A single nucleotide variant in the PPAR γ -homolog *Eip75B* affects fecundity in *Drosophila*

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ABSTRACT

Still little is understood about the nucleotide changes that underlie variation in complex phenotypes. Variation in the PPAR γ -homolog *Eip75B* has previously been suggested to be associated with longevity and life-history differences in the fruit fly *Drosophila melanogaster*. Using RNAi knockdown, we first demonstrate that reduced expression of *Eip75B* in adults affects lifespan, egg laying rate and egg volume. To then test the effect of a naturally occurring SNP variant within a cis-regulatory domain of *Eip75B*, we screened wildtype lines with alternative alleles and conducted precise genome editing using CRISPR/Cas9. These experiments revealed that this natural polymorphism has a significant effect on fecundity and egg-to-adult viability, but not on longevity or other life-history traits. These results provide a rare functional validation for the role of a natural allelic variant in adaptation of life-history traits directly linked to fitness at the single nucleotide level.

KEYWORDS: pleiotropy, *Drosophila*, ageing, reproduction, CRISPR/Cas9, RNAi

INTRODUCTION

Single nucleotide polymorphisms (SNPs) are the major constituent of genetic variation and fuel for evolutionary adaptation (Suh & Vijg, 2005). High-throughput sequencing technologies propelled the association of natural variants with complex, quantitative traits (Schlötterer, Tobler, Kofler, & Nolte, 2014), but functional tests demonstrating a causal effect at base-pair resolution are rare, especially for multicellular organisms (Mokashi et al., 2021; Ramaekers et al., 2019). Instead, functional analyses are often done at the gene level by generating (null) mutants or using RNAi in model organisms (Fabian et al., 2018; Huang et al., 2020; Martins et al., 2014; Parker et al., 2020; Zhou et al., 2011). Functional effects of natural alleles may, however, be more subtle or different altogether from mutant alleles (Mokashi et al., 2021; Stern, 2011). The aim of this study was to conduct functional analysis of a natural polymorphism of *Ecdysone-induced protein 75B* (*Eip75B*), a promising candidate gene for ageing and life-history evolution in fruit flies.

Eip75B encodes a nuclear hormone receptor homologous to peroxisome proliferator-activated receptor gamma (PPAR γ), a key regulator of adipogenesis and pregnancy-induced modifications in lipid metabolism in mice and humans (Hong & Park, 2010; Waite et al., 2000; Zipper, Jassmann, Burgmer, Gorlich, & Reiff, 2020). Nuclear hormone receptors, such as *Eip75B*, are evolutionarily conserved regulatory hub genes that integrate environmental factors, hormonal signaling, and metabolism and relay these inputs to downstream targets (Hoffmann & Partridge, 2015; Jagannathan & Robinson-Rechavi, 2011; Palanker et al., 2006). In the fruit fly, *Eip75B* also acts as an early-response gene in the ecdysone signaling pathway, which plays a critical role in the regulation of development, physiology and life-history of arthropods, similar to thyroid hormones in vertebrates (Belles & Piulachs, 2015; Bialecki, Shilton, Fichtenberg, Segraves, & Thummel, 2002; Caceres et al.,

2011; Flatt, Moroz, Tatar, & Heyland, 2006; Galikova, Klepsatel, Senti, & Flatt, 2011; Jaumouillé, Machado Almeida, Stähli, Koch, & Nagoshi, 2015; Magwire et al., 2010; Simon, Shih, Mack, & Benzer, 2003; Terashima & Bownes, 2006; Toivonen & Partridge, 2009; Yamanaka, Rewitz, & O'Connor, 2013).

Eip75B has been identified as an “ageing” candidate gene by two independent genome-wide association studies (Huang et al., 2020; Pallares et al., 2020) and three experimental evolution experiments (Carnes et al., 2015; Hoedjes et al., 2019; Parker et al., 2020), but each study detected different candidate SNPs. Experimental evolution of longevity has been intricately linked to changes in fecundity, suggesting a shared genetic basis of these two traits likely due to antagonistic pleiotropy (Carnes et al., 2015; Fabian et al., 2018; Flatt, 2020; May et al., 2019; Remolina, Chang, Leips, Nuzhdin, & Hughes, 2012). Moreover, evolutionary changes in longevity can be associated with changes in viability, development time, immune function, stress resistance, and even learning ability (e.g., (Baldal, Brakefield, & Zwaan, 2006; Chippindale, Hoang, Service, & Rose, 1994; Fabian et al., 2018; Flatt, 2020; Mery & Kawecki, 2005). Given its central role in hormonal signaling and its pleiotropic effects on numerous developmental and physiological processes, *Eip75B* may thus be involved in the concerted evolution of longevity and correlated life-history traits.

In this study, we first used RNAi silencing of *Eip75B* in adults to determine the effects of the gene on lifespan, egg laying and egg volume. We then selected a naturally occurring SNP in a cis-regulatory domain of *Eip75B*, previously identified as a candidate in an experimental evolution study (Hoedjes et al., 2019), for functional analyses by applying a combination of SNP-specific association studies and precise genomic modification using CRISPR/Cas9. Our results demonstrate that a single nucleotide variant can have a major effect on a complex fitness-related trait, namely fecundity.

