Chapter 2
Gadd45 Proteins in Aging and Longevity of Mammals and *Drosophila*

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Abstract Proteins of the GADD45 family play an essential role in the integration of cellular response to a wide variety of stressors and maintenance of homeostasis at the level of a cell, a tissue and an organism. The basic homeostatic processes are implicated in the determination of the progression of aging and development of major age-related disorders. Moreover, GADD45s mediate several well-known aging-associated signaling pathways through the interaction with such proteins as FOXO, p53, ATM, ATR, SIRT1, mTOR and some other. These reasons point out the role of the GADD45 proteins in the aging and life span regulation. Indeed, we have shown that constitutive and conditional (mifepristone-inducible) *D-GADD45* overexpression in *Drosophila melanogaster* nervous system extends median and maximum life span, and increases the resistance to genotoxic, oxidative, thermal stress, and starvation. The life span-extending effect was apparently due to more efficient recognition and repair of DNA damage, because the spontaneous DNA damage in the larva neuroblasts with *D-GADD45* overexpression was reduced. However, data obtained for flies with conditional ubiquitous *D-GADD45* overexpression demonstrates a negative effect of this intervention on the life span and stress resistance.

Keywords Aging · Age-related disease · Gadd45 proteins · Longevity · Stress resistance

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A.M. Vaiserman et al. (eds.), *Life Extension*, Healthy Ageing and Longevity 3,
DOI 10.1007/978-3-319-18326-8_2

2.1 Introduction

Proteins of the GADD45 family (Growth Arrest and DNA Damage-inducible) play an essential role in the integration of cellular response to a wide variety of stressors and maintenance of homeostasis at the level of a cell, a tissue and an organism (Liebermann and Hoffman 1994; Zhang et al. 1999; Fornace et al. 2002; Moskalev et al. 2012b; Salvador et al. 2013) as well as in the determination of aging-related processes and longevity (for review, see Moskalev et al. 2012b).

Majority of gerontogenes (genes whose activity determines organism life span) are members of conserved biological pathways across different groups of species (for review, see Moskalev et al. 2014). Likewise, GADD45 orthologs first appeared in molluscs, and were also found in anemones, polychaete worms, insects, fish, amphibians and mammals. The number of GADD45 proteins in each species varies from one in lower organisms to 5–6 in fish, and decreases to 2–3 in amphibians and mammals (*GADD45α*, *GADD45β* and *GADD45γ*). Additionally, several isoforms were described, which are generated as a result of alternative mRNA splicing (Flicek et al. 2011). This indicates that the GADD45 family is relatively "young" and has undergone duplications and deletions in the course of evolution (Moskalev et al. 2012b). In *Drosophila* there is a single ortholog of the *GADD45* family *D-GADD45* (*CG11086*) (Peretz et al. 2007).

The gerontogenes are classified as life span regulators, mediators, effectors, housekeeping genes, genes involved in mitochondrial function, and genes regulating cellular senescence and apoptosis (for review, see Moskalev et al. 2014). *GADD45s* can be relevant to the last category of regulatory genes. Protein products of *GADD45* genes are small (about 18–20 kDa), acidic (pH 4.0–4.2) proteins with high level of homology (55–57 % of identic aminoacids). Mainly, they have nucleus location and are associated with ribonucleoprotein speckles (Abdollahi et al. 1991; Zhang et al. 1999; Vairapandi et al. 2002; Sytnikova et al. 2011). GADD45 proteins form homo- and hetero-dimers and olygomers (Kovalsky et al. 2001; Schrag et al. 2008). The median half-life of the *GADD45* mRNA is unusually short (less than 1 h), suggesting a regulatory rather than metabolic function for GADD45 proteins (Sharova et al. 2009). They don't exercise enzymatic properties, and function through the interactions with other proteins and RNA (Sytnikova et al. 2011), or by way of modification of DNA/RNA accessibility for enzymes (Carrier et al. 1999; Moskalev et al. 2012b). The expression of the GADD45 proteins is detected in different tissues, including heart, brain, spleen, lungs, liver, skeletal muscles, kidneys, testicles, placenta (Zhang et al. 1999).

Most of the longevity genes described are related to stress response (for review, see Moskalev et al. 2014). GADD45 proteins are highly expressed after exposure to different physical, chemical and biological agents, and physiological factors. During stress response, they control such processes as DNA repair, cell cycle regulation, cellular senescence, apoptosis, inflammatory response, maintaining of the stem cell pool and cell differentiation. However, their inducibility is reduced with age (Edwards et al. 2004) which can be a reason of age-dependent descent of

resistance to spontaneous and induced stress influences, and organism survivability. Processes that provide basic homeostasis reactions are implicated in the determination of the progression of aging and age-related diseases (ARDs) (for review, see Moskalev et al. 2012b). Indeed, there are evidences that GADD45 proteins are involved in the development of major ARDs, including cancer, metabolic, cardiovascular and autoimmunity disorders (Budovsky et al. 2009; Wolfson et al. 2009; Moskalev et al. 2012b). Moreover, GADD45 activity is responsible for embryogenesis and ontogenesis, and its imbalance is implicated in the development of such pathologic reactions as preeclampsia (Xiong et al. 2009; Geifman-Holtzman et al. 2013). GADD45s mediate several well-known aging-associated signaling pathways through the interaction with such proteins as FOXO, p53, ATM, ATR, SIRT1, mTOR and some other (Furukawa-Hibi et al. 2002; Kobayashi et al. 2005; Bortoff et al. 2010; Moskalev et al. 2012b; Salvador et al. 2013). All these reasons point out the role of the GADD45 proteins in the aging and life span regulation. Recently, we have shown the life span-extending effects and stress resistance stimulation due to neuro-specific *D-GADD45* overexpression using the fruit fly *Drosophila melanogaster* (Plyusnina et al. 2011; Moskalev et al. 2012b; Plyusnina et al. 2012). This data confirm the involvement of GADD45 proteins in longevity determination, and suggest their ectopic expression can appear as beneficial method for the organism life span extension.

GADD45 family is evolutionary conserved in multicellular animals. In this chapter we considered participation of *GADD45* genes in different aging-related processes, based on the data obtained in different model objects. At the same time we focused on the results of our investigations, which demonstrate the role of *D-GADD45* gene in *Drosophila melanogaster* longevity.

2.2 Involvement of GADD45 Proteins in Stress Resistance Regulation

2.2.1 GADD45 in DNA Repair and Epigenetic Regulation

The expression of GADD45s is one of the critical conditions at the early stages of the DNA damage response with following activation of DNA repair machinery. The GADD45 promoters contain binding motifs for FOXO (AFX/FOXO4, FKHRL1/FOXO3A, and AKT/FOXO1 transcription factors), p53, p33 (ING1), p73, OCT-1, BRCA1. Their activation under stress conditions leads to GADD45 upregulation with subsequent stimulation of DNA repair, blockage of cell cycle in the G1/S and G2/M checkpoints, and apoptosis (Kastan et al. 1992; Guillouf et al. 1995; Vairapandi et al. 1996; el-Deiry 1998; De Laurenzi and Melino 2000; Jin et al. 2000; MacLachlan et al. 2000; Cheung et al. 2001; Jin et al. 2001; Furukawa-Hibi et al. 2002; Tran et al. 2002; Ju et al. 2014).

