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Original Article

Tequila Regulates Insulin-Like Signaling and Extends Life Span in *Drosophila melanogaster*

Cheng-Wen Huang,[1,2,*] Horng-Dar Wang,[2,3,4,*] Hua Bai,[5] Ming-Shiang Wu,[6] Jui-Hung Yen,[7] Marc Tatar,[5] Tsai-Feng Fu,[8] and Pei-Yu Wang[1]

[1]Graduate Institute of Brain and Mind Sciences, College of Medicine, National Taiwan University, Taipei, Taiwan. [2]Institute of Biotechnology, [3]Institute of Systems Neuroscience, and [4]Department of Life Science, National Tsing Hua University, HsinChu, Taiwan. [5]Department of Ecology and Evolutionary Biology, Brown University, Providence, Rhode Island. [6]Department of Internal Medicine, National Taiwan University Hospital, National Taiwan University, Taipei, Taiwan. [7]Department of Microbiology and Immunology, Indiana University School of Medicine, Fort Wayne, Indiana. [8]Department of Applied Chemistry, National Chinan University, Nantou, Taiwan.

*These authors contributed equally to this work.

Address correspondence to Pei-Yu Wang, PhD, No. 1, Sec. 1, Renai Road, Taipei 10051, Taiwan. Email: wangpeiyu@ntu.edu.tw

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Abstract

The aging process is a universal phenomenon shared by all living organisms. The identification of longevity genes is important in that the study of these genes is likely to yield significant insights into human senescence. In this study, we have identified *Tequila* as a novel candidate gene involved in the regulation of longevity in *Drosophila melanogaster*. We have found that a hypomorphic mutation of *Tequila* (*Teq*^{f01792}), as well as cell-specific downregulation of *Tequila* in insulin-producing neurons of the fly, significantly extends life span. *Tequila* deficiency–induced life-span extension is likely to be associated with reduced insulin-like signaling, because *Tequila* mutant flies display several common phenotypes of insulin dysregulation, including reduced circulating *Drosophila* insulin-like peptide 2 (*Dilp2*), reduced Akt phosphorylation, reduced body size, and altered glucose homeostasis. These observations suggest that *Tequila* may confer life-span extension by acting as a modulator of *Drosophila* insulin-like signaling.

Key Words: Aging—Longevity—Neurotrypsin—Glucose homeostasis

Metabolic processes are considered as central components of life-span regulation. Nutritional manipulation, such as dietary restriction, has been shown to prolong healthy life span across species, from yeast to flies to mammals (1–3). Insulin/IGF-like signaling is the major pathway responding to the energy and metabolic status of the body. In flies, there are eight genes encoding the *Drosophila* insulin-like peptides, *Dilp1-8*, which were originally identified based on a sequence similarity search using four conserved cysteine residues within the insulin A chain as bait (4–6). The sequence comparison reveals a 20%–35% similarity of the various *Dilps* to the mature human insulin peptide. *Dilps* are predicted to resemble preproinsulin at the structural level and require peptidase processing for their final activation. Four *Dilps* (*1*, *2*, *3* and *5*) are produced by median neurosecretory cells of the pars intercerebralis, also called insulin-producing neurons, which appear to function as pancreatic β cells in mammals (7).

Dilps signal through the insulin receptor and the insulin receptor substrate (*chico*, in flies), leading to activation of phosphoinositide-3-kinase and protein kinase B/Akt. This kinase cascade eventually phosphorylates the forkhead transcription factor (dFOXO), causing dFOXO retention in the cytoplasm via binding to 14-3-3 proteins. Despite the

fact that mutations resulting in reduced activity of insulin/IGF-like signaling have been shown to increase life span in several evolutionarily distant species, the role of insulin/IGF-like signaling in dietary restriction–induced life-span extension remains controversial (8–12).

Tequila was originally identified as a multiple-domain serine protease that is transcriptionally upregulated following fungal or bacterial infection (13). More recently, it has been recognized as a neurotrypsin ortholog that is involved in long-term memory (LTM) formation (14,15). Intriguingly, *Tequila* may regulate memory formation in a nutrient-dependent manner, because *Tequila* mutant flies display LTM deficiency when fed a normal diet but have normal LTM on a starvation diet (15,16). *Tequila* is highly expressed in the brain and fat body of adult *Drosophila melanogaster*, further suggesting that it may have a functional relationship with metabolic regulation. In this study, we report novel functions of *Tequila* that may modulate insulin-like signaling and extend life span in *D melanogaster*.

Materials and Methods

Flies and Life-Span Assays

w^{1118}, $Tequila^{f01792}$, *Elav*-Gal4, *PPL-Gal4*, *Dilp2-Gal4*, *c739-Gal4*, *Elav*-gene switch (GS), *MB-GS* driver, *UAS-GFP*, and *UAS-Tequila* RNAi (VDRC #45232) fly stocks were raised on standard sucrose/yeast/cornmeal food. *UAS-Tequila* was generated from a full-length *Tequila* D isoform cDNA and subcloned into the pUAST vector. We used the *Tequila* D isoform for overexpression experiments due to its higher expression level in adult flies compared with the other isoforms (13). The effectiveness of the *UAS-Tequila* RNAi and *UAS-Tequila* constructs used for *Tequila* knockdown and overexpression, respectively, was verified by quantitative polymerase chain reaction (qPCR, Figure 1 and Supplementary Figure 1). For life-span assays, all flies were backcrossed into the w^{1118} background for at least five generations, as described previously (17). Flies that had eclosed within 48 hours (approximately 100 males and 100 females) were transferred to a 1-L population cage and maintained in a humidified, temperature-controlled incubator with 12-hour on/off light cycle at 25°C (18). Fresh food was provided every other day, and the number and sex of dead flies were scored. Fly food contained 5% dextrose, 5% yeast, 2% agar, and 0.23% Tegosept (Apex). For the Gene Switch experiment, 200 μM RU486 (mifepristone) in ethanol, or ethanol alone as control, was added to the food.

