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Mitochondrial chaperone TRAP1 activates the mitochondrial UPR and extends healthspan in *Drosophila*

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ABSTRACT

The molecular mechanisms influencing healthspan are unclear but mitochondrial function, resistance to oxidative stress and proteostasis are recurring themes. Tumor necrosis factor Receptor Associated Protein 1 (TRAP1), the mitochondrial analog of Hsp75, regulates levels of reactive oxygen species *in vitro* and is found expressed at higher levels in tumor cells where it is thought to play a pro-survival role. While TRAP1-directed compartmentalized protein folding is a promising target for cancer therapy, its role at the organismal level is unclear. Here we report that overexpression of TRAP1 in *Drosophila* extends healthspan by enhancing stress resistance, locomotor activity and fertility while depletion of TRAP1 has the opposite effect, with little effect on lifespan under both conditions. In addition, modulating TRAP1 expression promotes the nuclear translocation of homeobox protein Dve and increases expression of genes associated with the mitochondrial unfolded protein response (UPR^{mt}), indicating an activation of this proteostasis pathway. Notably, independent genetic knockdown of components of the UPR^{mt} pathway dampen the enhanced stress resistance observed in TRAP1 overexpression flies. Together these studies suggest that TRAP1 regulates healthspan, potentially through activation of the UPR^{mt}.

1. Introduction

Age related impairment of locomotor, reproductive, and physiological functions is a universal phenomenon in animals. Intriguingly, several of the lifespan extension animal models are not healthy through their life (Bartke and Brown-Borg, 2004). In worms, mitochondrial mutants such as *isp-1* and *nuo-2* live long, but are slow growing and have a reduced brood size (Rea et al., 2007). Similarly, in long lived dwarf mice with deficiencies in growth hormone, prolactin and thyroid stimulating hormone, reproductive fitness is compromised (Bartke and Brown-Borg, 2004). Finally, *Drosophila* treated with the anti-convulsive agent Lamotrigine display significantly enhanced lifespan but poor locomotor performance at older ages (Avanesian et al., 2010). An emerging goal in aging research is developing interventions that not only increase longevity, but also increase the healthspan (Kirkland and Peterson, 2009; Selman and Withers, 2011).

The mechanisms that modulate healthy aging are unclear, but mitochondria are thought to play a central role (Wolff and Dillin, 2006). Mitochondria generate high levels of reactive oxygen species (ROS) that damage cellular macromolecules and gradually impair cellular function. Some evidence suggests that accumulation of ROS contributes to the physiological decline associated with age, though this theory is not universally accepted (Wolff and Dillin, 2006; Hekimi et al., 2011). Mice that overexpress catalase in their mitochondria have an enhanced lifespan, delayed age-associated pathologies, reduced mtDNA deletions with age and reduced levels of ROS (Schriner et al., 2005). Knockdown of components of the electron transport chain (ETC) not only decrease ATP production but surprisingly also increase lifespan (Wolff and Dillin, 2006; Copeland et al., 2009). Furthermore, overexpressing mitochondrial LON protease in the fungal aging

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model *Podospora anserina* results in enhanced healthspan, suggesting that mitochondrial proteostasis is an important regulator of organismal health (Luce and Osiewacz, 2009).

Mitochondrial function is tightly associated with energy sensing and stress response pathways that contribute to aging, in particular the mTOR and AMPK pathways (Haigis and Yankner, 2010). These pathways are also associated with oxidative stress resistance and regulation of cellular proteostasis (Salminen et al., 2012). Compartments such as the endoplasmic reticulum and mitochondria are especially vulnerable to diminished proteostasis because of their high intrinsic protein folding requirements. To regulate protein quality control, these organelles have dedicated stress response mechanisms called the unfolded protein response (UPR) (Ron and Walter, 2007; Haynes and Ron, 2010). Accordingly, it has been hypothesized that enhanced proteome maintenance would push the limits of the minimal proteostasis boundary (folding energetics necessary for folding of a protein at a defined proteostasis network capacity) towards health (Powers et al., 2009).

Mitochondrial chaperone Tumor necrosis factor Receptor Associated Protein 1 (TRAP1) is a member of the Hsp90 family and is the mitochondrial analog of Hsp75 (Felts et al., 2000). TRAP1, originally identified as a binding partner of the intracellular domain of TNFR1, also binds retinoblastoma protein during mitosis, and the tumor suppressors EXT1 and EXT2 (Altieri et al., 2012). Despite the high homology with Hsp90, TRAP1 does not interact with its traditional client proteins (Kang et al., 2007), indicating a distinct functional role for this mitochondrial chaperone. *In vitro* evidence suggests that TRAP1 regulates ROS levels and protects cells against oxidative damage (Hua et al., 2007). Furthermore, TRAP1 was found expressed at higher levels in tumor cells where it is thought to play a pro-survival role by inhibiting apoptosis (Kang et al., 2007; Costantino et al., 2009). Recent evidence also implicates TRAP1 in Parkinson's disease. PINK1 mediated phosphorylation of TRAP1 is considered important in suppressing cytochrome c release from mitochondria to regulate apoptosis (Pridgeon et al., 2007). TRAP1 also ameliorates cellular toxicity induced by α-synuclein in dopaminergic neurons (Butler et al., 2012) and expression of TRAP1 was able to rescue mitochondrial dysfunction in *Pink1* deficient flies (Zhang et al., 2013; Costa et al., 2013). In summary, TRAP1 lies at an exciting intersection between ROS, aging, mitochondrial proteostasis and human disease.

To determine whether modulating mitochondrial proteostasis would influence longevity and healthspan *in vivo*, we studied the effect of modulating TRAP1 expression levels in *Drosophila*. Here we demonstrate that TRAP1 regulates ROS levels, oxidative stress resistance and heat stress resistance in *Drosophila*, without significantly altering lifespan. Intriguingly, there is robust sex-specificity in TRAP1-mediated stress resistance. In addition, ubiquitous overexpression of TRAP1 enhances fertility and remarkably improves locomotor ability in aging flies. In contrast, loss of TRAP1 results in decreased fertility and locomotor ability, indicating strong modulation of healthspan by TRAP1. Finally, we show that dosage modulation of TRAP1 activates the mitochondrial UPR (UPR^{mt}) in males but not the females, indicative of similar sex-specificity as observed in stress resistance. Finally, impairment of the UPR^{mt} inhibits TRAP1 mediated resistance to stress. These findings suggest that TRAP1 mediated alterations in UPR^{mt} can modulate stress resistance and healthspan.

2. Material and methods

2.1. Drosophila stocks and culture

All fly stocks were maintained on standard cornmeal medium, unless stated otherwise, at 25 °C and 60% humidity under 12 h light: dark cycles. *w*; *TRAP1Δ4/Cyo*; *+/+* flies were obtained from Dr. Jessica Treisman, New York University. A full-length deletion of the *TRAP1* locus resulting from an imprecise p-element excision was confirmed by qPCR in *w*; *TRAP1Δ4/TRAP1Δ4*; *+/+* flies. To generate transgenic overexpression strains, *Drosophila TRAP1* cDNA was inserted in pUAST plasmid and microinjected into w^{1118}; +/+; +/+ embryos (BestGene Inc). Insertions were verified by inverse PCR and two independent strains were used, *w*; +/+; *UAS* $TRAP1^{4M}$/+ and *w*; +/+; *UAS* $TRAP1^{7M}$/+. Overexpression in the *w*; +/+; *UAS* $TRAP1^{4M}$/*Actin*Gal4 and *w*; +/+; *UAS* $TRAP1^{7M}$/*Actin*Gal4 was confirmed by qPCR. All strains were backcrossed 5–6 generations in the w^{1118}; +/+; +/+ background. w^{1118}; +/+; +/+, *Actin*Gal4 and *elav*Gal4 drivers were obtained from the Bloomington Stock Center, Indiana. *w*; *UAS CG5045 RNAi*/+; +/+ and *w*; *UAS DVE RNAi*; +/+ strains were obtained from the Vienna Drosophila Resource Center. Geneswitch-*tub5*Gal4 flies were a gift from Dr. Scott Pletcher (University of Michigan).

