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GADD45 proteins: central players in tumorigenesis

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Abstract

The Growth Arrest and DNA Damage-inducible 45 (GADD45) proteins have been implicated in regulation of many cellular functions including DNA repair, cell cycle control, senescence and genotoxic stress. However, the pro-apoptotic activities have also positioned GADD45 as an essential player in oncogenesis. Emerging functional evidence implies that GADD45 proteins serve as tumor suppressors in response to diverse stimuli, connecting multiple cell signaling modules. Defects in the GADD45 pathway can be related to the initiation and progression of malignancies. Moreover, induction of GADD45 expression is an essential step for mediating anti-cancer activity of multiple chemotherapeutic drugs and the absence of GADD45 might abrogate their effects in cancer cells. In this review, we present a comprehensive discussion of the functions of GADD45 proteins, linking their regulation to effectors of cell cycle arrest, DNA repair and apoptosis. The ramifications regarding their roles as essential and central players in tumor growth suppression are also examined. We also extensively review recent literature to clarify how different chemotherapeutic drugs induce GADD45 gene expression and how its up-regulation and interaction with different molecular partners may benefit cancer chemotherapy and facilitate novel drug discovery.

Keywords

GADD45 family; cancer; apoptosis; survival

Introduction

The Growth Arrest and DNA damage-inducible 45 (GADD45) gene family encodes three related GADD45 proteins, GADD45α, β, and γ. GADD45α was the first member identified

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Conflict of interest
The authors have no conflict of interest to declare.

[1] by screening a cDNA library of increased transcripts after ultraviolet (UV) irradiation of Chinese hamster (CHO) cells [1, 2]. GADD45β, also known as Myd118, was identified as a primary response gene transiently induced by IL-6 in myeloid leukemia cell lines [3]. The third member of the family, GADD45γ, was first described as an IL-2-inducible gene and named CR6 [4], but it is also known as OIG37, an oncostatin-M inducible gene [5], and as GRP17, gadd-related protein 17kDa [6].

GADD45 proteins are small (~18kD), with high homology among the multiple members, and localize to both the nucleus and cytoplasm, with low abundance in normal cells [3, 7, 8]. A comparison of the human GADD45 amino acid sequences demonstrates that they have around 55% similarity to each other (Figure 1A), and are evolutionary highly related (Figure 1B).

GADD45 proteins functions are similar, but not identical, and their induction differs under diverse physiological conditions or in different cell types. GADD45 proteins are ubiquitously detected in normal adult and fetal tissues, especially in quiescent cellular populations, and are highly expressed during the G1 phase of the cell cycle, with significantly reduced protein expression during the S phase [9]. Analysis of all GADD45 protein levels in a variety of murine tissues indicates that GADD45γ is highly expressed in skeletal muscle, kidney and liver, with low expression in heart, brain, spleen, lung and testis; GADD45β is detected predominantly in the lungs with moderate levels in skeletal muscle and liver, and low levels in kidney, spleen, brain, heart and testis; and GADD45α is highly expressed in kidney and skeletal muscle, moderately expressed in spleen, heart, lung, brain and liver, with low levels in testis [7]. GADD45 proteins play important roles in cellular genotoxic and non-genotoxic stress responses acting as stress sensors and tumor suppressors. After DNA damage all the GADD45 protein family members are rapidly induced, resulting in cell cycle arrest and/or apoptosis, or they actively participate in DNA repair mechanisms. GADD45 proteins have been extensively investigated due to their relevance in cancer development and progression [10]. Evidence has also been provided that the anti-cancer activity of chemotherapeutic agents [11] and Non-steroidal anti-inflammatory drugs (NSAIDS) [12] rely on GADD45 up-regulation for induction of cell cycle arrest and apoptosis in tumor cells.

In this review, we analyze in-depth the molecular structure of GADD45 proteins and the mechanism(s) by which interactions with their molecular partners modulate cancer cell cycle arrest, apoptosis and DNA repair. We review the relevance of GADD45 deregulation in different types of cancer and the molecular mechanisms involved in the induction of GADD45 expression by several chemotherapeutic drugs.

Protein-protein interactions of GADD45 proteins and the role of structure in mediating GADD45 function and activity

Insights into the function of any given protein are enhanced by elucidation of its molecular structure. In 2008, the structure of the human GADD45α protein was obtained by nuclear magnetic resonance (NMR) [13] and mouse GADD45γ protein was resolved by crystallography [14] demonstrating that these two proteins share similar secondary structures, presenting 5 α-helices and 5 β-sheets (Figure 2). These highly conserved proteins appear to exert similar functions and interact with similar sets of proteins, including CDK1 [15-17], PCNA [18-20], $p21^{waf/cip/mda-6}$ [17, 21], and MTK1 [22].

GADD45 dimerization

GADD45 proteins can interact with each other and form homo- and hetero-dimers, and these interactions may play a pivotal role in their functions. Initially the GADD45α region,

comprising the amino acids residues 33-61 and 132-165, was shown to be essential for oligomerization [23]. However, crystallography and mutational analyses indicate that mouse GADD45γ α-helices 2 and 3 in the central region (amino acids 43-87) of the protein are essential for dimerization, while mutation of L80, present in α-helix 3, blocks protein dimerization and inhibition of cell growth [14]. Schrag et al. (2008) point out that most of the interaction domains reported in the literature may not be actual interaction regions, but rather the dimerization domain present in the central region of the GADD45 proteins is crucial for protein interactions. As discussed further below, mutational analysis reveals that GADD45 protein interactions with CDK1, MTK1, $p21^{waf/cip/mda-6}$, p38 and PCNA map to the dimerization region. The relevance of GADD45 oligomerization has been demonstrated in experiments using GADD45 mutants lacking the region between amino acids 62-67 [17]. These mutants exhibit reduced interaction with $p21^{waf/cip/mda-6}$, CDK1 and PCNA.

