

The role of *D-GADD45* in oxidative, thermal and genotoxic stress resistance

Alexey Moskalev,^{1,3,4,*} Ekaterina Plyusnina,^{1,3} Mikhail Shaposhnikov,^{1,3} Lyubov Shilova,¹ Alexey Kazachenok² and Alexander Zhavoronkov^{2,4}

¹Laboratory of Molecular Radiobiology and Gerontology; Institute of Biology; Komi Science Center of Russian Academy of Sciences; Syktyvkar, Russia; ²Center for Pediatric Hematology, Oncology and Immunology; Moscow, Russia; ³Syktyvkar State University; Syktyvkar, Russia; ⁴The Biogerontology Research Foundation; London, UK

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There is a relationship between various cellular stress factors and aging. In earlier studies, we demonstrated that overexpression of the *D-GADD45* gene increases the lifespan of *Drosophila melanogaster*. In this study, we investigate the relationship between *D-GADD45* activity and resistance to oxidative, genotoxic and thermal stresses as well as starvation. In most cases, flies with constitutive and conditional *D-GADD45* overexpression in the nervous system were more stress-resistant than ones without overexpression. At the same time, most of the studied stress factors increased *D-GADD45* expression in the wild-type strain. The lifespan-extending effect of *D-GADD45* overexpression was also retained after exposure to chronic and acute gamma-irradiation, with doses of 40 cGy and 30 Gy, respectively. However, knocking out *D-GADD45* resulted in a significant reduction in lifespan, lack of radiation hormesis and radioadaptive response. A dramatic decrease in the spontaneous level of *D-GADD45* expression was observed in the nervous system as age progressed, which may be one of the causes of the age-related deterioration of organismal stress resistance. Thus, *D-GADD45* expression is activated by most of the studied stress factors, and *D-GADD45* overexpression resulted in an increase of stress resistance.

Introduction

Proteins of the GADD45 (growth-arrest and DNA damage inducible 45) family take part in gene expression regulation, cell cycle arrest, DNA repair and apoptosis.¹ In mammals, their expression is induced under conditions of stress, including oxidative and genotoxic stresses.² GADD45 interacts with proteins involved in DNA excision repair³ and also with proteins that are involved in the repair of double-stranded breaks in DNA.⁴ In *Drosophila melanogaster*, only one homolog of the *GADD45* gene is known—*D-GADD45*⁵—and its function is less studied. However, we have previously shown an increase in the lifespan of *D. melanogaster* (here referred to as *Drosophila*) as a result of *D-GADD45* overexpression.⁶

One of the probable mechanisms explaining the increase in longevity due to the overexpression of *GADD45* is that it confers resistance to stresses. There is much evidence for the relationship between stress resistance and longevity. As organisms age, there is a decrease in stress resistance.⁷ The overexpression of genes that improve stress resistance increases the lifespan.^{8,9} Furthermore, animals with mutations that lead to longevity are characterized by an increased resistance to oxidative and temperature stress.¹⁰ However, short-lived mutants of model organisms have decreased resistance to stress factors.¹¹ Gene activity contributing to the acceleration of the aging process also leads to endogenous stress

(e.g., p66).¹² Selection of animals on the basis of resistance to one stress factor increases resistance to other stress factors and leads to an increase in longevity.¹³ Species that have different life expectancies differ in stress resistance, especially to oxidative stress (for example, rodents and birds or bats).¹⁴ It is reported that the long-lived naked mole rat (*Heterocephalus glaber*) has an increased resistance to a broad spectrum of cytotoxins, including heat shock, heavy metals, genotoxicants and xenobiotics.¹⁵ In humans we see the same patterns; for example, human centenarians do not differ in having to follow a healthy lifestyle.¹⁶ Their extraordinary longevity can be explained by increased resistance to stress.

Mild stress results in an increase in lifespan.¹⁷ The mechanisms involved in increasing lifespan include the induction of protective systems, such as antioxidant defense,¹⁸ repair of DNA,¹⁹ synthesis of heat shock proteins,²⁰ activation of the immune system²¹ and selection of weakened cells.²² The aim of this work is to demonstrate the relationship between the induction of *D-GADD45* in *Drosophila*, and resistance to oxidative stress (paraquat), genotoxic stress (gamma-irradiation), heat shock (35°C) and starvation.

Results

Expression levels of the *D-GADD45* gene in flies with different genotypes. To confirm constitutive *D-GADD45* overexpression,

*Correspondence to: Alexey Moskalev; Email: amoskalev@ib.komisc.ru
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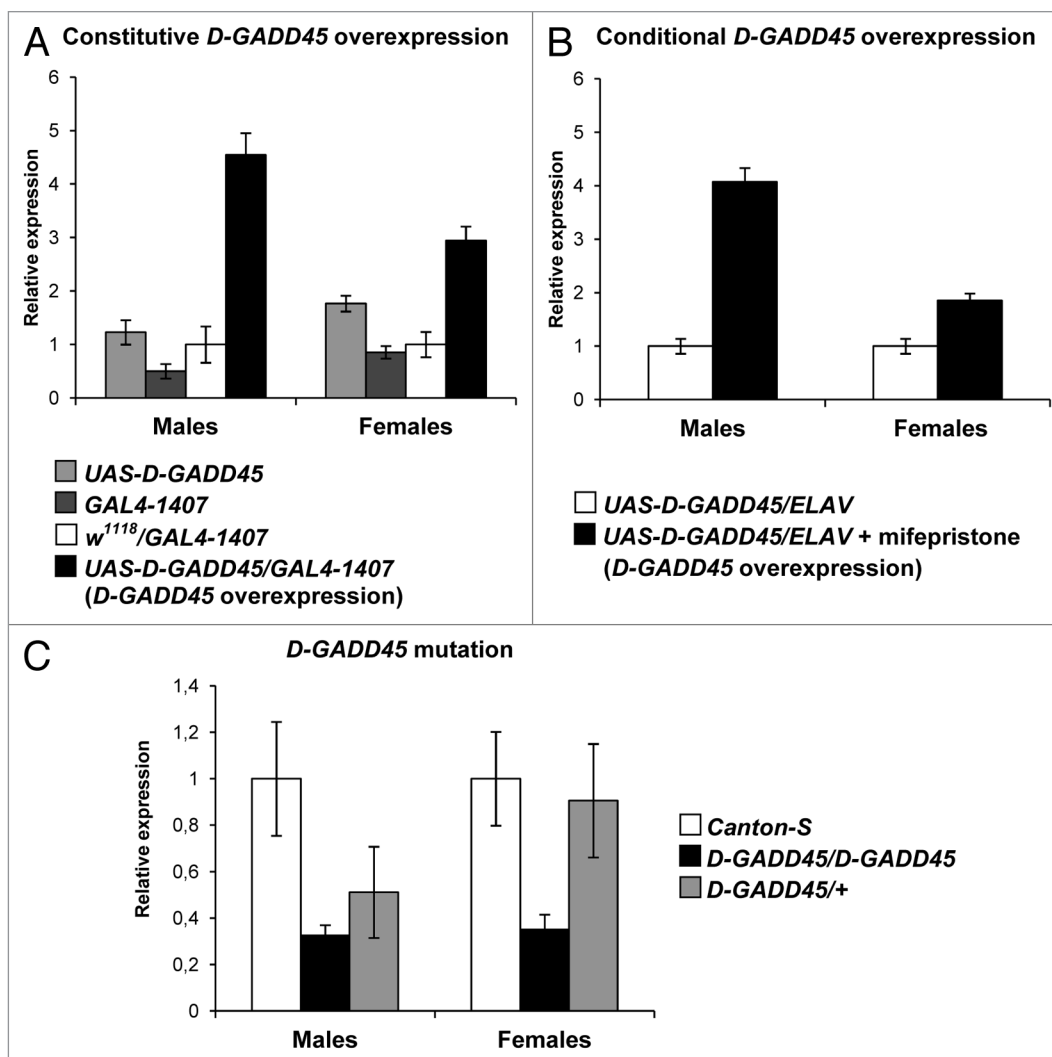


Figure 1. Expression levels of the *D-GADD45* gene in *Drosophila* with different genotypes. (A) Expression levels in the nervous tissue of *Drosophila* flies with the *UAS-D-GADD45/GAL4-1407* genotype (flies with expected constitutive *D-GADD45* overexpression), the *UAS-D-GADD45* and *GAL4-1407* parental genotypes, and the *w¹¹¹⁸/GAL4-1407* genotype. (B) Expression levels in the nervous tissue of flies with the *UAS-D-GADD45/ELAV* genotype kept on a medium with mifepristone (flies with expected conditional *D-GADD45* overexpression) and those not treated with mifepristone. (C) Ubiquitous expression in flies of the wild-type *Canton-S* strain, and flies with the homo- and heterozygous mutation in the *D-GADD45* gene (*D-GADD45/D-GADD45* and *D-GADD45/+* flies, respectively).

we compared expression levels of the *D-GADD45* gene in the nervous tissue of 5-d-old males and females with the *UAS-D-GADD45/GAL4-1407* genotype (flies with expected *D-GADD45* overexpression in the nervous system) and with the *UAS-D-GADD45* and *GAL4-1407* genotypes. In addition, we measured *D-GADD45* expression level in the F1 progeny of crosses between *w¹¹¹⁸* wild-type females and *GAL4-1407* males to avoid the heterosis effect (*w¹¹¹⁸/GAL4-1407* flies). Analysis of the relative *D-GADD45* expression values revealed 3–9-fold higher expression in *UAS-D-GADD45/GAL4-1407* males and 2–3-fold higher expression in females in comparison with flies without overexpression (Fig. 1).

To detect the conditional *D-GADD45* overexpression effect, F1 progeny of crosses between *UAS-D-GADD45* females and *ELAV* males were kept on a medium with mifepristone (inductor

of the *ELAV* driver). Comparison of the relative *D-GADD45* expression values in the nervous tissue of flies with the *UAS-D-GADD45/ELAV* genotype not treated with mifepristone exhibited a 4-fold higher expression in males and 2-fold higher expression in females when compared with wild-type flies (Fig. 1).

To analyze *D-GADD45* expression levels in flies with mutation in *D-GADD45*, 5-d-old *Drosophila* male and female with *D-GADD45/D-GADD45* (homozygous mutants) genotypes and *D-GADD45/+* (heterozygous mutants) were examined and compared with wild-type *Canton-S* flies as a control. We found a 3-fold decrease in *D-GADD45* expression both in males and females with homozygous insertion mutations in the gene *D-GADD45*, and a 2-fold decrease in heterozygous mutants (Fig. 1).

The impact of *D-GADD45* overexpression in the nervous system on resistance to oxidative stress, hyperthermia and starvation. It was recently demonstrated that overexpression of the *D-GADD45* gene in the nervous system leads to a significant increase in the lifespan of *Drosophila* without a corresponding decrease in fecundity or locomotor activity. This is apparently due to more efficient recognition and repair of DNA damage.⁶ In most cases reported in the literature, an increase in lifespan in model organisms is accompanied by an increase in stress resistance.^{10,11,13} We hypothesized that the overexpression of the *D-GADD45* gene in the nervous system would lead to an increase in resistance to stress in addition to its previously characterized role in increasing lifespan. For this reason, we examined the impact of *D-GADD45* overexpression on the survival of *Drosophila* during and after exposure to various stress factors—oxidative stress (20 mM paraquat), hyperthermia (35°C) and starvation.

Male *Drosophila* with constitutive overexpression of *D-GADD45* in the nervous system had higher resistance to superoxide-inducer paraquat, hyperthermia and starvation compared with males with UAS-*D-GADD45* and GAL4-1407 parental genotypes ($p < 0.001$) (Fig. 2). The percentage of dead flies after 48 h under stresses increased by 2.3–8.2 times for flies constitutively overexpressing *D-GADD45* ($p < 0.05$). In females, similar changes in survival rates were found, but it was significantly lower ($p < 0.05$) (Fig. 2). Differences in the percentage of dead flies between females with constitutive *D-GADD45* overexpression in the nervous system and females without overexpression were 1.3–2.7 times ($p < 0.05$). The exception is that the effects in females with constitutive overexpression of the *D-GADD45* gene were insignificant when compared with females of the GAL4-1407 strain under oxidative stress conditions.