2.2. Lifespan analysis and stress resistance

w^{1118}; +/+; +/+ strain was used as the background control for *TRAP1* mutants. For the TRAP1 overexpressing flies, the transgene alone and the driver alone strains were used as controls. In both lifespan and stress assays (oxidative and heat stress resistance), significant alteration was deemed only when $p < 0.05$ relative to all control strains. Statistical analysis was performed using JMP software and significance was determined using the standard chi-squared based log rank test. For detailed experimental design, see Supplementary information 'Material and methods'.

2.3. Feeding behavior

CAFE (Capillary Feeder) assay was modified from Ja et al. (2007). Briefly, 5 male flies of each genotype were starved for 3 h (same time duration as in oxidative stress resistance assay) before being transferred to a 2.5 × 9.5 cm vial with moistened filter paper at the base. Two 5 ml calibrated glass micropipettes (VWR) filled with 5% sucrose were inserted midway into the vial. Blue dye was added to the sucrose solution to facilitate readings that were taken every 2 h, for a total of 8 h. Amount of food consumed was corrected on the basis of evaporation in a blank vial and calculated per fly per hour. The experiment was repeated three times with independent cohort of flies and statistical significance determined using Student's *t*-test.

2.4. Locomotor ability

Locomotor ability was assessed using the negative geotaxis assay in a longitudinal study. A total of 50 male and 50 female flies of each genotype were assayed every 10 days, starting with 10-day old flies. On the day of experiment, flies were transferred to empty 2.5 × 9.5 cm vials. After 120 s of acclimatization, flies were gently tapped to the bottom three times and allowed to climb the walls. The number of flies that successfully crossed 7 cm in 10 s was scored. Each experiment was conducted with 5 independent groups, and each group was tested three times after a 5 min interval between each test. Statistical significance was determined by one-way ANOVA with Dunnett's post hoc comparison using SPSS software.

2.5. Fertility

To assess female fertility, five virgin females of each genotype were allowed to mate with five w^{1118} males, aged 3 days. Flies were transferred to fresh food every 3 days and total number of progeny eclosed from each vial was counted once every 3 days for 9 days.

Fresh 3-day old w^{1118} males were replaced at every time-point. Brood size was defined as the total progeny eclosed over 60 days. All genotypes were tested in parallel and three independent repeats were performed. To determine the fertility of males, one male of each genotype was allowed to mate with three virgin w^{1118} females aged 3 days. Parent flies were removed after 3 days and total number of progeny eclosed from each vial was counted. Fresh 3-day old virgin w^{1118} females were replaced at every time-point. The experiment was conducted with five males of each genotype in parallel and was repeated twice. Statistical significance was determined when $p < 0.05$, using Student's *t*-test.

2.6. ROS measurement

ROS levels were measured using MitoSox (Invitrogen). Adult brains were dissected in cold HBSS, transferred to 5 mM MitoSox and incubated for 30 min at room temperature. Brains were washed three times with cold PBS and mounted in Vectashield (Invitrogen) between a slide and a coverslip placed on double-sided tape to prevent squashing. For quantification purposes, z-stacks were acquired through the entire thickness of the brain, and total fluorescent intensity was measured from 3D reconstruction images using ImageJ (NIH).

2.7. Immunohistochemistry

Drosophila larvae were dissected and stained as described previously (Baqri et al., 2009). Adult *Drosophila* brains were dissected in cold PBS, fixed overnight in 0.8% paraformaldehyde at 4 °C, and washed thrice in PBS with 0.5% Triton and 0.5% BSA (PBST) for 15 min each. Samples were blocked in 10% BSA for 2 h, and incubated overnight in primary antibody at 4 °C. Samples were washed four times with PBST for 30 min each, and incubated with secondary antibody for 12 h at 4 °C. After three 30 min washes with PBST and a 15 min wash with PBS, brains were mounted in Vectashield with DAPI (Invitrogen). We used mouse anti-mitochondrial complex V monoclonal antibody at 1:500 (MitoSciences) and rabbit anti-Dve antibody at 1:1000 (Nakagoshi et al., 1998). All comparative images were either acquired on a Nikon TE swept field microscope or a Zeiss LSM 700 confocal microscope at identical laser settings, exposure and aperture. Fluorescently labeled secondary antibodies used were goat anti-mouse Alexa 568 (Invitrogen) and goat anti-rabbit Alexa 488 (Invitrogen).

2.8. Quantitative PCR

Total RNA was reverse transcribed using the Taqman kit by Applied Biosystems (Roche), according to manufacturer's recommendations. The quantitative PCR was done using the Power SYBr Master Mix (Applied Biosystems). Gene expression was normalized based on 28*S* RNA expression from three replicate experiments. For list of primers used, see Supporting information 'Materials and methods'.

3. Results

3.1. TRAP1 regulates ROS levels in vivo

In vitro evidence indicates that loss of TRAP1 results in increased accumulation of cellular ROS (Hua et al., 2007; Im et al., 2007; Yoshida et al., 2013). To determine whether TRAP1 regulates ROS levels *in vivo*, we assayed ROS levels in the brains of adult *Drosophila* with MitoSox, a cell permeable dye that fluoresces when oxidized by ROS and is rapidly and selectively targeted to the mitochondria. There is a marked increase in MitoSox staining in the brains of 5 day old males in *TRAP1* deletion mutant, *w*; *TRAP1Δ4/TRAP1Δ4*; +/+, but not when TRAP1 was overexpressed using the constitutive and ubiquitous driver *Actin*Gal4 (Fig. 1A, B, C and G). TRAP1 expression levels in mutant and overexpression strains were confirmed by qPCR (Supplementary Fig. 1A and B). Mitochondrial distribution was not significantly altered in the segmental nerves of these strains, indicating that increased MitoSox staining in the mutants was not due to changes in mitochondrial density (Fig. 1D, E and F). These data suggest that TRAP1 regulates generation of mitochondrial oxidants *in vivo*.

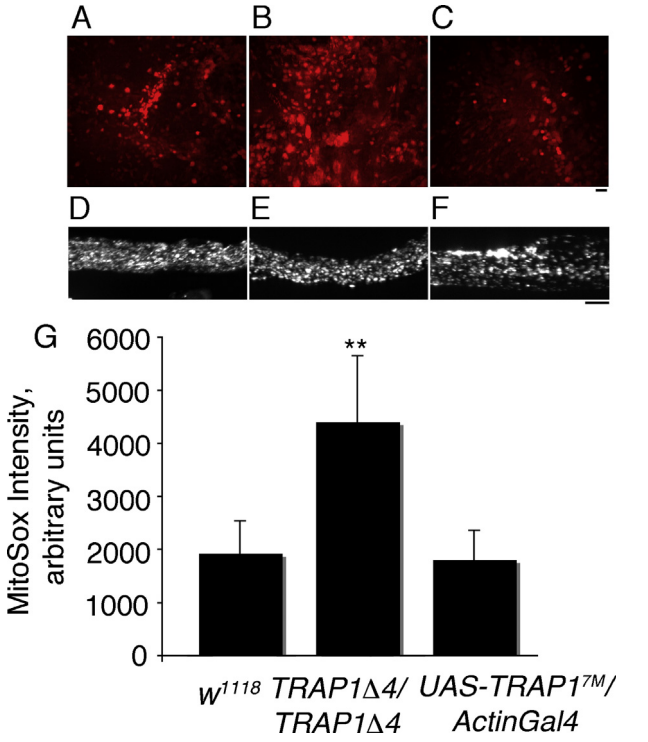

Fig. 1. *TRAP1* regulates ROS levels *in vivo*. (A–C) Optic lobes of young adult *Drosophila* brains stained with Mitosox, and (D–F) larval segmental nerves of corresponding genotypes stained with antibody against mitochondrial Complex V. (A and D) w^{1118}; +/+; +/+, (B and E) *w*; *TRAPΔ4/TRAPD4*; +/+, (C and F) *w*; +/+; *UAS-TRAP1^{7M}/Actin*Gal4. (G) MitoSox staining is significantly increased in *w*; *TRAPΔ4/TRAPΔ4*; +/+ mutants. While staining is marginally reduced in overexpression flies *w*; +/+; *UAS-TRAP1^{7M}/Actin*Gal4, it is not significantly different from wildtype. Error bars denote standard deviation of means. $N = 3–5$. (**) Indicate $p < 0.001$ from Student's *t*-test. Mitochondrial distribution appears normal in mutant and overexpression animals in segmental nerves of *Drosophila* larvae. In all cases, scale bars equal 10 μm.