Interaction of GADD45 with the upstream kinase MTK1/MEKK4

Interestingly, all three GADD45 family members directly interact with the upstream kinase MTK1/MEKK4 in response to environmental stresses, resulting in apoptosis induction through the p38/JNK pathways [22]. The GADD45β N-terminal amino acid residues (13-132) are essential to disrupt the auto-inhibitory domain of MTK1, leading to dimerization and phosphorylation at Thr1493, which activates the MTK1 kinase domain [22, 24, 25]. Additionally, GADD45α directly interacts with p38 MAPK kinase, which is dependent on the region comprising amino acid residues 71-96, and kinase activation is dependent on the residues between amino acids 71-124 [26].

Interaction of GADD45 with the upstream kinase MKK7

Contrary to its pro-apoptotic activity, GADD45β has been described to participate in pro-survival events through association and inhibition of another MAP kinase, MKK7, which is activated by MTK1/MEKK4, having the JNK kinase as a downstream target. GADD45β putative helices α–3 and α–4 and loops 1 and 2 (comprising amino acid residues 69-113 in the central region) are essential for the inhibition of MKK7. While helix α–3 seems to directly interact with MKK7, loops 1 and 2 and helix α–4 appear to inactivate MKK7 kinase activity by occupying the ATP-binding site, which results in a conformational change and inhibition of MKK7 catalytic function [27].

Interaction of GADD45 with PCNA

GADD45α and β participate in the DNA repair machinery, in nuclear excision repair (NER) through interaction with the Proliferating Cell Nuclear Antigen (PCNA), in recruitment to the DNA lesion sites and in modulating access of DNA to the repair proteins [28, 29]. The GADD45α N-terminal region (comprising amino acid residues 1-94) was identified as essential for binding with PCNA [18]. However, conflicting results from other studies demonstrated that the interaction of GADD45α and β with PCNA is mediated by their C-terminal regions (amino acids 137–165 and 114–156, respectively) [19]. Furthermore, it was shown that the central and C-terminal regions (residues 76-159) of GADD45γ are important for its interaction with PCNA [20]. One important aspect to be considered is that deletion of amino acids residues 1-94 results in disruption of the central portion of the protein responsible for GADD45 dimerization [14], and consequently causes a loss of activity and binding to other proteins, which can explain the differences observed in the various reports.

GADD45 activity is critical for genotoxic stress and DNA repair

The GADD45 family members are regulated by DNA damage mediated by alkylating agents, serum depletion or UV radiation [1, 2]. In this context, GADD45 proteins play a crucial role in preventing transformation of normal into malignant cells [1, 2]. Different

molecular contributors either inducing or stabilizing its transcripts regulate GADD45 gene expression. Among these proteins, p53, BRCA1 and several other tumor suppressors have been described to increase GADD45 expression [30-35].

Regulation of GADD45 by p53

Sequence comparison of the human, mouse and hamster GADD45α genes reveals a high level of conservation among the three species within the 1500 bp promoter region (75% homology) and in the third intron (92% homology) [36], where p53 can bind after treatment with UV, the alkylating agent methylmethane sulfonate (MMS) or ionizing radiation (IR) [30, 37-39]. Even though the GADD45α promoter region lacks binding sites for p53 it exhibits reduced levels of activity in cells with a negative p53 status after exposure to MMS, UV radiation and serum depletion [40].

GADD45 induction mediated by p53 appears to be dependent on the specific DNA damaging agent. GADD45α induction by IR is abrogated in myeloid leukemia cell lines with a deleted p53 or heterozygous allele [41], while MMS, UV radiation and serum depletion are still able to induce GADD45 expression in breast and colon cancer cell lines with a negative p53 status [40]. The GADD45 promoter contains binding sites for WT1 and Egr-1 transcription factors that interact with p53. GADD45 promoter activity is augmented by co-expression of p53 and WT1, but not by p53 alone [42].

Mutations in the p53 gene are common and range from 20 to 25% in advanced prostate cancers [43]. While p53 plays an important role in GADD45 induction, it has been demonstrated that activation of JNK-mediated phosphorylation of p53 by GADD45 is essential for the stabilization of a mutant form of p53 (altered in residues 274 and 223) and for mediating an apoptotic response [44]. In this context, GADD45α acts as an upstream effector in stabilizing p53 following DNA damage, thereby defining a positive feedback in the activation of the p53 pathway [45]. The co-dependence of p53 and GADD45α is evidenced by suppression of p53 phosphorylation (Ser15) mediated by zinc in GADD45α null cell lines [46], and reduced GADD45 gene induction mediated by zinc in cells treated with p53 inhibitors [47].

Regulation of GADD45 by BRCA1

The tumor suppressor BRCA1 is a nuclear phosphoprotein with a ring-finger in its N-terminal region and a transcription activation domain in the C-terminal region [48]. BRCA1 is a potent GADD45α inducer [31] relying on at least three essential motifs in the GADD45α gene sequence: (i) a BRCA1 binding site located in the first intron [31], (ii) a ZBRK1 binding site in the third intron [33], and (iii) Oct-1 and CCAAT-box elements in the proximal promoter region of the GADD45α gene between -121 to -75 [32]. GADD45 promoter induction by BRCA1 occurs through a p53-independent mechanism [32].