Quantification of mRNA

Total RNA was prepared from at least 30 flies using the NucleoSpin RNA Kit (Macherey-Nagel). The RNA was converted to cDNA using oligo-d$(T)_{15}$ (Invitrogen) and SuperScript III reverse transcriptase (Invitrogen) as described previously (19). qPCR was carried out using a StepOnePlus Real-Time PCR System (Applied Biosystems), SYBR Green Master Mix (Fermentas), and gene-specific primers 5′-CATTGGTGTGGAGCTGTGGTAAT-3′ and 5′-CTCG GCTATGTTTGCGTAGTGAT-3′ for *Tequila* and 5′-AATGGGTGT CGCTGAAGAAGTC-3′ and 5′-GACGAAATCAAGGCTAAGGT CG-3′ for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). A two-step PCR reaction was carried out with denaturation at 95°C for 15 seconds, annealing and extension combined at 60°C for 1 minute in a total of 40 cycles. The mRNA expression level of each target gene compared with GAPDH was quantified by subtraction: Ct (specific gene) – Ct (GAPDH) = ΔCt. A difference of

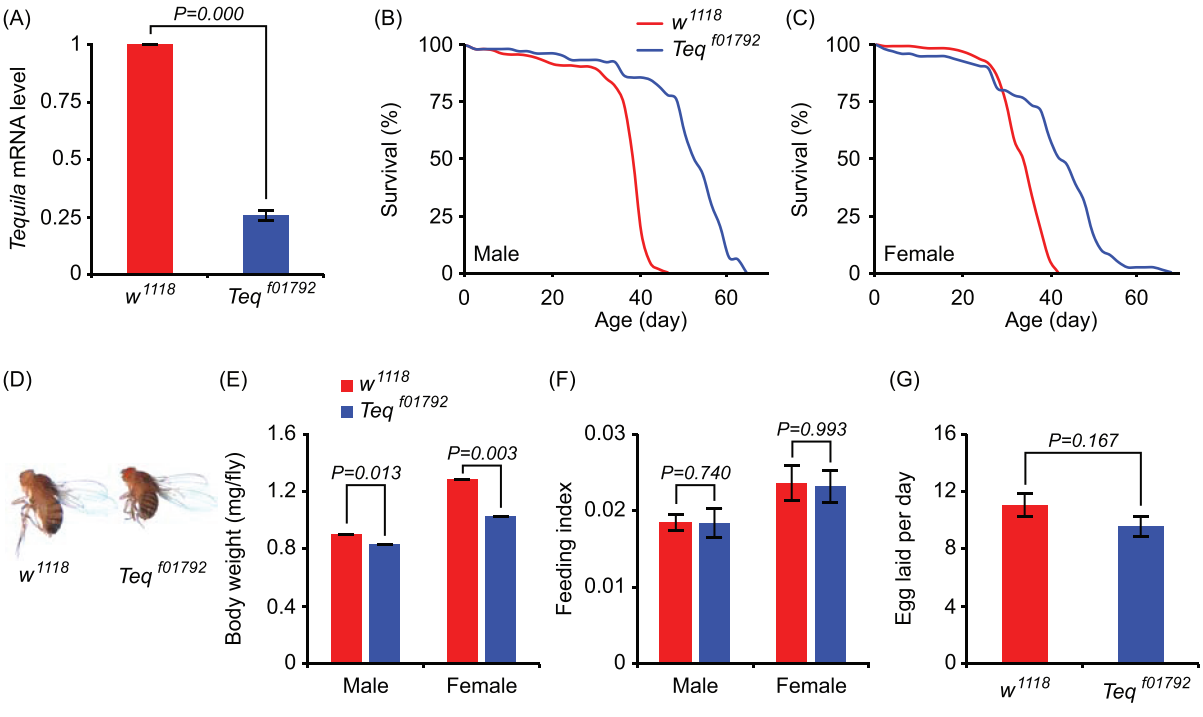

Figure 1. *Tequila* mutation induces life-span extension. (**A**) *Tequila* mRNA is significantly downregulated in Teq^{f01792} homozygous mutant flies (blue) compared with control flies (w^{1118}, red). Experiments were done in triplicate, and each replicate contained more than 30 flies for each group. (**B** and **C**) Extended life spans of male (**B**) and female (**C**) Teq^{f01792} homozygous mutant flies (blue) compared with control flies (w^{1118}, red). Statistical analysis of life spans is shown in Table 1. (**D** and **E**) Teq^{f01792} homozygous mutant flies show smaller body size (**D**) and reduced body weight (**E**). (**F** and **G**) Food intake and female fecundity are not altered in Teq^{f01792} homozygous mutant flies (blue) compared with control flies (w^{1118}, red). Data were collected from 8–10 replicates for each group. Each replicate contained 10 flies for the body weight (**E**) and feeding assays (**F**), and three mating pairs for the female fecundity measurements (**G**).

one PCR cycle equates to a twofold change in mRNA expression level. The uniqueness of amplicons was confirmed using dissociation curves.