In order to avoid the possible influence of heterosis and differences in genetic background, we examined the effect of conditional (mifepristone-inducible) *D-GADD45* overexpression in the nervous system of *Drosophila* under oxidative stress, thermal stress and starvation. Males with the UAS-*D-GADD45*/ELAV genotype treated with mifepristone (conditional *D-GADD45* overexpression in the nervous system) had higher survival rates compared with those kept without mifepristone induction ($p < 0.05$) during increased levels of oxidative stress and starvation (Fig. 3). In males with conditional *D-GADD45* overexpression, the percentage of dead flies after 48 h of oxidative stress and starvation increased by 1.6–1.8 times compared with males without overexpression ($p < 0.05$). Females, however, responded differently; they had a higher resistance to paraquat and lower survival rates under starvation conditions ($p < 0.05$). Conditional overexpression of the *D-GADD45* gene did not lead to significant alterations in resistance to hyperthermia in either males or females (Fig. 3).

Thus, in the majority of the experimental conditions examined here, constitutive and conditional overexpression of *D-GADD45* in the nervous system resulted in an increase in resistance to the superoxide inducer paraquat, hyperthermia and starvation in males, but led to lower or negative alterations in females. The dissimilarity in the response to stress factors between males and females is explained by the different levels of *D-GADD45*

activity. In males, 3–9-fold *D-GADD45* overexpression led to more efficient repair of cellular structure damage and caused an increase in resistance to oxidative stress, hyperthermia and starvation. In females, 2–3-fold overexpression of *D-GADD45* could be insufficient for the increase in stress resistance. Furthermore, it is possible that greater energy costs are associated with the generation of *D-GADD45* gene products and lead to a decrease in viability in females with *D-GADD45* overexpression.

Hormesis and radioadaptive response in *D-GADD45* mutants. We studied the role of *D-GADD45* in the formation of hormesis and the radioadaptive response. To do this, we examined the life expectancies of *Drosophila* flies of the wild-type Canton-S strain, as well as homo- and heterozygous strains with mutations in the *D-GADD45* gene.

Analysis of survival curves and lifespan parameters showed that wild-type Canton-S flies exposed to low-dose chronic gamma-irradiation at the pre-imago stages develop hormesis (Fig. 4). The median lifespan of flies that underwent chronic exposure to 40 cGy of gamma-irradiation was 12–15% ($p < 0.001$) higher in comparison with flies that were not exposed to the radiation (Table 1). Moreover, wild-type flies developed a radiation-induced adaptive response. In contrast, acute exposure at a dose of 30 Gy decreased the median lifespan by 51–57% ($p < 0.001$); however, the consistent effects of gamma-irradiation at a dosage of 40 cGy and 30 Gy decreased the median lifespan by only 6–21% ($p < 0.001$). As a result, the negative effect of irradiation at a dose of 30 Gy was reduced by 1.6–2.3 times ($p < 0.001$), with the help of pre-irradiation in low doses. Similar changes have been identified during the analysis of other factors affecting lifespan. For example, in male *Drosophila* undergoing chronic exposure to 40 cGy gamma irradiation, there was an increase in mortality rate (here, 90% mortality is considered an index of maximum lifespan) by 12% ($p < 0.05$), but pre-irradiation at the same dosage reduced the negative effect of acute exposure to 30 Gy gamma-radiation by 1.6–2.2 times in males and females of the Canton-S strain (Table 1).

In males and females with a heterozygous mutation in the *D-GADD45* gene, the median lifespan was 42–53% lower when compared with Canton-S wild-type flies ($p < 0.001$) (Table 1). In homozygous males and in females with heterozygous *D-GADD45* mutation, differences in the median lifespan were insignificant when compared with the wild-type strain Canton-S; however, maximum lifespan was expanded. Thus, the age of 90% mortality was increased by 17–40% in male and female *D-GADD45*/*D-GADD45* flies ($p < 0.001$) (Table 1). It is possible that this effect is the result of the genetic background of the strains. Nevertheless, in contrast to the wild-type strain Canton-S, both homo- and heterozygous flies with the mutation in *D-GADD45* had higher rates of mortality (MRDT increased by 1.6–2.1 times) and a reduced resistance to ionizing irradiation. Homo- and heterozygous males and females with a mutation in *D-GADD45* had a 4–32% ($p < 0.05$) decrease in median lifespan after chronic (40 cGy) or acute (30 Gy) gamma-irradiation exposure, or successive irradiation of both dose rates. It is important to point out that in flies with the *D-GADD45* mutation, both the effect of hormesis and a radioadaptive response were absent.

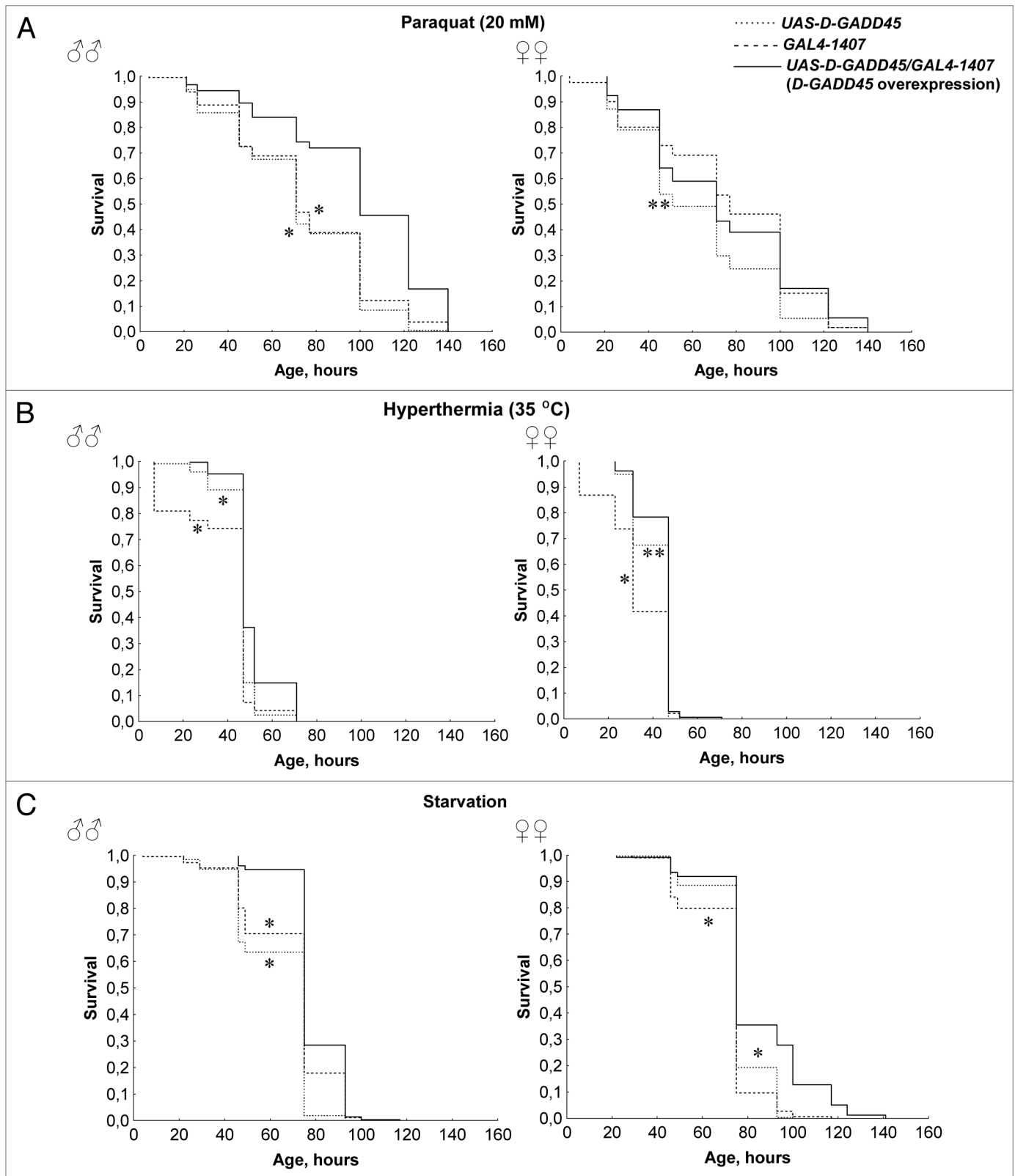


Figure 2. The influence of constitutive *D-GADD45* overexpression in the nervous system on stress resistance. (A–C) Survival curves of *Drosophila* males and females with constitutive *D-GADD45* overexpression in the nervous system and with the *UAS-D-GADD45* and *GAL4-1407* parental genotypes under oxidative stress (20 mM paraquat) (A), hyperthermia (35°C) (B) and starvation (C). Significant differences in survival are marked by * - $p < 0.001$, ** - $p < 0.05$ (Kolmogorov-Smirnov test), compared with flies with *D-GADD45* overexpression.

Instead, preliminary chronic irradiation at 40 cGy increased the negative effect of subsequent acute exposure in males by 13–21%. The most negative effect due to gamma-irradiation was observed after successive exposure at dosages of 40 cGy and 30 Gy. In female flies, the median survival rate after acute exposure to 30 Gy and sequential exposure at 40 cGy and 30 Gy did not differ significantly. However, in homozygous D-GADD45/D-GADD45 females, a 12% reduction of 90% mortality rate was observed, which indicates accelerated aging in *Drosophila* with this given genotype after exposure to low doses of pre-irradiation. These results confirm the analysis of the survival curve and other lifespan indicators (Fig. 4 and Table 1).

Therefore, a 2–3 times decrease in *D-GADD45* gene expression is enough to eliminate the effects of hormesis and radio-adaptive response. Therefore, *D-GADD45* plays a key role in the formation of response reactions to the influence of ionizing radiation.

Radiation resistance during *D-GADD45* overexpression in the nervous system. On the basis of our results (see above), we suggested that the overexpression of *D-GADD45* would lead to increased resistance to ionizing radiation and would cause an additional radioprotective effect. For this reason, we investigated lifespan changes in flies with constitutive *D-GADD45* overexpression in the nervous system following chronic exposure to ionizing radiation at a chronic (40 cGy) or acute (30 Gy) dose rate.

Comparison of the lifespan effects for different variants in our irradiation experiments demonstrated that flies with constitutive *D-GADD45* overexpression in the nervous system and parental strain flies UAS-D-GADD45 and GAL4-1407 either do not experience any significant changes in mortality rate, or show the effects of radiation hormesis after gamma-radiation exposure (Table 2). *Drosophila* females with *D-GADD45* overexpression showed a 6–13% increase in median lifespan after chronic and acute gamma-irradiation at 40 cGy and 30 Gy dose rates ($p < 0.05$). However, in males with overexpressed *D-GADD45* in their nervous system, gamma-irradiation had the opposite effect. The median lifespan of these flies was reduced by 17% after chronic irradiation at 40 cGy ($p < 0.001$), and did not observably change after acute exposure (Table 2). Therefore, *D-GADD45* gene overexpression in the nervous system did not cause any additional radiation resistance.

Recent experiments demonstrated a lifespan extension of 22–77% in males and females with constitutive overexpression of *D-GADD45* in the nervous system compared with flies of the parental strains UAS-D-GADD45 and GAL4-1407. It is important to mention that in conditions of chronic 40 cGy and acute 30 Gy gamma-irradiation doses, the lifespan of males and females with *D-GADD45* overexpression remained 20–40% higher compared with the lifespan of the parental strain flies ($p < 0.001$) (Fig. 5 and Table 2). Consequently, the overexpression of the *D-GADD45* in the nervous system increases the lifespan of *Drosophila* flies under standard conditions as well as under gamma-irradiation conditions.

Age-dependent dynamics of spontaneous activity of the D-GADD45 gene. We also observed the age-dependent dynamics of spontaneous activity of *D-GADD45* within the whole

body and nervous system of adult flies. During aging, flies of the wild-type Canton-S strain experienced a widespread increase in *D-GADD45* gene expression of 4.9–5.6 times homeostatic levels (Fig. 6B). At the same time, during evaluation of *D-GADD45* gene expression, an expression level increase of 2.4–3.3 times homeostatic levels up to the second week of life was noted, which was then followed by a reduction in expression to levels lower than those seen in the first days following adult emergence (Fig. 6C).