RESULTS

RNAi knock-down of Eip75B in adults reduces longevity, egg laying rate and egg volume

Recent studies have found that weak, constitutive knockdown of *Eip75B* throughout development and the adult stage increases lifespan in both males and females (Huang et al., 2020), whereas tissue-specific knockdown in the amnioserosa, ovaries and accessory glands causes decreased lifespan but increased fecundity in females (Parker et al., 2020). Given that *Eip75B* might have potentially confounding pleiotropic developmental effects on adult life-history, we investigated the effect of gene knockdown specifically in adults only. The effects of *Eip75B* knockdown on longevity, fecundity (measured as daily egg laying rate per female) and egg volume were assessed with four independent RNAi constructs expressed in the adult stage using the inducible, constitutive *daughterless*-GS-GAL4 driver (Osterwalder, Yoon, White, & Keshishian, 2001). RT-qPCR confirmed a significant, partial knockdown of *Eip75B* for three of the four constructs, E01, E03, and E44, but not for the fourth construct (E10) (Table 1). The fourth construct was nonetheless included in the study as it had been observed to affect lifespan and fecundity in previous studies (Huang et al., 2020; Parker et al., 2020).

Knockdown of *Eip75B* expression resulted in a significant reduction in lifespan only for constructs E01 (females) and E44 (both sexes), although we also observed a similar trend in lifespan reduction for construct E03 (Figure 1A). The effects of *Eip75B* knockdown on egg laying rate and egg volume were more consistent, with a significant reduction in both egg laying rate and egg volume for constructs E01, E03, and E44, and a weak trend for construct E10 (Figure 1B and 1C).

These results confirm the role of *Eip75B* in affecting lifespan and fecundity, in line with previous studies, but also suggest that the direction and the magnitude of the observed phenotypic effects depend on the timing, the tissue, and the strength of the genetic modification (Huang et al., 2020; Parker et al., 2020).

A natural polymorphism at Eip75B correlates with variation in fecundity

The *Eip75B* SNPs which have been identified as potentially affecting ageing and life-history are located throughout the gene, mostly in non-coding regions (Carnes et al., 2015; Hoedjes et al., 2019; Huang et al., 2020; Jha et al., 2015; Pallares et al., 2020; Parker et al., 2020; Turner, Stewart, Fields, Rice, & Tarone, 2011). *Eip75B* is a large (>100 kb) gene comprising seven transcript variants that are expressed from alternative promoters. Isoforms have different temporal and spatial expression patterns and fulfill different, even opposing, functions in insects (Bernardo, Dubrovskaya, Jannat, Maughan, & Dubrovsky, 2009; Cruz, Mane-Padros, Zou, & Raikhel, 2012; Keshan, Hiruma, & Riddiford, 2006; Li et al., 2016; Terashima & Bownes, 2006; Zipper et al., 2020). Given this complex genomic architecture and the pleiotropic effects of *Eip75B*, independent SNPs may have different regulatory functions. We focused on a set of 16 significantly diverged, intronic SNPs that were detected by experimental evolution of lifespan extension by selecting on the age at reproduction (Hoedjes et al., 2019; May et al., 2019) (at FDR<0.0005). One of these SNPs (3L:18026199, genome release 6) is located within a predicted cis-regulatory motif. This SNP is bi-allelic, with the "T" variant being more common in the late-reproducing, long-lived populations (average frequency: 0.84 "T", 0.16 "G") as compared to the early-reproducing populations (average frequency: 0.52 "T", 0.48 "G") (Hoedjes et al., 2019). These frequencies fall within

the range of frequencies observed in natural populations of *D. melanogaster* in Europe and North America (Appendix 1).

To test if the “T” allele is associated with a longer lifespan and/or changes in fecundity as compared to the “G” allele, we used a targeted SNP association study in which we assessed a set of F1 crosses of distinct lines of the *Drosophila Genetic Reference Panel* (DGRP) (Mackay et al., 2012) differing in allelic state (“T” vs. “G”). This heterozygous F1 approach was chosen as the DGRP strains are inbred and may thus suffer from reduced fitness due to recessive deleterious alleles. Previous studies of wildtype lines or recombinant outbred populations with alternative allelic states have already indicated significant effects for several natural SNP alleles in life-history evolution (Betancourt et al., 2021; Durmaz et al., 2019; Glaser-Schmitt & Parsch, 2018; Glaser-Schmitt, Wittmann, Ramnarine, & Parsch, 2021).

F1 crosses homozygous for the “G” allele (and with a diverse genomic background) showed a significantly and consistently higher egg laying rate than crosses homozygous for the “T” allele (Figure 2). However, there was no significant association between the candidate SNP and longevity. These findings suggest that the “G” allele may have played a role in facilitating a higher (early) fecundity in the populations selected for early reproduction, but that this SNP is not responsible for differences in lifespan and late reproduction previously reported in the experimental evolution experiment (May et al., 2019).

