cancer, cardiovascular and neurodegenerative diseases. Aging is malleable in animal models, and pharmacological interventions can reduce the incidence of age-related pathologies. Here, we show that trametinib, an anticancer agent, extends lifespan and improves gut health in female fruit flies. This effect is mediated by the inhibition of RNA polymerase III, a conserved enzyme that synthetizes short, non-coding RNAs such as tRNAs (transfer RNAs). Our findings advance the understanding of the anti-aging properties of trametinib in animals and confirm its potential as a geroprotective intervention.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2311313121/-/DCSupplemental.

Published January 19, 2024.

extends lifespan in *Drosophila* and the budding yeast *Saccharomyces cerevisiae*, indirect inhibition of HRas extends lifespan in wild-type and tumor-free mice, and variants of *HRAS1* in combination with *APOE* and *LASS1* variants are associated with human longevity and healthy aging (5, 14–17). Furthermore, pharmacological inhibition of the Ras/MAPK pathway with trametinib not only extends lifespan in *Drosophila* but also reduces cellular senescence in normal human dermal fibroblasts in vitro (18). Thus, emerging evidence suggests that Ras/MAPK pathway plays an essential role in the aging process and makes trametinib an interesting candidate geroprotector. However, little is known about the molecular and cellular processes that mediate this effect.

Recently, RNA polymerase III (Pol III) has been described as a potential target for anti-aging treatments, as its inhibition in *S. cerevisiae*, the worm *Caenorhabditis elegans,* and *Drosophila* is sufficient to extend lifespan (19). Pol III is an evolutionarily conserved complex composed of 17 subunits and controls the transcription of different short untranslated RNAs, including transfer RNAs (tRNAs), which play an essential role in the incorporation of the correct amino acid during translation (20, 21). Pol III has been shown to act downstream of TORC1, but whether its activity mediates the effects of other signaling pathways on aging remains unaddressed (19).

Here, we have used *Drosophila* to study the geroprotective effects of trametinib, in both females and males, and to analyze the molecular mechanisms at work. We show that trametinib consistently extends lifespan and ameliorates aging-related gut pathology in females, while in males it has minor effects on lifespan and no detectable impact on gut health. Further, we show that in females, trametinib reduces Pol III activity in intestinal stem cells (ISCs) and that the full life-extending effect of trametinib requires Pol III inhibitor Maf 1 in ISCs. These findings show that the inhibitory effect of trametinib on Pol III activity in ISCs mediates, at least partially, its pro-longevity effect and the reduction of gut pathology in aging females.

Results

Trametinib Robustly Extends Lifespan in Females But Not Males. Drugs and the pathways they affect often have sexually dimorphic effects on lifespan (22–24). Pharmacological inhibition of the Ras/MAPK pathway with trametinib extends lifespan in *Drosophila* females (5). To examine the relative effects of trametinib on male and female flies, we performed seven independent experimental measurements of the response of lifespan to the drug. At a concentration of 15.6 μM, previously described as optimal for maximizing the pro-longevity effect (5), trametinib significantly extended lifespan in all female trials (Fig. 1). In males, 15.6 μM trametinib significantly extended lifespan only in two of the seven trials, and mixed effects Cox Proportional Hazard (CPH) analysis showed significant interaction between the sex and the effect of trametinib, demonstrating that the drug extended lifespan significantly more in females than in males (mixed effects CPH: trametinib $P < 10^{-15}$***; sex $P < 10^{-15}$***; interaction $P < 10^{-15}$***) (Fig. 1). Males eat approximately half the amount eaten by females (25, 26), and the lack of response in males fed 15.6 μM trametinib could therefore have been attributable to lower drug uptake. However, doubling the dose to 31.2 μM extended lifespan only in the trials where the 15.6 μM dose also did so (*SI Appendix*, Fig. S1), suggesting that the much weaker effect of the drug in males was not attributable to lower drug uptake.

To confirm that trametinib reduced Ras/MAPK signaling in both sexes, despite the lower food intake of males, we fed female and male flies with 15.6 μM of the drug and measured the levels of activated, phosphorylated ERK (pERK), relative to total ERK, in dissected midguts by western blot. Midguts from trametinib-treated female flies showed significantly lower levels of pERK/ERK compared to control female guts, confirming that trametinib effectively decreased Ras/MAPK signaling in this sex (Fig. 1*B*). In males, total ERK levels were much lower, consistent with the lower ISC proliferation in their guts (27), but trametinib nonetheless reduced the levels of pERK/ERK (Fig. 1*B*).

Altogether, these results show that trametinib consistently extends lifespan in *Drosophila* females, while it has a variable and much smaller effect in males.

Trametinib Ameliorates Aging-Associated Gut Pathology in Females. In *Drosophila*, similar sexual dimorphisms in response to other geroprotective drugs, such as rapamycin, have been attributed to an effect on the gut, where age-related pathology and functional decline occur to a much greater extent in females (23, 27). Indeed, Ras/MAPK signaling plays a central role in maintaining gut homeostasis during adulthood under both basal conditions and during stress-induced intestinal regeneration (28–30). For these reasons, we analyzed the effect of trametinib on gut health in aged females and males.