Moreover, flies with constitutive *D-GADD45* overexpression in the nervous system showed a reduction in the level of increased transcriptional activity of the transgene. Between 0–5 d of age, *Drosophila* with the parental genotypes UAS-D-GADD45/GAL4-1407 showed increased levels of *D-GADD45*: expression in males was higher by 4.5–5.3 times, and in females by 2.2–2.9 times, levels in flies without the transgene. However, by age 29 d, levels had decreased in transgenic males to only 2.7 times higher than that of flies without the transgene, while in females there was no statistically significant difference between transgenic and non-transgenic flies (Fig. 7).

The influence of oxidative stress, genotoxic stress, hyperthermia and starvation on the *D-GADD45* gene expression. To understand the mechanisms involved in *D-GADD45*-mediated stress resistance in *Drosophila*, we studied the way in which the expression of the gene changes in response to various stress factors. The 5-d-old wild-type Canton-S flies were exposed to paraquat, hyperthermia and starvation for 24 h followed by quantitative real-time RT-PCR and the calculation of the relative levels of *D-GADD45* expression.

Treatment with 20 mM paraquat enhanced the *D-GADD45* expression by 7.6–7.8-fold both in *Drosophila* males and in females. The lower 1.6–3.9-fold increase was detected after 35°C hyperthermia. However, starvation increased the expression level of the *D-GADD45* gene 1.5-fold in males only. In females, the transcriptional activity of investigated gene was 1.5 times lower in starved flies compared with flies that lived in standard conditions (Fig. 6A).

Age-dependent dynamics of *D-GADD45* gene expression were observed in *Drosophila* adults of the wild-type Canton-S strain, within a standard environment and after chronic ionizing irradiation (genotoxic stress) in the pre-imago developmental stages at levels of 40 cGy. We described that ionizing irradiation at this dosage does not affect the expression of *D-GADD45*, neither in the nervous system nor in the whole body, throughout the life of adults (Fig. 6B and C).

Stress-induced *D-GADD45* gene expression and the induction of D-GADD45-dGFP. The expression of *D-GADD45* in vivo was evaluated using the reporter construct D-GADD45-dGFP. Inflammation arose as a response to damage to the chitinous exoskeleton after 1 h (data not shown). This induced the expression of D-GADD45-dGFP in both males and females. However, acute gamma-irradiation exposure (at a dosage of 30 Gy) and chronic (40 cGy) gamma-irradiation exposure, as well as oxidative stress (20 mM paraquat), thermal stress (35°C), dehydration and starvation, did not lead to any changes in the activity of GADD45 protein, as measured by the reporter construct D-GADD45-dGFP (data not shown).

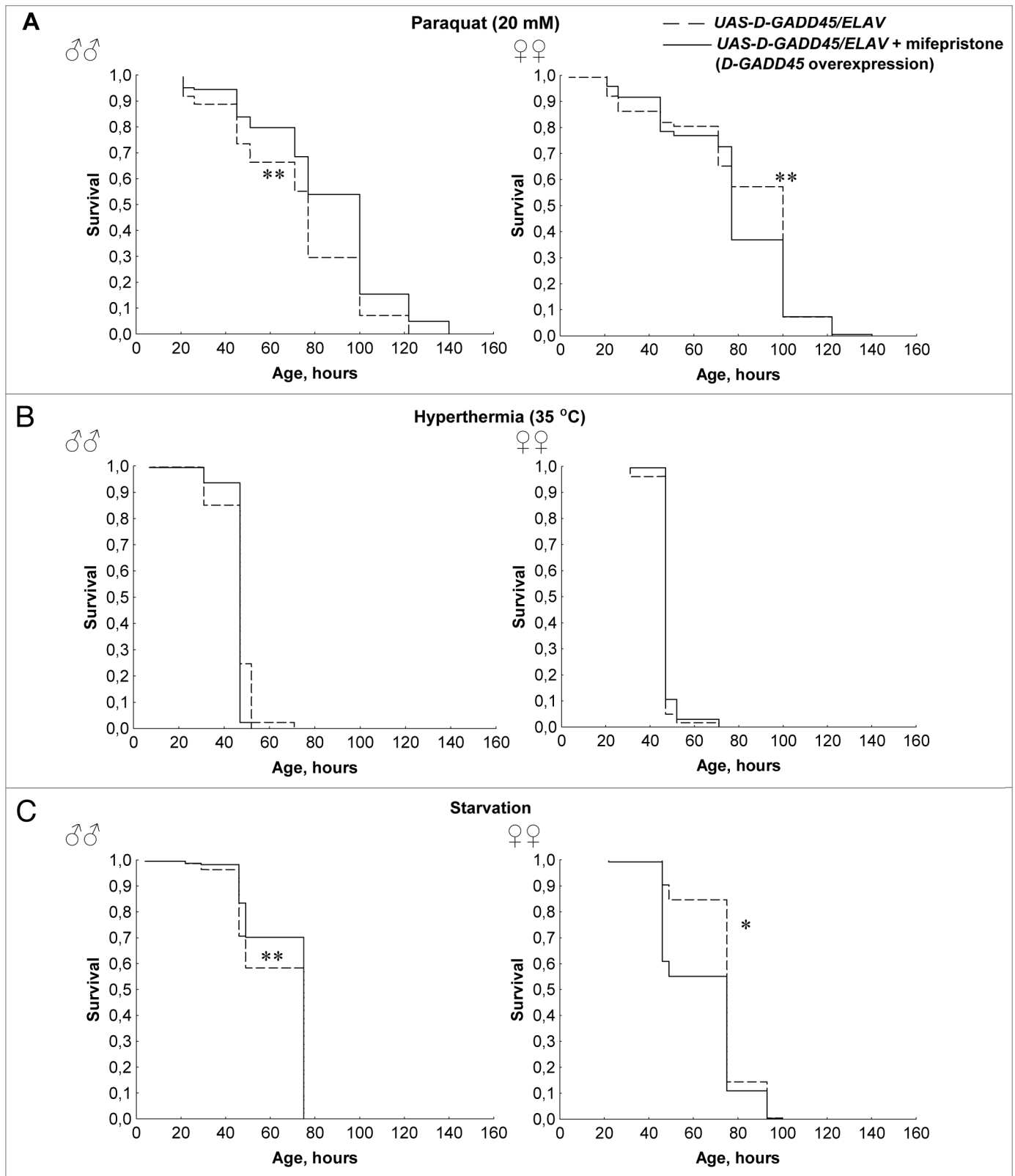


Figure 3. The influence of conditional *D-GADD45* overexpression in the nervous system on stress resistance. (A–C) Survival curves of *Drosophila* males and females with conditional *D-GADD45* overexpression in the nervous system and without overexpression under oxidative stress (20 mM paraquat) (A), hyperthermia (35°C) (B) and starvation (C). Significant differences in survival are marked by * - $p < 0.001$, ** - $p < 0.05$ (Kolmogorov-Smirnov test).

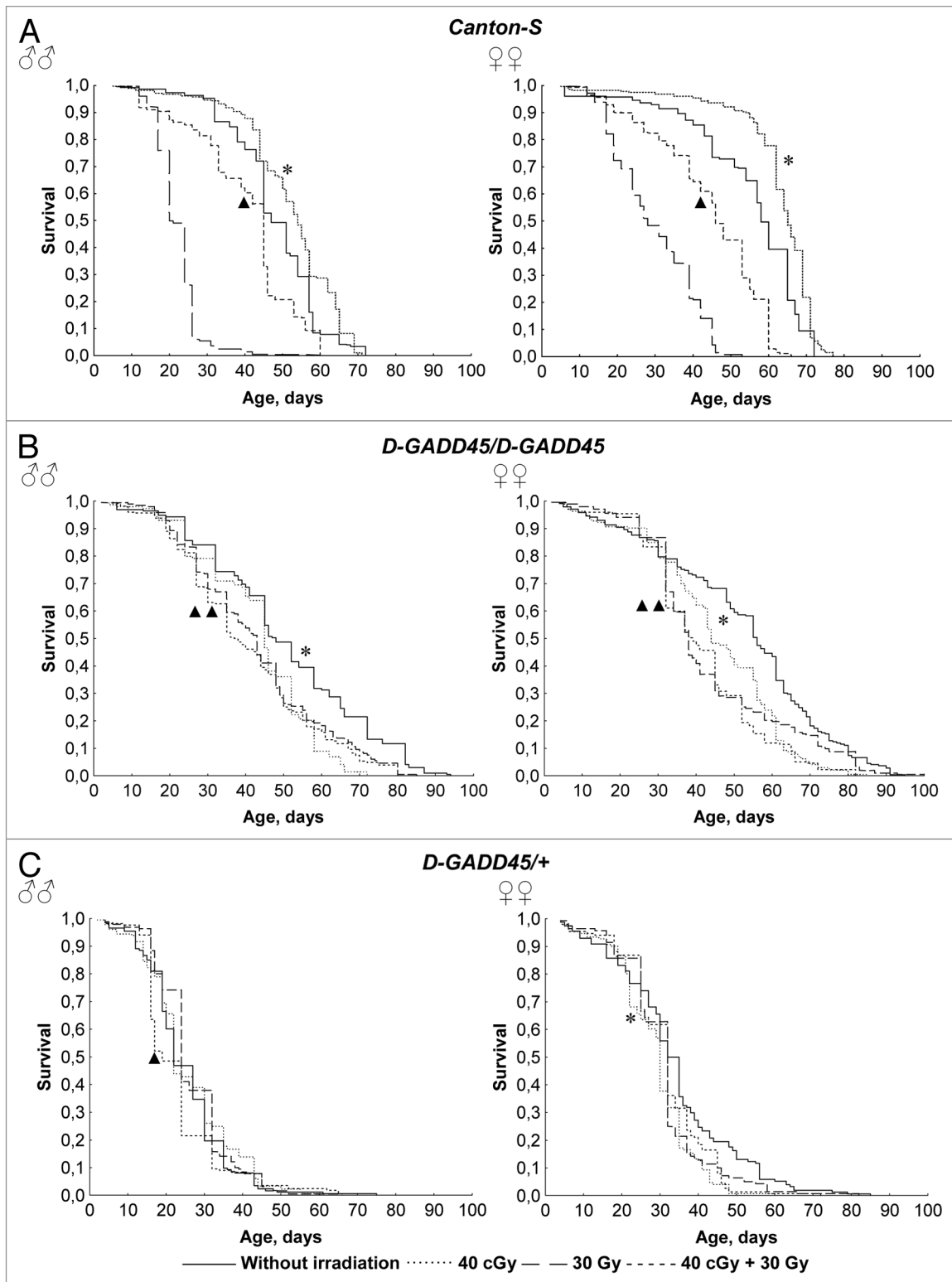


Figure 4. Survival of the wild-type Canton-S flies and flies with the *D-GADD45* mutation under different irradiation conditions. (A–C) Survival curves of *Drosophila* males and females of the wild-type Canton-S strain (A), and flies with the homo- and heterozygous mutation in the *D-GADD45* gene (*D-GADD45/D-GADD45* and *D-GADD45/+* flies, respectively) (B and C) under different irradiation conditions. Significant differences in survival are marked by asterisks (for the variants “without irradiation” and “40 cGy”) or by triangles (for the variants “30 Gy” and “40 cGy + 30 Gy” variants), * - $p < 0.001$, ** - $p < 0.05$ (Kolmogorov-Smirnov test).