3.2. TRAP1 regulates resistance to oxidative stress in young and old Drosophila

Increased ROS levels are associated with oxidative damage and diminished stress resistance in several animal models (Hekimi et al., 2011). To examine if TRAP1 regulates resistance to oxidative stress, we exposed 5-day old *TRAP1Δ4* mutant flies to paraquat and assayed for survival. Paraquat is a methyl viologen that undergoes redox cycling to generate superoxide. In congruence with a recent study (Costa et al., 2013), we found that young *TRAP1Δ4* mutant males were significantly more susceptible to oxidative stress as compared to males of the w^{1118} wildtype background strain (Fig. 2A). In contrast, resistance to oxidative stress was significantly increased when TRAP1 was ubiquitously overexpressed in two independent transgenic strains, *w*; +/+; *UAS-TRAP1^{7M}/Actin*Gal4 and *w*: +/+; *UAS-TRAP1^{4M}/Actin*Gal4 (Fig. 2B and Supplementary Fig. 2A). Interestingly, this response was sex specific: males were especially responsive to modulation of TRAP1

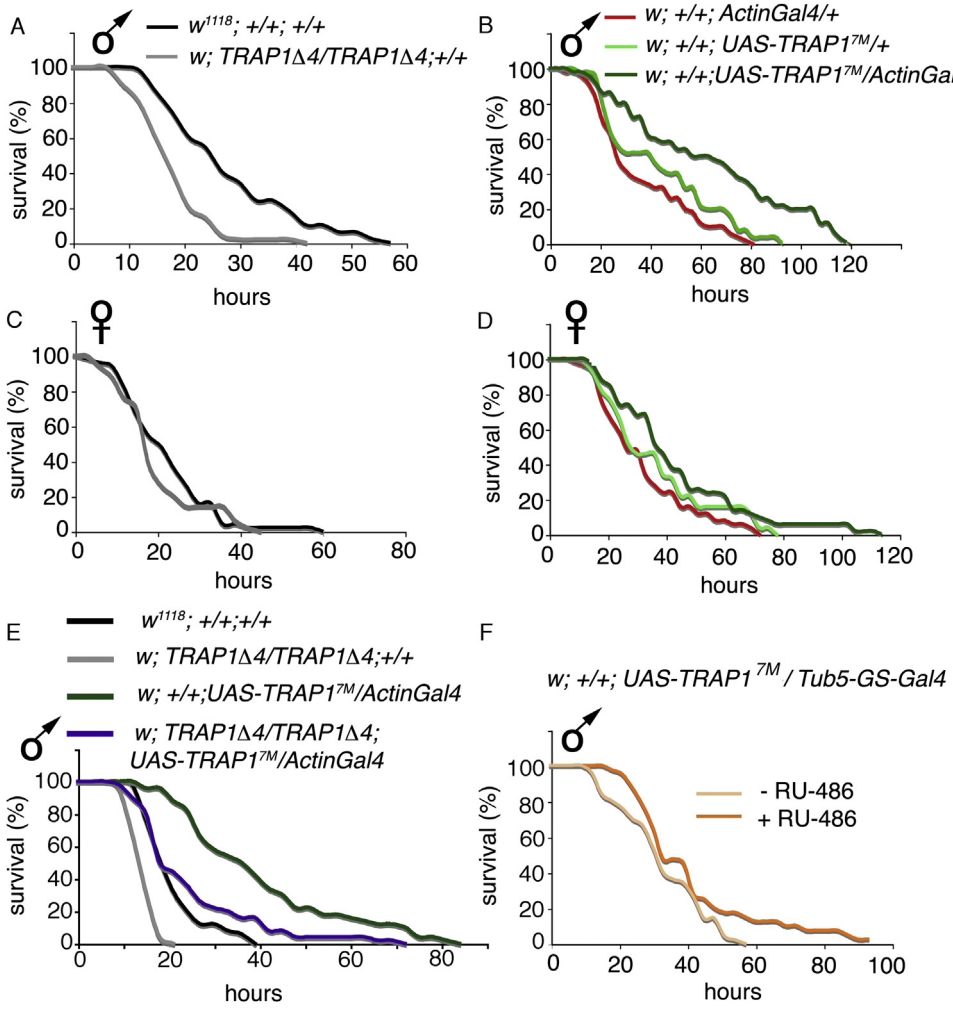

Fig. 2. TRAP1 regulates oxidative stress resistance in young and old *Drosophila* males. (A–E) Survival curves of young flies (5 days) of indicated genotypes exposed to 20 mM paraquat. (A) Median survival of male *w; TRAP1Δ4/TRAP1Δ4*; +/+ (18.5 h ± 0.9) is significantly less than that of w^{1118}; +/+; +/+ (28.4 h ± 1.6; $p < 0.0001$). (B) Median survival of male *w*; +/+; *UAS-TRAP1*^{7M}/*Actin*Gal4 (63.2 h ± 4.8) is significantly more than that of *w*; +/+; *UAS-TRAP1*^{7M}/+ (43.9 h ± 3.2; $p = 0.0002$), and that of *w*; +/+; *Actin*Gal4 (36.2 ± 2.8; $p < 0.0001$). (C) Median survival of female *w*; *TRAP1Δ4/TRAP1Δ4*; +/+ (20.3 h ± 1.4) is comparable to that of w^{1118}; +/+; +/+ (22.4 h ± 1.4; $p = 0.4$). (D) Median survival of female *w*; +/+; *UAS-TRAP1*^{7M}/*Actin*Gal4 (43.9 h ± 3.3) is significantly higher than that of *w*; +/+; *Actin*Gal4/+ (32.4 h ± 2.2; $p = 0.004$) but comparable to that of *w*; +/+; *UAS-TRAP1*^{7M}/+ (36.9 h ± 2.7; $p = 0.1$). (E) Median survival of young *w*; *TRAP1Δ4/TRAP1Δ4*; *UAS-TRAP1*^{7M}/*Actin*Gal4 males (25.26 h ± 1.88) is significantly higher than *w; TRAP1Δ4/TRAP1Δ4*; +/+ (15 h ± 0.4; $p < 0.0001$). (F) Median survival is increased in old (40 days) *w*; +/+; *UAS-TRAP1*^{7M}/*GS-tub5*Gal4 males maintained on 5 mM RU-486 (41.47 h ± 2.99), relative to control flies of the same genotype, sex and age maintained on vehicle (32.88 ± 1.78; $p = 0.03$). In all cases, errors denote standard deviation. Statistical significance was determined using the standard chi-squared based log-rank test.

levels while female flies in both the mutant and overexpression strains did not show pronounced changes in oxidative stress resistance (Fig. 2C, D, and Supplementary Fig. 2B).

To confirm the role of TRAP1 in regulating oxidative stress resistance, we rescued loss of function by over-expressing TRAP1 in the *TRAP1Δ4/TRAP1Δ4* mutant background. Median survival on paraquat was significantly enhanced in *w*; *TRAP1Δ4/TRAP1Δ4*; *UAS-TRAP1*^{7M}/*Actin*Gal4 flies as compared to *w*; *TRAP1Δ4/TRAP1D4*; +/+ mutants (Fig. 2E). The observed decrease of stress resistance in the overexpression strain itself, when endogenous TRAP1 is depleted in this background, also suggests that changes in survival are the result of TRAP1 dosage modulation as opposed to the effects of hybrid vigor.