Immunostaining

Fly brains or *Drosophila* Schneider (S2) cells (Invitrogen) were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 minutes at room temperature, treated with PBS containing 0.25% Triton X-100 (PBST) for 1 hour, blocked with PBST and 10% normal goat serum for 1 hour, and incubated with primary antibodies diluted in blocking solution for 1 day at 4°C. After being washed in PBST three times, samples were incubated with secondary antibody for 1 day at 4°C. Samples were washed and incubated with 1:250 Alexa Fluor 635 streptavidin (Molecular Probes). Primary antibodies used were rabbit anti-hemagglutinin (1:250, Bethyl) and rabbit anti-*Tequila* (1:50, a kind gift from Dr. T. Preat). Secondary

Table 1. The Effect of *Tequila* on *Drosophila* Life Span (LS)

Gender	Strain	Number	Mean LS (day/hr)	Extension (%)
Male	w^{1118}	424	35.7	
	Teq^{f01792}	199	47.4	32.8*
Female	w^{1118}	430	33.3	
	Teq^{f01792}	399	43.1	29.1*
Male	*Elav-Gal4*>+	149	47.5	
	+>*UAS-Teq* RNAi	216	52	
	Elav-Gal4>*UAS-Teq* RNAi	197	60.7	21.7*/14.3*
Female	*Elav-Gal4*>+	152	45.2	
	+>*UAS-Teq* RNAi	217	47.3	
	Elav-Gal4>*UAS-Teq* RNAi	215	55.1	18.0*/14.2*
Male	*Elav*-GS> *UAS-Teq* RNAi (RU–)	239	43.9	
	Elav-GS >*UAS-Teq* RNAi (RU+)	208	57.9	31.9*
Female	*Elav*-GS> *UAS-Teq* RNAi (RU–)	165	40.5	
	Elav-GS >*UAS-Teq* RNAi (RU+)	215	58.4	44.2*
Male	*PPL-Gal4*>+	288	44.2	
	+>*UAS-Teq* RNAi	315	45.4	
	PPL-Gal4>*UAS-Teq* RNAi	403	36.8	–20.1*/–23.4*
Female	*PPL-Gal4*>+	332	46.8	
	+>*UAS-Teq* RNAi	415	44.9	
	PPL-Gal4>*UAS-Teq* RNAi	394	32	–46.3*/–40.3*
Male	*Dilp2-Gal4*>+	195	42.4	
	+>*UAS-Teq* RNAi	215	45.8	
	Dilp2-Gal4>*UAS-Teq* RNAi	208	59.3	28.5*/22.8*
Female	*Dilp2-Gal4*>+	169	40.2	
	+>*UAS-Teq* RNAi	216	37.5	
	Dilp2-Gal4>*UAS-Teq* RNAi	215	59.3	32.2*/36.8*
Male	*c739-Gal4*>+	218	50.3	
	+>*UAS-Teq* RNAi	317	50.4	
	c739-Gal4>*UAS-Teq* RNAi	220	49.8	–1.0/–1.2
Female	*c739-Gal4*>+	224	48.7	
	+>*UAS-Teq* RNAi	218	47.4	
	c739-Gal4>*UAS-Teq* RNAi	220	48.4	–0.6/–2.1
Male	MB-GS> *UAS-Teq* RNAi (RU-)	211	55.3	
	MB-GS >*UAS-Teq* RNAi (RU+)	208	55.9	1.1
Female	MB-GS> *UAS-Teq* RNAi (RU–)	157	68.5	
	MB-GS >*UAS-Teq* RNAi (RU+)	213	70.1	2.3
Male	*Dilp2*>+	184	54.7	
	Dilp2>*Teq*	190	55.2	0.9
Female	*Dilp2*>+	171	56	
	Dilp2>*Teq*	213	51.4	–8.2*
Starvation				
Male	w^{1118}	99	41.6 hr	
	Teq^{f01792}	98	54.6 hr	31.3*
Female	w^{1118}	100	71.1 hr	
	Teq^{f01792}	100	78.5 hr	10.4*
Oxidative stress				
Male	w^{1118}	49	20.9 hr	
	Teq^{f01792}	50	27.9 hr	33.5*
Female	w^{1118}	49	22.5 hr	
	Teq^{f01792}	50	24.5 hr	8.9

*p Value <0.01 compared with genetic matched control by log-rank test.

antibody was biotinylated anti-rabbit (1:200 Molecular Probes). Nuclei were labeled with 4′,6-diamidino-2-phenylindole (The Jackson Laboratory). Brain samples were mounted in FocusClear (CelExplorer Labs), and images were captured using confocal microscopy (Zeiss LSM 700).

Cell Culture, Plasmid Transfection, and Western Blot Analysis

Drosophila S2 cells were cultured in M3 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and penicillin/streptomycin (Gibco) at 25°C. For transfection, 5×10^6 S2 cells per well were subcultured in a 6-well culture plate, and plasmids were introduced into cells by Effectene transfection reagent (Qiagen) according to the manufacturer's instructions. Full-length *Dilp2* and *Tequila* (D isoform) cDNAs were subcloned into pENTR-D/TOPO (Invitrogen) and recombined into the destination vectors, pAWH (DGRC) and pUWR (DGRC), respectively, using LR Clonase (Invitrogen). Cell lysates were harvested 48 hours after transfection. For Western blot analysis, fly tissues or S2 cells were lysed in radioimmunoprecipitation assay buffer (Thermo); proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes using standard procedures (20). The antibodies used were rabbit anti-phospho-Akt (Ser505, 1:1000, Abcam), mouse anti-tubulin (1:1000, Developmental Studies Hybridoma Bank), and rabbit anti-hemagglutinin (HA) (1:4000, Bethyl). Protein signals were visualized with horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence reagent (Thermo).