In old female flies, age-related ISC hyperproliferation and subsequent accumulation of undifferentiated and mis-differentiated cells transform the usual single-layer epithelium, leading to epithelial dysplasia (27, 31). To analyze a potential effect of trametinib on this pathology, we quantified the number of mitotic

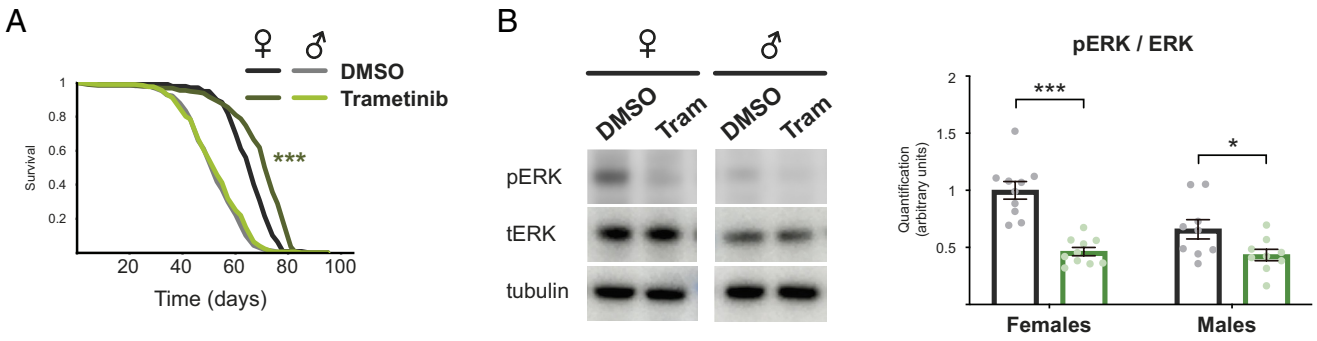

Fig. 1. Trametinib extends lifespan in females but only sometimes and to a much lesser extent in males. (*A*) Survival curves showing trametinib significantly extends female lifespan (P = 2.06E-12***; n = 150; log-rank test) and does not extend male lifespan (P = 0.36; n = 170; log-rank test), representative of the seven trials. Trametinib feeding significantly extended female lifespan in all seven trials, while it did not extend male lifespan in five out of seven trials (see *SI Appendix*, Fig. S1 for representation of all the trials performed). (*B*) Trametinib feeding significantly decreased pERK levels in the gut compared to Dimethyl sulfoxide (DMSO)-fed flies (control), both in females (Welch's *t* test, $P < 0.0001$***) and males (Welch's *t* test, P = 0.039*). Western blot (*Left*) and quantification of the bands (*Right*) showing the ratio pERK/ERK after normalization with Tubulin. Bar charts show mean ± SEM, n = 9 to 10 biological replicates per condition with 5 guts per replicate.

cells in the midgut of old female flies and observed a significant reduction of ISC proliferation under trametinib treatment (Fig. 2*A*). Trametinib also significantly decreased the proportion of dysplasia in the anterior region of the midgut, and the incidence of severe pathology (intestines carrying large tumors and significant epithelial disruption) in old females expressing a fluorescent epithelial marker (*Resille-GFP*) (Fig. 2*B*).

Female age-related gut pathology is also characterized by a decrease in the stability of the intestinal barrier, frequently tested by feeding flies with a non-absorbable blue dye that can spread all over the fly body when the integrity of the intestinal barrier is compromised, leading to blue "smurf" flies (32). Trametinib significantly reduced the number of female flies with leaky guts at 60 d of age compared to control flies of the same age (Fig. 2*C*), showing an improved maintenance of the gut barrier in aged females. Trametinib thus ameliorated aging-associated gut pathology in female flies, reducing ISC hyperproliferation, proportion of epithelial dysplasia, and disruption of the gut barrier.

The incidence of gut pathology is usually much less severe in old males, which show lower levels of age-related increases in ISC division, proportion of dysplasia, tumor formation, and barrier disruption compared to old females (27). While the extent of ISC proliferation was indeed lower in old control males compared to females, trametinib significantly further reduced ISC proliferation in old males (Fig. 2*A*). Despite this, we could not detect an effect of trametinib treatment on the extent of dysplasia, the incidence of severe pathology, or gut leakiness, all of which were substantially lower in control males than in control females (Fig. 2 *B* and *C*). These results show that trametinib treatment, while having an effect on ISC proliferation, did not significantly affect the overall lower aging-associated gut pathology in males.

Altogether, our data show a sexually dimorphic effect of trametinib on age-related pathology and decline of gut function, which correlates with the effects observed on lifespan. The effect of trametinib on the female gut may, therefore, in part explain its effects on longevity. For this reason, our subsequent investigation