It is known that oxidative stress resistance declines as a function of age, which we have verified in wild-type flies (Supplementary Fig. 2C and D). To determine whether TRAP1 over-expression in older flies could confer protection against oxidative stress, we transiently over-expressed TRAP1 in 40-day old flies using the inducible GeneSwitch-*tub5*Gal4 driver and assessed oxidative stress resistance against paraquat. GeneSwitch drivers allow temporal and spatial control because the expression of UAS effector lines is controlled by a chimeric Gal4 protein that is activated only in the presence of the steroid RU-486 which is not naturally expressed in *Drosophila*. Therefore, interpretation of these experiments relies on comparable ingestion of paraquat and RU-486 in wildtype, mutant and overexpression strains. To assess feeding behavior, we used a modified CAFE assay and found similar consumption of food across all groups (Supplementary Fig. 3). Median survival was significantly increased in *UAS-TRAP1*^{7M}/*GS-tub5*Gal4 males maintained on RU-486 as compared to control flies of the same genotype, sex and age maintained on the vehicle (Fig. 2F). These results suggest that a high transient dosage of TRAP1 is sufficient to improve resistance to oxidative stress in older flies. Together, these data implicate TRAP1 as an important modulator of oxidative stress resistance in young and aged *Drosophila*.

3.3. TRAP1 confers protection against heat stress

Because TRAP1 is an Hsp we subjected TRAP1 deletion and overexpression strains to acute heat stress as a second measure of

stress resistance. Heat shock was applied twice, at 5 and 19 days of age, and survival was measured 24 h later in both cases. Younger flies are largely resistant to heat shock; there is only a moderate decrease in survival of *TRAP1D4* mutant males and females as compared to wildtype controls on the 6th day. Similarly, there is only a modest improvement in survival of *UAS-TRAP1*^{4M}/*Actin*Gal4 males and females after the first heat shock (Fig. 3). However, the differences are significant after the second heat shock on the 20th day. In both male and female *TRAP1D4* mutants, survival was significantly decreased as compared to the wildtype control. In contrast, *UAS-TRAP1*^{4M}/*Actin*Gal4 males and females overexpressing TRAP1 display significant improvement in survival compared to control animals (Fig. 3). These results are consistent with TRAP1 being an important regulator of stress resistance.

3.4. Loss or overexpression of TRAP1 does not significantly alter lifespan

While recent reports indicate that ROS levels may not regulate longevity directly, overexpression of chaperones and enhanced resistance to oxidative stress are both associated with lifespan extension (Walker and Lithgow, 2003; Chen et al., 2006; Sanz et al., 2010; Hekimi et al., 2011). It has been reported previously in multiple model systems that overexpressing small heat shock proteins (Hsps) such as Hsp16, Hsp 27 and Hsp22 leads to a sizeable extension in lifespan (Walker and Lithgow, 2003; Morrow et al., 2004; Wang et al., 2004). However, overexpression of larger Hsps such as Hsp70 and Hsp60 do not affect lifespan dramatically (Tatar et al., 1997; Wadhwa et al., 2005), and in the case of induced Hsp70 expression can even be deleterious for growth in *Drosophila* cells (Feder et al., 1992). To examine whether TRAP1-mediated oxidative stress resistance is associated with alteration in longevity, we conducted lifespan analyses. A recent study suggested that TRAP1 mutant flies have reduced lifespan (Costa et al., 2013). However, when we separated the sexes in the lifespan analysis, we found that survival of TRAP1 mutant males and females is not significantly different from control groups (Fig. 4A and B). We did observe a marginal increase in median lifespan in females of one of the TRAP1 overexpressing strains, *UAS-TRAP1*^{7M}/*Actin*Gal4 (Fig. 4D). However, this effect was relatively minor (~4–14% increase in median lifespan), unlike the substantial increase in oxidative stress resistance in TRAP1 overexpressing flies (~44% increase in median survival in males). We did not observe any increase in lifespan of males and females in a second overexpression strain, *UAS-TRAP1*^{4M}/*Actin*Gal4 (data not shown). As described in Section 2, all transgenic strains and drivers used in these studies were backcrossed for at least 5 generations. However, lifespan can be significantly influenced by the slightest heterogeneity in genetic background. To control for this possibility, we overexpressed TRAP1 using the GeneSwitch-*tub*5Gal4 driver which increases *TRAP1* mRNA levels by 5 and 10 fold in males and females respectively (Supplementary Fig. 1C and D). Average lifespan of *UAS-TRAP1*^{4M}/*GS-tub5*Gal4 males and females maintained on RU-486 (Waskar et al., 2009) was comparable to controls maintained on vehicle only (Fig. 4E and F). Collectively, these observations suggest that knockout or overexpression of TRAP1 has little effect on lifespan.

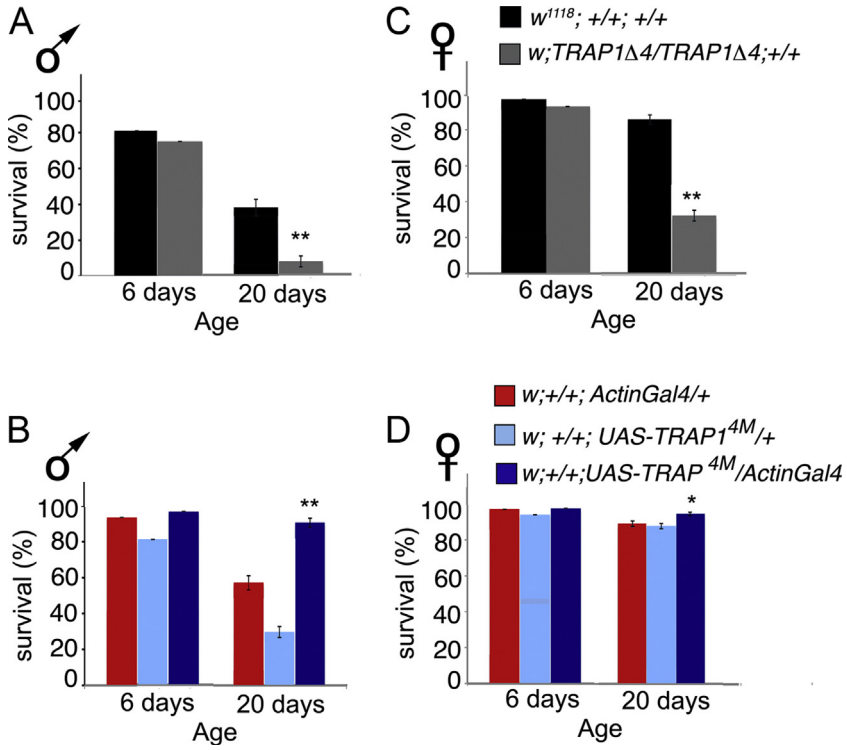

Fig. 3. TRAP1 confers resistance against heat stress. Fly survival at 6 days and 19 days of age, 24 h after each heat stress is delivered. Data is presented as the percentage of flies subjected to the stress that survives. (A) On the 6th day, survival of male *w*; *TRAP1 Δ4/TRAP1 Δ4*; +/+ (75 ± 6%) trends toward being lower than w^{1118}; +/+; +/+ (81 ± 8%; $p = 0.05$), but is significantly decreased (7.14 ± 0.66%) at 20 days of age relative to control (37.41 ± 0.77%; $p < 0.001$). (B) Survival of female *w*; *TRAP1 Δ4/TRAP1 Δ4*; +/+ (93 ± 1%) is comparable to that of w^{1118}; +/+; +/+ (97 ± 1%; $p = 0.1$) on the 6th day, but significantly decreased at 20 days (32.05 ± 7%) compared to control (85.62 ± 3.5%; $p < 0.001$). (C) On 6th day, survival of male *w*; +/+; *UAS-TRAP1*^{4M}/*Actin*Gal4 (96.5 ± 1.5%) is significantly higher than *w*; +/+; *Actin*Gal4/+ (81 ± 6%; $p = 0.01$), but comparable to *w*; +/+; *UAS-TRAP1*^{4M}/+ (93.5 ± 0.5%; $p = 0.1$). However, on the 20th day, survival of male *w*; +/+; *UAS-TRAP1*^{4M}/*Actin*Gal4 (90.64 ± 1%) is remarkably higher than *w*; +/+; *Actin*Gal4/+ (29.71 ± 2.16; $p < 0.0001$), and *w*; +/+; *UAS-TRAP1*^{4M}/+ (57.14 ± 0.75%, $p < 0.001$). (D) Survival of female *w*; +/+; *UAS-TRAP1*^{4M}/*Actin*Gal4 (97.5 ± 1.5%) is not significantly different from *w*; +/+; *Actin*Gal4/+ (94 ± 1%; $p = 0.1$), and *w*; +/+; *UAS-TRAP1*^{4M}/+ (97 ± 1%; $p = 0.2$) at 6 days. However at 20 days, survival of *w; +/+; UAS-TRAP1*^{4M}/*Actin*Gal4 (94.62 ± 2%) is significantly more than *w; +/+; Actin*Gal4/+ (87.18 ± 2.5%; $p < 0.001$), and *w*; +/+; *UAS-TRAP1*^{4M}/+ (89.18 ± 2.5%; $p = 0.01$). In all cases, errors denote standard errors of mean. Statistical significance was determined using one-way ANOVA.