Body Weight, Feeding, and Fecundity Measurements

For body weight measurements, 10 male or female 5-day-old flies were anesthetized (CO_2) and weighed immediately, using a microbalance (Sartorius). In feeding assays, 10-day-old flies maintained on high calorie food were transferred to fresh vials of the same food, with addition of 0.5% FD&C no. 1 blue food dye. After 6 hours, 10 flies were homogenized in a single tube containing PBS, and the amount of ingested dye was determined by spectrophotometer for dye absorbance at 620 nm. Female fecundity was determined by daily counting of eggs produced by three mating pairs. Flies were passed daily to new vials containing appropriate food, and the number of eggs laid was counted and recorded for the first 20 days of adult life.

Circulating Dilp2 and Oral Glucose Tolerance Test

Hemolymph *Dilp2* level was quantified following our recently developed enzyme immunoassay (EIA) (21). Briefly, about 0.5 µL of hemolymph was collected from 15 decapitated female flies (5-day-old). Hemolymph was diluted in PBS and incubated overnight in a 96-well EIA plate (Corning Incorporated). After the incubation with anti-DILP2 antibody (1:2500) and a horseradish peroxidase–conjugated secondary antibody (1:2500), the plate was developed by adding TMB solution (3,39,5,59-teramethylbenzidine; American Qualex antibodies) and incubated for 15 minutes at room temperature. The reaction was stopped by addition of 1 M phosphoric acid, and the absorbance was recorded at 450 nm using a plate reader. For the glucose tolerance test, female flies were fasted for 16 hours on 2% agar before being transferred to vials containing 10%

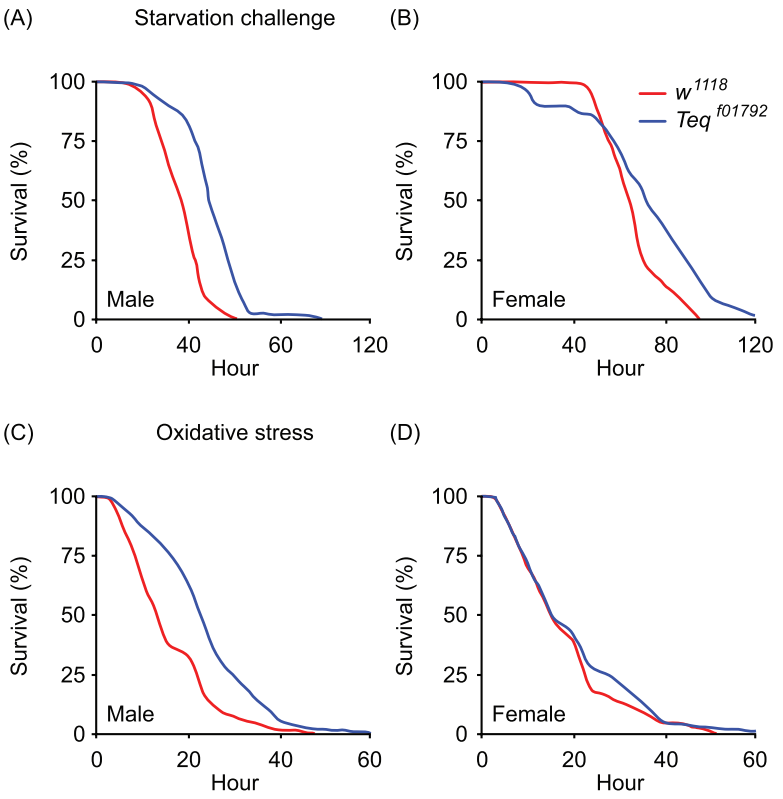

Figure 2. *Tequila* mutation induces stress resistance. (**A** and **B**) Enhanced survival of male and female Teq^{f01792} homozygous mutant flies (blue) on starvation challenge compared with control flies (w^{1118}, red). (**C**) Enhanced survival of male Teq^{f01792} homozygous mutant flies (blue) on paraquat-induced oxidative stress challenge compared with control flies (w^{1118}, red). (**D**) No difference in survival is observed between female control (w^{1118}, red) and Teq^{f01792} homozygous mutant flies (blue) on paraquat-induced oxidative stress. Statistical analysis is shown in Table 1.

dextrose-soaked filters for 1 hour. Flies were then transferred to vials containing water-soaked filters for 30 and 60 minutes, respectively. A total of 30 flies were collected in a microcentrifuge tube and centrifuged at 5000*g* for 10 minutes to obtain the hemolymph. The amount of circulating glucose was measured using a glucose assay kit (Sigma).

Starvation Challenge and Paraquat-Induced Oxidative Stress

Flies that were 10-day-old were transferred to vials containing either 2% agar (starvation) or filter paper soaked in a solution of 20 mM paraquat and 6% dextrose. The number of dead flies was counted every 3–4 hours.

Statistics

All data are expressed as mean ± *SEM*. Survival curves were analyzed by the Kaplan–Meier procedure and log-rank test. Data for all other assays were analyzed using Student's *t* test.