CRISPR/Cas9 editing reveals pleiotropic effects of the Eip75B SNP on fecundity and viability but not longevity

To minimize potential confounding effects of genetic background on lifespan and fecundity, we used CRISPR/Cas9-induced genomic editing to modify the candidate SNP in an isogenic background without introducing any markers or off-target modifications that might interfere with gene function in the genomic region surrounding the focal SNP. We constructed two edited lines with the “G” allele and two lines with the “T” allele. The lines carrying the “G” allele exhibited a significantly higher egg laying rate and higher egg-to-adult viability as compared to lines with the “T” allele (Figure 3). Lines with the “G” allele also showed a small but significant reduction in starvation resistance in females. No significant differences between the two allelic states were observed for lifespan, egg-to-adult development time, adult dry weight, and egg volume.

DISCUSSION

The PPAR γ -homolog Eip75B plays a significant role in the evolution of fecundity

Our results demonstrate a clear pleiotropic effect of a naturally occurring nucleotide polymorphism at the *Eip75B* locus on egg-to-adult viability and fecundity. The effect of this SNP on reproduction may be linked to the known role of *Eip75B* in oogenesis (Ables, Hwang, Finger, Hinnant, & Drummond-Barbosa, 2016; Belles & Piulachs, 2015), but could also indicate that allelic variants at this locus modify nutrient availability or metabolic processes required for egg production. Ecdysone titers in the ovaries themselves control overall food intake, but *Eip75B* also plays a role in the mating-induced remodeling of the gut, which prepares the female body for the increasing energy demands associated with egg laying (Carvalho-Santos & Ribeiro, 2018; Reiff et al., 2015; Zipper et al., 2020). Further functional analyses of the two allelic variants studied here are necessary to disentangle the mode of action of this polymorphism.

Functional assays of natural alleles are required for understanding longevity and life-history evolution

Even though RNAi-induced knockdown of *Eip75B* affects longevity, as shown here and by others (Huang et al., 2020; Parker et al., 2020), no effect on lifespan was observed for a natural allelic variant previously identified as an “ageing” candidate in an experimental evolution study (Hoedjes et al., 2019). Instead, we found that this polymorphism affects viability and fecundity. No effects on various other life-history traits, known to be regulated by the *Eip75B*, were detected for this SNP, perhaps suggesting that natural alleles have fewer pleiotropic effects than null alleles of the same gene (Stern, 2011). Hence, other allelic variants of *Eip75B* may play a role in longevity. In addition, the effects of an individual SNP – like the one we have tested here – might depend on the haplotype and genetic background, given the major role linkage and epistasis can play in affecting quantitative traits (Mackay, 2014; Magwire et al., 2010).

In conclusion, a vast number of studies have identified naturally occurring candidate polymorphisms associated with complex traits, but experimental demonstrations of direct, causal effects of such variants still remain scarce (Mokashi et al., 2021; Ramaekers et al., 2019). Our study provides a rare example of functional characterization of a natural nucleotide polymorphism with pleiotropic effects on two complex fitness-related traits, viability and fecundity. Our results further suggest that this molecular polymorphism has, as one might expect for regulatory variants (Stern 2011), fewer pleiotropic effects than the knockdown of the entire gene or null alleles at this locus.

METHODS

RNAi

Transgenic RNAi was performed using the ubiquitously expressing, mifepristone (RU486)-inducible *daughterless (da)*-GeneSwitch(GS)-GAL4 (Tricoire et al., 2009) (courtesy of Véronique Monnier, Paris) to drive the expression of *Eip75B* UAS-RNAi constructs during the adult stage only, to avoid adverse effects on development. Two independent UAS-RNAi constructs obtained from the Vienna *Drosophila* RNAi Center (VDRC) (#108399 [E10] and #44851 [E44]), and two additional independent constructs obtained from Bloomington *Drosophila* Stock Center (BDSC) (#TRiP.GLC01418 [E01] and #TRiP.JF02257 [E03]) were used. These constructs target a domain of *Eip75B* that is shared by all isoforms. The isogenic host strain for the VDRC RNAi library, *w¹¹¹⁸* (#60000), was used to test for the potential effects of mifepristone treatment. All lines were kept and assays were performed at 25°C, 65% humidity and 12h:12h light:dark cycle, on a cornmeal-yeast-sucrose-agar medium (per 1 liter of food: 7 g agar, 50 g sucrose, 50 g cornmeal, 50 g yeast, 6 ml propionic acid, 10 ml of a 20% nipagin stock solution). After emergence, flies were kept on medium containing either 100 or 200 µg/ml (233 or 466 µM, respectively) mifepristone dissolved in ethanol, or on control medium (i.e., ethanol without mifepristone).