Oct-1 and NF-YA transcription factors have been shown to be important in GADD45 promoter activation by UV, MMS [34, 49], and Trichostatin A [50]. GADD45 third intron activation mediated by BRCA1 is regulated by Zinc finger and BRCA1 interacting protein with a KRAB domain 1 (ZBRK1), which encodes a 60-KD protein containing a BRCA1 binding site, a zinc finger domain, and a KRAB (Krüppel-associated box) domain. ZBRK1 interacts directly with BRCA1 and represses the transcriptional activation of GADD45 [33]. ZBRK1 is degraded in the presence of UV and MMS, establishing a link between environmental stress and GADD45 expression during both UV exposure and MMS treatment [51]. Similar to ZBRK1, BRCA1 associated RING domain (BARD1), another inhibitor of BRCA1 transcriptional activity, represses BRCA1-mediated trans-activation of

the GADD45 promoter, even though BARD1 increases the accumulation of BRCA1 in the nucleus [35].

Regulation of GADD45 by NF-κB

NF-κB has been implicated in tumorigenesis, cancer cell survival, invasion and metastasis [52]. Regulation of GADD45α and γ expression by NF-κB is mediated by upregulation of c-myc, another oncogene and transcription factor frequently overexpressed or translocated in a variety of cancers [53]. C-myc inhibits the GADD45 promoter by its interaction with the GC-rich region, which contains the WT1 and Egr motifs [54], by a post-RNA polymerase II recruitment mechanism [55]. C-myc also inhibits induction of GADD45 mediated by CCAAT/enhancer-binding protein alpha (C/EBPa) [56]. In addition, inhibition of NF-κB has also been demonstrated to increase GADD45α mRNA stability by interacting with nucleolin [57]. The main players involved in GADD45 expression are depicted in Figure 3.

In contrast to GADD45α and γ, GADD45β expression is induced by NF-κB via direct interaction within NF-κβ elements present in the GADD45β promoter [58]. However, GADD45β gene expression is also induced by other transcription factors. Analysis of colon cancer cell lines showed that activation of the GADD45β gene by MMS relies on the transcription factors NFY, SP1 and Egr1 [59].

GADD45 and DNA repair

The role of GADD45 proteins in the DNA repair machinery is still not clear. It has been reported that GADD45 null mice display increased sensitivity to radiation carcinogenesis, genomic instability and chromosome abnormalities, characterized by aneuploidy, centrosome amplification, aberrant mitosis and cytokinesis [60]. Additionally, GADD45 null mice exhibit a reduction in NER activity, an increase in mutation levels and enhanced sensitivity to chemically-induced carcinogenesis [61]. These deleterious features are postulated to be related to the lack of GADD45-mediated DNA repair.

GADD45-mediated DNA repair events may involve the interaction of GADD45 proteins with PCNA. PCNA changes its nuclear localization from DNA replication sites to DNA damage sites after exposure to DNA damaging agents [62], provoking changes in NER, mismatch repair (MMR) and base excision repair (BER) machinery [63]. While GADD45 interaction with PCNA stimulates the process [29], silencing of GADD45 genes abrogates PCNA presence in the DNA damage sites and reduces DNA-repair [28].

In another mechanism, GADD45 is recruited to mononucleosomes, which have been altered by histone acetylation or UV radiation, where it interacts with core histones causing alteration in DNA accessibility and facilitating the relaxation and cleavage activity of topoisomerase [64].

GADD45 effects on methylation

The role of the GADD45 family of genes in demethylation processes is controversial and appears linked to NER. The DNA demethylation process occurs by conversion of the cytosine group to thymine, and excision of the mismatched base followed by DNA repair [65]. GADD45 activates the expression of methylation-silenced reporter genes and induces global DNA hypomethylation, while GADD45 inhibition induces DNA hypermethylation. Interestingly, GADD45-mediated DNA demethylation is an active mechanism, which does not rely on cell replication. GADD45 interacts with the DNA repair endonuclease XPG, which acts as an endonuclease during NER activating methylation-silenced reporter genes [66]. Analysis of DNA demethylation in a Zebrafish model enhanced understanding of this process. The Zebrafish contains 6 GADD45 family members, GADD45α, β, and γ, and

GADD45α, β, and γ-like proteins. Overexpression of GADD45α increased the level of demethylation, while silencing of the GADD45 and GADD45-like proteins reduced demethylation [65].

Activation induced deaminase (AID) and Apolipo-protein B RNA-editing catalytic component-1 (Apobec-1) are members of the cytidine deaminase family. These proteins participate in the deamination of 5-meC, generating a thymine in just one of the DNA strands and causing a mismatch that should be repaired [67]. Co-expression of GADD45, AID and methyl-binding domain protein 4 (Mdb4) increases the levels of demethylation, and the overexpression of GADD45 upregulates the expression of the AID/Apobec family [65]. Rai et al. (2008) proposed a model of DNA demethylation, in which GADD45 interacts with AID, which deaminates the methylated cytosine and converts it into a thymine. After Mdb4 excises the mismatched base, the DNA repair machinery promotes the replacement of the unmethylated base [65]. Recruitment of GADD45α to methylation sites may involve TAF12, a TATA-binding protein (TBP)-associated factor, which has been shown to recruit GADD45α to rDNA-methylated sites. GADD45α then recruits the DNA repair machinery promoting demethylation [68]. A role for GADD45 in mediating demethylation is still an area of debate, since neither the demethylation activity of GADD45 nor the correlation between expression of GADD45 and DNA demethylation could be reproduced in subsequent studies [69, 70].