Table 1. The influence of different irradiation conditions on the longevity parameters of *Drosophila* individuals of the wild-type Canton-S strain and with mutation in the *D-GADD45* gene

Genotype	Sex	Treatment	M	$x \pm \Delta m$	90%	Min	Max	MRDT	α	R_0	N
<i>Canton-S</i>	♂♂	W/o irradiation	47.0	47.9 ± 0.6	58	6	72	7.4	0.09	0.0006	379
		40 cGy *	54.0 *	52.2 ± 0.6	65 **	5	71	6.7	0.10	0.0003	478
		30 Gy	20.0	22.0 ± 0.3	26	7	60	6.7	0.10	0.0088	399
		40 cGy + 30 Gy *	45.0 *	40.2 ± 0.7	56 *	7	60	8.0	0.09	0.0016	358
	♀♀	W/o irradiation	58.0	55.0 ± 0.8	68	6	72	7.1	0.10	0.0002	328
		40 cGy *	65.0	63.0 ± 0.6	71	5	77	4.4	0.16	0.0000	378
		30 Gy	28.0	29.8 ± 0.5	45	7	53	7.5	0.09	0.0039	420
		40 cGy + 30 Gy *	46.0 *	44.4 ± 0.7	60 *	7	66	7.8	0.09	0.0010	397
<i>D-GADD45/D-GADD45</i>	♂♂	W/o irradiation	48.0	50.2 ± 1.5	82	6	94	15.3	0.05	0.0033	195
		40 cGy *	45.0 *	43.0 ± 1.2	58 *	4	72	9.2	0.08	0.0018	144
		30 Gy	43.0	42.7 ± 1.3	69	2	85	14.9	0.05	0.0050	197
		40 cGy + 30 Gy **	37.5 **	40.5 ± 1.2	67	5	85	15.7	0.04	0.0060	228
	♀♀	W/o irradiation	56.0	52.6 ± 1.4	80	2	95	14.5	0.05	0.0026	242
		40 cGy *	44.0 *	45.1 ± 1.2	63 *	4	85	11.6	0.06	0.0027	193
		30 Gy	38.0	43.8 ± 1.3	74	4	100	18.4	0.04	0.0063	203
		40 cGy + 30 Gy **	39.0	41.5 ± 1.2	65 *	4	82	12.9	0.05	0.0043	175
<i>D-GADD45/+</i>	♂♂	W/o irradiation	22.0	24.9 ± 0.8	35	4	75	12.2	0.06	0.0137	173
		40 cGy	22.0	25.8 ± 0.9	43	2	63	10.9	0.06	0.0108	180
		30 Gy	24.0	26.6 ± 0.7	38	4	65	9.0	0.08	0.0079	190
		40 cGy + 30 Gy *	19.0 *	23.1 ± 0.8	32	4	66	15.0	0.05	0.0188	167
	♀♀	W/o irradiation	33.5	33.8 ± 1.2	56	4	85	15.2	0.05	0.0088	154
		40 cGy *	30.0 *	28.8 ± 0.8	41*	4	61	7.6	0.09	0.0046	151
		30 Gy	32.0	31.1 ± 1.0	45	4	82	12.3	0.06	0.0087	140
		40 cGy + 30 Gy	32.0	31.4 ± 0.9	45	4	72	9.0	0.08	0.0051	152

M, median lifespan; $x \pm \Delta m$, mean lifespan and standard error of the mean; 90%, age of 90% mortality; Min and Max, minimum and maximum lifespan; α and R_0 , Gompertz equation parameters; MRDT, mortality rate doubling time ($\ln 2/\alpha$); N, number of individuals in the population; * - $p < 0.001$, ** - $p < 0.05$ (between "w/o irradiation" and "40 cGy" variants, and between "30 Gy" and "40 cGy + 30 Gy" variants; treatment, Kolmogorov-Smirnov test; median lifespan, Gehan-Breslow-Wilcoxon and Mantel-Cox tests; age of 90% mortality, Wang-Allison test).

Discussion

GADD45 is a key regulator of stress resistance in mammals and acts in the pathogenesis of many age-dependent diseases, including tumors, atherosclerosis and neurodegeneration.¹ Previously, we demonstrated that the overexpression of the *Drosophila* ortholog to *GADD45*, *D-GADD45*, in both male and female flies, caused an increase in their median and maximum lifespan.⁶ At the same time, no deterioration in fertility or neuro-muscular activity was detected.⁶ The spontaneous level of damaged DNA measured within larval neuroblasts of the third generation that had *D-GADD45* overexpression was reduced.⁶ Thus, the overexpression of *D-GADD45* is associated with an increase in efficiency DNA damage detection and repair.

The role of GADD45 in signal transduction pathways of DNA damage and repair is generalized in **Figure 8**. Gadd45 proteins function in DNA damage, and repair is mediated by a complex interplay of physical interactions with other cellular proteins (**Fig. 8**). These include PCNA, ATM, ATR and BRCA1.^{3,23,24} In response to DNA damage, GADD45 may be activated by

ATF-2,²⁵ Oct-1²⁶ and p53²⁷ transcription factors. Additionally, the DNA repair process can be activated through intermediaries such as MutS α ²⁸ or through the transcription factor p53 and MSH-family proteins.²⁹ The binding of GADD β with the kinase CDK1 (p34)³⁰ or with cyclin B1 results in G₂/M checkpoint arrest.

Besides, it is found that under certain conditions, p53 suppress senescence.^{31,32} While arresting cell cycle, p53 may simultaneously suppress the senescence program, thus causing quiescence. p53 suppresses senescence presumably by inhibition of mTOR (mammalian target of rapamycin).^{33,34} Senescence occurs when p53 fails to inhibit mTOR. Low concentrations of DNA damaging drugs induce p53 at levels that do not inhibit mTOR, thus causing senescence.^{33,34} In this context, the p53-dependent activation of GADD45 may complete the anti-aging and stress-resistance effects of p53.

At an organismal level, one possible mechanism for the increase in longevity associated with *D-GADD45* overexpression is an increased resistance to oxidative stress. The involvement of GADD45 in oxidative stress response is summarized in

Table 2. The influence of different irradiation conditions on the longevity parameters of *Drosophila* individuals with the *D-GADD45* overexpression in the nervous and without overexpression

Genotype	Sex	Treatment	M	$x \pm \Delta m$	90%	Min	Max	α	R_0	MRDT	N
<i>UAS-D-GADD45</i>	♂♂	W/o irradiation	43.0	45.0 ± 1.2	71	3	84	0.05	0.0041	3.7	258
		40 cGy *	54.0 *	51.3 ± 1.1	70	6	84	0.07	0.0011	3.6	190
		30 Gy **	49.0 **	50.0 ± 1.1	67 **	10	81	0.08	0.0011	3.6	134
	♀♀	W/o irradiation	57.5	56.6 ± 1.2	76	3	84	0.07	0.0007	3.6	211
		40 cGy	56.0	55.0 ± 1.2	71 *	3	79	0.08	0.0006	3.7	170
		30 Gy *	53.0 **	53.0 ± 1.0	73 **	5	81	0.08	0.0009	3.5	180
<i>GAL4-1407</i>	♂♂	W/o irradiation	44.0	45.6 ± 0.8	56	4	73	0.10	0.0006	3.2	136
		40 cGy *	41.0 **	43.5 ± 1.1	57	3	71	0.09	0.0012	3.3	108
		30 Gy *	40.0 **	44.0 ± 1.6	66 *	10	81	0.07	0.0024	3.4	168
	♀♀	W/o irradiation	48.0	45.0 ± 1.0	56	6	64	0.11	0.0004	3.2	138
		40 cGy *	51.0 **	48.8 ± 0.6	57	9	71	0.15	0.0001	3.0	147
		30 Gy **	46.0	45.8 ± 1.1	56	5	73	0.11	0.0005	3.6	179
<i>UAS-D-GADD45/GAL4-1407</i>	♂♂	W/o irradiation	76.0	74.2 ± 1.4	89	34	98	0.09	0.0001	3.5	186
		40 cGy	65.0 **	66.7 ± 1.7	85 **	4	98	0.07	0.0003	3.6	187
		30 Gy **	73.0	67.1 ± 2.3	87	3	95	0.07	0.0003	3.9	178
	♀♀	W/o irradiation	70.0	67.0 ± 1.1	84	15	98	0.08	0.0003	3.5	173
		40 cGy *	79.0 *	74.8 ± 1.1	85	4	90	0.12	0.0000	3.7	142
		30 Gy *	74.0 **	66.8 ± 1.9	84	3	95	0.07	0.0003	4.1	158

M, median lifespan; $x \pm \Delta m$, mean lifespan and standard error of the mean; 90%, age of 90% mortality; *Min* and *Max*, minimum and maximum lifespan; α and R_0 , Gompertz equation parameters; *MRDT*, mortality rate doubling time ($\ln 2/\alpha$); *N*, number of individuals in the population; * - $p < 0.001$, ** - $p < 0.05$ (treatment, Kolmogorov-Smirnov test; median lifespan, Gehan-Breslow-Wilcoxon and Mantel-Cox tests; age of 90% mortality, Wang-Allison test).

Figure 9. The expression levels of antioxidant enzymes SOD1 and GPX1 are regulated by the transcription factors PPAR γ ³⁵ and RXRA,³⁶ respectively. All three forms of the protein GADD45 have a positive effect on these transcription factors by increasing their transcription levels. Downstream of the oxidative pathway, SOD1 activates thioredoxin and glutaredoxin, redox enzymes that inhibit ASK1,³⁷ a protein that leads to the dissociation of microtubules. Thus, the activity of GADD45 family proteins prevents cellular damage.

GADD45 β acts through the protein kinases MEKK4 and JNK.^{38,39} Activation of these cascades increases levels of the protein ASK1,⁴⁰ which acts in opposition to the protein SOD1. Moreover, JAK and MEK kinase cascades inhibit the activity of the transcription factors PPAR and RXRA,^{41,42} thus reducing the level of SOD1 and GPX1 expression. The overexpression of SOD1⁴³ and GPX1⁴⁴ increases the resistance of cells to oxidative stress in transgenic mouse models. However, the equilibrium of SOD1 and GPX1 activity is also critical for the cell fate. On the one hand, too little SOD relative to GPX could lead to an accumulation of O₂⁻, which is toxic to macromolecules; on the other hand, too much SOD relative to GPX could lead to increased production of the H₂O₂ intermediate.⁴⁵ Thus, in the process of oxidative stress response, GADD45 family proteins are involved in the control of the activity and maintenance of the balance between antioxidant enzymes and can both positively and negatively affect the fate of cells. It can also be noted that the ASK1 protein has a mutually inhibitory relationship with the kinase

PDK,⁴⁶ which inhibits AKT⁴⁷ and thereby reduces the chances of cell survival in response to oxidative stress.

Paraquat (N,N'-dimethyl-4,4'-bipyridinium dichloride, C₁₂H₁₄N₂) is a molecular compound that causes the formation of free radicals within the cell. By receiving an electron from cellular redox enzymes, paraquat interacts with molecular oxygen, forming the superoxide free radical O₂⁻.⁴⁸ It has been previously established that in adult *Drosophila*, treatment with paraquat leads to a decrease in longevity. At the same time, selection for longevity increases resistance to paraquat.¹¹ In our experiment, adult mortality under the effects of paraquat was significantly lower during constitutive and conditional overexpression of *D-GADD45* in the nervous system in males as well as females. Moreover, we observed the 7.6–7.8-fold increase in expression level of the *D-GADD45* gene after treatment of wild-type flies with paraquat. These results indicate the involvement of GADD45 in oxidative stress response and resistance. GADD45 family proteins in mammals can increase resistance to oxidative stress through the activation of nucleotide and base excision repair mechanisms (for example, by binding to PCNA, XPC and XPG)^{3,49,50} (Fig. 8) or through JNK-dependent longevity that is activated in response to an increased level of pro-oxidants (Fig. 9).⁵¹

The negative effect of thermal stress factors was reduced when *D-GADD45* was overexpressed in the nervous system. Heat shock causes damage within the tertiary structure of proteins, negatively impacts their aggregation and causes endoplasmic stress.¹⁷ GADD45 proteins affect the activity of heat shock proteins in