The role of GADD45 in DNA repair is supported by the studies on in vitro and in vivo models. *GADD45α*-null mouse embryo fibroblasts and *GADD45α*-deficient

human colon cancer cells exhibited slow base excision repair and delays removal of AP sites after treatment with methyl methanesulfonate (MMS) (Jung et al. 2007). The *GADD45a*$^{-/-}$ mice demonstrate genomic instability, reduced nucleotide excision repair, increased level of mutations and high susceptibility to chemical oncogenes (Hollander et al. 1999, 2001). The disrupted expression of *GADD45β* caused by the hepatitis C viral infection suppresses the DNA excision repair as well (Higgs et al. 2010). GADD45 proteins participate in base and nucleotide excision repair through the links with DNA repair enzymes and regulation of their activity. GADD45α and GADD45β interact with the apurinic/apyrimidinic endonuclease 1 (APE1) (Jung et al. 2007), xeroderma pigmentosum proteins XPC and XPG (Hartman and Ford 2002; Chang et al. 2003; Ma et al. 2009; Le May et al. 2010; Schäfer et al. 2010), and proliferating cell nuclear antigen (PCNA) (Smith et al. 1994; Hall et al. 1995; Vairapandi et al. 2000).

Another way of GADD45 involvement in DNA repair is its involvement in the repair-mediated active DNA demethylation (Barreto et al. 2007; Rai et al. 2008; Cortellino et al. 2011; Schomacher 2013). A possible GADD45-mediated demethylation mechanism involves nucleotide excision repair associated with the endonuclease activity of XPG protein. Specifically, 5-methylcytosine containing nucleotides could be recognized and removed through GADD45–XPG complex, ultimately resulting in the demethylation of CpG dinucleotides (Ma et al. 2009; Schmitz et al. 2009; Le May et al. 2010; Schäfer et al. 2010; Schomacher 2013). Additionally, GADD45 and XPG are involved in base excision repair, which could be another DNA repair mechanism associated with removal of methylated DNA (Jung et al. 2007). Additionally, GADD45 is able to bind histones and modify accessibility of damaged DNA for repair enzymes, and participates in chromatin decondensation (Carrier et al. 1999; Ma et al. 2009; Schomacher 2013). Thus, GADD45 recruits nucleotide and/or base excision repair factors to gene-specific loci and acts as an adapter between repair factors and chromatin, thereby creating a nexus between epigenetics and DNA repair (for review, see Niehrs and Schäfer 2012).

Recently, GADD45α was shown to bind RNA, forming ribonucleoprotein particles. GADD45 was detected inside nuclear speckles which are sites of active transcription, RNA splicing and processing (Sytnikova et al. 2011). Thus, GADD45 could exert its epigenetic effects both through active DNA demethylation, chromatin remodeling and post-transcriptional RNA regulation.

2.2.2 *GADD45 in Cell Cycle Regulation and Cellular Senescence*

ATM- and p53-mediated activation of GADD45 is essential for a DNA damage-induced G1 and G2/M cell cycle arrest during stress response (Wang et al. 1999). Thus, human and mouse GADD45-dificient fibroblasts and lymphocytes failed to arrest at G2/M after exposure to stress stimulus (Wang et al. 1999). Microinjection

of the *GADD45α* expression vector into human primary fibroblasts arrests the cells in G2/M phase (Wang et al. 1999). On the same hand, ectopic expression of GADD45α, GADD45β or GADD45γ in cancer cells (M1 human myeloblastic leukemia and H1299 lung carcinoma) leads to accumulation of cells arrested in the G1 phase that later underwent apoptosis (Zhang et al. 2001). Additionally, it was found that GADD45α expression and G1 cell cycle arrest are activated by c-Jun N-terminal kinase (SAPK/JNK) under fucoxanthin treatment of LNCap prostate cancer cells, while the inhibition of SAPK/JNK attenuated the induction of G1 arrest and GADD45α expression (Satomi 2012). To achieve cell cycle arrest, GADD45 proteins interact with the protein kinase cell division cycle 2 (Cdc2), Cyclin B1, PCNA and cyclin-dependent kinase inhibitor p21 (Liebermann and Hoffman 2003). The interaction of GADD45α and GADD45β with the Cdc2/Cyclin B1 kinase complex leads to its dissociation and following G2/M cell cycle arrest as well as the inhibition of Cdc2 kinase activity (Zhan et al. 1999; Zhang et al. 1999; Vairapandi et al. 2002; Hsu et al. 2014). The interaction of all three GADD45 proteins with p21 induces both the G1 and G2/M arrest (Smith et al. 1994; el-Deiry 1998; Xiong et al. 2009; Zhang et al. 2014a).

GADD45 mediates cellular senescence in the case of unrepaired DNA damages, through the cell cycle arrest in the G1 phase with following unresponsiveness to growth factors (for review, see Moskalev et al. 2012b). A significant increase in *GADD45α* expression was observed upon stress-induced cellular senescence triggered by hydrogen dioxide (Duan et al. 2005). On the other hand, ectopic expression of GADD45γ robustly elicited senescence in hepatocellular carcinoma cells and suppressed tumor growth in vivo (Zhang et al. 2014b). Induction of GADD45 expression with subsequent cellular senescence can be activated by p53-dependent and JAK/STAT3 signaling pathways (Jackson and Pereira-Smith 2006; Zhang et al. 2014b). GADD45-mediated cellular senescence involves an increased expression of p21, mitochondrial dysfunction and generation of reactive oxygen species through the GADD45/p38 MAPK/GRB2/TGFBR2/TGFβ signaling pathway (Passos et al. 2010; Zhang et al. 2014a).

2.2.3 GADD45 Role in Cell Death and Survival

GADD45 family members play a dual role during mediation of apoptosis associated with two major components—p38/JNK mitogen-activated kinase (MAPK) and NF-κB signaling pathways (Takekawa and Saito 1998; Harkin et al. 1999; Lu et al. 2001; Hildesheim et al. 2002; Yoo et al. 2003; Tront et al. 2006). In fact, the MAPK/GADD45/NF-κB axis responds to a variety of extracellular stimuli, converting them to intracellular responses (Yang et al. 2009; Moskalev et al. 2012b). It is noteworthy that GADD45 proteins and stress kinases form a feedback regulatory loop: the expression of GADD45 is also under the control from p38 and JUNK MAPKs. GADD45γ and GADD45β bind to MEK kinase 4 (MEKK4) and promote phosphorylation and activation of the p38 and JNK MAP kinases by

MEKK4 (Takekawa and Saito 1998). However, specific inhibitor of p38 MAPK SB202190 suppresses the expression of all three *GADD45* genes (Oh-Hashi et al. 2001). NF-κB and *GADD45s* form a positive feedback regulatory loop as well (Gupta et al. 2006).

In the case of irreparable damages, GADD45 proteins exert a pro-apoptotic function. For example, the GADD45 proteins mediate the endoplasmic reticulum stress-induced apoptosis in mouse liver cells (Ji et al. 2005). Ectopic expression of GADD45 triggers apoptosis via the TGFβ/MEKK4/p38/JNK pathway in human leukemic cells or in mouse hepatocytes (Selvakumaran et al. 1994; Yoo et al. 2003). At the same time, blocking of early expression of GADD45β suppresses the apoptosis induced by TGFβ in myeloid leukemia cells (Selvakumaran et al. 1994).