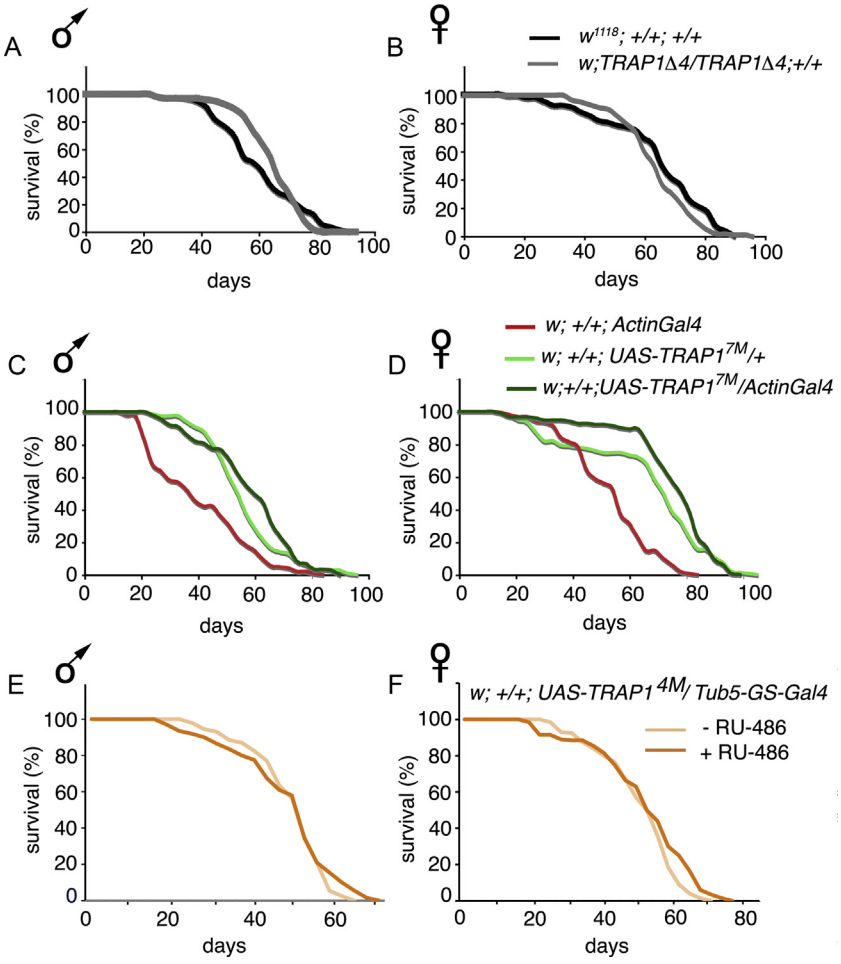

Fig. 4. TRAP1 has a marginal influence on lifespan. Lifespan curves of indicated genotypes. (A) Median lifespan of male w^{1118}; +/+; +/+ (60.4 ± 1.4 days) is comparable to that of *w*; *TRAP1Δ4/TRAP1Δ4*; +/+ (64.9 ± 1.1 days; $p = 0.48$). (B) Median lifespan of female w^{1118}; +/+; +/+ (64.6 ± 1.44 days) is comparable to *w*; *TRAP1Δ4/TRAP1Δ4*; +/+ (64.32 ± 0.98 days; $p = 0.08$). (C) Median lifespan of male *w*; +/+; *UAS-TRAP1*^{7M}/*Actin*Gal4 (58.6 ± 1.3 days) is significantly more than *w*; +/+; *Actin*Gal4/+ (40.7 ± 1.4 days; $p < 0.001$), and marginally more than that of *w*; +/+; *UAS-TRAP1*^{7M}/+ (56.5 ± 1.1 days; $p = 0.05$). (D) Median lifespan of female *w*; +/+; *UAS-TRAP1*^{7M}/*Actin*Gal4 (75.5 ± 1.4 days) is significantly more than that of *w*; +/+; *Actin*Gal4/+ (53.5 ± 1.2 days; $p < 0.001$), and marginally more than that of *w*; +/+; *UAS-TRAP1*^{7M}/+ (66.5 ± 1.9; $p = 0.044$). (E) Median lifespan of *w*; +/+; *UAS-TRAP1*^{4M}/*GS-tub5*Gal4 males maintained on RU-486 (43.66 ± 0.88 days) is similar to control flies of the same genotype, sex and age maintained on vehicle (44.86 ± 0.64 days; $p = 0.15$). (F) Median lifespan of *w*; +/+; *UAS-TRAP1*^{4M}/*GS-tub5*Gal4 females on RU-486 (47.24 ± 0.97 days) is also comparable to controls (44.84 ± 0.81 days; $p = 0.04$). In all cases, errors denote standard deviation. Statistical significance was determined using the standard chi-squared based log-rank test.

3.5. TRAP1 regulates locomotor performance and fertility in aging Drosophila

Recent studies on LON protease in the fungal aging model *P. anserina* have uncovered a role for mitochondrial proteostasis in healthspan regulation (Luce and Osiewacz, 2009). Because TRAP1 is a mitochondrial chaperone, we sought to determine whether TRAP1 would regulate healthspan. In *Drosophila*, negative geotaxis (the ability of flies to climb vertically when startled) is an established paradigm to assess age-related locomotor impairment (Rhodenizer et al., 2008). In a longitudinal study, we found that locomotor performance in TRAP1 mutant flies was only moderately decreased at older age (Fig. 5A and B). In contrast, the effect on male and female TRAP1 overexpressing flies (*UAS-TRAP1*^{7M}/*Actin*Gal4 and *UAS-TRAP1*^{4M}/*Actin*Gal4) was much clearer and they climb significantly better than the driver alone and transgene alone controls at 60 and 70 days of age (Fig. 5C and D, Supplementary Fig. 4A and B). As an independent indicator of healthspan, we determined the fertility of TRAP1 mutant and overexpression flies. TRAP1 mutant females show severely compromised fecundity at all age-points (Fig. 5E). In contrast, overexpression of TRAP1 results in increased fecundity (Fig. 5F). In addition, TRAP1 mutant males appear to be less fertile than control males (Supplementary Fig. 4C), whereas males overexpressing TRAP1 produced significantly more progeny at older ages than control strains (Supplementary Fig. 4 D). Together, these results indicate strongly that TRAP1 promotes healthspan in both male and female *Drosophila*.

3.6. Pan-neuronal overexpression of TRAP1 attenuates age-related decline in locomotor performance but does not affect lifespan or oxidative stress resistance

Modifying mitochondrial physiology in one tissue may influence function in others through cell non-autonomous stress response pathways (Durieux et al., 2011). In addition, Butler and colleagues showed that overexpression of human TRAP1 in cultured rat cortical neurons increased resistance to rotenone induced oxidative stress (Butler et al., 2012). To test whether overexpressing TRAP1 exclusively in the nervous system would be beneficial, we used the pan-neuronal driver *elav*Gal4 for tissue specific overexpression. In contrast to the *in vitro* result, pan-neuronal overexpression of TRAP1 in adult *Drosophila* does not significantly enhance oxidative stress resistance to paraquat