Results

Tequila Mutants Are Long-Lived Flies

To test whether any alteration in *Tequila* gene expression affects life span in flies, we obtained a mutant fly line carrying a hypomorphic mutation of *Tequila* (*Teq*^{f01792}) (15). The *Teq*^{f01792} flies were backcross to w^{1118} flies for at least five generations for genetic background isogenization, and we further confirmed that *Teq*^{f01792} homozygous mutant flies showed reduced *Tequila* mRNA expression (Figure 1A). The physical appearance of *Teq*^{f01792} flies was smaller, with reduced body weight in both male and female flies (Figure 1D and E). The life span of the mutant flies was examined in a 1-L demographic cage, and we found that both male and female homozygous *Teq*^{f01792} flies displayed significant life-span extension compared with genetically matched w^{1118} control flies (Figure 1B and C and Table 1). The homozygous *Teq*^{f01792} mutation did not show noticeable trade-offs for longer life span that are commonly seen in other long-lived mutant flies, such as decreased female fecundity and feeding behavior (Figure 1F and G).

Long-lived mutants are generally more stress resistant than their wild-type counterparts (22–24). In agreement with this, we found that both male and female *Teq*^{f01792} homozygous mutant flies were more resistant to starvation-induced mortality compared with w^{1118} flies (Figure 2A and B and Table 1). Male *Teq*^{f01792} homozygous mutants were also resistant to paraquat-induced oxidative stress, but female mutant flies showed only a trend of increased survival that did not reach statistical significance (Figure 2C and D and Table 1).

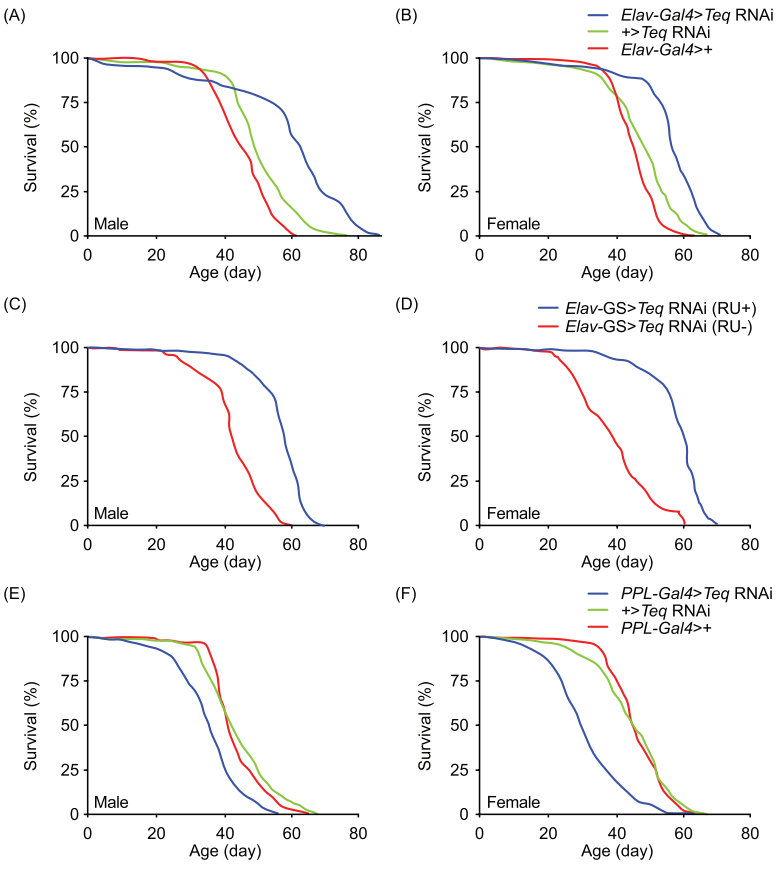

Figure 3. Neuron-specific inactivation of *Tequila* induces life-span extension. (**A** and **B**) Extended life spans in male (**A**) and female (**B**) mutant flies with constitutive, neuron-specific knockdown of *Tequila* (*Elav-Gal4>TeqRNAi*, blue) compared with control flies (*+>TeqRNAi*, green; *Elav-Gal4>+*, red). (**C** and **D**) Extended life spans of male (**C**) and female (**D**) mutant flies carrying the RU486-inducible driver for neuron-specific knockdown of *Tequila* (*Elav-GS>TeqRNAi*; RU+, blue) compared with uninduced control flies (RU-, red). RU486 was fed to adult flies throughout the life span. (**E** and **F**) Shortened life spans in male (**E**) and female (**F**) mutant flies with constitutive, fat body knockdown of *Tequila* (*PPL-Gal4>TeqRNAi*, blue) compared with control flies (*+>TeqRNAi*, green; *PPL-Gal4>+*, red). Statistical analysis of life spans is shown in Table 1.

Tequila-Induced Life-Span Extension Is Associated With Insulin-Producing Neurons

Tequila is known to be expressed in *Drosophila* brain and fat body, which are involved in cognitive and immune regulation, respectively (13,15). In order to identify which tissue might mediate the *Tequila* deficiency–induced life-span extension, we employed the Gal4/UAS binary system to specifically knockdown *Tequila* mRNA expression in either brain or fat body. Flies carrying a normal allele (+) and either a constitutive pan-neuronal driver (*Elav-Gal4*), an inducible pan-neuronal driver (*Elav*-GS), or a constitutive fat body–specific driver (*PPL-Gal4*), were crossed to UAS-*Tequila* RNAi (*TeqRNAi*) flies, and life spans of the progeny were analyzed. We found that constitutive *Tequila* inactivation in neurons extended the life span of both male and female flies (Figure 3A and B and Table 1). Life-span extensions were also observed in both male and female mutant flies carrying *Elav*-GS and UAS-*Tequila* RNAi when food was supplemented with RU486 after eclosion (Figure 3C and D and Table 1). Thus, adult neuronal inactivation of *Tequila* is essential for the observed increased longevity in flies. Constitutive *Tequila* inactivation in fat body, on the other hand, shortened the life span of both male and female flies (Figure 3E and F and Table 1).