However, GADD45α and GADD45β also can fulfil an anti-apoptotic function. For example, their activity increases hematopoietic cell survival under UV-irradiation or treatment with certain chemotherapeutic drugs (Gupta et al. 2005). Bone marrow cells obtained from *GADD45α*$^{-/-}$ and *GADD45β*$^{-/-}$ mice show an impaired ability for differentiation and increased sensitivity to the induction of apoptosis after being stimulated by cytokines (Gupta et al. 2006). *GADD45α*-deficient E1A + Ras cells treated with HDAC inhibitors demonstrated a higher level of pro-apoptotic signals, whereas the anti-apoptotic program is suppressed (Igotti Abramova et al. 2014). Anti-apoptotic function of GADD45 is realized through two mechanisms: activation of the p38/NF-κB anti-apoptotic pathway by GADD45α (Zhang et al. 2005; Gupta et al. 2006) and inhibition of the MKK7/JNK pro-apoptotic pathway by GADD45β (Papa et al. 2004b; Tornatore et al. 2008). Additionally, interactions of GADD45 with PCNA may promote cell survival, apoptosis inhibition together with DNA repair (Vairapandi et al. 2000; Azam et al. 2001).

It should be noted that the MAPK-mediated effect of GADD45 activation on the apoptosis onset is cell type specific. For example, activation of p38 and JNK kinases by GADD45 is associated with apoptosis in endothelial and epithelial cells (Harkin et al. 1999; Hildesheim et al. 2002), whereas it increases survival of hematopoietic cells (Platanias 2003). Additionally, induction of GADD45β by NF-κB downregulates pro-apoptotic JNK signaling in mouse embryonic fibroblasts (De Smaele et al. 2001) and in hepatocytes during liver regenerations after partial hepatectomy (Papa et al. 2008).

2.2.4 GADD45 in Antioxidant System Regulation and Heat Shock Response

GADD45 proteins can be involved in the prevention of cellular damages and heat shock response induction. Indeed, it was found that *D-GADD45* gene was upregulated in fly heads after treatment with free radical inductor paraquat and high temperatures. Furthermore, flies with *D-GADD45* overexpression in the nervous system were more resistant to this stressor compared with ones without

overexpression (Moskalev et al. 2012a). In mammalian cells GADD45β-mediated activation of the protein kinases MEKK4 and JNK (Takekawa and Saito 1998; Papa et al. 2004a) increases the level of the ASK1 (Ko et al. 2001), which acts in opposition to the SOD1 protein. The GADD45 proteins affect the expression level of the transcription factors PPARγ (Hamza et al. 2009) and RXRA (Wu et al. 2004) as a part of JAK and MEK kinase signaling cascades, and participate in the induction of downstream antioxidant systems (SOD1, thioredoxin and glutaredoxin enzymes). 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 determine the fate of cells (Moskalev et al. 2012a).

Additionally, the transcription factors PPAR-γ and RXRA increase the expression of the heat shock protein HSP22 (Hamza et al. 2009). HSP22 is responsible for activating another heat shock protein, HSP27 (Sun et al. 2004) and following HSP70 induction (Whitlock et al. 2005). For example, GADD45 proteins can participate in the heat shock response through PPAR-γ and RXRA. Another way of involvement of the GADD45 family members in the heat shock protein activation is mediated by its interaction with the CDK1 protein kinase. All three GADD45 proteins bind and inhibit CDK1, which phosphorylates the transcription factor SP1 (Chuang et al. 2012). The tanscription factor SP1 activates the expression of heat shock proteins HSP27 and HSP60 (Reed et al. 2008; Friedman et al. 2009). Finally, GADD45s activates the transcription factor HSF1 by inhibiting the p38 protein kinase (Moskalev et al. 2012a). HSF1 is a key element for pathways activating heat shock proteins, such as HSP60, HSP90, HSPA4, HSP70, HSP27 and HSP22.

2.2.5 GADD45 in Inflammatory Response and Immunity

GADD45 proteins also contribute to cellular inflammation response and organism survival by modulation of the immune response (for review, see Schmitz 2013). It was shown using the *Drosophila* model, that the inflammation induced by bacterial infection increases both the level of *D-GADD45* mRNA and protein (Peretz et al. 2007; Moskalev et al. 2012a). The *GADD45* genes are induced by the pro-inflammatory transcription factor NF-κB (Balliet et al. 2001), cytokines including interleukins TNFα, TNFβ, IL-2, IL-6, IL-8, IL-12, IL-18 (Fan et al. 1999; Zhang et al. 1999; Yang et al. 2001; Salerno et al. 2012) and oncostatin M (Nakayama et al. 1999). The main function of GADD45 in the inflammation response is determined by its interactions with mitogen-activated protein kinase p38, cyclin-dependent kinase p34 (Yang et al. 2000), and PCNA (Smith et al. 1994). For example, after IL-12 and IL-18 treatment GADD45β activated p38 and selectively increased cytokine-induced interferon γ (IFNγ) production (Yang et al. 2001). Additionally, the GADD45 proteins affect the transcription of IFNγ by interacting with PCNA-p300 family (Nakayama et al. 2001). Regulation of this pathway

is mediated by interaction of GADD45s with the transcription factors PPARα, C/EBPβ and c-Jun (Moskalev et al. 2012a).

The GADD45β and GADD45γ proteins provide proliferation of T helper 1 (Th1) cells and induce the production of IFNγ in these cells (Yang et al. 2001). The essential role of GADD45 in elevating the level of IFNγ is evidenced by the absence of this process in *GADD45γ*- (Lu et al. 2001) and *GADD45β*-deficient mice (Ju et al. 2009). Furthermore, GADD45 proteins play an important role in the process of Th1-mediated anti-tumor immune responses (Ju et al. 2009) and in autoimmunity reaction development (Liu et al. 2005).

It was found that GADD45α and GADD45β are also essential for differentiation of bone marrow cells into macrophage and granulocyte. The *GADD45α*- and *GADD45β*-deficient mice were characterized by increased apoptosis during differentiation and reduced clonogenicity (Gupta et al. 2006). Additionally, GADD45s activation of the p38 kinase is implicated in the response of granulocytes to lipopolysaccharide (a component of gramm-negative bacterial cells) mediated chemotaxis, whereas Gadd45α and Gadd45β curtailment of JNK activation was linked to chemotaxis of macrophages in response to this inflammatory stimulus (Salerno et al. 2012). Moreover, Gadd45β regulates the autophagy process, a catabolic pathway that also degrades intracellular pathogens (Schmitz 2013). The Gadd45β-MEKK4 pathway specifically directs p38 to autophagosomes and mediate phosphorylation of the autophagy regulator autophagy-related 5 (ATG5) protein. This process results in an accumulation of autophagosomes through the p38-mediated inhibition of lysosome fusion (Keil et al. 2013).