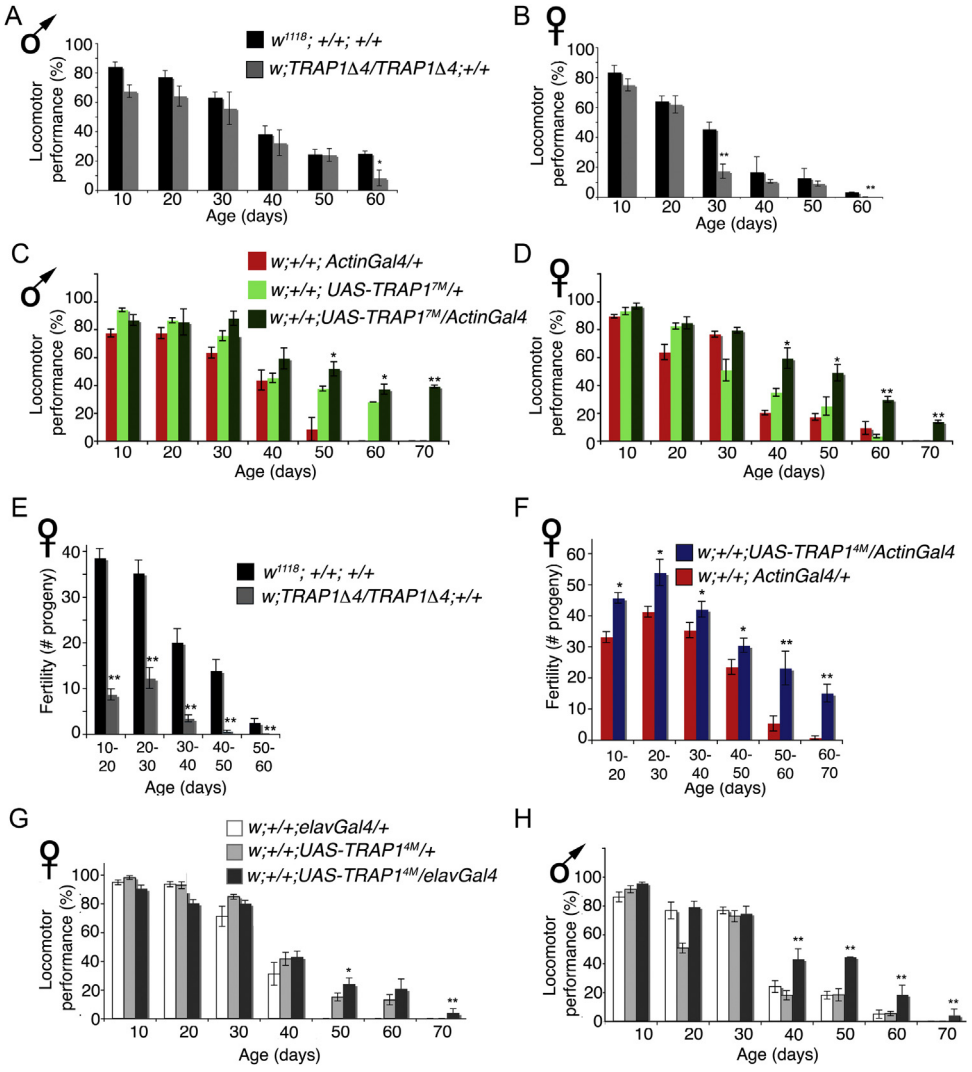

Fig. 5. TRAP1 modulates healthspan. (A and B) TRAP1 mutant flies tend to climb more poorly than controls in the negative geotaxis assay and the difference becomes significant at older ages. There is a significant drop in performance of mutant females at 30 days. By 60 days, fewer male and female *w; TRAP1Δ4/TRAP1Δ4; +/+* flies climbed successfully as compared to w^{1118}; +/+; +/+. (C and D) Locomotor performance after 40 days of age is well maintained in *w*; +/+; *UAS-TRAP1*^{7M}/*Actin*Gal4 males and females on ubiquitous overexpression of TRAP1. (E) *w*; *TRAP1Δ4/TRAP1Δ4*; +/+ females produce fewer progeny than controls at all stages of their life. (F) *w*; +/+; *UAS-TRAP1*^{7M}/*Actin*Gal4 females produce more progeny than controls at all stages of their life. (G and H) Male and female flies overexpressing TRAP1 exclusively in the nervous system displayed significant improvement in locomotor ability at older age-points in *w*; +/+; *UAS-TRAP1*^{4M}/*elav*Gal4. In all cases error bars denote standard error of means; (*) indicates $p < 0.05$, (**) indicates $p < 0.001$. Statistical significance was determined by one-way ANOVA with Dunnett's post hoc comparison. (See also Fig. S5).

(Supplementary Fig. 5A and B), nor does it appreciably change lifespan (Supplementary Fig. 5C and D). Nonetheless, overexpression of TRAP1 in the fly nervous system was sufficient to improve locomotor performance of both sexes at older age-points in *w*; +/+; *UAS-TRAP1*^{4M}/*elav*Gal4 strain (Fig. 5G and H), although less significantly in the females. Given previous work implicating TRAP1 in Parkinson's disease *via* its interaction with PINK1 (Pridgeon et al., 2007; Costa et al., 2013; Zhang et al., 2013) and α-synuclein (Butler et al., 2012), these data indicate that augmenting TRAP1 expression in the nervous tissue may be beneficial in human neurological diseases that impair motor behavior.

3.7. Dosage modulation of TRAP1 activates the mitochondrial unfolded protein response

The mitochondrial unfolded protein response (UPR^{mt}) is a protective response pathway between mitochondria and the nucleus that is initiated in response to a mitochondrial stress signal (Haynes and Ron, 2010). Induction of the UPR^{mt} promotes expression of chaperones and proteases that enhance mitochondrial proteostasis in mammalian cell culture and *C. elegans* (Zhao et al., 2002; Yoneda et al., 2004). Because TRAP1 is a component of the mitochondrial proteostasis machinery by virtue of being a chaperone, we hypothesized that it is involved in the regulation of the UPR^{mt}.

As part of the UPR^{mt} in worms, transcription factors such as DVE-1 translocate from the cytoplasm to the nucleus to promote downstream transcription of chaperones and proteases (Haynes and Ron, 2010). Immunohistochemical analysis of adult brains with antibody against Dve, the *Drosophila* homolog of DVE-1, reveals a ring like cytosolic localization pattern around the nucleus of neuronal cell bodies of wildtype flies (Fig. 6A–C). In contrast, Dve in TRAP1 mutants displayed a more diffuse staining pattern indicating nuclear translocation (Fig. 6D–F). We also note a similar change in sub-cellular localization of Dve in the TRAP1 overexpressing flies (Fig. 6G–I). These observations were verified by quantifying the ratio of cytosolic to nuclear intensity of DVE