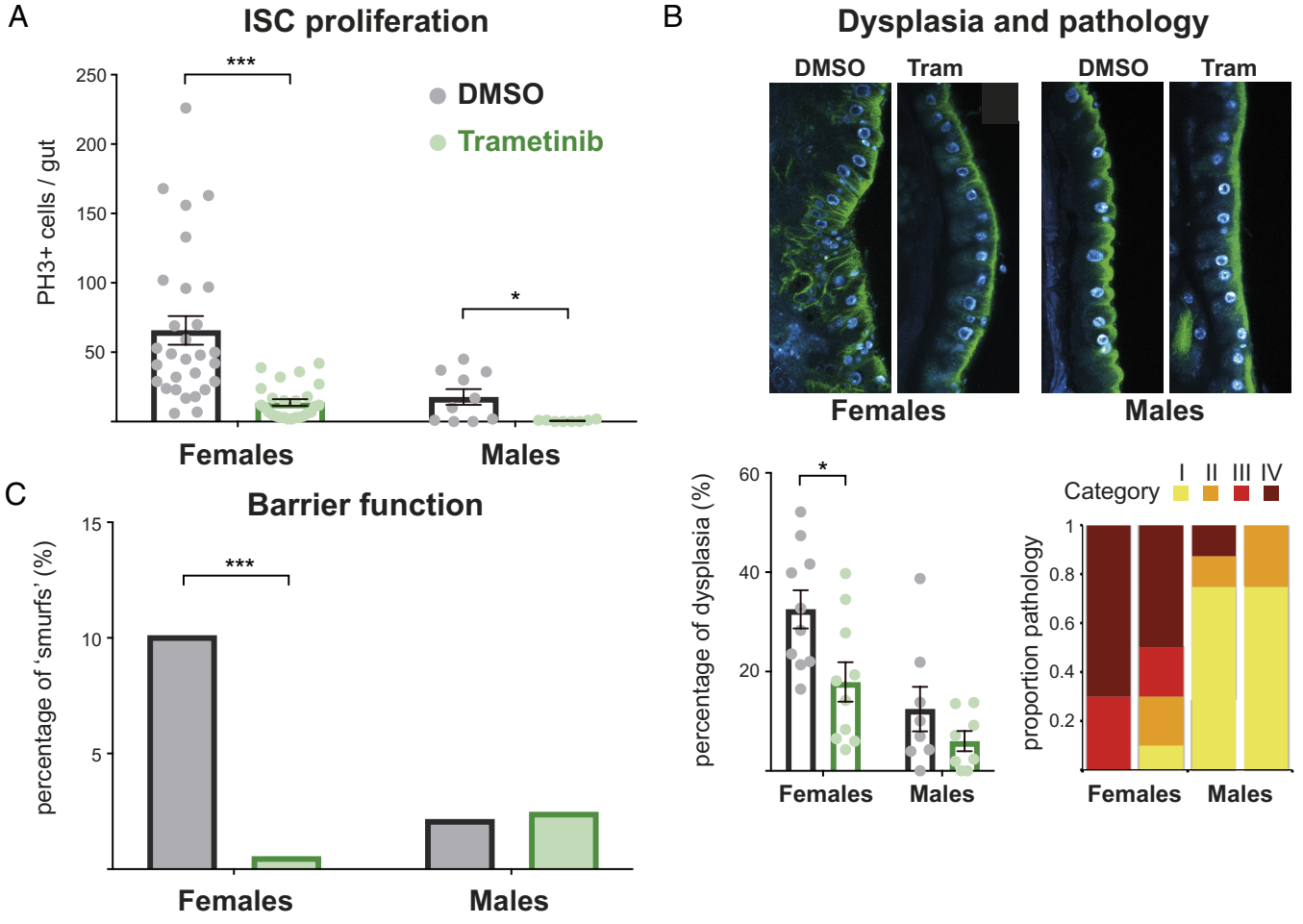

Fig. 2. Trametinib decreases aging-associated gut pathology in females. Effect of trametinib on ISC proliferation, gut pathology, and barrier function in females and males. (*A*) Trametinib reduced ISC proliferation in midguts of 35-d-old females and males, measured by staining with a $PH3^{+}$ antibody to detect mitotic cells in whole midguts. Bar charts show quantification of number of PH3+ cells/gut, mean ± SEM. Guts per condition in females: DMSO n = 29, trametinib n = 26; $P < 0.001$***, Mann–Whitney test. Guts per condition in males: DMSO n = 8; trametinib n = 10; $P = 0.022$*, Mann–Whitney test. ISC proliferation is significantly lower in males than in females (two-way ANOVA: sex, $P = 0.0031$**; trametinib, $P = 0.0009$***; interaction, $P = 0.087$). (*B*) Trametinib treatment reduced intestinal dysplasia and epithelial pathology in *Resille-GFP* females, but not in males. Length of dysplasia quantified from luminal sections of approximately 800 µm of the R2 region from DMSO and trametinib-treated intestines. Bar charts show percentage of dysplastic regions (mean ± SEM). Guts imaged and used for quantification in females: DMSO n = 10, trametinib n = 10; $P = 0.0162$*, *t* test with Welch's correction. Guts imaged and used for quantification in males: DMSO n = 8, trametinib, n = 8; $P = 0.22$, *t* test with Welch's correction. Percentage of dysplasia is significantly lower in males than in females (two-way ANOVA: sex, $P = 0.0002$***; trametinib, $P = 0.0089$**; interaction, $P = 0.29$). Pathology scored and binned into categories: I = no pathology; II = sporadic small tumors; III = epithelial wounds and small tumors; IV = large tumors, severely disrupted epithelium. (*C*) Treatment with trametinib ameliorated intestinal barrier function decline in 60-d-old females, measured by quantification of "smurf" flies. Males are not significantly affected. Data from 2 independent experiments. Number of female flies scored: DMSO n = 257, trametinib n = 352; $P = <0.0001$***, Fisher's exact test. Number of males scored: DMSO n = 138, trametinib n = 201; $P = 0.999$, Fisher's exact test.

focused on deciphering the molecular mechanisms whereby trametinib ameliorates gut aging in females.