2.3 Role of GADD45 Proteins in Aging and Life Span Regulation

The GADD45 family members are deeply implicated in the maintaining of cellular, tissue and organism homeostasis which determines the aging rate and longevity. Indeed, some well-known regulators of aging-associated processes and longevity are partner proteins for GADD45s (Budovsky et al. 2009; Wolfson et al. 2009; Moskalev et al. 2012b). The GADD45 proteins contain the FOXO- and p53-binding motifs (Kastan et al. 1992; Guillouf et al. 1995; el-Deiry 1998; Furukawa-Hibi et al. 2002; Tran et al. 2002; Ju et al. 2014), and are activated by the ATM- and ATR-dependent way (Kastan et al. 1992; O'Prey et al. 2003; Jang et al. 2010). In particular, RNA interference of FOXO3a leads to inhibition of *GADD45* stress-induced expression (Tran et al. 2002). Another example demonstrated that in human epithelial cells an inhibitor of ATM/ATR prevented induction of GADD45 and growth arrest by flavonoid treatment (O'Prey et al. 2003). The SIRT1 histone deacetylase is another key longevity regulator (Guarente 2011; Satoh et al. 2013; Hubbard and Sinclair 2014), that is involved in the GADD45 functioning regulation (Kobayashi et al. 2005; Scuto et al. 2013). The FOXO-mediated GADD45 induction was markedly impaired in cells, which depleted

SIRT1 expression by RNA-interference (Kobayashi et al. 2005). Additionally, there are evidences that *GADD45* expression is linked with the target of rapamycin (TOR) signaling. Insulin induces *GADD45β* transcription by activating the mTOR pathway (Bortoff et al. 2010), well known for its association with aging, longevity, and ARDs (for review, see Blagosklonny 2008; Zoncu et al. 2011). These examples of the relationship between *GADD45* family members and longevity-associated genes confirm their immixture in the life span control.

Another way of GADD45 participation in the aging and longevity determination at the tissue level is subjected by the role in stem cell pool maintenance (for review, see Moskalev et al. 2012b). The Gadd45 proteins participate also in the maintenance of the pool of myeloid quiescent stem cells. *Gadd45α* or *Gadd45β* deletion was shown to suppress the quiescent stem cell population or lower the survival rate of progenitor cells, leading to the depletion of the stem cell compartment, and to affect the clonogenic potential of these cells (Hoffman and Liebermann 2007). Gadd45γ is involved in stem cell pool maintenance as well. A possible regulatory mechanism of stem cell pool maintenance is mediated by the Nucleus accumbens-1 (NAC-1) protein which is important for self-regeneration and pluripotency of embryonic stem cells, negatively regulates the expression of Gadd45γ-interacting protein 1 (Gadd45γ-ip1), preventing its suppressive activity towards Gadd45γ (Jinawath et al. 2009).

Aging negatively affects the ability of cells to express GADD45 proteins in response to stress conditions. For example, treatment of cardiomyocytes of young mice with free radical inducer paraquat led to the significantly increased expression of GADD45 isoforms, but did not stimulate its expression in the myocardium of old animals (Edwards et al. 2004). Decreased inducibility of GADD45 members may have far-reaching consequences including genome instability, accumulation of DNA damage, and disorders in cellular homeostasis—all of which may eventually contribute to the aging process (for review, see Moskalev et al. 2012b).

The *GADD45* family members as well as longevity-associated genes are concerned with ARDs. One of the main implications of the GADD45 proteins in the ARDs is associated with the cancerogenesis determination (for review, see Liebermann et al. 2011; Hoffman and Liebermann 2013). It was shown that the *GADD45α*-deficient mice were characterized by genomic instability, increased sensitivity to cancerogenes, and high aptitude to ovarian, hepatocellular and vascular tumors (Hollander et al. 1999, 2001; Tront et al. 2006). Mice with the *GADD45β* gene knockout are more susceptible to ionizing radiation and chemical carcinogens, and display a lower immune response against implanted melanoma cells (Ju et al. 2009). In the in vivo model of Ras-overexpressing mice with different GADD45α expression levels (*Ras/GADD45α*$^{+/+}$, Ras/GADD45α$^{+/-}$, and Ras/GADD45α$^{-/-}$), it was shown that Ras-driven genesis and growth of breast tumors is a GADD45α-dependent process (Tront et al. 2006). Clinical patients with solid and hematopoietic cancers including breast, lung, nasopharyngeal, brain, liver, prostate cancer, and lymphoma showed disruption in *GADD45* expression pattern (Hoggard et al. 1995; Sun et al. 2003; Jiang and Wang 2004; Qiu et al. 2004; Ying et al. 2005; Cretu et al. 2009; Na et al. 2010; Liebermann et al. 2011; Hoffman and Liebermann 2013).

The main cause of *GADD45* expression loss in cancers is epigenetic modifications, particularly, DNA methylation (for review, see Moskalev et al. 2012b). For example, the methylation of the *GADD45γ* promoter was significantly higher in different types of cancers than in normal tissues (Zhang et al. 2010). On the other hand, treatment of cancer cells with DNA methyltransferase inhibitors restored *GADD45β* expression to its level in the non-tumorous cells (Qiu et al. 2004). One of the pathways that determine *GADD45* methylation is NF-κB signaling. This is supported by the effect of NF-κB inhibition in cancer cells which leads to the GADD45α- and γ-dependent induction of apoptosis and reduction in tumor growth (Zerbini et al. 2004). The NF-κB transcription factor induces the expression of proto-oncogene c-Myc, which binds to the GC-rich sites of the *GADD45* promoters and significantly reduces the *GADD45* inducibility in response to genotoxic stress (Amundson et al. 1998; Zerbini et al. 2004; Zerbini and Libermann 2005). It is known that hipermethylation of gene promoters provides the aging process as well (Muñoz-Najar and Sedivy 2011), thus methylation of the *GADD45* promoters can be involved in the age-dependent reduction of its expression and inducibility.

Conversely, ectopic expression of the GADD45 members blocks cell growth by arresting the cells in the G2/M phase (Zhu et al. 2009) and G1/G0 phase (Higgins et al. 2009), and/or induces apoptosis in human tumor cell lines (Zhan et al. 1994; Vairapandi et al. 1996; Zhang et al. 2001; Sun et al. 2003; Jiang and Wang 2004; Ying et al. 2005). For example, *GADD45β* overexpression in LβT2 mouse gonadotrope cells blocked tumor cell proliferation and increased rates of apoptosis in response to growth factor withdrawal (Michaelis et al. 2011). Anti-cancer activity of the GADD45 proteins is conditioned by its role in apoptosis and cell cycle control as well as in negative regulation of oncogenes, such as p63 and β-catenin (Hildesheim et al. 2004).

However, in some cases, GADD45α may exert a pro-cancer action, depending on the type of the oncogenic stimuli. For example, the Myc-driven breast cancer is promoted by GADD45α activity, which dramatically decreased level of the enzyme MMP10 and led to angiogenesis. In Myc expressing tumors loss of GADD45α was accompanied by apoptosis or cellular senescence (Tront et al. 2010).