Quantitative real-time PCR (qRT-PCR) was used to confirm knockdown efficiency of the different constructs. For this, cohorts of F1 offspring between *da*-GS-GAL4 females and males carrying one of the UAS-RNAi constructs were collected upon emergence, and placed in vials with either control medium or medium with mifepristone (100 or 200 µg/ml) for four days in groups of 10 females and 10 males. The flies were then flash frozen in liquid nitrogen and three females were pooled together per sample and homogenized for whole-body RNA extraction. RNA extractions were done using a MagMax robot, using the

MagMax-96 total RNA isolation kit (ThermoFisher Scientific), following the instructions of the manufacturer. cDNA was prepared following the GoScript Reverse Transcriptase system (Promega); qRT-PCR was done on a QuantStudio 6 real-time PCR machine (ThermoFisher Scientific), using Power SYBR Green PCR Master mix (ThermoFisher Scientific). We analyzed ten biological samples per construct and mifepristone treatment, with three technical qRT-PCR replicates each. The expression levels of *Eip75B* and four reference genes (*TBP*, *Cyp1*, *Ef1a48D* and *Rap2l*; see Table 2 for primer sequences) were measured. The reference genes had a stability of $M < 0.25$ as determined a priori from a set of female whole-body samples of various strains and mifepristone concentrations using the GeNorm algorithm incorporated in qbasePLUS v.1.5 (Biogazelle). The three technical replicates were analyzed for reproducibility, and measurements were accepted if none or only one of the technical replicates differed > 0.5 Cq from the other two replicates. In the latter case the divergent Cq value was removed from the dataset. All measurements fulfilled these criteria. The technical replicates were averaged and a single gene expression value per gene per sample was calculated using qbasePLUS v.1.5. The gene expression levels of the four reference genes were then averaged geometrically to obtain an accurate normalization factor for each sample (Vandesompele et al., 2002). The relative gene expression levels of *Eip75B* for the four RNAi constructs were then calculated compared to the expression of the control treatment (i.e. no mifepristone) for each construct separately, respectively. The gene expression levels were analyzed in R (v.3.3.1) using ANOVA with mifepristone concentration as a fixed effect (continuous variable).

We detected a significant reduction in whole-body *Eip75B* expression three out of four constructs when being fed mifepristone (E01: $F = 6.11$, $P = 0.020$; E03: $F = 19.66$, $P = 0.00013$; E44: $F = 4.51$, $P = 0.043$) (Figure 1 and Table 1). The average reduction in *Eip75B*

expression was 18%, 28%, and 18%, respectively, at a mifepristone concentration of 200 $\mu\text{g/ml}$. No significant reduction in the expression of *Eip75B* was detected for construct E10 ($F = 1.95$, $P = 0.04$), even though phenotypic effects on lifespan had been observed when using this construct in previous studies (Huang et al., 2020)

We examined the effects of *Eip75B* knockdown on lifespan, fecundity and egg volume. To measure the effects of lifespan, cohorts of F1 offspring between crosses of *da-GS-GAL4* virgin females and males carrying one of the four UAS-RNAi constructs or the isogenic control strain were collected within a 24-hour window; the flies were sexed under mild CO_2 exposure and transferred at random to 1-liter demography cages with food vials (with 0, 100, or 200 $\mu\text{g/ml}$ mifepristone) attached to the cages. For each genotype and mifepristone concentration, we set up three replicate cages, each containing 75 flies per sex. Dead flies were scored and fresh food was provided every two days. Differences in lifespan between mifepristone-induced RNAi and uninduced controls were analyzed in R (v.3.3.1) for the two sexes separately using mixed-effects Cox (proportional hazards) regression with mifepristone concentration as fixed effect and with 'replicate cage' as a random effect using the R package *coxme* (v2.2-5) for each construct separately. In addition, to test the statistical significance of similar trends in lifespan changes across the four constructs, we used a mixed-effects Cox regression on all data, with mifepristone concentration, construct, and their interaction as fixed effects and with 'replicate cage' as random effect. Bonferroni correction was applied to account for multiple testing ($0.05/8 = 0.00625$).

The effect of *Eip75B* knockdown on fecundity was assessed by measuring daily egg laying rates. For this, virgin females were collected from the F1 offspring of *da-GS-GAL4* females and males from a UAS-RNAi strain or the isogenic control strain. After 24 hours, two

virgin females and two *w¹¹¹⁸* males were placed together in vials with 0 (i.e. control) or 100 µg/ml mifepristone. The analyses were limited to mifepristone concentrations of 100 µg/ml, as a significant effect of 200 µg/ml of mifepristone on the control was observed (Mifepristone: $\chi^2 = 28.9$, $P = 5.3e^{-7}$; Mifepristone*Day: $\chi^2 = 22.6$, $P = 2.0e^{-6}$) (Figure 1 – figure supplement 1). Ten replicate vials were prepared per genotype and mifepristone concentration. The flies were left for 48 hours to ensure mating and consumption of mifepristone before the start of the experiment. Then the flies were transferred to a fresh vial with food (with 0 or 100 mifepristone, respectively) to lay eggs for 24 hours. The number of eggs laid per pair of flies was counted under a microscope. The daily egg laying rate was measured at day 3, 6, 9, 12 and 15.

After day 15, all flies were transferred to vials with medium without mifepristone for 24 hours. The eggs laid were collected and 30 randomly chosen eggs per strain and mifepristone concentration were photographed with a Canon EOS 60D camera under a Leica dissection microscope. The length and the width of each egg were measured using ImageJ (v.1.51h) software and the volume of each egg was approximated following the formula described by (Jha et al., 2015): $\text{volume} = 1/6\pi(\text{width})^2 \cdot \text{length}$. Variation in egg laying rates was analyzed in R (v.3.3.1) using generalized linear mixed models with a Poisson distribution and with mifepristone concentration, day and their interaction as fixed effects and with 'replicate vial' as a random effect using the R package *lme4* (v.1.1-13). Egg volume was analyzed in R using ANOVA with mifepristone concentration as fixed effect. A Bonferroni correction was applied to account for multiple testing ($0.05/4 = 0.0125$).