Downregulation of Ras/MAPK Signaling in ISCs Is Sufficient to Extend Lifespan and Improve Gut Pathology. We next tested the role of the gut on the pro-longevity effect of trametinib. Downregulation of the Ras/MAPK pathway in the gut by knocking down MEK, using an inducible, gut-specific driver (19, 33) and a *MEK* RNA interference (RNAi) (*TIGS* > *MEK*^{RNAi}), extended female lifespan (Fig. 3*A*), supporting the hypothesis that trametinib acted in the gut to extend female lifespan.

In young flies, Ras/MAPK signaling is mainly active in ISCs, as shown by immunostaining guts with a pERK antibody (28, 29, 34) (*SI Appendix*, Fig. S2*A*). To test the role of ISCs on the lifespan-extending effect of trametinib, we decreased Ras/MAPK signaling in this cell type during adulthood using the ISC-specific gene-switch driver *5961GS* (31, 35). Knockdown of MEK (*5961GS* > *MEK*^{RNAi}), ERK (*5961GS* > *ERK*^{RNAi}), or ectopic expression of the Raf inhibitor PEBP1 (*5961GS* > *PEBP1*) all increased fly lifespan (Fig. 3*B* and *SI Appendix*, Fig. S3), showing that a decrease in Ras/MAPK signaling in ISCs is sufficient to extend lifespan and supporting the hypothesis that trametinib acts directly in ISCs. Moreover, ISC-specific knockdown of MEK reduced ISC proliferation and gut permeability in old flies (Fig. 3 *C* and *D*), confirming that Ras/MAPK signaling in ISCs is causal in age-related hyper-proliferation and deterioration of the gut barrier.

Altogether, these data strongly suggest that the effect of trametinib on Ras/MAPK signaling in ISCs is, at least partially, responsible for its effect on gut health and survival of older female flies.

Trametinib Decreases Pol III Activity in ISCs to Extend Lifespan. Previous work has shown that, in the *Drosophila* fat body (functionally equivalent to adipose tissue and liver in mammals), the effects of Ras/MAPK inhibition on lifespan are mediated by activation of an E-twenty six (ETS) transcriptional repressor, Aop (5). However, Aop activity in the ISCs is not sufficient to promote longevity (5, 36). RNA Pol III has also been described as one of the effectors of the Ras/MAPK pathway that controls growth and metabolism in *Drosophila* (37), and Pol III inhibition in ISCs extends fly lifespan and ameliorates aging-associated gut pathology (19). Thus, we hypothesized that Pol III could be the effector of the lifespan-extending effect of trametinib in ISCs. To test this, we first analyzed the effect of trametinib on Pol III activity, by measuring the expression levels of several precursor tRNAs (pre-tRNAs) that are products of Pol III, namely *pre-tRNA*^{His}, *pre-tRNA*^{Ile}, and *pre-tRNA*^{Leu}, as these are indicative of in vivo Pol III activity (19). Feeding flies with trametinib significantly decreased the expression levels of these pre-tRNAs in whole flies (Fig. 4*A*), confirming that trametinib reduced Pol III activity.

Heterozygous female mutants in the Pol III-specific subunit D, *Polr3D*$^{\Delta}$/+, live longer than the corresponding controls (19). To further test the role of Pol III in the pro-longevity effect of trametinib, we combined the two interventions and measured their combined effect on lifespan. As previously described, *Polr3D*$^{\Delta}$/+ mutants and +/+ trametinib-fed flies lived longer than the +/+ DMSO-fed controls (Fig. 4*B* and *SI Appendix*, Fig. S4*A*). Interestingly, the combination of the two interventions in *Polr3D*$^{\Delta}$/+ mutants fed with trametinib did not show a fully additive effect, as trametinib extended lifespan less in the presence of the mutant, and CPH analysis showed significant interaction between *Polr3D*$^{\Delta}$ mutation and trametinib (CPH: *Polr3D*$^{\Delta}$/+, P = 8.09E-16***; trametinib, P = 1.76E-11***; interaction, P = 0.000799***) (Fig. 4*B*