Additionally, other ARDs are associated with changes in *GADD45* expression. The GADD45 proteins participate in the development of the nervous system during ontogenesis and provide long-term memory formation, as well as their deregulation results in neuronal pathologies including brain cancers, ischemia, insults, seizures, memory decline, autism, Alzheimer's disease, psychosis (for review, see Sultan and Sweatt 2013). For example, Alzheimer's disease patients are characterized by highly increased level of *GADD45* expression in neurons, that prevents neuronal cells from apoptosis induced by accumulation of β-amyloid (Torp et al. 1998; Santiard-Baron et al. 1999, 2001). The upregulation of GADD45 was also observed in the in vitro model (human neuroblastoma cells) of dopamine-induced neurotoxicity, which is a part of some neurodegenerative disorders (for example, Parkinson's disease) and normal brain aging (Stokes et al. 2002). The same

changes were found in cultures of human endothelial cells derived from atherosclerotic aorta or coronary arteries, as well as in the mouse model of atherosclerosis (Thum and Borlak 2008). Thus, the GADD45 proteins apparently are induced during neurodegenerative processes and atherosclerosis providing a vicarious protective mechanism.

Chronic inflammation is largely attributed to an age-related increase in pro-inflammatory cytokines TNFα, IL-1β, IL-6 and NF-κB (Finch 1990; Chung et al. 2009; Coppé et al. 2010), that induce GADD45 proteins. For example, the induction of GADD45 was observed in the course of liver inflammation (Gant et al. 2003). Additionally, the GADD45 proteins can be involved in the process of the epithelial to mesenchymal transition (EMT). The EMT is a crucial process in the development of different tissues in the embryo and its reactivation in the adult is a part of inflammatory responses useful for the healing damaged tissue. However, abnormality of its control leads to tumorogenesis and organ fibrosis development (López-Novoa and Nieto 2009). GADD45s closely cooperate with key EMT regulators NF-κB, β-catenin, and matrix metalloproteases (Moskalev et al. 2012b).

Aging-dependent induction of oxidative stress and inflammation processes contributes to a process known as immunosenescence. Immunosenescence manifests in a decreased immune responsiveness to foreign and self-antigens, leading to an increased susceptibility to infection, cancer and autoimmune diseases. A decreased ability to maintain tolerance against self-antigens may result in autoimmune disorders (for review, see Moskalev et al. 2012b). Mice with deficiency in *GADD45β* and *GADD45γ* spontaneously develop signs of autoimmune lymphoproliferative syndrome and systemic lupus erythematosus. The reduced inducibility of GADD45s in immune cells is one of the possible factors that increase frequency of autoimmune conditions in aging (Liu et al. 2005).

High GADD45 expression may sustain the age-related immune dysfunctions, particularly, rheumatoid arthritis (for review, see Lindstrom and Robinson 2010). It is known that infiltrated Th1 cells in the synovial fluid of patients with rheumatoid arthritis are resistant to apoptosis. This resistance is accompanied by the high levels of GADD45β resulting from stimulation by pro-inflammatory cytokines TNFα and IL-12 (Du et al. 2008). The activated Th1 cells avoid utilization, which leads to chronic inflammation and tissue destruction. The important role of GADD45β in this process also follows from the finding that silencing of GADD45β by RNA interference abolished the anti-apoptotic effect of rheumatoid arthritis synovial fluid (for review, see Moskalev et al. 2012b). Another disorder associated with elevated expression of GADD45α protein is preeclampsia. Inflammatory and immune activation in preeclampsia may function in a feedback loop to maintain elevated expression of GADD45α protein (Geifman-Holtzman et al. 2013). GADD45α activates Mkk3-p38 and/or JNK signaling that leads to immunological and inflammatory changes as well as to triggering the production of circulating factors such as sFlt-1 (Xiong et al. 2009; Geifman-Holtzman et al. 2013; Xiong et al. 2013).

In hepatocytes both injury and growth stimulation remarkably increase the expression of the GADD45β protein. In liver cancer, promoter methylation

frequently silences GADD45β, demonstrating a suppressive proapoptotic function. This contrasts with normal hepatocytes, where GADD45β facilitates cell survival, growth, and proliferation. GADD45β protects the liver through two ways: binding MKK7 to block damaging signal transduction or binding CAR to coactivate anabolic transcription (Tian et al. 2011; Tian and Locker 2013). Furthermore, the GADD45γ protein deregulation may be a reason of liver hypertrophy and liver tumor as well through the interaction with cyclins and cyclin-dependent kinase inhibitors (Ozawa et al. 2011).

Thus, GADD45 proteins are involved in major aging-associated conditions including oxidative stress, chronic inflammation, immunosenescence and fibroproliferative repair that contribute to the development of ARDs and aging progression (for review, see Moskalev et al. 2012b).

Aging-related changes in organism fertility can be caused by GADD45 expression alterations as well. In a model mice with deficiency of GADD45 isoforms, it was shown that GADD45s determine male fertility, testis development, and primary sex determination (Johnen et al. 2013).

In a view of its functions, it seems reasonable that *GADD45* overexpression might promote longevity, in particular, by increasing the efficiency of DNA repair (for review, see Moskalev et al. 2012b). Recently, we have confirmed this hypothesis in the *Drosophila melanogaster* model and have shown a life span-extending effects of *D-GADD45* overactivation in the nervous system (Plyusnina et al. 2011; Moskalev et al. 2012a; Plyusnina et al. 2012).

2.4 Life Span and Stress Resistance in Fruit Flies with *D-Gadd45* Overexpression

Research of the life span and stress resistance in model animals such as fruit fly *Drosophila melanogaster* with overexpressed investigated genes is a promising method to reveal their life span-extending properties. Therethrough, we investigated the effect of conditional and constitutive overexpression of the GADD45 gene both in the nervous system and the whole body on the life span and some age-dependent physiological parameters. To realize this aim the UAS/GAL4 system was used.

It was obtained that despite the fact that the overall spontaneous activity of the *D-GADD45* gene increases with age, the level of expression of this gene in the nervous system is practically eliminated (Moskalev et al. 2012a). 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. Moreover, flies with constitutive *D-GADD45* overexpression in the nervous system showed a reduction in mRNA levels of *D-GADD45* with age as well (Moskalev et al. 2012a) that may indicate an epigenetic cause of the low level of activity of this gene in old flies.

Peretz et al. (2007) have shown that *D-GADD45* overexpression in *Drosophila melanogaster* has diverse phenotypic manifestations depending on the target

tissue. Ubiquitous overexpression of this gene in *Drosophila* flies from the first stages of life cycle is lethal. Our investigations revealed that flies with conditional (mifepristone-inducible) ubiquitous *D-GADD45* overexpression in the imaginal developmental stage are viable, but characterized by 22–46 % decreased life span (Fig. 2.1) (our unpublished data) and low resistance to the acute γ-irradiation and oxidative stress induced by paraquat treatment. The reason of this effect may be an insufficient epigenetic regulation of *D-GADD45* activity. For example, in human fibroblasts increased activity of DNA repair genes slows the replicative senescence only under simultaneous overexpression of histone deacetylase SIRT6 (Mao et al. 2012). The *GADD45* expression is depends on the sirtuins activity as well (Kobayashi et al. 2005; Scuto et al. 2013). Another reason could be associated with a high energy rate of repair processes (Halmosi et al. 2001). Ubiquitous *D-GADD45* overexpression can lead to excessive energy expenditure, which violates other processes.