Evaluation of a *da-GS-GAL4/w¹¹¹⁸* indicated that that the mifepristone concentrations used did not significantly affect lifespan, egg laying rate and egg volume (Figure 1 – figure supplement 1)

SNP association studies

Strains from the *Drosophila* Genetic Reference Panel (DGRP), obtained from the Bloomington *Drosophila* Stock Center (BDSC) were used for a SNP association experiment. The SNP genotypes of our candidate SNP in *Eip75B* of each line were obtained from the genome database available for this panel. The genotype was confirmed by sequencing a small genomic region surrounding the SNP using Sanger sequencing. We generated ten independent F1 crosses from twenty DGRP strains per allelic variant, and then assessed lifespan and fecundity of these crosses. The ten F1 crosses per allelic variant were generated by crossing virgin females of one strain with males from another strain (Table 3). The F1 crosses were reared and assays were performed at 25°C, 65% humidity and 12h:12h light:dark cycle, on a cornmeal-yeast-sucrose-agar medium as described above.

Lifespan was assessed in demography cages as described earlier with the regular medium provided in food vials. Flies that emerged within a 24-hour window were collected and 75 males and 75 females were placed in a single demography cage. For the experiment using F1 crosses, a single demography cage per cross was setup. Differences in lifespan between the two allelic states of each SNP were analyzed in R (v.3.3.1) using mixed-effects Cox (proportional hazards) regression with allele, sex and their interaction as fixed effects and with 'F1 cross' as a random effect using the R package *coxme* (v2.2-5).

Egg laying rate over a period of 30 days after emergence was measured to provide insight in both early (peak) and late (post-peak) fecundity. Again, flies that emerged within a 24-hour window were collected, and two females and two males were placed together in a vial containing regular medium. The experiment with F1 crosses, three replicate vials per cross were setup. Every third day (i.e. day 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30), the number

of eggs laid per pair of flies during a period of 24 hours was counted under a microscope.

Variation in egg laying rates was analyzed in R (v.3.3.1) using generalized linear mixed

models with a Poisson distribution and with allelic state, day and their interaction as fixed

effects and with 'replicate vial' as a random effect using the R package *lme4* (v.1.1-13).

CRISPR/Cas9 induced SNP modification

For editing the SNP in *Eip75B* at position 3L:18026199, plasmids containing the gRNA

sequence and dsDNA donor templates were prepared and injected into *yw;nos-Cas9* (II-

attP40)/*CyO* embryos. This strain has a "T" allele at position 3L:18026199, hence the goals

of the experiment was to modify it to a "G" allele. In addition to editing the candidate

Eip75B SNP, a dsRED marker was inserted to chromosome 2L, into the C-terminus of *I(2)gl*,

to facilitate screening. For this, gRNA sequences were cloned into bicistronic pDCC6

plasmids (*Eip75B*: 5' CACTGCTTGCAATAAGTGATGGG 3'; *I(2)gl*: 5'

TGTTAAAATTGGCTTTCTTCAGG 3'). Homology directed repair was facilitated by constructing

donor templates matching the flanking sequences of the injection line for 500bp on either

side of the dsDNA cleavage point (i.e. 1 kb in total). These donor templates were cloned into

pcDNA3.1. The injection mix consisted of 35 µg of the *Eip75B* donor plasmid, 35 µg of the

I(2)gl dsRED donor plasmid, 15 µg *I2gl* gRNA plasmid, and 15 µg *Eip75B* plasmid per 100µl.

The CRISPR-Cas9 editing was done by InDroso (Rennes, France). A total of 300 embryos

were injected, resulting in 60 surviving larvae and 30 fertile adults. The flies were crossed to

a balancer line (*w;+;Dr/TM6,Sb,Tb*) and their offspring was screened visually for the dsRED

marker and molecularly, by sequencing, for the *Eip75B* candidate SNP. A total of seven

fertile lines with the desired modification in *Eip75B* ("G") were obtained in the F1. Lines that

were positive for the dsRED marker, but negative for the modification in *Eip75B* (hence,

they carried the “T” allele) were kept to serve as controls. The *Eip75B*-modified and control lines were backcrossed to the balancer line for three generations to remove the dsRED marker and to obtain homozygous lines for the candidate SNP: *w;;Eip75B*^{T18’026’199G} (edited) and *w;;Eip75B*^T (control). We generated two homozygous edited lines with the “G” allele (“E5” and “E6”) and two lines with the “T” allele (“C4” and “C6”). The sequence surrounding the *Eip75B* SNP, including the full length of the homology arms, were sequenced to confirm the identity of the SNP and to monitor for any potential undesired genomic modifications. No genomic modifications, apart for the candidate SNP, were detected in any of the lines. An additional set of control lines was generated by backcrossing uninjected *yw;nos-Cas9* (II-attP40)/CyO flies into a *w*¹¹¹⁸ background, using the balancer line and the same crossing scheme as applied for the injected flies.