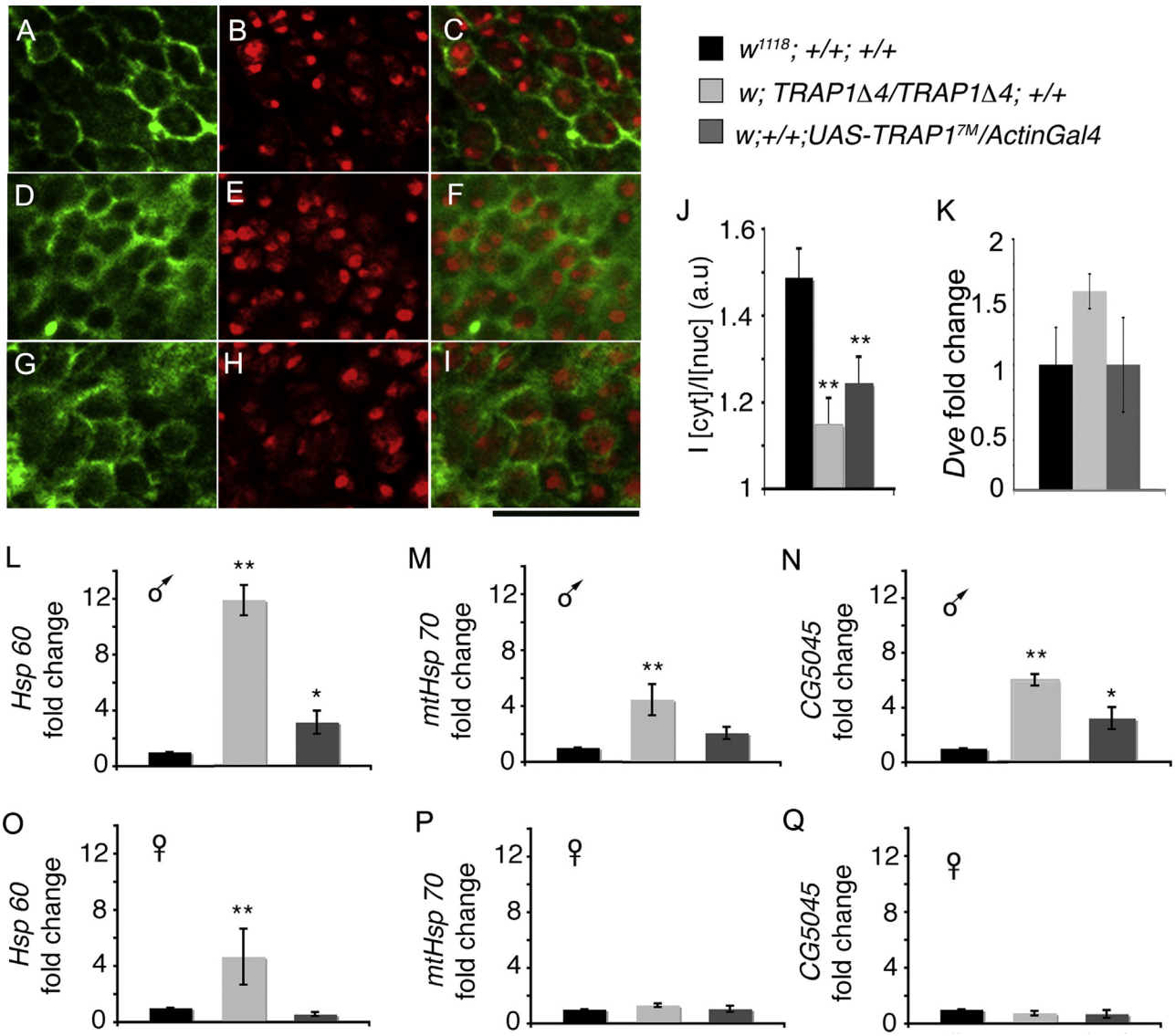

Fig. 6. Loss and overexpression of TRAP1 activates the UPR^{mt} pathway. (A–C) Anti-Dve antibody (green) reveals tight cytosolic localization of Dve in neuronal cell bodies in brains of adult w^{1118}; +/+; +/+ males. Nuclear dye DAPI (red) stains the entire nucleus although it appears brighter in the nucleolus. The merged image of anti-Dve and DAPI stain reveals crisp cytosolic sub-cellular localization of Dve. (D–F) Dve staining appears diffuse in neuronal cell bodies of male *w; TRAP1Δ4/TRAP1Δ4*, +/+ flies and exhibits considerable overlap with the nucleus. (G–I) Dve staining appears similarly diffused and overlaps with nuclear stain in neuronal cell bodies of male *w*; +/+; *UAS-TRAP1^{4M}/Actin*Gal4 flies. Scale bar equals 3.5 μm. (J) Ratio of staining intensity of Dve in the cytoplasm relative to the nucleus in indicated genotypes. (K) mRNA expression of *Dve* in w^{1118}; +/+; +/+ females is not significantly different from mutant ($p = 0.2$) and over expression ($p = 0.9$) strains. (L, M and N) Male *w; TRAP1Δ4/TRAP1Δ4*, +/+ and *w*; +/+; *UAS-TRAP1^{7M}/Actin*Gal4 flies exhibit significantly increased mRNA expression of *Hsp60, mtHsp70* and *CG5045* as compared to w^{1118}; +/+; +/+. (O, P and Q) Female *w*; *TRAP1Δ4/TRAP1Δ4*; +/+ exhibit significantly increased mRNA expression of *Hsp60* as compared to w^{1118}; +/+; +/+, but not that of *mtHsp70* and *CG5045*. In all cases, data are presented as fold change from control. Error bars denote standard error of means; statistical significance was determined using one-way ANOVA. (*) Indicates $p < 0.05$, (**) indicates $p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

staining which corroborates a change in sub-cellular localization of DVE (Fig. 6J). Importantly, comparison of *Dve* mRNA levels between the strains by qPCR reveals that these changes in staining pattern are not due to an overall reduction in *Dve* transcription in TRAP1 mutants (Fig. 6K). These results demonstrate that dosage modulation of TRAP1 influences nuclear translocation of DVE in *Drosophila*, suggesting an induction of UPR^{mt}.

To further test if TRAP1 regulates the UPR^{mt}, we examined expression levels of genes known to be associated with the UPR^{mt}, including *Hsp60*, *mtHsp70* and a putative *Drosophila* protease *CG5045* that is 77% identical to the worm ClpP (Flybase). In males, loss of TRAP1 led to strong upregulation of *Hsp60*, *mtHsp70* and *CG5045* (Fig. 6L–N). As with resistance to oxidative stress, this response is sex-specific: in females only *Hsp60* was increased significantly (Fig. 6O–Q). These results suggest that depletion of TRAP1 leads to induction of a robust UPR^{mt}. In the TRAP1 overexpression strains we found expression of the UPR^{mt} genes were modestly elevated in the overexpression strain in a similar sex dependent transcription profile as mutants but the magnitude increase was smaller in comparison (Fig. 6L–Q). These results suggest that overexpression of TRAP1 also induces an UPR^{mt} response in males, albeit weaker than that in TRAP1 depletion.

This result led us to ask if the increased resistance to stressors in TRAP1 overexpression animals is due to induction of the UPR^{mt}. To address this, we sought to impair UPR^{mt} induction in flies overexpressing TRAP1 and test their response to acute heat stress. We used the inducible GeneSwitch-*tub*5Gal4 driver to drive RNAi against transcription factor *Dve* and the protease *CG5045* independently in *UAS-TRAP1^{4M}* background. As with constitutive overexpression of TRAP1 (Fig. 3), conditional overexpression also