The role of GADD45 proteins in cell cycle arrest

Cell cycle arrest is an essential cell-defense mechanism to preserve genome integrity and prevent replication of damaged DNA and propagation of misleading information. Moreover, genomic instability due to loss of cell cycle checkpoints is frequently associated with tumorigenesis [71, 72]. The progression of cell cycle through G1, S, G2 and M phases occurs in an orderly fashion and is controlled by regulatory proteins, such as the Ser/Thr kinases, cyclin-dependent kinases (CDK) and Cyclins [73]. While CDK protein expression is maintained at a stable rate, cyclin levels fluctuate during cell cycle, activating the CDKs and promoting phosphorylation of proteins that lead to progression of cell cycle [74-77].

Interaction of GADD45 with the CDK1/Cyclin B1 complex induces G2/M cell cycle arrest

The CDK1/Cyclin B1 complex, which is responsible for the G2/M checkpoint [78], is the main target of GADD45-mediated cell cycle arrest. GADD45 induction of cell cycle arrest leads to a reduced cell proliferation rate, in both normal and cancer cells. GADD45α, β [79] and γ [7] have been shown to be firmly involved in this process. Overexpression of GADD45 α, β and γ suppresses cell proliferation in numerous cell lines, without evidence of apoptosis induction [5, 80].

Silencing of the GADD45α gene in human and murine cell lines leads to an impaired G2/M arrest mediated by UV or MMS, but not by IR. Indeed, the most prominent action of GADD45 proteins in regulation of the cell cycle is observed at the G2/M checkpoint by disrupting the CDK1/Cyclin B1 complex [81]. GADD45 proteins interact directly with CDK1, but not with Cyclin B1, inhibiting the kinase activity of the CDK1/Cyclin B1 complex, but not the CDK2/Cyclin E complex [15]. GADD45α and β disrupt the CDK1/CyclinB1 complex, while GADD45γ inhibits the complex without disrupting interaction [8]. A GADD45α mutant lacking the central region (amino acids 71-124) of the protein is unable to interact with the CDK1/Cyclin B1 complex and inhibit its kinase activity [15]. It was later found using GADD45 deletion mutants that interaction of GADD45 with the CDK1/CyclinB1 complex involves a central region (amino acids 65-84) of the GADD45 proteins, which is also essential for interaction with $p21^{waf/cip/mda-6}$ [21]. Additionally, GADD45 proteins lacking the region between amino acid residues 50-76 are unable to

induce G2/M arrest in fibroblast cells and lack inhibitory effects on the CDK1/CyclinB1 complex [17]. However, further work is necessary to comprehend the molecular mechanisms used by GADD45 proteins to inhibit CDK1/CyclinB1 complex activity and induce G2/M arrest. Initial studies indicated that this process was p38-independent [80]. However, conflicting reports demonstrated that GADD45α- and γ-mediated cell cycle arrest depends on the activation of the JNK and p38 pathways [82]. Furthermore, GADD45β and γ have been shown neither to induce cell cycle arrest nor to inhibit CDK1 kinase activity [17]. The different results observed may be due to disparate experimental conditions as well as dissimilar cell types employed by the investigators.

GADD45 induces G1/S cell cycle arrest through CRIF1

The modulation of the G1/S transition by GADD45 proteins relies on a different mechanism. CR6 interacting factor 1 (CRIF1) co-localizes in the nucleus with GADD45γ, and its overexpression inhibits cell cycle progression at the G1/S phase, and increases GADD45α- and γ mediated inhibition of the CDK1/Cyclin B1 complex [83]. CRIF1 has also been found to inhibit androgen-induced proliferation and cell cycle progression at the G1/S phase in prostate cancer cells [84].

The cyclin-dependent kinase $p21^{waf/cip/mda-6}$ has been implicated in cell cycle checkpoint regulation through interaction with GADD45 proteins. $p21^{waf/cip/mda-6}$ interacts with CDK/Cyclin complexes [85], activating the G1/S checkpoint. GADD45α, β and γ [9, 86, 87] interact with $p21^{waf/cip/mda-6}$ leading to cell cycle arrest at both the G1/S and G2/M transitions.

Apoptosis and cell survival are regulated by GADD45 proteins

Cells exposed to stresses that impair cell growth or cause damage to DNA, normally undergo growth arrest until the damage is repaired. Nevertheless, if the damage cannot be repaired, cells will undergo apoptosis. As already highlighted in this review, GADD45 proteins also play an important role in apoptosis induction.

GADD45 effects on JNK and p38 signaling

In breast cancer, GADD45 induction by BRCA1 leads to programmed cell death through the JNK pathway via interaction with the upstream kinase MTK1/MEKK4 [31]. JNK has been shown to be involved in the induction of apoptosis after genotoxic stress and UV radiation [88]. However, JNK and p38 may exert antagonistic effects depending on cell context, cross-talk with other signaling pathways and intensity and duration of the stimulus [89]. The importance of JNK in induction of apoptosis is controversial. Originally, JNK activation and inhibition experiments indicated a pro-apoptotic role for JNK, such as in UV-induced apoptosis [90, 91]. However, other studies provide evidence for an anti-apoptotic role for JNK [92, 93]. This controversy about JNK may be related to the different systems and inducers being used in the various studies.

GADD45α null mouse skin and keratinocyte cell lines demonstrate impaired activation of p38, JNK and p53 and are resistant to UV-induced apoptosis substantiating JNK and GADD45 proteins pro-apoptotic activity [94]. Induction of apoptosis mediated by TGF-β relies on GADD45β expression in a Smad 2, 3 and 4 dependent manner [95]. Silencing the GADD45β gene delays TGF-β-mediated apoptosis in myeloid leukemia and pancreatic cancer cell lines and mouse hepatocytes [95-97].