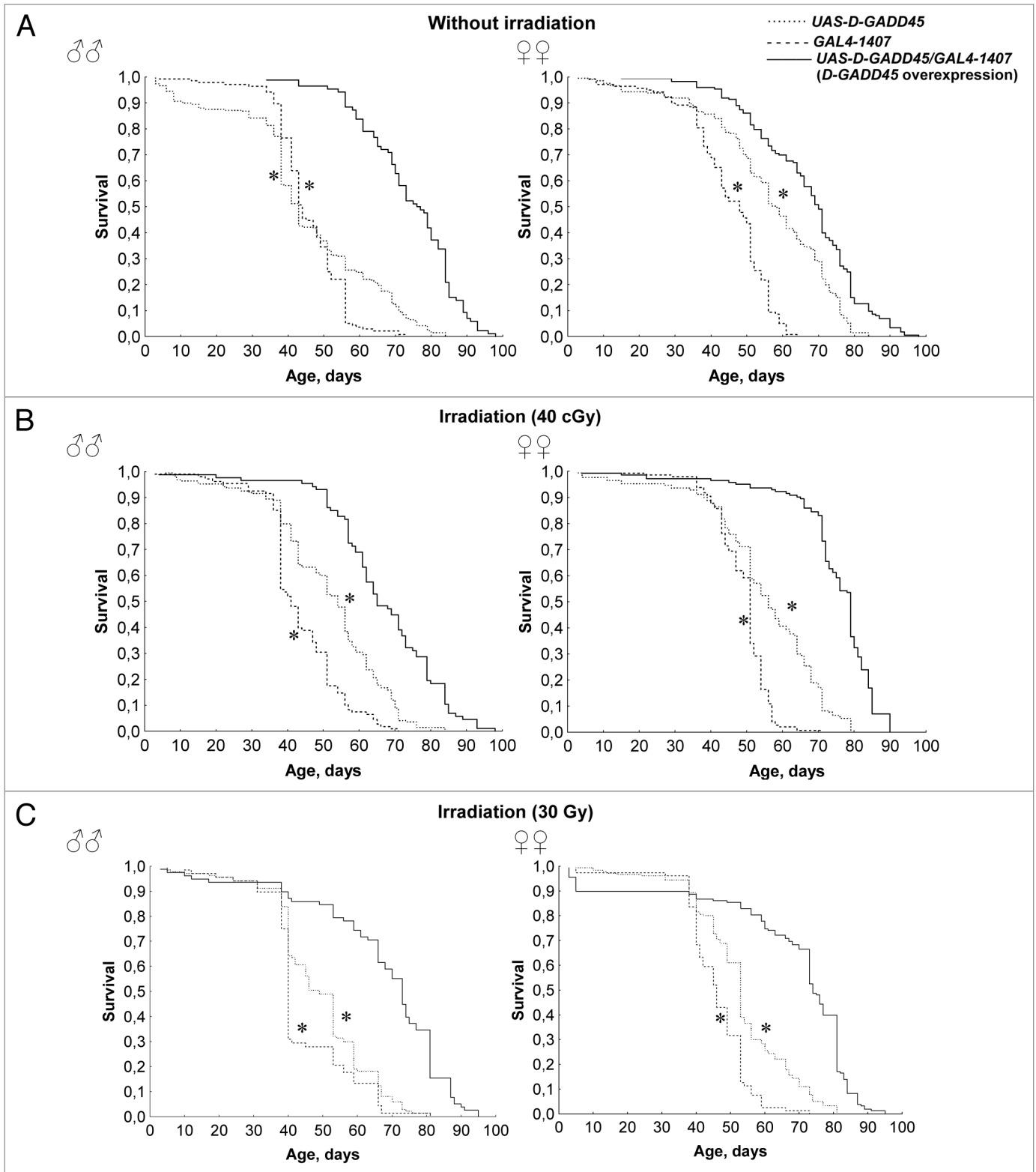


Figure 5. Survival of flies with *D-GADD45* overexpression and with parental genotypes under different irradiation conditions. (A–C) Survival curves of *Drosophila* males and females with constitutive *D-GADD45* overexpression in the nervous system, and with the UAS-*D-GADD45* and GAL4-1407 parental genotypes under standard conditions (A), and chronic 40 cGy (B) and acute 30 Gy gamma-irradiation (C). Significant differences in survival are marked by * - $p < 0.001$, ** - $p < 0.05$ (Kolmogorov-Smirnov test).

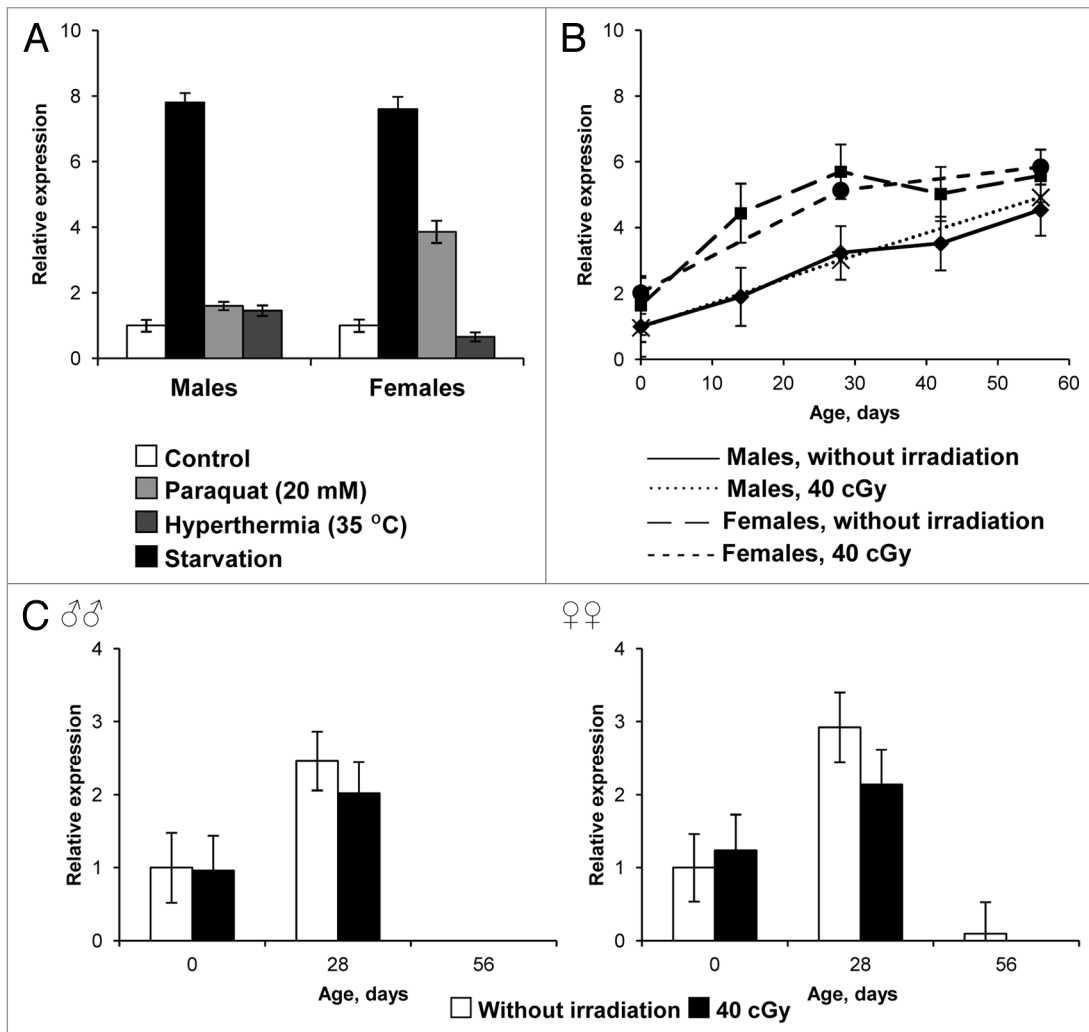


Figure 6. The influence of stress factors on the expression levels of the *D-GADD45* gene. (A) Expression levels in wild-type Canton-S flies after oxidative stress (20 mM paraquat), hyperthermia (35°C) and starvation. (B) The age-dependent changes in ubiquitous *D-GADD45* gene expression of wild-type Canton-S flies. (C) The age-dependent changes in *D-GADD45* gene expression in the nervous system of the wild-type Canton-S males and females.

several ways (Fig. 10). All three orthologs of GADD45 inhibit the protein kinase CDK1, which phosphorylates the transcription factor SP1, thereby inhibiting its activity.⁵² The transcription factor SP1 activates the expression of heat shock protein HSP27;⁵³ thus, GADD45 proteins increase the activity of HSP27 by preventing its inactivation. In turn, this increases the activity of another heat shock protein, HSP70.⁵⁴ SP1 also increases the expression of another heat shock protein, HSP60.⁵⁵

The GADD45 family of proteins also activates the transcription factor PPAR- γ , which increases the expression of the heat shock protein HSP22,⁵⁵ which can also be activated by the upregulation of RXRA by GADD45 proteins. HSP22 is responsible for activating another heat shock protein, HSP27.⁵⁶ Thus, when activated, members of the GADD45 family are able to trigger a cascade of effects on heat shock proteins.

GADD45, by inhibiting the kinase p38MAPK, activates the transcription factor HSF1,⁵⁷ which is a cornerstone element for pathways activating heat shock proteins, such as HSP60, HSP90,

HSPA4, HSP70, HSP27 and HSP22 (Fig. 10). *D-GADD45* overexpression through MAPKs could cause an increase in HSF-1 and heat shock protein activity, contributing to an organism's resistance to stressors. In addition, due to the intensification of metabolism, heat shock results in the appearance of additional reactive oxygen.⁵⁸ In our experiment, the *D-GADD45* expression increased 1.6–3.9-fold at 35°C, confirming its role in thermal stress response.

In our experiments, increased resistance to starvation was demonstrated in males with *D-GADD45* overexpression in the nervous system, compared with flies without overexpression. Starved wild-type males but not females demonstrated a slight increase (1.5-fold) in GADD45 expression. Recent studies demonstrated that GADD45 is a critical protein in the starvation-induced pathway that reprograms muscle gene expression to cause skeletal muscle atrophy.⁵⁹ Gadd45 α represses genes involved in anabolic signaling and energy production, and it induces pro-atrophy genes. As a result, Gadd45 α reduces multiple barriers to muscle

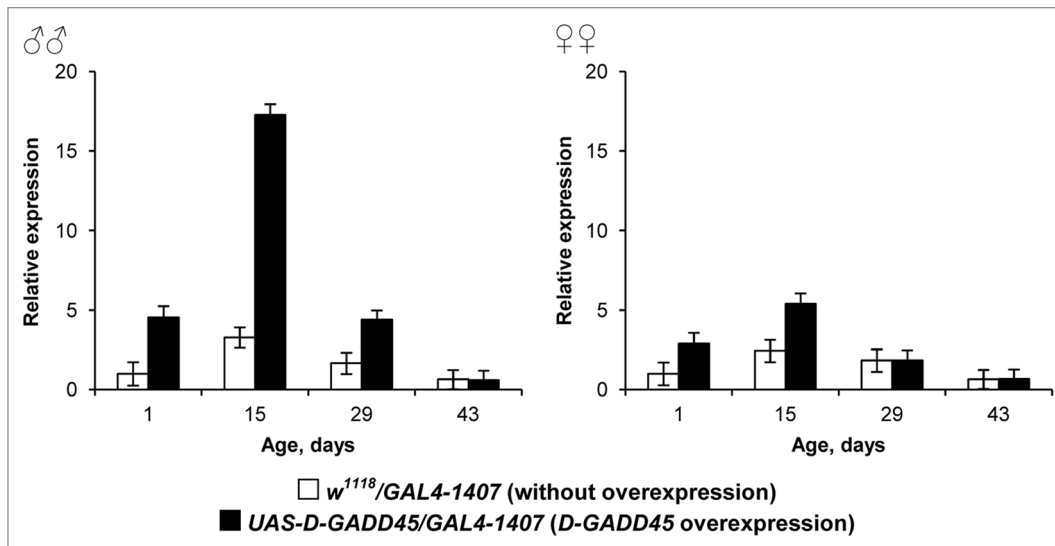


Figure 7. The age-dependent changes in *D-GADD45* expression in flies with constitutive *D-GADD45* gene overexpression in the nervous system.

atrophy (including PGC-1 α , Akt activity, and protein synthesis) and stimulates pro-atrophy mechanisms (including autophagy and caspase-mediated proteolysis).⁵⁹ *GADD45*'s implication in the response to starvation is shown in Figure 11. *GADD45* α activity is redundant with respect to β and γ forms. However, the α form of *GADD45* inhibits p38MAPK, and the β form activates it. Starvation results in the oppression of amino acid composition and affects protein levels of endophilin B1 and BAD. The reduction in amino acids begins with the protein GCN2, which inhibits the eIF family proteins that regulate translation in the cell.⁶⁰ *GADD45* affects this process through the intermediaries PCNA⁶¹ and HDAC1,⁶² which have negative effects on the entire family of eIF proteins⁶³ through the p300 protein. Thus, *GADD45* protein can inhibit the activity of translation during starvation. Proteins such as DMNT1 and p38MAPK, are also involved in this process as additional mediators of regulation of translation by *GADD45* protein.