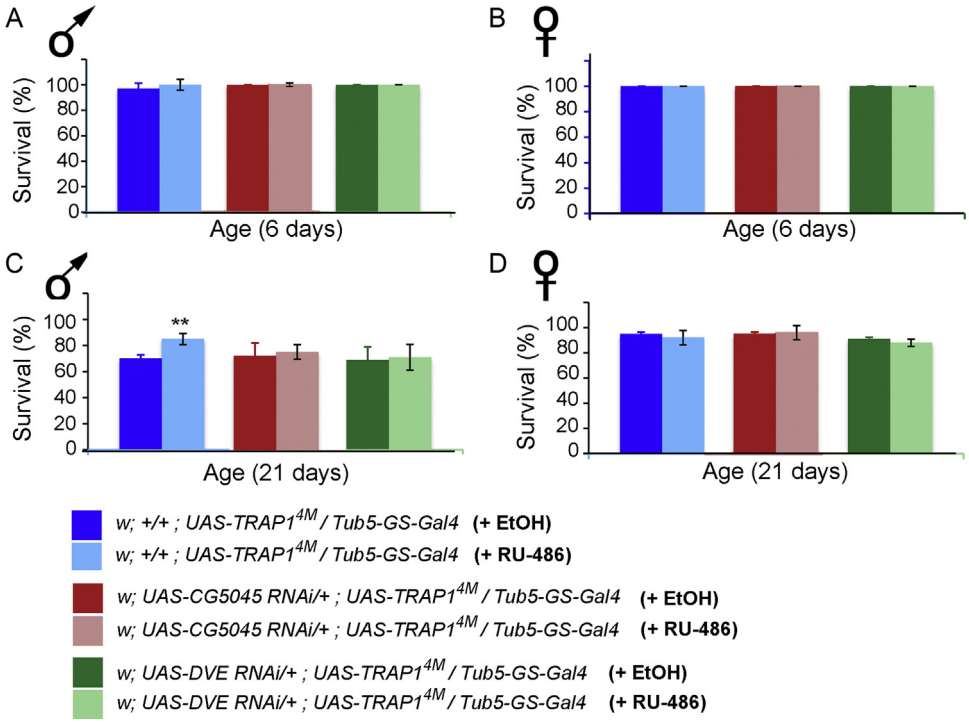

Fig. 7. Activation of the UPR^{mt} is required for TRAP1 mediated heat stress resistance in older males. Fly survival at 6 days and 21 days of age, 24 h after each heat stress is delivered. (A) In 6-day old male flies with conditional Gal4, survival of *w*; +/+; *UAS TRAP1*^{4M}*/GS-Tub5*Gal4 +/+ in control group (97 ± 4.2%) is comparable to identical flies maintained on RU-486 (99 ± 1.4%; $p = 0.6$), *w*; *UAS CG5045 RNAi/+*; *UAS TRAP1*^{4M}*/GS-Tub5*Gal4 in control group (100%) are the same as identical flies maintained on RU-486 (100%), and *w*; *UAS DVE RNAi/+*; *UAS TRAP1*^{4M}*/GS-Tub5*Gal4 in control group (100%) are the same as identical flies maintained on RU-486 (100%). (B) In 6-day old female flies with conditional Gal4, survival of *w*; +/+; *UAS TRAP1*^{4M}*/GS-Tub5*Gal4 +/+ in control group (100%) is the same as identical flies maintained on RU-486 (100%), *w*; *UAS CG5045 RNAi/+*; *UAS TRAP1*^{4M}*/GS-Tub5*Gal4 in control group (100%) is the same as identical flies maintained on RU-486 (100%), and *w*; *UAS DVE RNAi/+*; *UAS TRAP1*^{4M}*/GS-Tub5*Gal4 in control group (100%) are the same as identical flies maintained on RU-486 (100%). (C) In 21-day old male flies with conditional Gal4, survival of *w*; +/+; *UAS TRAP1*^{4M}*/GS-Tub5*Gal4 +/+ of flies maintained on RU-486 (85 ± 4.2%) is significantly higher than controls (70 ± 2.8%; $p = 0.05$). However, *w*; *UAS CG5045 RNAi/+*; *UAS TRAP1*^{4M}*/GS-Tub5*Gal4 in control group (73 ± 9.8%) is comparable as identical flies maintained on RU-486 (76 ± 5.6%; $p = 0.7$), and *w*; *UAS DVE RNAi/+*; *UAS TRAP1*^{4M}*/GS-Tub5*Gal4 in control group (69 ± 1.4%) are similar to identical flies maintained on RU-486 (71 ± 2.8%; $p = 0.8$). (D) In 21-day old female flies with conditional Gal4, survival of *w*; +/+; *UAS TRAP1*^{4M}*/GS-Tub5*Gal4 +/+ in control group (95 ± 1.4%) is comparable to identical flies maintained on RU-486 (92 ± 5.6%; $p = 0.5$), *w*; *UAS CG5045 RNAi/+*; *UAS TRAP1*^{4M}*/GS-Tub5*Gal4 in control group (95 ± 1.5%) is similar to identical flies maintained on RU-486 (96 ± 5.6%; $p = 0.8$), and *w*; *UAS DVE RNAi/+*; *UAS TRAP1*^{4M}*/GS-Tub5*Gal4 in control group (91 ± 1.4%) are the comparable to identical flies maintained on RU-486 (88 ± 2.8%; $p = 0.3$). In all cases, errors denote standard deviation. Statistical significance was determined using one-way ANOVA.

significantly improves resistance to acute heat stress in 20-day old males but there is no significant difference in 5-day old flies as younger flies handle heat stress better (Fig. 7). However, when *Dve* or *CG5045* expressions were knocked down while simultaneously overexpressing TRAP1, the improvement in stress resistance was considerably dampened (Fig. 7). Together, these data strongly suggest that the protective influence of TRAP1 overexpression is at least in part due to activation of the UPR^{mt} pathway.

4. Discussion

The molecular mechanisms influencing healthspan remain unclear (Tatar, 2009; Yu and Driscoll, 2011), but mitochondrial proteostasis is evolving as a central determinant. We find that dosage modulation of the mitochondrial chaperone TRAP1 in *Drosophila* regulates oxidative stress and heat stress resistance in males and modulates healthspan of both sexes, yet only has a negligible effect on lifespan. To gain a better understanding of the mechanism underlying these changes, we examined the effect of TRAP1 dosage on the UPR^{mt}. We found that modulation of TRAP1 expression induces the nuclear translocation of transcription factor Dve and drives expression of *Hsp60*, *mtHsp70* and a putative protease, *CG5045*. These results provide evidence for the presence of the UPR^{mt} in *Drosophila*, confirming the conservation of this stress response pathway across phylogeny. Furthermore, dampening of TRAP1 mediated stress response upon impairment of the UPR^{mt} indicates that alterations in mitochondrial proteostasis can influence stress resistance and healthspan,

Recently published studies have also indicated a similar role of TRAP1 in regulating stress resistance in flies (Costa et al., 2013; Zhang et al., 2013). However, we further demonstrate that dosage modulation of TRAP1 has remarkable sex-specific influences and is correlated with the sex-specific activation the UPR^{mt}. Sex specificity has been reported previously in stress survival studies involving mitochondrial proteins (Magwere et al., 2006; Mourikis et al., 2006). One possible explanation is that mitochondria in female *Drosophila* have dissimilar bioenergetics from males, with higher oxygen consumption, higher hydrogen peroxide production and lower levels of catalase, along with higher mtDNA copy number (Yin et al., 2004; Ballard et al., 2007). In our oxidative stress and heat stress survival assays (Figs. 2 and 3), TRAP1 mutant and overexpression animals displayed considerable sex-specificity that correlated directly with the expression of *mtHsp70* and *CG5045*, but not *Hsp60* in males and females (Fig. 6). These findings suggest that sex-specific differences in activation of proteostasis pathways underlie sex dependent differences in resistance to stress.

Our finding that both loss and overexpression of TRAP1 activate the UPR^{mt} is indicative of the complex regulation of this response system. Because TRAP1 is a chaperone, induction of the UPR^{mt} in *TRAP1* mutants is likely a compensatory response to protect against excess unfolded proteins. While this response pathway may allow the *TRAP1* mutants to have a near normal lifespan, the absence of TRAP1 function has obvious negative consequences on fitness. In

contrast, the induction of the UPR^{mt} in TRAP1 overexpressing flies could occur by two non-exclusive mechanisms. One possibility is that the mild stress of over-abundant TRAP1 in the mitochondrial matrix induces the UPR^{mt}. Alternatively, given the potential role of TRAP1 in multiple signaling pathways involving Rb, myc, TNF, cyclophilin D (Kang et al., 2007; Altieri et al., 2012) overexpression of TRAP1 may lead to efficient protein folding, direct suppression of apoptosis, and the coordinated induction of other UPR genes through a TRAP1 mediated stress response. In the context of a minimal proteostasis boundary (Powers et al., 2009), either an increase in TRAP1 activity or the indirect induction of UPR^{mt} would result in enhanced protein quality control in TRAP1 overexpression flies and would shift the proteostasis boundary towards health. Altogether, our results suggest that because of the critical roles played by TRAP1 in regulating several cellular processes, it is important that TRAP1 expression itself is finely regulated.

The role of TRAP1 in activation of the UPR^{mt} has broad clinical relevance. TRAP1 is reportedly expressed at higher levels in cancer cells where it has been suggested to play a pro-survival role by inhibiting apoptosis (Kang et al., 2007; Costantino et al., 2009; Leav et al., 2010). Hsp90 molecules, including TRAP1, antagonize the cyclophilin D-dependent mitochondrial permeability transition, and this cytoprotective pathway is a potential target in cancer therapy (Kang et al., 2007). Our results demonstrate that complete loss of function of TRAP1 has little effect on organismal lifespan (Fig. 4) and there is no discernible delay in development (data not shown). While caution must be used when speculating how results in model organisms translate to therapeutics in humans, these results suggest that aggressive disruption of TRAP1 in the context of cancer treatment may provide a means to sensitize tumors to chemotherapy without significantly reducing the lifespan of patients.

In addition, we show that overexpression of TRAP1, ubiquitously or in the nervous system, extends healthspan in both sexes. These results suggest that augmentation of TRAP1 expression may help offset normal age-related decline of physiological and motor capacity, as well as the pathological decline associated with neurological disorders.

Author contributions

R.M.B and K.E.M designed the study; R.M.B, A.V.P, R.H.G collected data; R.M.B and A.W.S analyzed data; B.A.T and S.K contributed reagents; R.M.B, L.S.K, A.W.S, and K.E.M wrote the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2004.08.011.

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