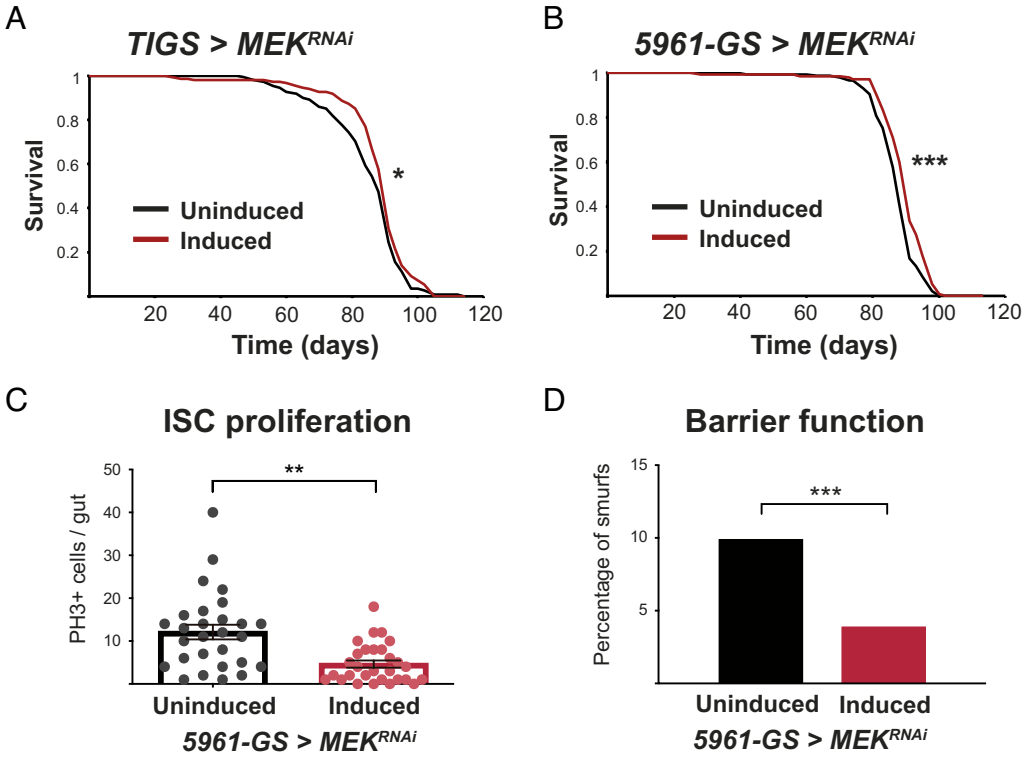

Fig. 3. Downregulation of Ras/MAPK in ISCs extends lifespan and reduces age-dependent gut pathology. (*A*) Knockdown of MEK specifically in the gut (*TIGS* > *MEK*^{RNAi}) after RNAi induction with RU486 significantly extended female lifespan (n = 150 flies per condition, P = 0.034*, log-rank test). (*B*) Knockdown of MEK specifically in ISCs (*5961-GS* > *MEK*^{RNAi}) after induction with RU486 significantly extended female lifespan (n = 150 flies per condition, P = 0.00035***, log-rank test). (*C*) Knockdown of MEK in ISCs reduced ISC proliferation in midguts from 37-d-old females. Bar charts show the quantification of PH3+ cells per midgut, mean ± SEM. n = 28 (−RU) and 29 (+RU) guts. P = 0.0001***, Mann–Whitney test. (*D*) Knockdown of MEK in ISCs reduced intestinal barrier disruption in 64-d-old females. Bar charts show percentage of smurf flies in 24 h. n = 376 (−RU) and 392 (+RU) flies. P = 0.0009***, Fisher's exact test.