The starvation response is also associated with mitochondrial proteins; in particular, Bcl 2, BAD and Blx-x. Apoptosis is activated during starvation by these proteins. *GADD45* family of proteins can affect the activity of mitochondrial apoptotic proteins through the intermediaries Cdk1 (p34)³⁰ and p38MAPK,¹⁷ as well as the transcription factor p53. In this way, the pathway influences the susceptibility of cells to starvation and, consequently, the fate of cells.

Starvation can also lead to autophagy, mediated by the proteins UVRAG and Beclin 1.⁶⁴ In turn, the Beclin 1 is affected by mitochondrial proteins⁶⁵ as well as the protein DAPK1.⁶⁶ DAPK1, through intermediaries such as p38MAPK and DMNT1,⁶⁷ is directly regulated by the *GADD45* family. In addition, excessive reduction in food intake causes oxidative stress and leads to a decrease in the efficacy of DNA repair mechanisms.⁶⁸ It is possible that *D-GADD45* overexpression contributes to a reduction in the negative effects of a given stress factor and contributes to survival of the organism by modulating cellular metabolism (Fig. 11) and DNA repair (Fig. 8).

The inflammation is another stress factor that increases not only the level of *D-GADD45* mRNA⁵ but also protein (our data). The implication of *D-GADD45* in inflammation is shown in Figure 12. The inflammatory process is thought to be mediated through three major pathways: the IKK-NF pathway, the arachidonic acid and prostaglandin pathway and the MEK-MAPK pathway. The main function of *GADD45* in the inflammatory processes is determined by its interactions with other proteins, such as p38MAPK,⁶⁹ p34CDK³⁰ and PCNA.³ The interaction of *GADD45* with the transcription factors PPAR, C/EBP β and c-Jun can also trigger activation of the inflammatory response.

The *GADD45* family of proteins affects the transcription of IFN gamma by interacting with PCNA-p300 and some representatives of the IL (receptor)⁷⁰ family. Mediation through this pathway adjusts the force of the inflammatory process that is distributed along the IKK-NF pathway. Regulation of this pathway can occur indirectly by transcription factors such as C/EBP β ,⁷¹ PPAR α ⁷² and c-Jun.⁷³ The inflammatory pathway first involves the activation of PPAR α ,⁷² which activates the downstream transcription factors C/EBP β and c-Jun.

GADD45 proteins can also act through a prostaglandin-endoperoxid synthase COX-2 (PRGS2). COX-2 converts arachidonic acid into prostaglandin endoperoxide. *GADD45* family proteins affect COX-2 (PTGS2) through p38MAPK,⁷⁴ or indirectly through the transcription factors PPAR α and C/EBP β .⁷⁵

We demonstrated that flies with constitutive and conditional *D-GADD45* gene overexpression in the nervous system were more resistant to the actions of stress factors (such as paraquat, hyperthermia and starvation) than flies without overexpression, and the wild-type strain showed increased expression of *D-GADD45*. This does not confirm the previous reports that demonstrated that *D-GADD45* gene expression in *Drosophila* is only induced during inflammation in response to microbial exposure and wounds, but not to paraquat, arsenite, UV radiation and X-rays.⁵

Ionizing irradiation is genotoxic and leads to oxidative stress.⁷⁶ In the wild-type strain Canton-S, hormesis was observed during

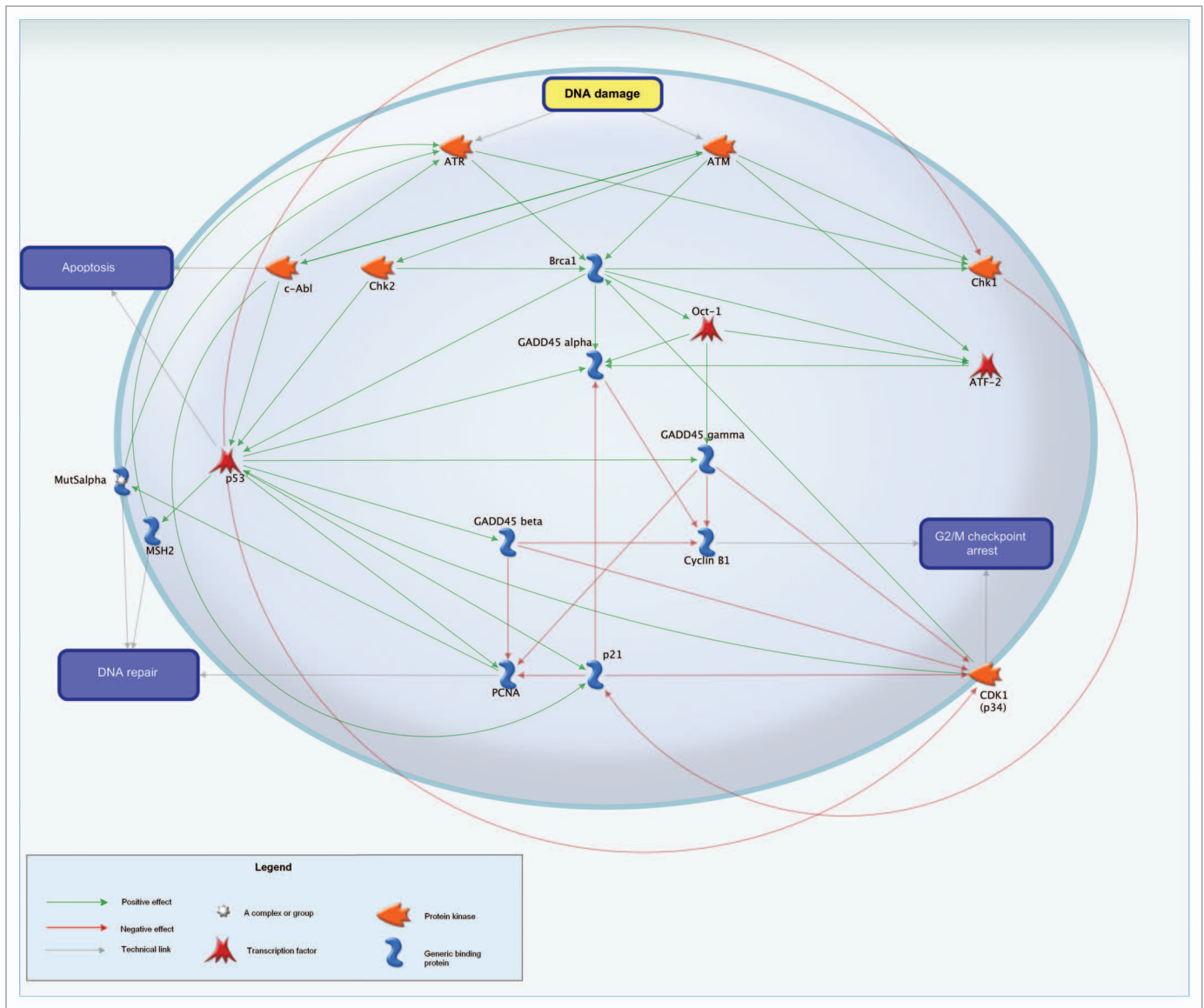


Figure 8. The role of GADD45 in DNA repair.

chronic exposure of low doses of gamma-irradiation, as indicated by an increase in median lifespan of irradiated flies. In addition, there was a change in radioadaptive response: following acute exposure at 30 Gy after chronic pre-irradiation at 40 cGy, exposed flies showed a 1.6- to 2.3-fold reduction in the damaging effect of irradiation. In contrast, in the homo- and heterozygous *D-GADD45* mutants, chronic irradiation caused a decrease in lifespan, while pre-irradiation did not cause radioadaptation to the subsequent acute irradiation. Therefore, *D-GADD45* appears to play a role in the adaptive response to ionized irradiation. At the same time, chronic irradiation at a dose rate of 40 cGy did not induce *D-GADD45* expression, which is congruent with reports in the literature.⁵

Despite the fact that acute exposure at a high dose rate of 30 Gy caused a reduction in lifespan of flies with constitutive *D-GADD45* overexpression, the lifespan of irradiated flies

remained higher overall compared with the lifespan of non-irradiated flies (without overexpressed *D-GADD45*). Consequently, *D-GADD45* gene expression in the nervous system prolongs lifespan in *Drosophila* both in the control environment and after gamma-irradiation.

It is known that with aging there is a change in the level of the expression of stress-resistance genes. The expression of one type of gene reduces the response to the accumulation of damage (for example, DNA damage response and DNA repair genes),¹⁹ while, at the same time, the expression of other genes compensatively increases this response (for example, heat shock response genes, oxidative stress inducible genes and DNA damage-inducible genes).^{19,77} Previous studies demonstrated that aging negatively affects the ability of cells to express GADD45 proteins in response to various stress factors. For example, exposure of cardiomyocytes of young mice to paraquat led to the increased expression of

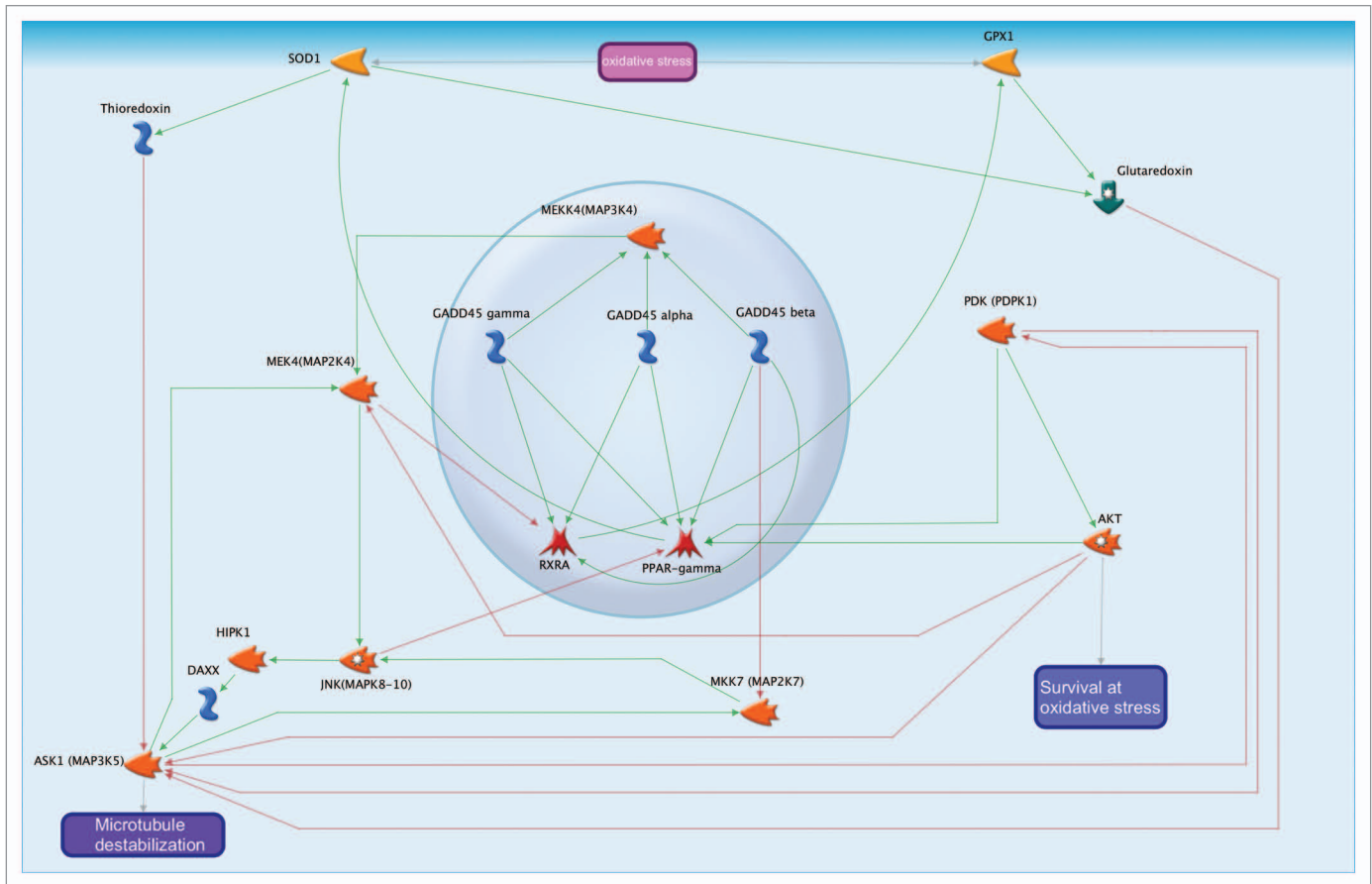


Figure 9. GADD45 in the oxidative stress pathway.