We further investigated which neuronal cell type might be involved in *Tequila*-induced life-span extension. We first used constitutive (*c739-Gal4*) and inducible (*MB-GS*) mushroom body drivers to reduce *Tequila* expression, because *Tequila* mRNA and protein have been shown to be expressed in the mushroom body following LTM conditioning (15). However, we found no effect on the life span of mutant flies carrying *Tequila* knockdown in LTM-related mushroom body (Figure 4A–D and Table 1). Because manipulation of insulin-producing neurons in *Drosophila* brain affects the life span of flies (25,26), we examined the expression pattern of *Tequila* in the insulin-producing neurons of *Drosophila* brain. Using immunohistochemistry, we found that anti-*Tequila* immnuoreactivity was indeed associated with insulin-producing neurons (Figure 4E–H). We also found that knockdown of *Tequila* mRNA expression specifically in the insulin-producing neurons (*Dilp2>TeqRNAi*) induced significant life-span extension in both male and female flies (Figure 4I and J and Table 1). These data suggest that *Tequila*-induced life-span extension is associated with insulin-producing neurons.

Tequila Regulates Insulin-Like Signaling

To investigate the functional relationship of *Tequila* and insulin-like peptides, *Drosophila* S2 cells were transfected with pAWH-*Dilp2*-HA and/or pUWR-*Tequila*-red fluorescent protein constructs (Figure 5A). Successfully transfected cells were identified by colocalization of red fluorescent protein and/or anti-hemagglutinin immunoreactivity (Figure 5B–E). We subsequently lysed and harvested the cells and found that there were elevated levels of smaller *Dilp2* fragments in cells that had been cotransfected with both pAWH-*Dilp2*-HA and pUWR-*Tequila*-red fluorescent protein constructs in Western blot analysis, implying that *Dilp2* might act as substrate for the peptidase activity of *Tequila* in transfected *Drosophila* S2 cells (Figure 5F). We postulated that this putative peptidase digestion process might be essential for activation of the *Dilps*, because both circulating *Dilp2* and Akt phosphorylation, a downstream molecular signature of insulin signaling, are reduced in Teq^{f01792} homozygous mutant flies (Figure 5G–I). Moreover, we also tested the glucose homeostasis of flies in an oral glucose tolerance test. In control (w^{1118}) flies, typical glucose clearance kinetics were observed when the flies were subjected to fasting and subsequent addition of glucose solutions to food (Figure 5J). Teq^{f01792} homozygous mutant flies showed slower glucose clearance kinetics, indicated by much higher circulating glucose levels even at 60 minutes after glucose injection (Figure 5J).