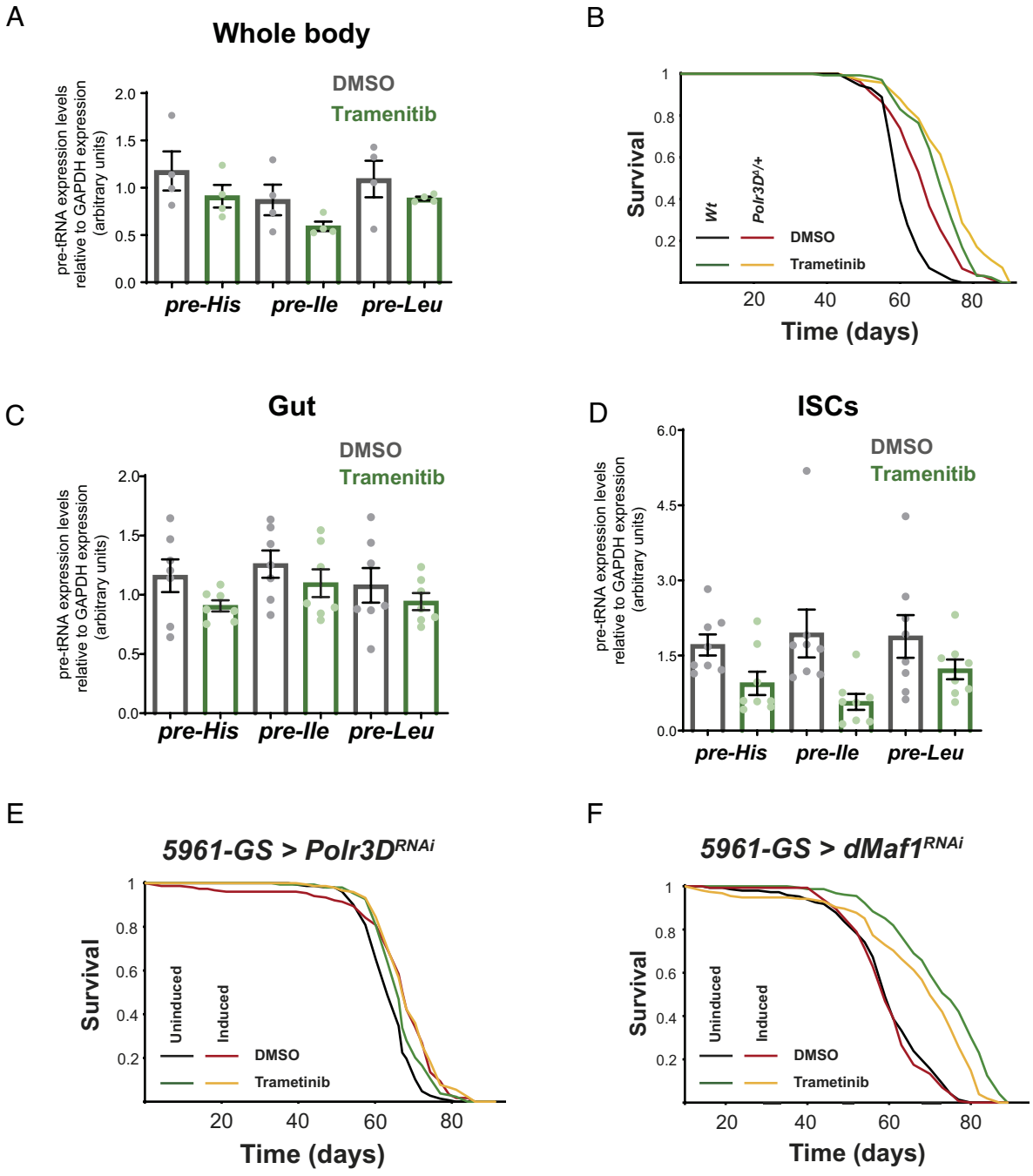

Fig. 4. Trametinib reduces Pol III activity and interacts with Pol III inhibition. (*A*) Trametinib feeding reduced levels of *pre-tRNA^{His}*, *pre-tRNA^{Ile}*, and *pre-tRNA^{Leu}*, measured by qPCR in whole female flies compared to DMSO (control) flies. Bar charts show mean ± SEM of relative expression levels normalized with *GAPDH* expression. n = 4 biological replicates per condition with 5 flies per replicate (P = 0.0099** for effect of trametinib, Linear mixed-effects model). (*B*) Survival curves showed significant lifespan extension in heterozygous female mutants of *Polr3D, Polr3D$^{\Delta}$*/+, and wild-type +/+ trametinib-fed flies, compared to control +/+ flies (P = 2.27E-12 and P = 3.01E-38, respectively, log-rank test), but their pro-longevity effects were not additive when combined together (CPH: *Polr3D$^{\Delta}$*/+, P = 8.09E-16***; trametinib, P = 1.76E-11***; interaction, P = 0.000799***). (*C*) Trametinib reduced levels of *pre-tRNA^{His}*, *pre-tRNA^{Ile}*, and *pre-tRNA^{Leu}* in dissected midguts, measured by qPCR. Bar charts show mean ± SEM of relative expression levels normalized with GAPDH expression. n = 7 biological replicates per condition with 10 guts per replicate (P = 0.0269*, Linear mixed-effects model). (*D*) Trametinib reduced levels of *pre-tRNA^{His}*, *pre-tRNA^{Ile}*, and *pre-tRNA^{Leu}*, measured by qPCR in ISCs isolated by FACS sorting from *esg-gal4 > GFP* flies. Bar charts show mean ± SEM of relative expression levels normalized with GAPDH expression. n = 8 biological replicates per condition with 15 guts per replicate (P = 0.0003***, Linear mixed-effects model). (*E*) Induction of *Polr3D* RNAi in ISCs with RU486 (*5961-GS > Polr3D^{RNAi}* +RU/DMSO) extended lifespan (P = 0.00046***, log-rank test), but this effect was not additive with the pro-longevity effect of trametinib (CPH: RU486, P = 0.00051***; trametinib, P = 4.96E-08***; interaction, P = 0.044*). (*F*) Knockdown of Maf1 in *5961-GS > Maf1^{RNAi}* flies inducing Maf1 RNAi with RU486 had no effect on female lifespan (*5961-GS > Maf1^{RNAi}* -RU/DMSO vs. *5961-GS > Maf1^{RNAi}* +RU/DMSO, P = 0.48, log-rank test), but significantly reduced pro-survival effect of trametinib (*5961-GS > Maf1^{RNAi}* -RU/trametinib vs. *5961-GS > Maf1^{RNAi}* +RU/trametinib, P = 1.97E-05***, log-rank test) (CPH: RU486, P = 0.462; trametinib, P < 2E-16***; interaction, P = 0.007**).

and *SI Appendix*, Fig. S4*A*). These results indicate that the two treatments act, at least partially, in the same longevity pathway.

We next analyzed the effect of trametinib on Pol III activity in guts and ISCs, since at least part of the pro-longevity action of trametinib was directly mediated by this cell type. Trametinib-treated flies showed lower expression levels of *pre-tRNA^{His}*, *pre-tRNA^{Ile}*, and *pre-tRNA^{Leu}* in whole guts from wild-type flies (Fig. 4*C*) and in FACS (Fluorescence-Activated Cell Sorting)-isolated ISCs from

flies expressing GFP in ISCs (*esg-gal4* > *GFP*) (Fig. 4*D*), indicating that trametinib inhibited Pol III specifically in the intestine and its resident stem cells. At the same time, reduction of Pol III activity specifically in the ISCs, achieved by knocking down *Polr3D* (*5961-GS>Polr3D*^{RNAi}), which extends lifespan (19), did not show an additive pro-longevity effect when combined with trametinib (CPH: RU486, P = 0.00051***; trametinib, P = 4.96E-08***; interaction, P = 0.044*) (Fig. 4*E*), suggesting that trametinib and ISC-specific Pol III inhibition act in part through the same longevity pathway. Importantly, pERK levels were not affected in the guts of the Pol III heterozygous mutant, supporting the hypothesis that Ras/MAPK signaling controls Pol III activity and not the other way around (*SI Appendix*, Fig. S2*B*). Altogether, these data strongly suggest that trametinib extends lifespan in part by reducing Pol III activity in the ISCs.