The following phenotypes were tested in the CRISPR-edited and control lines: lifespan, egg laying rate, egg volume, egg-to-adult development time, egg-to-adult viability, starvation resistance, and adult dry weight. Before the onset of phenotyping experiments flies that were used to generate the cohorts for the experiments were also screened for the identity of the *Eip75B* SNP using PCR-based screening. For this, gDNA was isolated from individual flies using Chelex extraction (Biorad). Flies were placed individually in 100 ul Chelex solution (5g of Chelex mixed with 95ml TE buffer) with 5 ul proteinase K (Promega), and incubated overnight at 56°C. The resulting gDNA was then analyzed with two alternative reverse primers to distinguish the two allelic states: forward primer 5’ CCGGGCACAGTCTCAGTGTT 3’, in combination with reverse primers 5’ GCGCCAAAGCTTTCCCGTCA 3’ or 5’ GCGCCAAAGCTTTCCCGTCC 3’. The PCR was done with GoTaq2 polymerase following the instructions of the manufacturer (Promega) and the following PCR protocol: 94°C for 3 min; 32 cycles with 94°C for 15 sec, 62°C for 30 sec, and

68°C for 1 min; 68°C for 6 min. This procedure facilitated rapid and high-throughput screening by gel electrophoresis. A subset of the samples was sequenced using Sanger sequencing for confirmation. All phenotyping experiments were done in parallel using the same cohort of flies.

Lifespan was measured as described above, by placing 75 females and 75 males that emerged in a 24h window in demography cages with standard medium provided. Five replicate cages were set-up per strain. Differences in lifespan between the two allelic states in *Eip75B* were analyzed in R (v.3.3.1) using mixed-effects Cox (proportional hazards) regression with allele, sex and their interaction as fixed effects and with ‘line’ and ‘replicate cage’ as random effects using the R package *coxme* (v2.2-16).

Egg laying rate was measured as described above. Two females and two male flies, emerged within a 24-hour window, were placed together in a vial containing standard medium. On days 5, 10, 15, 20, 25, and 30 the number of eggs laid per pair of flies during a period of 24 hours was counted under a microscope. Egg laying rates was analyzed in R (v.3.3.1) using generalized linear mixed models with a Poisson distribution and with allelic state, day and their interaction as fixed effects and with ‘line’ and ‘replicate vial’ as random effects using the R package *lme4* (v.1.1-26). The effect of allelic state for each day separately were analyzed in models with only allelic state as fixed effect and ‘replicate vial’ as random effect.

Egg volume was assessed by collecting eggs from females, age 7 days after emergence, laid overnight. We randomly picked 30 eggs, which were photographed and measures as described above. Egg volume was analyzed in R using ANOVA with allele as fixed effect.

To assay egg-to-adult development time ~400 flies were placed in bottles with regular medium and yeast added to lay eggs during two consecutive periods of 3 hours (“batch 1” and “batch 2”). Eggs were collected and distributed to vials at density of 40 eggs per vial, with 16 replicate vials per line. Emergence of adult flies was scored three times per day, at 08:00 (“lights on”), 14:00, and 20:00 (“lights off”). For each vial, the mean time of emergence per sex per vial was calculated and analyzed using linear mixed models with allele, sex and their interaction as fixed effects and batch and line as random effects using the *R* package *lme4*. The egg-to-adult viability was calculated as the proportion of flies that emerged from 40 eggs per vial. It was analyzed using linear mixed model with allele as fixed effect and batch and line as random effects using *lme4*.

Starvation resistance was measured by collecting flies from a 24h window of emergence and placing them in vials with standard medium at a density of 10 females and 10 males for 5 days. Then the two sexes were placed in separate vials. At day 7 after emergence, the flies were transferred to vials with 0.5% agar. Survival was scored three times daily (08:00 (“lights on”), 14:00, and 20:00 (“lights off”)) until all flies has died. The mean time to death was calculated per vial and analyzed for both sexes separately using linear mixed models with allele as fixed effect and line as random effect.

Adult dry weight was assayed by collecting flies from a 24h window of emergence and placing them in vials with standard medium at a density of 10 females and 10 males. On day 7 after emergence, the flies were frozen at -20°C. Flies were dried individually by placing them in 96-wells plates in an incubator at 65°C for three days. This was done in two batches. Adult dry weight was then determined in pools of six flies, with 16 pools of flies per line and sex, on a Mettler Toledo MT5 microbalance. The mean time to death was calculated per vial

and analyzed for both sexes separately using linear mixed models with allele as fixed effect
and line as random effect.

ACKNOWLEDGEMENTS

We thank Tadeusz Kawecki for generously allowing us to use his *Drosophila* facilities; the
Flatt and Keller labs for discussion and support; Véronique Monnier for the *da*-GS-GAL4
strain; and the Bloomington *Drosophila* Stock Center (BDSC) and the Vienna *Drosophila* RNAi
Center (VDRC) for providing stocks. This work was supported by H2020-MSCA-IF-2015 (grant
701949 to KMH), the European Research Council (grant 741491 to LK), and the Swiss
National Science Foundation (grants PP00P3_165836, PP00P3_133641/1, and 310030E-
164207 to TF, grant 310030B_176406 to LK).