GADD45 proteins, but did not lead to the increased expression in the myocardium of old animals.⁷⁸ Our data shows that, despite the fact that the overall spontaneous activity of the *D-GADD45* gene increases with age (Fig. 6B), the level of expression of this gene in the nervous system is practically eliminated (Figs. 6C and 7). As the overexpression of *D-GADD45* within the nervous system increases lifespan and increases the resistance to various forms of stress, the dramatic decrease of its activity within the nervous system with age might be one of the reasons for the decrease in the organism's stress resistance. The demonstrated reduction in mRNA levels of *D-GADD45* with age and during artificial, constitutive overexpression in the nervous system may indicate an epigenetic cause of the low level of activity of this gene in old flies.

Thus, we have demonstrated an interconnection between the induction of *D-GADD45* and resistance to oxidative (paraquat), genotoxic (gamma-irradiation) and heat stress (35°C), as well as to starvation in *Drosophila*. At the same time, we demonstrated that most stress factors induce the mRNA expression of *D-GADD45*, and that the effect of an increase in stress resistance in *Drosophila* is associated with the *D-GADD45* gene activity.

There are some possible mechanisms by which *GADD45* may play a role in stress resistance. Research performed on mammals demonstrates that *GADD45* family proteins take part in protein-protein interactions, along with cell cycle and DNA replication regulators, mitogen-activated protein kinases and nuclear

hormone receptors.¹ *GADD45a* participates in demethylation of CpG in DNA, and is associated with the nucleosome.⁷⁹ Finally, *GADD45* binds RNA but not single- or double-stranded DNA or methylated DNA in vitro.⁸⁰ *GADD45a* also displays RNase-sensitive co-localization in nuclear speckles with the RNA helicase p68 and the RNA-binding protein SC35.⁸⁰

Materials and Methods

***Drosophila melanogaster* strains.** The following laboratory strains of *Drosophila melanogaster* were used: Canton-S and w¹¹¹⁸ as a wild-type strains; D-*GADD45*/D-*GADD45* [homozygous flies with y* w*; P(GawB)Gadd45NP0351 genotype] and D-*GADD45*/+ [heterozygous flies with y* w*; P(GawB)Gadd45NP0351/CyO, P(UAS-lacZ.UW14)UW14 genotype] with P transposable element insertion site in the coding region of D-*GADD45* (provided by Kyoto Stock Center); UAS-D-*GADD45*, containing an additional copy of the *D-GADD45* gene controlled by the UAS promoter (a kind gift from Dr. Uri Abdu, Ben-Gurion University); GAL4-1407 [genotype: w*; P(GawB)1407], carrying the constitutively expressed GAL4 promoter in the nervous system (kindly provided by the Bloomington Stock Center); ELAV [genotype: y w; P(ELAV-GeneSwitch)] with the mifepristone-inducible GAL4 driver in the nervous system (kindly granted by Dr. Haig Keshishian, Yale University).

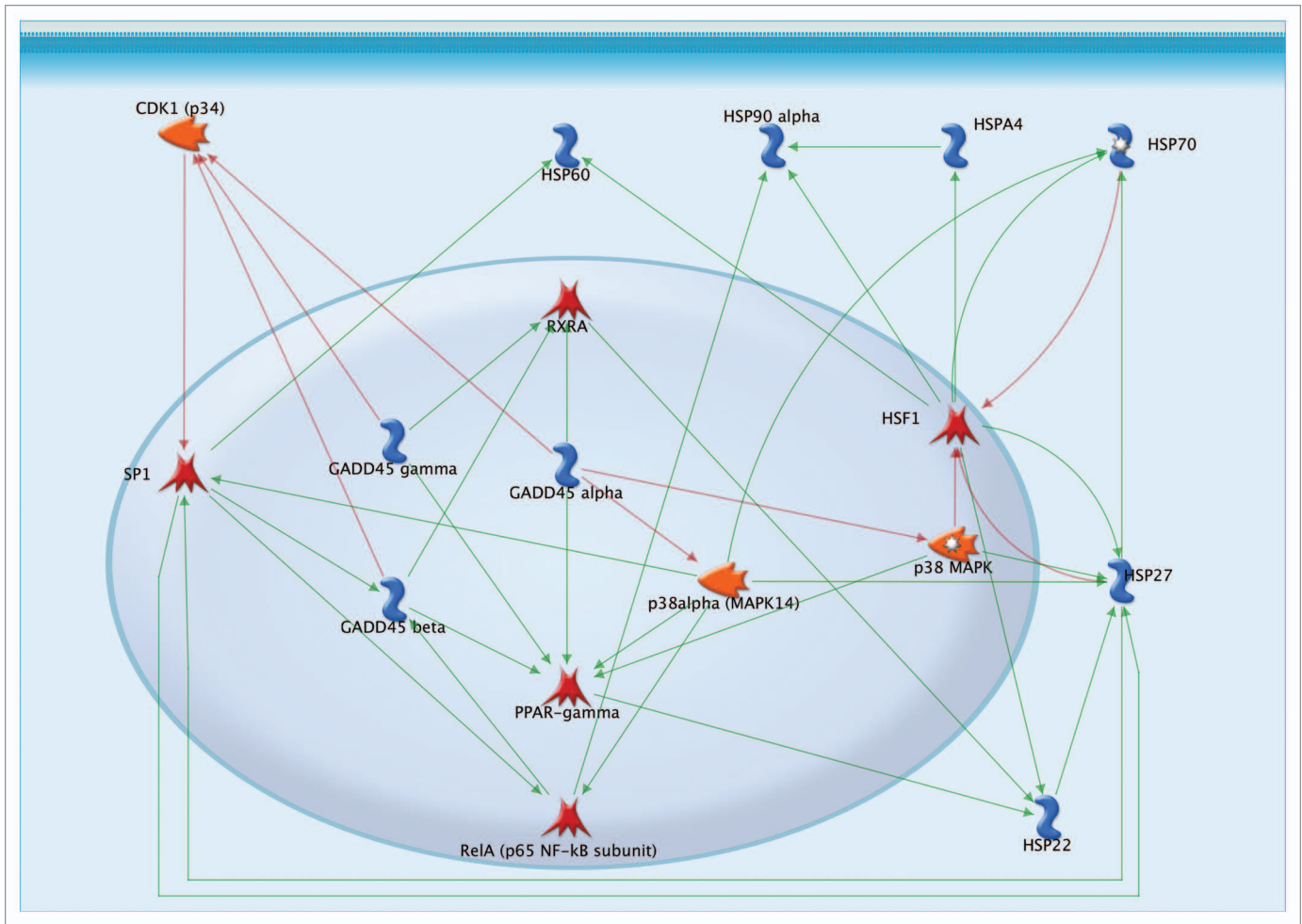


Figure 10. Involvement of GADD45 in heat shock response.

Overexpression of the *D-GADD45* gene. For constitutive overexpression of *D-GADD45* in the *Drosophila* nervous system, UAS-*D-GADD45* females were crossed with GAL4-1407 males. Parental strain individuals were used as controls.

For conditional overexpression of *D-GADD45* in the *Drosophila* nervous system, UAS-*D-GADD45* females were crossed with ELAV males. The offspring were kept on a medium with mifepristone (a conditional inducer of GAL4 expression). It is known that mifepristone on its own does not affect the lifespan of *Drosophila*.⁸¹ Therefore, we can infer that responses observed in the experimental animals were caused by *D-GADD45* overexpression and/or genetic background. Flies obtained by crossing UAS-*D-GADD45* females with ELAV males and kept on a medium without mifepristone were used as a control when examining conditional *D-GADD45* gene overexpression.

Mifepristone treatment. For mifepristone treatment, a stock solution of RU486 (Mifepristone, Sigma) with a 25 mg/ml concentration in 100% ethanol was used. It was mixed with yeast paste (60.4% water, 39% active yeast, 0.5% acetic acid, 0.1% ethanol) in a proportion of 0.1 ml initial RU486 solution per 100 ml of yeast paste. 0.3 ml of prepared yeast paste was then added to the surface of the medium. For experiments without

mifepristone, the medium surface was covered with yeast paste that was prepared in the same way, but with an equivalent amount of ethyl alcohol added in place of the RU486 solution.

***Drosophila* maintenance.** Flies were kept under standard conditions at 25°C in a 12:12 h light-dark regime, on an agar/semolina/sugar/yeast medium (but see above for mifepristone treatment).⁸² Twenty-five pairs of parents, with synchronized 24 h egg laying, were used to obtain experimental flies. The flies were extracted from vials immediately after imago eclosion.

Irradiation conditions. Flies were subjected to one of four different irradiation conditions: (1) no irradiation; (2) chronic exposure to 40 cGy of ionizing radiation from a gamma-source (Ra²²⁶) at the preimaginal stages of development. Treatment was conducted for 10 consecutive days per generation; (3) acute exposure to a gamma-source (Co⁶⁰) for a period of 30 min, at a dosage of 30 Gy immediately after adults emerged; (4) flies that were already exposed to ionizing radiation earlier, at both doses, were re-exposed to determine if earlier exposure had preadapted individuals for subsequent exposure.