The induction of GADD45α and γ expression is regulated by NF-κB. NF-κB is a dimeric transcription factor activated by inflammatory cytokines (such as tumor necrosis factor-α interleukin-1 and lipopolysaccharide) and environmental stresses (such as UV light and γ-

irradiation). NF-κB is found constitutively activated in different cancer cell types, and has been implicated in tumorigenesis, invasion, angiogenesis, metastasis, and as a mechanism triggered to allow tumor evasion from the host immune system [98]. NF-κB increases c-myc expression that in turn inhibits GADD45α and γ gene expression, but not GADD45β expression. In this scenario, JNK-mediated apoptosis can be blocked by silencing of GADD45α and γ, demonstrating the important role of NF-κB regulation in GADD45 expression and cancer cell survival [53].

GADD45 effects on mitochondria-mediated cell death

GADD45α is also linked to mitochondria-mediated cell death. GADD45α interacts with elongation factor 1-α (EF1-α) and disrupts cytoskeletal stability, which causes Bim dissociation from microtubules-associated components and translocation to the mitochondria. Furthermore, its interaction with Bcl-2 relieves Bax, increasing cytochrome-c into the cytoplasm and consequently inducing apoptosis of HeLa cells [99].

Even with evidence supporting pro-apoptotic activity and a well-established mechanism of GADD45 induction of apoptosis, there are still some controversies. Shaulian and Karin (1999) demonstrated that induction of GADD45α transcripts mediated by cellular stress or DNA damaging agents lagged behind the activation of JNK in 3T3 fibroblasts [100]. Furthermore, Wang et al. (1999) were not able to detect differences in JNK activation after cellular stress in GADD45α null MEFs compared to GADD45 wild type cells [101]. Likewise, the apoptosis induction after UV treatment is not impaired in GADD45α null lymphocytes or MEFs [60], suggesting that activation of the JNK pathway is GADD45-independent. A pro-survival activity of GADD45β in hematopoietic cell lines has also been shown [102].

Cross-talk between NF-κB and JNK

GADD45β, X-chromosome linked IAP (XIAP), A20 and blockers of reactive oxygen species (ROS) have been linked in the cross-talk between NF-κB and JNK [98]. GADD45β up-regulation is induced by NF-κB, and is involved in the down-regulation of JNK activation mediated by TNF-α [103]. The cytokine TNF-α regulates immune responses, inflammation and apoptosis [104]. JNK activation induced by TNF-α is prolonged in NF-κB deficient cells and triggers apoptosis [103]. In contrast to GADD45α and GADD45γ pro-apoptotic function, GADD45β plays a role in inhibiting JNK activation of apoptosis mediated by TNF-α by binding to and inhibiting MKK7 [103]. In addition, GADD45β is essential for suppression of TNF-α-induced cytotoxicity [105, 106]. In hematopoietic cells, both GADD45α and β have a distinct role in cell survival. GADD45β plays a role in JNK pathway inactivation by interaction with MKK4, while GADD45α activates p38 and subsequently inhibit IKβ, releasing NF-κB, and consequently leading to cell survival [107].

Pro-survival role of GADD45β

Several reports validate the pro-survival role of GADD45β protein. Bone marrow cells from GADD45β deficient mice are more sensitive to UVC-, VP-16- and daunorubicin-induced apoptosis [108]. Infiltrating Type 1 T helper (Th1) cells in the synovial fluid of patients with rheumatoid arthritis express high levels of GADD45β and are resistant to apoptosis, which was abrogated by silencing GADD45β gene expression [109]. Indeed, GADD45β overexpression in fibroblasts protects cells from programmed cell death [110]. However, MEFs from normal or GADD45β null mice are not susceptible to TNF-α-mediated apoptosis and do not demonstrate differences in JNK activity [111].

GADD45 proteins respond to environmental stresses mediating the activation of both p38 and JNK pathways, and are considered crucial mediators of cellular stress responses.

However, the cell's decision to undergo survival or apoptosis is complex, and dependent not only on GADD45 proteins activation, but also on the environmental stimuli and the cell type involved. MAPK pathways may act together in apoptosis induction or play different roles depending on the molecular participants involved. In cancer cells, the activation of GADD45 proteins induces pro-apoptotic pathways, while in some normal and particularly in hematopoietic cell lines, GADD45 proteins play a role in pro-survival pathways. In sum, these studies demonstrate the heterogeneity of mechanisms involved in GADD45 protein activation. Our current understanding of pro-survival and apoptotic activities of GADD45 proteins are summarized in Figures 4 and 5.

GADD45 expression is deregulated in various types of cancer

Mutations of GADD45 in cancer

Mutation and gene expression analyses of the GADD45 family have been performed in different types of tumors to identify sequence alterations and/or deregulated expression. GADD45α is located on chromosome 1p31.1-21.2 [36], which has been shown to contain a high incidence of deletions in breast cancer [112]. No mutations in the GADD45 gene have been observed in familial breast cancer [113, 114]. Different tumor cell lines, including lung, breast, bladder, testis, cervix, leukemia, ovarian, epidermis and sarcoma also do not contain mutations in the GADD45α gene [115], and no mutation is present in atypical fibroxanthoma [116]. However, 13% of the clinical samples from patients with invasive pancreatic ductal carcinomas harbor point mutations in exon 4 between codons 141-159 of the GADD45α gene [117]. Furthermore, 9.1% of Egyptian and 4.5% of American pancreatic adenocarcinoma patients were found with GADD45 gene mutations in exons 1 and 4 [118]. GADD45β is located at chromosome 19p13.3. This locus was found to be a common integration site in polyclonal tumors developed by retroviral insertion, and this integration leads to increased levels of GADD45β expression and inhibition of apoptosis in tumor cells [110]. GADD45γ is located at chromosome 9q22.1-q22.2 and at the present time no mutations have been found in this gene. The current information regarding GADD45 gene mutations in human cancer cells is summarized in Table 1.