Maf1 Mediates the Effect of Trametinib in ISCs. Ras/MAPK signaling promotes Pol III activity by phosphorylation and consequent nuclear exclusion of the Pol III repressor Maf1 both in *Drosophila* cell culture and in vivo (37). We therefore hypothesized that Maf1 could be mediating the longevity effect of trametinib in ISCs. To test this, we knocked down Maf1 in ISCs (*5961-GS* > *Maf1*^{RNAi}), which alone did not affect lifespan (Fig. 4*F*). However, when fed trametinib, flies expressing *Maf1* dsRNA showed a lesser extension of lifespan than did trametinib-fed controls (–RU/trametinib vs. +RU/trametinib, P = 1.97E-05, log-rank test), and CPH analysis confirmed significant interaction between the two treatments (CPH: RU486, P = 0.462; trametinib, P < 2E-16***; interaction, P = 0.007**). This suggests that Maf1 is required for the full effects of trametinib in ISCs. Interestingly, the knockdown of Maf1 using the ISC-active gal4 driver *esg-gal4* (*esg-gal4* > *Maf1*^{RNAi}) not only reduced the pro-longevity effect of trametinib but induced a synthetic detrimental effect on the flies (*SI Appendix*, Fig. S4). Taken together, these results show that Maf1, and the subsequent inhibition of Pol III activity, is required in ISCs for trametinib to extend lifespan.

Discussion

Trametinib is a Food and Drug Administration (FDA)-approved anticancer drug with the potential to be repurposed as a geroprotector (5). We have now shown that it reduces age-related ISC hyperproliferation in both sexes, decreases epithelial dysplasia and tumor formation, and maintains gut integrity in females. The Ras/MAPK pathway controls cell proliferation in multiple tissues across organisms (10, 13). In *Drosophila* guts, it plays a central role in preserving gut homeostasis, as it is necessary for maintaining ISC proliferation both in unchallenged conditions and under stress in young flies (28, 29, 34), where reduced activity of Ras/MAPK signaling leads to reduced ISC proliferation, while activation triggers high proliferation levels (28, 34, 38). Our results extend previous studies in young flies and show that Ras/MAPK signaling is necessary for age-related hyperproliferation leading to dysplasia and tumor formation in old flies, contributing to organismal aging.

Trametinib treatment or genetic inhibition of Ras/MAPK pathway in ISCs also reduced the disruption of the gut barrier in old flies. The deterioration of the intestinal barrier with age is a conserved pathology among different animal models including worms, fish, mice, and monkeys, and some markers of intestinal disruption have been observed in elderly humans (39–44). In *Drosophila*, loss of intestinal barrier stability correlates with gut dysbiosis, including increased bacteria in the gut, changes in microbiota composition and systemic inflammation (32, 45, 46). Similarly, microbial spread and systemic inflammation following intestinal dysfunction have been observed in aged mice and vervet monkeys (41, 47). Thus, impaired intestinal function is closely related to health decline and disease in aged organisms, supporting further studies to confirm the effect of trametinib on intestinal homeostasis in higher animals.

The increase in ISC proliferation in old flies is significantly lower in males than in females, leading to a lower rate of dysplasia and tumor formation and contributing to healthier guts at old ages with a better maintenance of barrier function (23, 27). Although trametinib significantly reduced ISC proliferation in males, we did not detect a significant effect of the treatment on intestinal dysplasia, tumor formation, or barrier function. Moreover, the effect of trametinib on male lifespan, although significant in a minority of trials, was much weaker. Increasing the concentration of the drug in the food and measurements of Ras/MAPK pathway activity in the gut indicated that the much lower effects of trametinib on gut pathology and lifespan in males could not be solely attributed to their lowered food consumption relative to females (25, 26). The more likely explanation, and one consistent with previous observations, is that trametinib increases survival in part by reducing gut pathology, which is limiting for lifespan in females but not in males. In those trials where male lifespan was extended, it is possible that some micro-environmental condition (e.g., microbes) induced some level of pathology in males that was rescued by the drug. Loss of intestinal homeostasis and barrier dysfunction is common in both sexes in mammals, contributing to the onset of aging-related inflammatory and metabolic disorders. Thus, the effect of trametinib on gut health should be investigated in mammalian models, as it could be beneficial in both sexes.

Pol III, an RNA polymerase essential for protein translation and cell growth, limits lifespan in yeast, worms, and flies, and reducing its activity specifically in ISCs extends lifespan and ameliorates gut pathology in old female flies (19). The Ras/MAPK pathway controls growth and proliferation promoting Pol III activity and tRNA synthesis through phosphorylation of the Pol III repressor Maf1 in the fruit fly (37). This inhibitory mechanism is conserved in mammals to promote protein synthesis, cell proliferation, and tissue growth (48). Here, we have shown that trametinib decreases Pol III activity in ISCs, and our results suggest that Pol III acts downstream of the Ras/MAPK in ISCs to limit survival. Moreover, we have found that the life-extending effect of trametinib partially depends on Maf1 expression in ISCs. Thus, we propose a model in which the inhibition of MEK in ISCs decreases pERK signaling, allowing unphosphorylated Maf1 to bind and inhibit Pol III in the nucleus, preventing its transcriptional activity (Fig. 5). This contributes to extending lifespan and ameliorating the age-associated gut pathology.