Quantitative real-time RT-PCR. Gene expression levels were tested using quantitative real-time RT-PCR (qRT-PCR), and evaluated in males and females separately. Ten whole flies (for

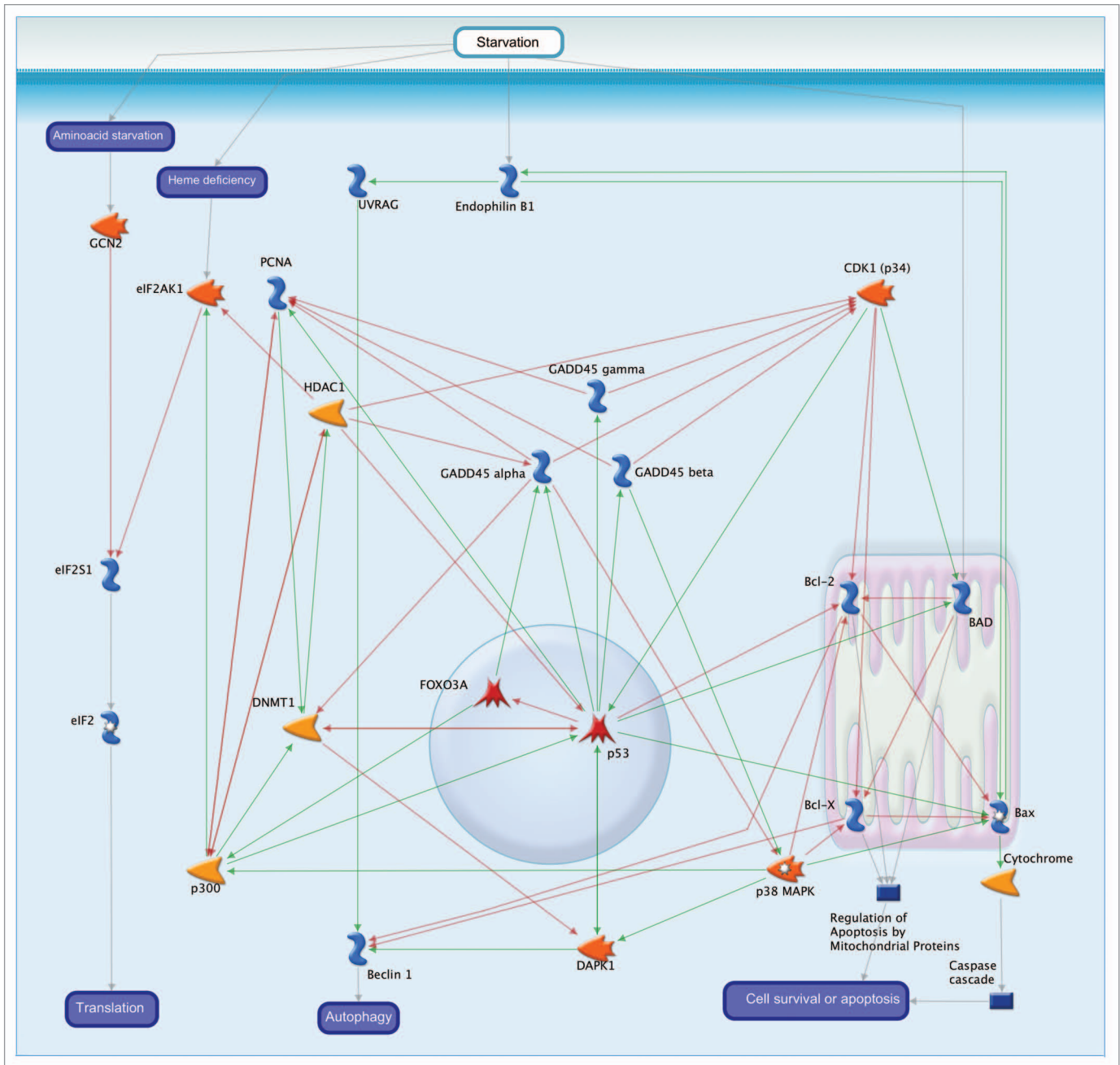


Figure 11. Starvation and GADD45.

analysis of ubiquitous expression level) or 30 heads (for analysis of expression level in the *Drosophila* nervous system) removed from their bodies, were placed in TRIzol Reagent (Invitrogen) and homogenized using a SilentCrusher S homogenizer (Heidolph). RNA was then extracted according to the manufacturer's instructions. RNA was separated using phenol-chloroform technique, followed precipitation with isopropyl alcohol. cDNA synthesis was performed with an Oligo(dT) primer (Invitrogen) and SuperScript III First-Strand cDNA Synthesis reverse transcriptase (Invitrogen) according to the manufacturer's protocol.

In order to test the background level of DNA contamination in RNA samples, a separate transcription reaction without

enzyme was performed. PCR was performed using β -*tubulin* primers (forward primer: 5'-GCA ACT CCA CTG CCA TCC-3'; reverse primer: 5'-CCT GCT CCT CCT CGA ACT-3').

Real-time PCR was performed in a 30- μ l volume, in 200- μ l PCR tubes, according to the manufacturer's instructions, using a reaction mixture containing SYBR Green PCR Master Mix dye (Applied Biosystems) and the following primers (Syntol, Russia): *D-GADD45* (forward primer: 5'-GCA AAC GCA CAA CCA AAC-3'; reverse primer: 5'-GGC CAT CAG GCA GAA GAG-3'), β -*tubulin* primers (forward primer: 5'-GCA ACT CCA CTG CCA TCC-3'; reverse primer: 5'-CCT GCT CCT CCT CGA ACT-3'). qRT-PCR was performed in a CFX96 amplifier

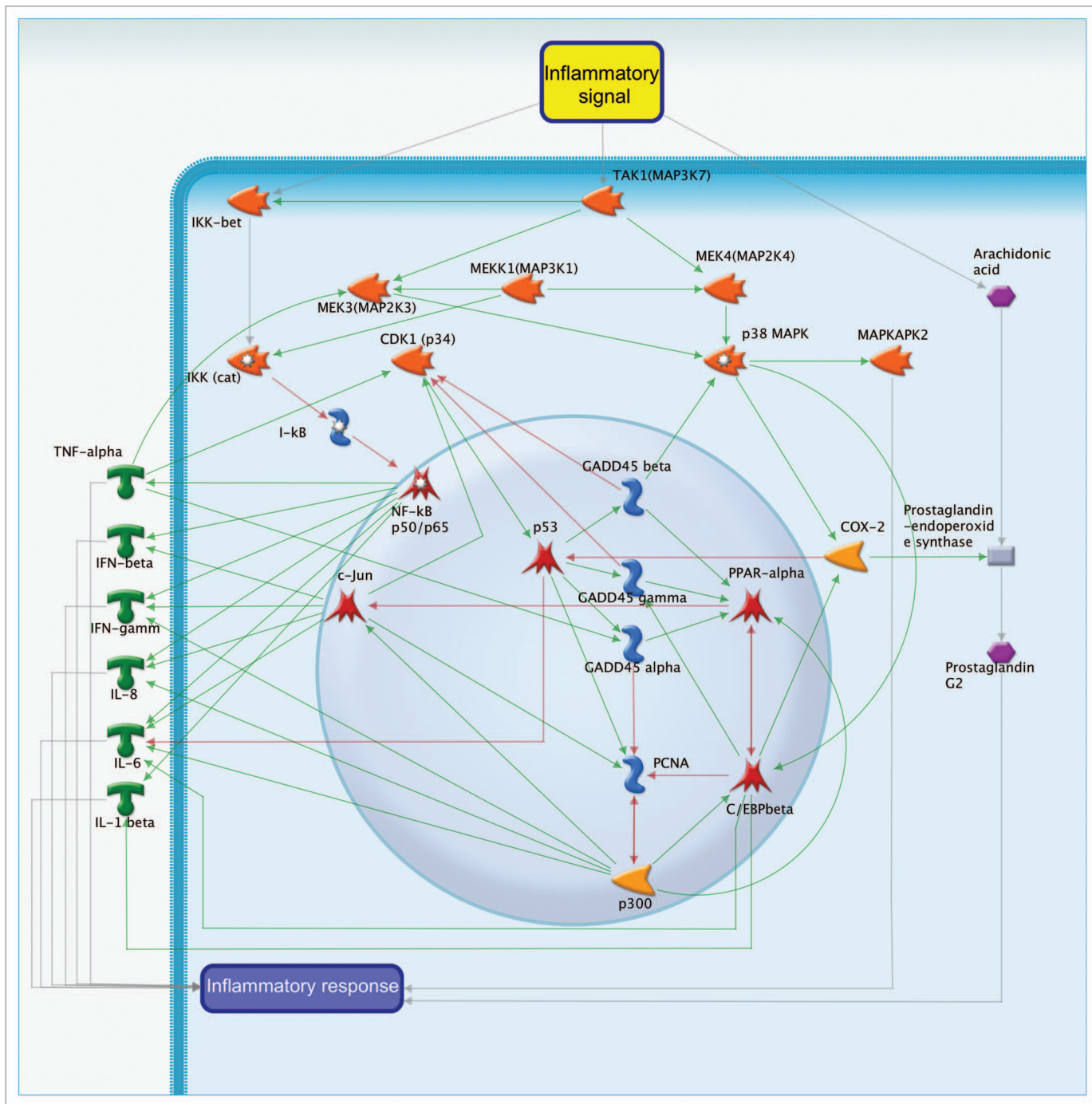


Figure 12. Inflammation and GADD45.

(BioRad) using the following program: (1) denaturing at 95°C for 10 min, (2) denaturing at 95°C for 15 sec, (3) annealing at 60°C for 30 sec, (4) elongation at 60°C for 30 sec, (5) stages 2–4 were repeated 50 times. *D-GADD45* expression was normalized against the β -*tubulin* housekeeping gene. Amplification of *D-GADD45* and β -*tubulin* was performed in individual tubes. Four to eight measurements were performed for each experiment. The relative level of *D-GADD45* gene expression was calculated using CFX96 software (BioRad). The relative level

of *D-GADD45* expression was calculated by the $2^{-\Delta\Delta C_t}$ method⁸³ using cycle threshold (C_t). The $\Delta\Delta C_t$ value was calculated as $\Delta C_t(\text{test sample}) - \Delta C_t(\text{control sample})$, and each value of $\Delta C_t = C_t(D-GADD45) - C_t(\beta-Tubulin)$.

Lifespan assay. To estimate longevity, 150–250 flies were collected (approximately 30 adult flies per 120 ml vial) for each experiment. Males and non-virgin females were kept separately. Flies were transferred to fresh medium twice weekly. Dead flies were counted and removed daily.

Mortality data was used to plot survival curves and to calculate the mean, median, minimum and maximum lifespan, as well as the age of 90% mortality. In order to estimate the significant statistical differences between experimental and control groups, nonparametric methods were used. The Kolmogorov-Smirnov test was used for the comparison of survival functions between sample groups,⁸⁴ and the Gehan-Breslow-Wilcoxon⁸⁵ and Mantel-Cox tests⁸⁶ were used for the comparison of median lifespan values. The significance of differences in maximum lifespan between groups was evaluated using the Wang-Allison test.⁸⁷ For this test, each animal in each experiment was categorized into one of two groups: either lifespan above the 90th percentile or lifespan below the 90th percentile. A two-by-two contingency table was used to record data. An ordinary χ^2 -test was used for independent testing of two groups. In addition, the α and R_0 parameters of the Gompertz equation [$l(x) = R_0 e^{-\alpha x}$] and the mortality rate doubling time [MRDT = $\ln(2)/\alpha$] were calculated, and the trajectories of the logarithmic mortality rate were plotted. The significance of differences in the age-dependent mortality rate and initial mortality rate (parameters α and R_0 in the Gompertz equation) were evaluated using the maximum likelihood method.⁸⁸ Statistical analyses was performed using Statistica version 6.1 (StatSoft, Inc.) and WinModest version 1.0.2⁸⁸ software.

Analysis of stress resistance. To evaluate stress resistance, 200–300 flies were selected (30 flies per vial) for each experiment. Males and females were examined separately. After 2–3 d, individuals were exposed to conditions of intense stress. To evaluate oxidative stress resistance, flies were starved for 2 h and then moved into vials where a filter paper had been soaked in a 20 mM paraquat (Methyl Viologen, Sigma) solution in 5% sucrose (1 ml), instead of the animals' normal nutrient environment. To evaluate hyperthermia resistance, flies were placed at a

temperature of 35°C in vials containing the standard agrarian-yeast nutrient environment. To estimate starvation resistance, *Drosophila* individuals were kept in vials where a water-soaked filter paper was used instead of the nutrient environment. Dead flies were counted twice daily. Survival curves were constructed and the percentages of dead flies after 48 h of exposure to specific stressors was calculated. To evaluate the difference between samples, the Fisher ϕ -test (for the comparison of percentage of dead flies after 48 h)⁸⁹ and the Kolmogorov-Smirnov test (to compare the distribution of mortality within the samples) were used.⁸⁴

***D-GADD45* in vivo reporter.** We used a 2-kb fragment upstream of the start site of the *D-GADD45* coding region to drive expression of the destabilized form of GFP (dGFP) in vivo. dGFP is only stable for 8 h and is therefore a suitable temporal marker of transcriptional activity.⁹⁰ To obtain transgenic flies containing a dGFP reporter of *D-GADD45*, 2 kb of DNA 5' of *D-GADD45* was cloned. This region included the ATG initiation codon located upstream of the *D-GADD45* gene. dGFP was followed with the SV40 transcriptional stop signal commonly used in most fly vectors. This reporter construct was cloned into an attB vector for site-specific integration into the second chromosome. Cloning, site-specific integration, balancing and embryo injections were performed by GenetiVision. The resulting *Drosophila* strain with the *D-GADD45* in vivo reporter was called D-GADD45-dGFP.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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