Methylation of GADD45 promoters in cancer

Comparison of normal and tumor cell lines as well as evaluation of clinical samples have provided new insights about GADD45 deregulation in cancer. Analysis of hepatocellular carcinoma clinical samples indicates down-regulation of GADD45γ mRNA in 65% of the cases [119] and GADD45α mRNA in 77% of the cases [120]. The expression of GADD45α is 10 times lower in non-small cell lung carcinoma (NSCLC) compared to normal lung tissues [121] and is also down-regulated in pituitary tumors [122].

Down-regulation of GADD45 genes is usually, but not always, correlated with methylation of the gene's promoter region. The DNA methylation process consists of the addition of a methyl moiety to a DNA cytosine base in its fifth position (5-methycytosine), leading to the insertion of a new coding element which may play a role in transfer of genomic information through modulation and alteration in gene expression. The region where most of the methylation events occur in mammals are in CpG dinucleotides. The DNA methylation may take place in both silenced and actively transcribed genomic regions and, in general, is not an event readily reversible [123].

Methylation frequencies in NSCLC are 1.4% for GADD45α, 7.2% for GADD45β, and 31.6% for GADD45γ [124]. In breast cancer cell lines DNA demethylation mediated by 5-azacytidine (5-Aza) induces cell growth arrest, which does not occur in normal breast cell lines [125]. 5-Aza induces DNA hypomethylation [126] and has been approved by the U.S. Food and Drug Administration (FDA) for the treatment of all subtypes of myelodysplastic

syndromes [127]. Using a methylation-sensitive transcriptome approach, GADD45α was one of the genes identified to be expressed after DNA demethylation in breast cancer cells [125]. In addition, reduced GADD45α expression in prostate cancer cell lines correlates with methylation of a 4CpG region upstream of the GADD45α proximal promoter [128].

GADD45β expression is restored by 5-Aza in hepatocellular carcinoma cell lines [129]. Decreased GADD45β expression is demonstrated in individuals infected by hepatitis C virus, and is associated with hypermethylation of the GADD45β promoter upon viral infection [130].

GADD45γ is heavily methylated in cancer cell lines. GADD45γ CpG island methylation is present in 75% of carcinoma and lymphoma cell lines, 85% of non-Hodgkin's lymphomas, 50% of Hodgkin's lymphomas, 73% of nasopharyngeal carcinomas, 50% of cervical carcinomas, 29% of esophageal carcinomas, and 40% of lung carcinomas, while no methylation is present in any immortalized normal epithelial cell line, normal tissue, or peripheral blood mononuclear cells [131, 132]. Furthermore, recent findings demonstrated that methylation of the GADD45γ gene is also more frequent in gastric, colorectal and pancreatic cancers cells compared to normal cells [133], and the loss of GADD45γ expression in pituitary tumors is associated with methylation of CpG islands in the GADD45γ gene [134].

In contrast, clinical samples of colon carcinoma demonstrate down-regulation of ZBRK1 and up-regulation of the BRCA1 and GADD45 genes [10]. Although these genes are deregulated, there are neither promoter hypermethylation in GADD45 and BRCA1 genes, or mutations in GADD45 and ZBRK1 interaction regions [10]. GADD45α and p53 expression in clinical samples of invasive pancreatic ductal carcinomas correlated with lower survival rates for patients with GADD45α (+) subgroup compared to GADD45α (-) subgroup, suggesting a link between GADD45α and p53 expression and poor prognosis for pancreatic cancer patients [117]. Supporting this data, GADD45 proteins are highly detected in pancreatic cancer samples and those patients with positive expression of p53 and GADD45α have shorter post-operative survival rates [135]. Moreover, in pancreatic ductal adenocarcinoma, GADD45α knockdown reduces cell proliferation and induces apoptosis [136]. In these cases, the high malignant potential of the tumor cells might be able to overcome the high levels of GADD45α and p53 expression. The alterations of GADD45 gene expression in different types of human cancers are summarized in Table 2.

Inhibition of GADD45 family of genes through promoter methylation or NF-κB activation is considered a critical step in cancer development, with essential implications in survival, and resistance to environmental stresses and DNA damage, through inhibition of cell cycle arrest and diminished DNA excision repair.

GADD45 proteins are new targets for antitumor therapy

Several chemotherapeutic drugs induce the expression of GADD45 proteins *in vivo* and *in vitro*, either by promoting transcription or by stabilizing the transcripts, which may contribute to the observed drug effect on cell growth and survival.

CD437 effects on GADD45

A great number of drugs have been shown to induce cell cycle arrest and inhibit cancer cell growth by targeting GADD45α expression. Among these compounds, CD437 (6-[3-adamantyl-4-hydroxyphenyl]-2-naphthalene) causes G1 arrest and apoptosis in breast cancer cell lines [137, 138]. The mechanism underlying CD437's effect in breast cancer depends on increased expression of GADD45α through stabilization of its transcripts [11]. CD437 has

also been shown to induce GADD45α mRNA stabilization in other carcinoma cell lines, including fibrosarcomas and lung, mammary, bladder and colorectal carcinomas [139]. A similar mechanism of action has been described for arsenic chloride. In human bronchial epithelial cell lines, arsenic chloride stabilizes GADD45α transcripts through the RNA binding protein, nucleolin [140], promoting GADD45 mRNA translation through an internal ribosome entry site located in the 5′ –untranslated region of the GADD45 gene [141].