Opposite to ubiquitous overexpression, tissue-specific *D-GADD45* overexpression in the nervous system leads to life span prolongation. Our data indicate that constitutive *D-GADD45* overexpression in the *Drosophila* nervous system leads to median life span extension (by 22–77 %) in comparison with flies without overexpression (Fig. 2.2a). Furthermore, the maximum life span was also increased, which is an evidence of slowing down aging (Plyusnina et al. 2011). To avoid effect of heterosis we also studied the life span effects of the conditional (mifepristone-inducible) *D-GADD45* overexpression in the nervous system. We found that the median life span of individuals with conditional overexpression of the *D-GADD45* gene in the nervous system was higher in comparison with animals with the same genotype kept on a medium without mifepristone (by 3–102 %) (Fig. 2.2b) (Plyusnina et al. 2011). It must be noted that the life span-extending effect of *D-GADD45* overexpression in *Drosophila* nervous

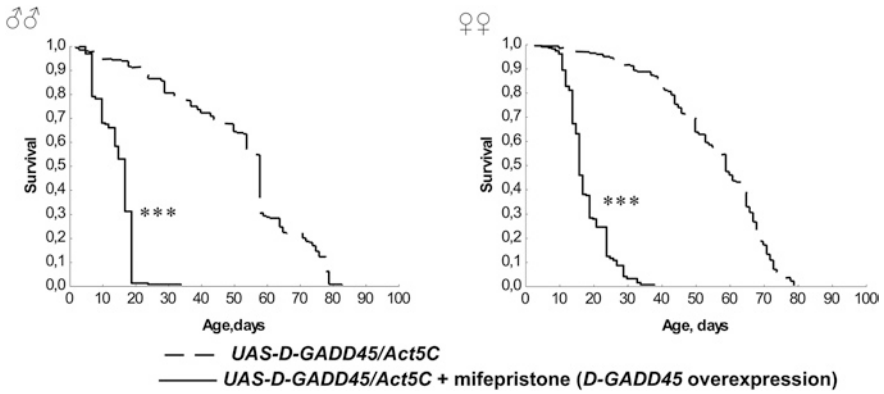

Fig. 2.1 Survival curves for *Drosophila melanogaster* males (♂♂) and females (♀♀) with the *UAS-D-GADD45/Act5C* genotype not treated with mifepristone and treated with mifepristone (conditional ubiquitous *D-GADD45* overexpression) (combined results of two replications), *** $p < 0.001$ (Kolmogorov–Smirnov test)

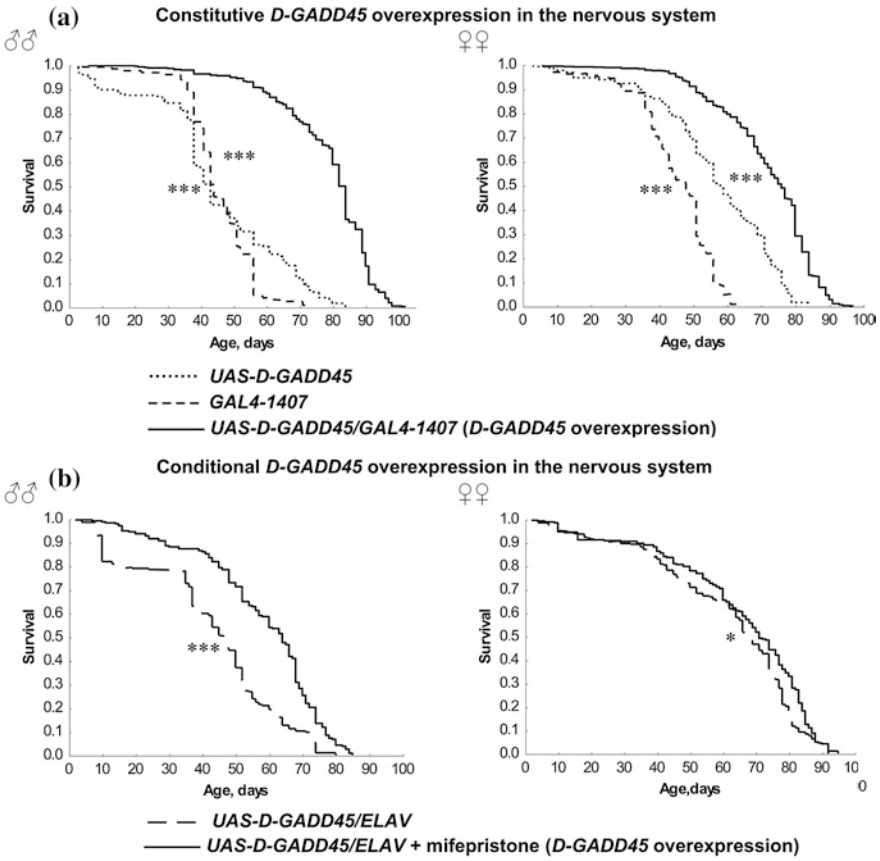

Fig. 2.2 Survival curves for *Drosophila melanogaster* flies with and without *D-GADD45* overexpression in the nervous system: **a** Survival curves for *Drosophila melanogaster* males (♂♂) and females (♀♀) with the parental *UAS-D-GADD45* and *GAL4-1407* genotypes and constitutive *D-GADD45* overexpression in the nervous system (combined results of three replications), **b** Survival curves for *Drosophila melanogaster* males (♂♂) and females (♀♀) with the *UAS-D-GADD45/ELAV* genotype not treated with mifepristone and treated with mifepristone (conditional *D-GADD45* overexpression in the nervous system) (combined results of two replications), * $p < 0.05$, *** $p < 0.001$ (Kolmogorov–Smirnov test) (Plyusnina et al. 2011)

system was not accompanied by decreases in fertility and locomotor activity parameters (Plyusnina et al. 2011). We proposed that *D-GADD45* overexpression causes more effective functioning of stress response mechanisms. Indeed, the DNA comet assay showed that neuroblasts of third-instar larvae with *D-GADD45* overexpression had the decreased DNA damage level (by 21–27 %). Therefore, overexpression of the *D-GADD45* gene in *Drosophila* nervous system results in more efficient recognition and elimination of spontaneous DNA damage caused by physiological processes and environmental factors (Plyusnina et al. 2011).

In further experiment we found additional evidences of increased resistance of *Drosophila melanogaster* individuals with constitutive and conditional *D-GADD45* overexpression in the nervous system. In most cases, these flies are characterized by increased survival under conditions of genotoxic stress (chronic and acute γ-irradiation), oxidative stress (paraquat), hyperthermia and starvation (Figs. 2.3, 2.4 and 2.5) (Moskalev et al. 2012a). Additionally, the involvement of the *D-GADD45* gene in the formation of biological responses to γ-irradiation was shown in the experiment on the fruit flies with homozygous and heterozygous *D-GADD45* mutation. Our results revealed the effects of hormesis and radioadaptive response for the wild-type flies irradiated by the low 40 cGy dose. Over against, the *D-GADD45* mutations led to elimination of these reactions (Moskalev et al. 2012a).