Several studies have shown that mTORC1 directly phosphorylates and inactivates Maf1 to stimulate Pol III activity and tRNA synthesis (49, 50). Moreover, Pol III exerts a role on the pro-longevity effect of rapamycin, mTORC1 inhibitor, in *Drosophila* (19). This means Ras/MAPK signaling is not the only pathway to regulate Maf1 and tRNA synthesis in ISCs, and rapamycin and trametinib probably share this mechanism of action to extend lifespan. However, the fact that the pro-longevity effects of rapamycin and trametinib are almost completely additive suggests that other mechanisms of their action exist and that they may be complementary (6).

As well as consistently extending lifespan in *Drosophila* females, trametinib reduces translational errors in vitro in S2R+ cells, decreases insulin resistance in obese wild type and genetically obese mice, diminishes the proportion of senescent cells in senescent human dermal fibroblast cultures, and increases autophagy in pancreatic ductal adenocarcinoma cells, among others (18,

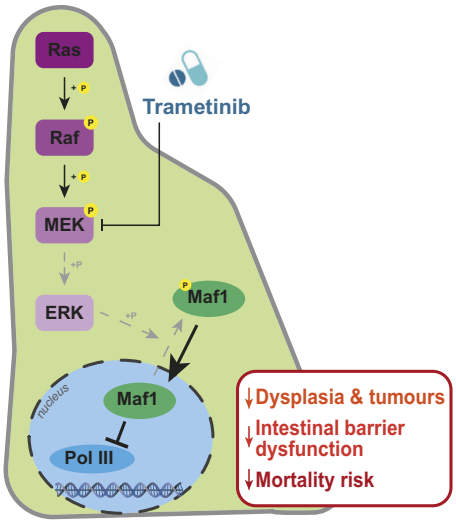

Fig. 5. The Ras/MAPK-Maf1-Pol III axis mediates the life-extending effect of trametinib in ISCs. Graphical model representing the mechanism of action of trametinib in ISCs. Trametinib inhibits MEK, which reduces pERK levels and the subsequent phosphorylation of Maf1. Increased presence of Maf1 in the nucleus inhibits Pol III transcriptional activity, reducing tRNA synthesis. This results in a decreased proportion of dysplasia, tumor formation, and barrier dysfunction in old flies and contributes to the pro-longevity effect of trametinib in females.

51–54). Furthermore, its anti-inflammatory effects have been widely described in different diseases including cancers, cystic fibrosis, acute lung injury, or traumatic brain injury (55–58). The effect of trametinib on Pol III activity in ISCs and gut pathology described in this work adds another piece of evidence to its anti-aging effect, paving the way for further analysis in higher animal models while presenting trametinib as a solid candidate for future geroprotective treatments. Meanwhile, further experiments will be necessary to fully understand the impact of trametinib in all tissues and pathologies, as well as the molecular mechanisms responsible. This knowledge would be greatly beneficial to advance toward the potential repurposing of trametinib as a new anti-aging therapy.

Materials and Methods

Fly Husbandry and Lifespan. *Drosophila* stocks were maintained and experiments conducted at 25 °C on a 12 h:12 h light:dark cycle at 60% humidity, with SYA food containing 10% (w/v) brewer's yeast, 5% (w/v) sucrose, and 1.5% (w/v) agar, with propionic acid and Nipagin as preservatives. For lifespans, the indicated number of flies was sorted into experimental vials at a density of 15 flies per vial. Flies were transferred to fresh vials every 2 to 3 d, and deaths/censors were scored during transferral. Further details on fly husbandry, lifespan experiments, and stocks used in this work are detailed in *SI Appendix*.

Analysis of Gut Pathology. Fly guts were dissected in cold Phosphate Buffered Saline (PBS) and immediately fixed in 4% formaldehyde for 15 min. Guts were mounted in mounting medium (Vectashield) containing DAPI and then imaged immediately with a Zeiss (UK) LSM 700 confocal laser scanning microscope. Details on gut pathology measurements and smurf assay are described in *SI Appendix*.

qRT-PCR Analysis. Total RNA was isolated from either whole adult flies, whole guts, or ISCs using standard TRIZOL (Invitrogen) protocols. Total RNA was treated with Turbo DNase (Invitrogen) and converted to cDNA using random hexamers (ThermoFisher) and Superscript II reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed using Power SYBR Green PCR Master Mix (ABI) in the Quant Studio 6 Flex system, and relative quantities of transcripts were determined using the relative standard curve method normalized to GAPDH.

Western Blots, Immunofluorescence, and FACS. These procedures and the antibodies used are described in *SI Appendix*.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

ACKNOWLEDGMENTS. We thank Mumtaz Ahmad, James Shilland, and Sam Yee Low for their work in the Institute of Healthy Ageing (IHA) fly facility; Giovanna Vinti, Michael Wright, and Muslima Chowdhury for organizational and administrative assistance; and Jente Zeubring for drawing the graphical model in Fig. 5. Stocks obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537) and Vienna Drosophila Resource Center (VDRC, https://shop.vbc.ac.at/vdrc_store/) were used in this study. This research has been funded by Biotechnology and Biological Sciences Research Council (BBSRC) (BB/V006541/1), a Wellcome Trust Strategic Award (098565/Z/12/Z), EU grant (FP6-518230, IDEAL), the European Research Council under the European Union's Seventh Framework Programme (FP7/2007–2013)/ERC grant agreement no. 268739, and the European Union's Horizon 2020 research and innovation programme (no. 741989). This research was funded in whole, or in part, by the Wellcome Trust [098565/Z/12/Z]. For the purpose of Open Access, the author has applied a CC BY public copyright licence to any Author Accepted Manuscript version arising from this submission.

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