Genistein effects on GADD45

Genistein is an isoflavone that has been linked to inhibition of tyrosine kinases [142] and to decreased risk of mortality for prostate and breast cancer [143, 144]. Genistein's growth arrest in prostate cancer cell lines relies on the induction of the GADD45α gene through the CCAAT-box motif present in the GADD45α promoter region [145].

Trichostatin A effects on GADD45

Trichostatin A (TSA) inhibits the histone deacetylase (HDAC) family, which regulates expression of key genes involved in apoptosis, cell differentiation and cell cycle arrest [146]. In human osteosarcoma cell lines, TSA induces GADD45α expression and causes G2/M arrest [50]. GADD45α and γ induction by TSA requires the Oct-1 and CCAAT-box motifs in the promoter region of these genes [147]. Other histone deacetylase inhibitors demonstrate additive effects in inducing GADD45α expression and inhibiting cancer cell growth in a p53-dependent manner [148].

(-)-Xanthatin effects on GADD45

(-)-Xanthatin is an exo-Methylene lactone group-containing compound, which is present in a large variety of biologically active natural products and shows anti-inflammatory, antimalarial and cytotoxic activities in cancer cells [149]. In a farnesyltransferase-inhibitor (FTI) resistant breast cancer cell line (-)-Xanthatin induced GADD45γ and consequently promoted p38 and JNK activation causing reduced proliferation and caspase-independent apoptosis [150].

NSAID effects on GADD45

NSAIDs have emerged as potential drugs for chemoprevention in cancer. Several epidemiological studies indicated that the use of NSAIDs at clinically relevant concentrations reduce colorectal [151], breast [152] and ovarian cancer risk [153-155], although there are still some controversies [156, 157]. One major target of NSAID action is through inhibition of cyclooxygenases (COX), which are responsible for the conversion of arachidonic acids into prostaglandins. The two COX genes, COX-1 and COX-2, are almost identical; however, one relevant difference is that COX-1 expression is constitutive, whereas COX-2 expression is induced by growth factors and pro-inflammatory stimuli [158].

High COX-1 expression in ovarian cancer strongly correlates with high levels of Vascular Endothelial Growth Factor (VEGF) [159-161] and NSAIDs inhibit VEGF production in ovarian cancer cell lines [162, 163] indicating that COX-1 may regulate VEGF expression. Angiogenesis and VEGF expression are implicated in ascites formation [164] and metastasis of ovarian cancer [165], while its inhibition prevents ascites formation and inhibits disseminated cancer growth [166].

Along with COX and VEGF inhibition, NSAIDs mechanism of action involves induction of GADD45 proteins [12]. The intraperitoneal administration of NSAIDs, such as Sulindac and Indomethacin, results in the up-regulation of GADD45α gene expression, leading to gastric mucosal injury and apoptosis in mice, while the administration of NSAIDs in GADD45-null

mice shows reduced cell apoptosis [167]. Moreover, the suppression of the GADD45α gene reduces caspase 9 activation-induced by NSAIDs in human gastric mucosal cells [167].

We have shown that in ovarian, prostate, renal, breast and stomach cancer cell lines structurally diverse NSAIDs induce apoptosis through activation of melanoma differentiation associated gene-7/Interleukin-24 (*mda-7*/IL-24) [12, 168-170]. *mda*-7/IL-24 induction and activation by NSAIDs leads to upregulation of GADD45α and GADD45γ that is essential for cancer programmed cell death via c-Jun NH(2)-terminal kinase (JNK) activation [12]. *mda*-7/IL-24 is expressed in cells of the immune system and normal melanocytes [171]. However, when *mda*-7/IL-24 is overexpressed it induces apoptosis only in cancer cells sparing the normal cells and therefore has been highlighted as a “magic bullet” for cancer [172-174]. Some promising studies indicate that induction of *mda-7*/IL-24 by a recombinant adenovirus results in apoptosis of non-small cell lung carcinoma [175], lung carcinoma [176], malignant gliomas [177-179], renal carcinomas [180], pancreatic carcinoma [181] and ovarian cancer [182, 183].

Effect of the thymidine kinase (TK) gene coupled with the antiviral drug gancyclovir (GCV) on GADD45

The efficacy of another therapy that combines a recombinant adenovirus expressing the thymidine kinase (TK) gene coupled with the antiviral drug gancyclovir (GCV) in human pancreatic adenocarcinomas relies on CCNE1 and GADD45 genes to induce cell death [184]. In another recombinant adenoviral approach, the combination of adenoviral-mediated expression of GADD45α and anticancer drugs such as etoposide, cisplatin, and 5-fluorouracil, resulted in chemosensitivity in pancreatic ductal adenocarcinoma cancer-derived cell lines [185].

Effect of Fucoxanthin and curcumin on GADD45

The carotenoid Fucoxanthin exhibits tumor cell growth inhibition by cell cycle arrest and apoptosis induction as a result of GADD45α and β gene activation [186, 187]. In hepatocellular carcinoma cells the inhibition of the MAPKs, p38 and ERK enhances the expression of GADD45α and GADD45β, respectively, mediated by Fucoxanthin, while the inhibition of JNK suppresses GADD45α gene expression induced by Fucoxanthin in prostate cancer cell lines [186]. Another recent finding indicates that Curcumin, a molecule isolated from the plant *Curcuma longa* (LINN), increases expression of GADD45α in a p53-independent manner, inducing cell cycle arrest and concurrently apoptosis in human lung cancer cell lines [188].