Thus, ubiquitous *D-GADD45* overexpression leads to decrease of life span and stress resistance. At the same time, neuron-specific overexpression of the

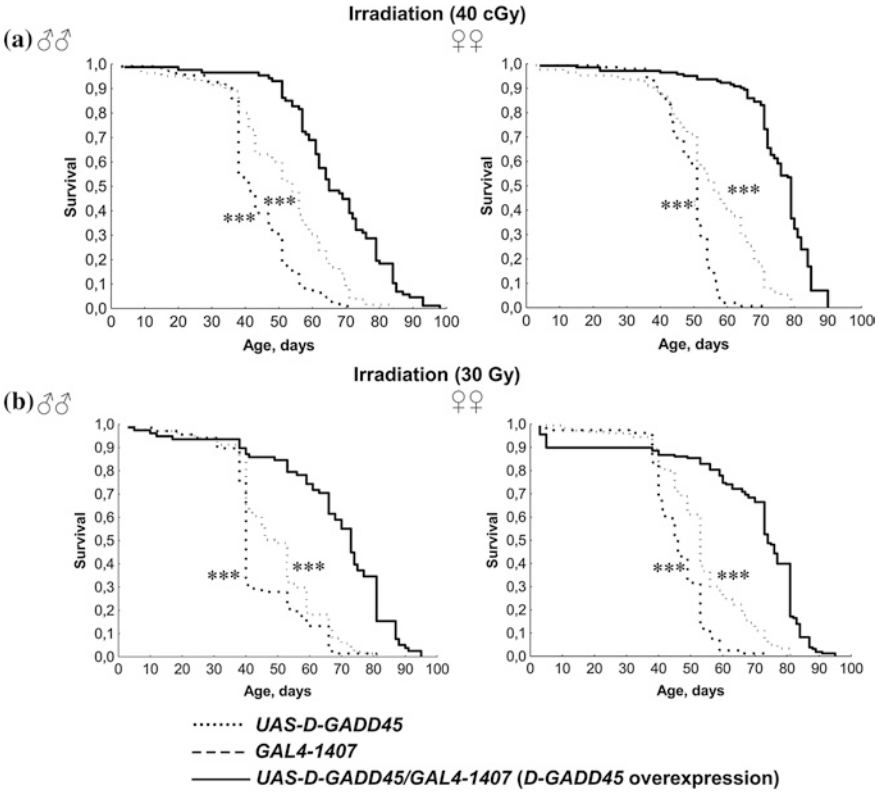

Fig. 2.3 Survival curves of *Drosophila* males (♂♂) and females (♀♀) with the parental *UAS-D-GADD45* and *GAL4-1407* genotypes and constitutive *D-GADD45* overexpression in the nervous system under different irradiation conditions: **a** chronic 40 cGy γ-irradiation, **b** acute 30 Gy γ-irradiation, *** $p < 0.001$ (Kolmogorov–Smirnov test) (Moskalev et al. 2012a)

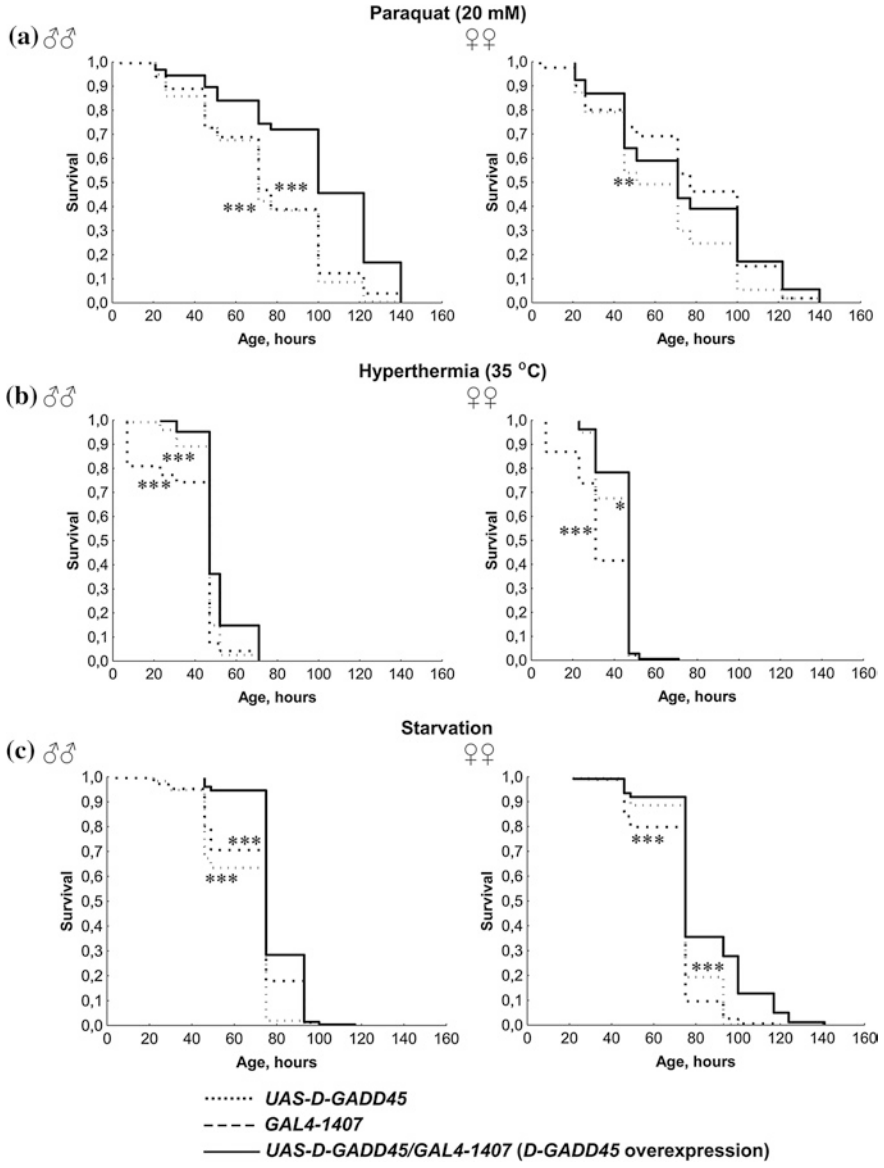

Fig. 2.4 Survival curves of *Drosophila* males (♂♂) and females (♀♀) with the parental *UAS-D-GADD45* and *GAL4-1407* genotypes and constitutive *D-GADD45* overexpression in the nervous system under different stress conditions: **a** paraquat (20 mM), **b** hyperthermia (35 °C), **c** starvation, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Kolmogorov–Smirnov test) (Moskalev et al. 2012a)