COMPETING INTERESTS

The authors declare that there are no competing interests

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TABLES

Table 1: Confirmation of knockdown of *Eip75B* by RNAi. The relative expression of *Eip75B* after mifepristone application (100 µg/ml or 200 µg/ml), was compared to the expression level in the control (no mifepristone) using quantitative real-time PCR.

Acronym	Construct	<i>Eip75B</i> relative expression		<i>F</i>	<i>P</i>
		100 µg/ml	200 µg/ml		
E01	TRiP.GLC01418	0.86	0.82	6.11	0.020
E03	TRiP.JF02257	0.83	0.72	19.66	0.00013
E10	VDRC #108399	1.08	0.87	1.95	0.17
E44	VDCR #44851	0.85	0.82	4.51	0.043

620 **Table 2: Primers used to quantify *Eip75B* knockdown using quantitative real-time PCR.**

Gene	Forward primer	Reverse primer	Amplicon (bp)	Primer efficiency*
<i>Eip75B</i>	GGAGGATGTCCCTGCGCAACC	GAGTATTCCGATCTGCTGCTGCTG	168	1.88
<i>TBP</i>	CGGTTTCCCTGCAAAGTTCCTCGA	CACGATTCGAGGTCGCACCATACG	172	1.92
<i>Cyp1</i>	GTCGGCAGCGGCATTTCAGAT	CTGCACGCTGACGAAGCTAGG	78	1.91
<i>Ef1a48D</i>	TGCCACACCGCTCACATTGCT	CACGCACAGGGGCTTAGAGG	150	1.96
<i>Rap2l</i>	TATTGGACACCGCGGGCACA	TGGGTGCTGGCTGACTTCCT	168	1.96

*qPCR protocol: 95°C for 10 min; 40 cycles with 95°C for 15 sec and 60°C for 1 min; melting analysis from 60°C until 95°C (+0.05 °C/s).

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622

Table 3: F1 crosses setup for assessing the correlation of the focal SNP identity with lifespan and fecundity. Ten F1 crosses were setup per allelic variant. The area surrounding this SNP was sequenced to confirm SNP identity. The table indicates from which DGRP lines females and males were taken to generate the F1 crosses.

Allele	Cross	Females	Males
GG	1	142	787
GG	2	304	852
GG	3	319	379
GG	4	356	821
GG	5	386	324
GG	6	555	105
GG	7	73	377
GG	8	796	832
GG	9	83	790
GG	10	908	158
TT	1	239	362
TT	2	306	208
TT	3	340	375
TT	4	385	508
TT	5	391	853
TT	6	405	129
TT	7	426	566
TT	8	443	843
TT	9	716	355
TT	10	819	761

630 **FIGURES**

635 **Figure 1: Effects of RNAi knockdown of *Eip75B* on lifespan, fecundity, and egg volume** Effects of
636 RNAi on *Eip75B* in the adult stage were assessed using four different UAS-RNAi constructs (E01, E03,
637 E10, and E44) under control of the mifepristone-inducible *da*-GS-GAL driver. The effects of
638 mifepristone concentration (0, 100, or 200 µg/ml), as measure for RNAi knockdown, on lifespan
639 were considered significant when $P < 0.0042$ (Bonferroni: 0.05/12), whereas effects on egg laying rate
640 and egg volume were considered significant when $P < 0.01$ (Bonferroni: 0.05/5); asterisks indicate

641 significant results. (A) *Eip75B* knockdown resulted in a significant reduction in lifespan for the
642 constructs E44 (female: $P < 0.0001$, male: $P = 0.0021$) and E01 (female: $P = 0.0002$, male: $P = 0.22$),
643 but not E03 (female: $P = 0.44$, male: $P = 0.1$) and E10 (female: $P = 0.071$, male: $P = 0.28$). (B) *Eip75B*
644 knockdown had a significant effect on fecundity for constructs E01 ($P < 0.0001$), E03 ($P < 0.0001$) and
645 E44 ($P < 0.0001$), but not E10 ($P = 0.18$). (C) Also, *Eip75B* knockdown had a significant effect on egg
646 volume for constructs E01 ($P = 0.0028$), E03 ($P < 0.0001$) and E44 ($P < 0.0001$), but not E10 ($P =$
647 0.066).
648

(A) Lifespan

(B) Fecundity

(C) Egg volume (mm³)

Figure 1 – figure supplement 1: No significant effects of mifepristone application on *da-GS-*

GAL4/w¹¹¹⁸. Mifepristone application did not have a significant effect on lifespan (Batch 1 - female:

$P = 0.039$, male: $P = 0.37$; Batch 2 - female: $P = 0.3$, male: $P = 0.0052$), nor on egg volume ($P = 0.44$).

A mifepristone concentration of 200 µg/ml did, however, have a significant effect on egg laying rates

($P < 0.0001$). The concentration of mifepristone was therefore limited to 100 µg/ml for the fecundity

experiments ($P = 0.045$). The effects of mifepristone concentration (0, 100, or 200 µg/ml) were

considered significant when $P < 0.0042$ (Bonferroni: 0.05/12), whereas effects on egg laying rate and

egg volume were considered significant when $P < 0.01$ (Bonferroni: 0.05/5).