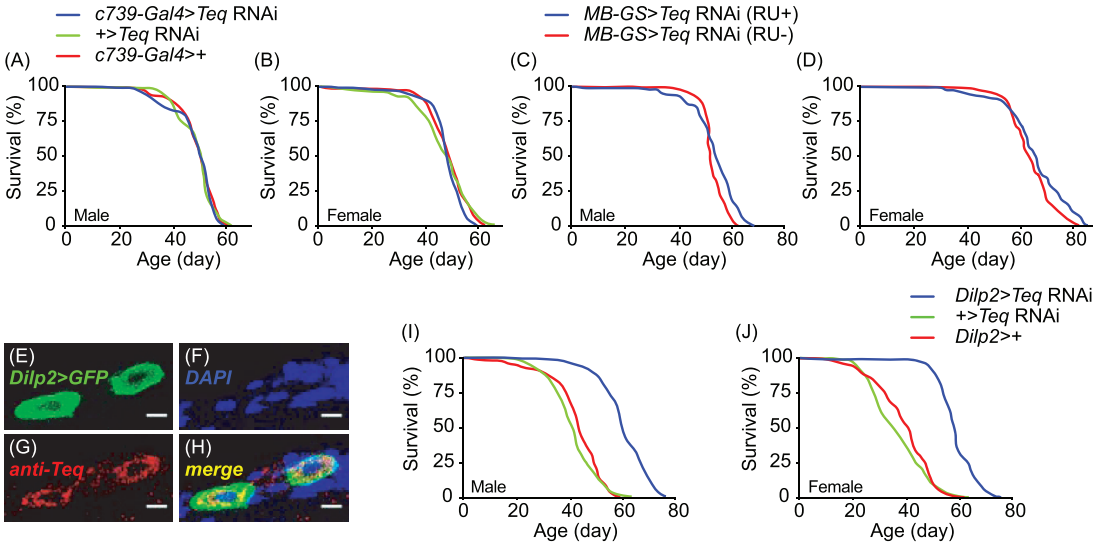

Figure 4. Insulin-producing neurons, but not mushroom body, mediate *Tequila*-induced life-span extension. (**A** and **B**) No life-span extension is seen in male (**A**) and female (**B**) mutant flies carrying constitutive, mushroom body knockdown of *Tequila* (*c739-Gal4>TeqRNAi*, blue) compared with control flies (*+>TeqRNAi*, green; *c739-Gal4>+*, red). (**C** and **D**) No life-span extension is seen in male (**C**) and female (**D**) mutant flies carrying the RU486-inducible driver for mushroom body knockdown of *Tequila* (*MB-GS>TeqRNAi*; RU+, blue) compared with uninduced control flies (RU-, red). RU486 was fed to adult flies throughout the life span. (**E–H**) Confocal images showing that *Tequila* immunoreactivity (anti-*Teq*, red) colocalizes with the insulin-producing neurons (green). The insulin-producing neurons are labeled by green fluorescence (*Dilp2>GFP*). Cell nuclei are labeled with 4′,6-diamidino-2-phenylindole (blue). Scale bars = 5 μm. (**I** and **J**) Extended life spans of male (**I**) and female (**J**) mutant flies carrying constitutive, insulin-producing neuron knockdown of *Tequila* (*Dilp2>TeqRNAi*; blue) compared with control flies (*+>TeqRNAi*, green; *Dilp2>+*, red). Statistical analysis of life spans is shown in Table 1.

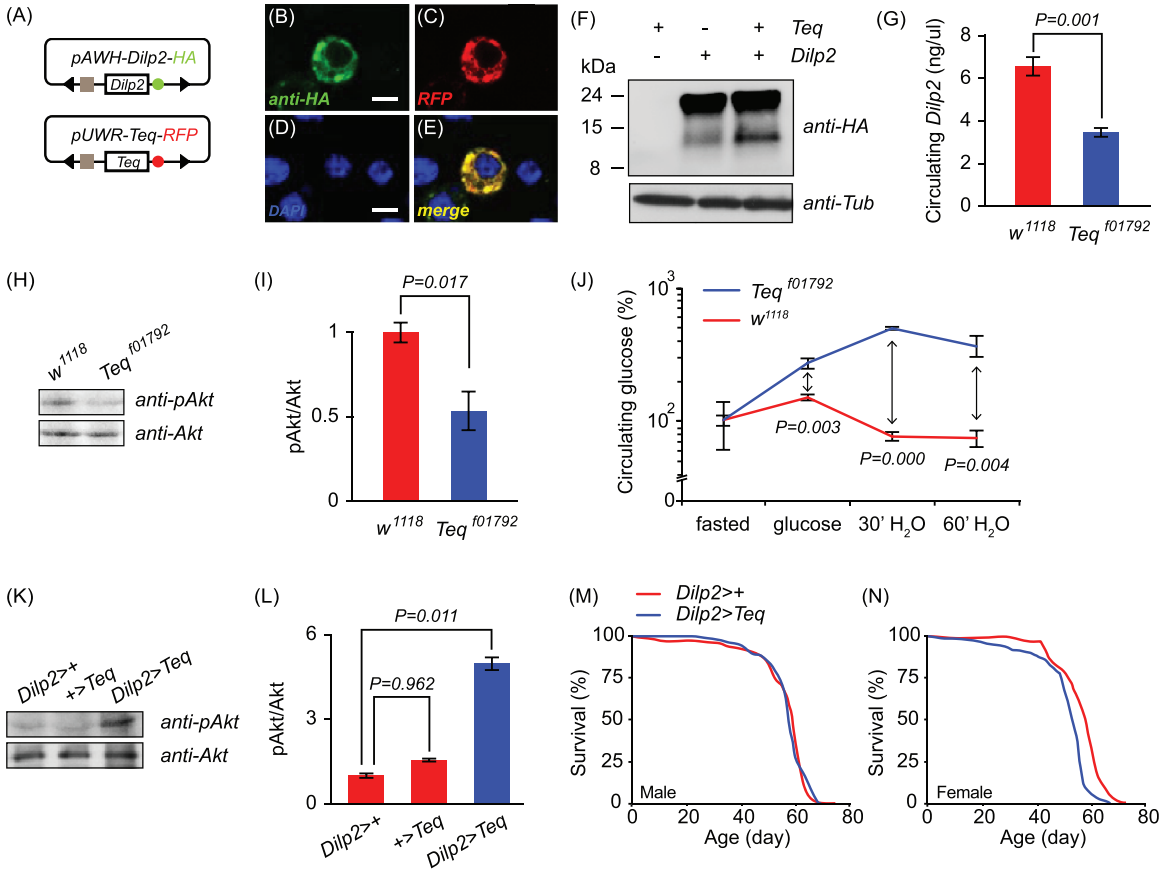

Figure 5. *Tequila* regulates insulin-like signaling. (**A–E**) *Drosophila* S2 cells transfected with pAWH-*Dilp2*-HA (green) and/or pUWR-*Tequila*-red fluorescent protein (red) constructs can be observed under a fluorescence microscope. Cell nuclei are labeled with 4′,6-diamidino-2-phenylindole (blue). Scale bars = 5 μm. (**F**) Increased smaller molecular weight *Dilp2* fragments were detected in cell lysates of *Tequila* and *Dilp2* cotransfected *S2* cells using an anti-hemagglutinin antibody in Western blot analysis. Anti-α-tubulin (Tub) is shown as an internal control. (**G**) Reduced circulating *Dilp2* were detected in the hemolymph of Teq^{f01792} homozygous mutant flies (blue) compared with control flies (w^{1118}, red). Experiments were done in eight replicates, and each replicate contained 15 flies for each group. (**H** and **I**) Akt phosphorylation (*anti-p-Akt*) is decreased in Teq^{f01792} homozygous mutant flies (blue) compared with control flies (w^{1118}, red) in Western blot analysis. Experiments were done in triplicate, and each replicate contained 15 flies for each group. (**J**) An oral glucose tolerance test shows that Teq^{f01792} homozygous mutant flies have abnormal kinetics for glucose clearance. Experiments were done in triplicate, and each replicate contained 30 flies for each group. (**K** and **L**) Akt phosphorylation (*anti-p-Akt*) is increased in mutant flies carrying constitutive, insulin-producing neuron overexpression of *Tequila* (*Dilp2>Teq*, blue) compared with control flies (*+>Teq* or *Dilp2>+*, red) in Western blot analysis. Experiments were done in triplicate, and each replicate contained 15 flies for each group. (**M** and **N**) Normal life span in male (M) and slightly shortened life span in female (N) mutant flies carrying constitutive, insulin-producing neuron overexpression of *Tequila* (*Dilp2>Teq*, blue) compared with control flies (*Dilp2>+*, red). Statistical analysis of life spans is shown in Table 1.