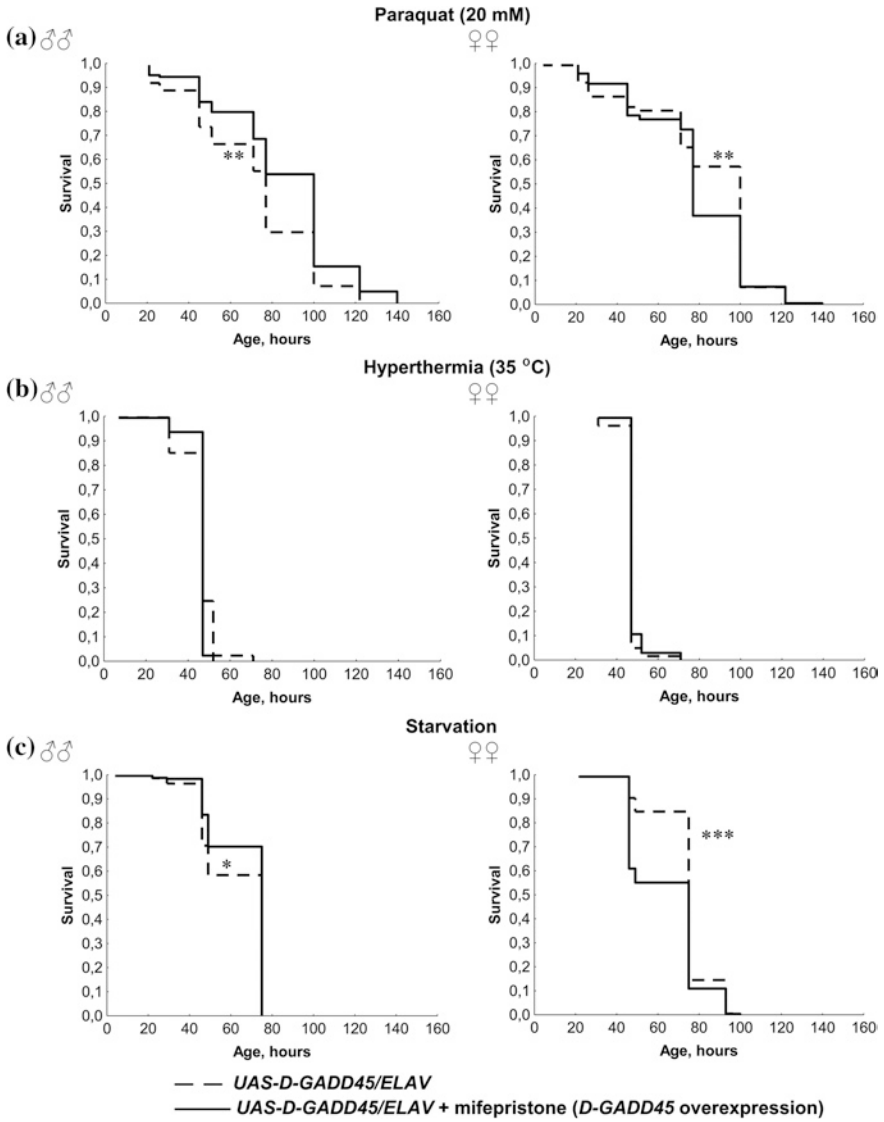

Fig. 2.5 Survival curves for *Drosophila melanogaster* males (♂♂) and females (♀♀) with the *UAS-D-GADD45/ELAV* genotype not treated with mifepristone and treated with mifepristone (conditional *D-GADD45* overexpression in the nervous system) under different stress conditions: **a** paraquat (20 mM), **b** hyperthermia (35 °C), **c** starvation, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Kolmogorov–Smirnov test) (Moskalev et al. 2012a)

D-GADD45 gene demonstrates a high life span-extending potential of controlled manipulation with this gene.

2.5 Concluding Remarks

Proteins of the GADD45 family are essential for stress resistance, and display antiaging and prolongevity activities (Fig. 2.6). Particularly, GADD45s provide a maintenance of basic homeostatic reactions and regulate a balance between cell (DNA) repair, eliminating (apoptosis) or preventing the expansion of potentially dangerous cells (cell cycle arrest, cellular senescence), maintaining of the stem cell pool and cellular differentiation. These processes provide survival of cells from different tissues and contribute to tissue regeneration. In turn, a decreased inducibility of the GADD45 family members may have far reaching consequences including genome instability, accumulation of DNA damage, and disorders in cellular homeostasis. All these negative processes may eventually lead to the age-dependent decline of organism system and organ functioning, aging progression, and promotion of carcinogenesis and development of other ARDs. It must be noted, that the GADD45 protein members are deeply involved in major longevity-associated signaling pathways, which confirm their role in aging and longevity determination.

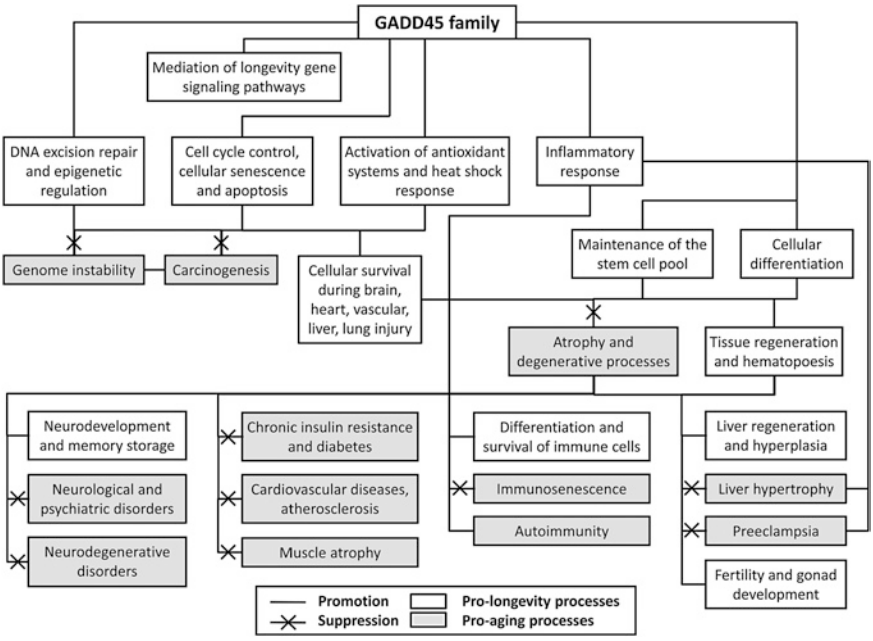

Fig. 2.6 The main anti-aging and pro-longevity activities of Gadd45 family

Investigations carried out in *Drosophila melanogaster* model disclosed the life span-extending activity of the *D-GADD45* gene due to its neuron-specific overactivation (Plyusnina et al. 2011, 2012) but not ubiquitous overexpression. It was shown that both constitutive and conditional *D-GADD45* overactivation in *Drosophila* nervous system extends median and maximum life span without negative changes in fertility and locomotor activity. This effect is apparently conditioned by elevated efficiency of recognition and elimination of spontaneous DNA damages. Additionally, neuron-specific *D-GADD45* overexpression can stimulate the resistance to different stress agents including genotoxic (γ-radiation), oxidative (paraquat) and thermal stressor as well as starvation.

Obtained results suggest that controlled manipulations of GADD45s and its interacting partners may also bring benefits to humans. Indeed, the increased GADD45 expression can be induced by several anti-tumor, anti-oxidant, anti-inflammatory pharmacological agents with potential life span-extending action, such as troglitazone (Yin et al. 2004), arsenic trioxide (Li et al. 2003), cucurbitacin E (Hsu et al. 2014), xanthatin (Takeda et al. 2011, 2013), quercetin (Yoshida et al. 2005), fucoxanthin (Kumar et al. 2013), epicatechin (Saha et al. 2010), ibuprofen (Bonelli et al. 2011). Thus, future studying the GADD45 family may provide prosperous therapeutic targets for promoting longevity and combating ARDs, as well as for stimulation of organism stress-resistance and enhancement of survivability.

Acknowledgments This work was supported by Russian Science Foundation grant N 14-50-00060.

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