Figure 2: Association of a SNP in a cis-regulatory motif of *Eip75B* with (A) fecundity and (B) lifespan in wildtype lines. Correlation of the “T” versus “G” identity of the biallelic *Eip75B* SNP at position 3L:18026199 with fecundity and lifespan was tested by measuring F1 crosses of the *Drosophila* Genetic Reference Panel. (A) There was a significant effect of SNP identity on fecundity (allele: $P < 0.0001$, allele*day: $P < 0.0001$), but (B) not on lifespan (female: $P = 0.47$; male: $P = 0.66$). Asterisks indicate significant results.

Figure 3: CRISPR-Cas9 modification of the *Eip75B* SNP in an isogenic background reveals its effects on life history. Two lines with the “G” allele (E5 and E6) and two lines with the “T” allele (C4 and C6) were constructed using CRISPR-Cas9 and tested for differences in a range of life history traits known to be regulated by *Eip75B* from mutant analyses and RNAi studies. (A) There was a significant effect of SNP identity on fecundity, as measured by daily egg laying rates per female up to day 30 after emergence (allele: $P = 0.0013$, allele*day: $P = 0.052$). Also, there was a significant effect on (D) egg-

677 to-adult viability ($P = 0.0004$), and (G) starvation resistance in females (females: $P = 0.0006$; males: P
678 $= 0.41$). This SNP allele did not have an effect on (B) lifespan (females: $P = 0.0006$; males: $P = 0.41$),
679 (C) egg volume ($P = 0.12$), (E) egg-to-adult development time (females: $P = 0.16$; males: $P = 0.24$),
680 and (F) adult dry weight (females: $P = 0.58$; males: $P = 0.052$). Asterisks indicate significant results.
681

A single nucleotide variant in the PPAR γ -homolog *Eip75B* affects fecundity in *Drosophila*

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APPENDIX 1

Population genetic analysis of the candidate SNP in *Eip75B*

Methodology

The frequency of the "T" allele of the candidate SNP in *Eip75B* (3L:18026199, release 6) ranged from an average of 0.52 to 0.84 in early reproducing *versus* late reproducing, long-lived EE populations, respectively (Hoedjes et al., 2019). These allele frequencies were compared to the allelic variation that exists in populations from Europe, North-America, and Africa similar to (Hoedjes et al, in prep). For this, we analyzed whole genome sequencing data from multiple geographic populations available from the DEST dataset (Kapun et al., 2021). We included both single individual sequencing data and Pool-Seq data from various sources. For the single individual sequencing data, the "T" allele frequency of the *Eip75B* allele was calculated when at least ten individuals were sequenced for a given population. Data from pooled populations was included when the coverage for each *Eip75B* SNP was at least 10. We tested the data from the 173 samples collected in Europe for clinal distribution patterns of the allele frequencies. For this, we used generalized linear models (GLMs) based on the allele counts of the *Eip75B* SNP, with either longitude or latitude as explanatory factor. False discovery rate was estimated by testing clinal distribution patterns among a set

of 21'008 neutral SNPs (Clemente & Vogl, 2012; Parsch, Novozhilov, Saminadin-Peter, Wong, & Andolfatto, 2010). Empirical cumulative density functions (ECDF) were generated based on the P values of these neutral SNPs. The area of the upper tail confined by the χ^2 values of the *Eip75B* SNP indicates the percentile of neutral SNPs with χ^2 values equal or higher than the *Eip75B* SNP. This parameter provides a corrected estimate of significance of clinality of each SNP compared to genome-wide neutral estimates (Ramaekers et al., 2019).

Distribution of the Eip75B allele

Analysis of the allele frequencies of the *Eip75B* SNP in multiple geographic populations from Europe (173 samples, "T" allele frequency: 0.53-0.92) and North America (55 samples: 0.53-0.82) indicates a variable allele frequency for this SNP in natural populations, and demonstrates that the allele frequencies observed by (Hoedjes et al., 2019) fall within the range of naturally occurring variation in Europe and North America (Supplementary Table 1). Limited data on the allele frequencies of the candidate *Eip75B* SNP in African populations suggests that both alleles were present in ancestral populations already, although the frequency of the "G" allele may have been limited ("T" allele frequency: 0.8 in Gabon, 1.0 in Cameroon, 0.99 in Zambia). Analysis of the allele frequency data from the European populations provided no indication for a clinal distribution of the *Eip75B* SNP (latitude: $\chi^2 = 1.13$, $P = 0.29$, $P_{\text{corrected}} = 0.61$; longitude: $\chi^2 = 9.45$, $P = 0.002$, $P_{\text{corrected}} = 0.43$).

Appendix 1 – figure 1: Allele frequency distribution of the "T" allele at position 3L: 18026199 across Europe. The samples are ordered by latitude (left) and longitude (right).

(A) Lifespan

(B) Fecundity

(C) Egg volume (mm3)

(A) Lifespan

(B) Fecundity

(C) Egg volume (mm³)

(A) Fecundity

(B) Lifespan

(A) Fecundity **(B) Lifespan** **(C) Egg volume (mm3)** **(D) Viability** **(E) Development time (hours)** **(F) Adult dry weight (mg)** **(G) Starvation resistance (hours)**