If *Tequila* is required for *Dilp2* maturation, we would expect that *Tequila* overexpression might increase insulin-like signaling. We overexpressed *Tequila* in insulin-producing neurons (*Dilp2>Teq*) and found that Akt phosphorylation was significantly increased in these mutant flies compared with control flies (Figure 5K and L). However, *Tequila* overexpression in insulin-producing neurons (*Dilp2>Teq*) only slightly reduced life span in female flies and had no effect on male flies (Figure 5M and N). It is possible that both the control and mutant flies in our experimental setting already sustained a high level of insulin-like signaling, in which case, enhanced *Tequila* expression would not necessarily have a large influence on *Drosophila* life span.

Discussion

This study was undertaken with the goal of identifying novel longevity genes, and as one of these genes, *Tequila* was evaluated for its potential to regulate longevity. Despite its action in LTM formation, and possibly in the immune response (13–15), relatively little is known about the biological function of *Tequila* in aging. The observation that *Tequila* inactivation induced life-span extension was verified by a number of different genetic approaches. We showed that a hypomorphic mutation of *Tequila* (Teq^{f01792}) was capable of inducing life-span extension. Moreover, cell-specific RNAi expression confirmed that reduction of *Tequila* expression in insulin-producing neurons could function to extend life span. Thus, the insulin/IGF-signaling pathway appears to be critical for *Tequila*-mediated life-span extension.

In mammalian cells, maturation of insulin first requires conversion of preproinsulin to proinsulin by removal of an N-terminal signal peptide. Proinsulin then undergoes proteolytic processing during protein secretion to become the bioactive insulin hormone (27). Although the maturation process of *Dilps* remains unknown, analysis of their amino acid sequence predicts several likely cleavage sites (4). Because *Tequila* is recognized as a neurotrypsin ortholog, we examined the possibility that *Dilps* might

be a substrate for the putative protease activity of *Tequila*. Our data provide some evidence supporting this possibility, demonstrated by (i) an increase in smaller *Dilp2* fragments in transfected *Drosophila* S2 cells, (ii) reduced circulating *Dilp2*, (iii) altered Akt phosphorylation, and (iv) disrupted glucose homeostasis in *Tequila* mutant flies. Although we do not currently have evidence of the specific cleavage sites or for bioactivity of the processed *Dilp2*, our data are suggestive that *Tequila* could be involved in activation of *Dilp2*-mediated signaling.

In contrast to the action of *Tequila* in the insulin-producing neurons, fat body–specific inactivation of *Tequila* resulted in reduced life span in flies. One possible explanation for this is that *Tequila* inactivation in fat body may cause altered *Dilp6*-mediated signaling, and *Dilp6* has been shown to regulate life span by repressing *Dilp2* expression and secretion from the insulin-producing neurons of *Drosophila* brain (21). Our future studies will include further examination of *Dilp6* expression or secretion from fat body in *Tequila* mutant flies. The proposed function of *Tequila* may also be involved in immune response (13), and chronic fat body–specific upregulation of immune response has been shown to induce enhanced pathogen resistance but reduced life span (28). Although it is unclear at what level *Tequila* may be involved in immune regulation in flies, the amino acid sequence and functional analysis of *Sp22D* protein, a *Tequila* ortholog in the mosquito *Anopheles*, suggest that it may serve as a signaling molecule upon pathogen intrusion or wounding in insects (29). These observations support the hypothesis that *Tequila* may have multiple functions in different biological contexts.

It is well established that metabolic trade-offs can modulate the life history evolution of growth, reproduction, and somatic maintenance. Analysis of mutant flies having reduced insulin/IGF-like signaling has identified several common phenotypes that include growth retardation, reduced fecundity, altered metabolism, increased resistance to starvation and oxidative stress, and extended life span (4,7,25). *Tequila* mutant flies display several of these characteristic phenotypes, but we did not find any reduction in female fecundity. Thus, we are not able to demonstrate a strong connection linking *Tequila* between insulin/IGF-like signaling and reproductive regulation. Testing under multiple food conditions will be needed in order to establish the contribution of *Tequila* to fly reproductive regulation, because fly fecundity is known to fluctuate significantly in order to permit *Drosophila* to adapt to varying nutritional status.

In summary, our studies have identified *Tequila* as a novel longevity-regulating gene in *Drosophila*. We have shown that *Tequila* has the potential to act as a modulator of fly insulin/IGF signaling, possibly through proteolytic processing of *Dilps*, and that it can regulate life span.

Supplementary Material

Supplementary figure can be found at: http://biomedgerontology.oxfordjournals.org/

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