Effect of docetaxel on GADD45

Docetaxel is a chemotherapeutic drug that binds to microtubules and inhibits cancer cell proliferation [189]. The sensitivity of prostate cancer cell lines to docetaxel is enhanced by overexpression of GADD45α or treatment with the demethylation agent 5-Aza [128]. Another demethylation agent, decitabine, also induces GADD45α expression and apoptosis in osteosarcoma cell lines, and silencing of GADD45α gene expression abrogates its apoptotic response [190]. Multidrug resistant human osteosarcoma cell lines have been shown to have direct links with GADD45 gene expression. The authors demonstrated that Paclitaxel or Doxorubicin impairment of apoptosis induction due to multidrug resistance is dependent of GADD45 defective gene expression. However, drug resistance can be overcome by transient expression of GADD45α, increasing cell sensitivity to chemotherapeutic drugs [191]. The effect of different drugs in GADD45 expression in several cancer cell lines is listed in Table 3.

Some of the drugs mentioned above are currently used as treatment options for several forms of cancer. Docetaxel, for instance increases the mean survival time of patients with castration-resistant prostate cancer (CRPC) and was approved by the FDA in 2004 and has been used since then for the treatment of CRCP [192]; 5-AZA has been used for the therapy of all subtypes of myelodysplastic syndromes [127]. Some natural compounds, like Genistein and curcumin have been studied for both chemoprevention and treatment of cancer; soy food has high content of isoflavones, like Genistein and its intake has been hypothesized to contribute to the lower incidence of breast cancer in Asian populations [193]. Development of viral vectors expressing tumor supressors genes that induce GADD45 expression, like MDA-7/IL-24 and p53, have produced promising results and represent an alternative to traditional drug therapies. The dependence of several natural and synthetic drugs and tumor suppressor genes on activation of GADD45 proteins highlights the importance of this family of proteins in cancer therapy.

Concluding remarks

GADD45 proteins have been shown to mediate the activation of several molecules involved in crucial steps of tumorigenesis. Our current understanding of the mechanism of action of GADD45 proteins supports the suggestion that these molecules may serve as potential targets to be utilized in novel cancer therapeutic strategies. Therapies involving activation of GADD45 expression, mRNA stabilization and modulation of any of its upstream or downstream effectors, may provide the requisite promising strategies. Future work, resulting in a better understanding of GADD45 pathways regulated by the tumor microenvironment and in cancer stem cells, will provide additional entry points to interfere with GADD45 function. Additionally, high throughput screening strategies with natural and synthetic libraries to identify and develop small molecule drugs with potential to selectively induce GADD45 pathways in cancer cells is clearly an avenue of research worth exploring.

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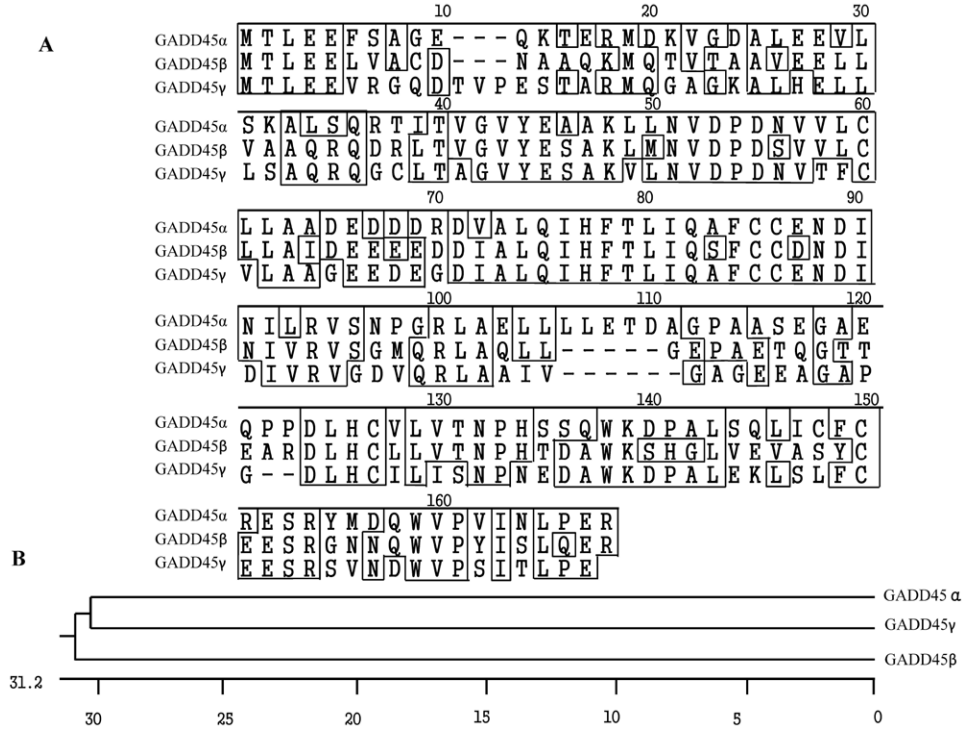

Figure 1.

Comparison of the human GADD45α, GADD45β and GADD45γ proteins. (**A**) Alignment of human GADD45α, GADD45β and GADD45γ amino acid sequences. (**B**) Phylogenetic tree of GADD45α, GADD45β and GADD45γ proteins.

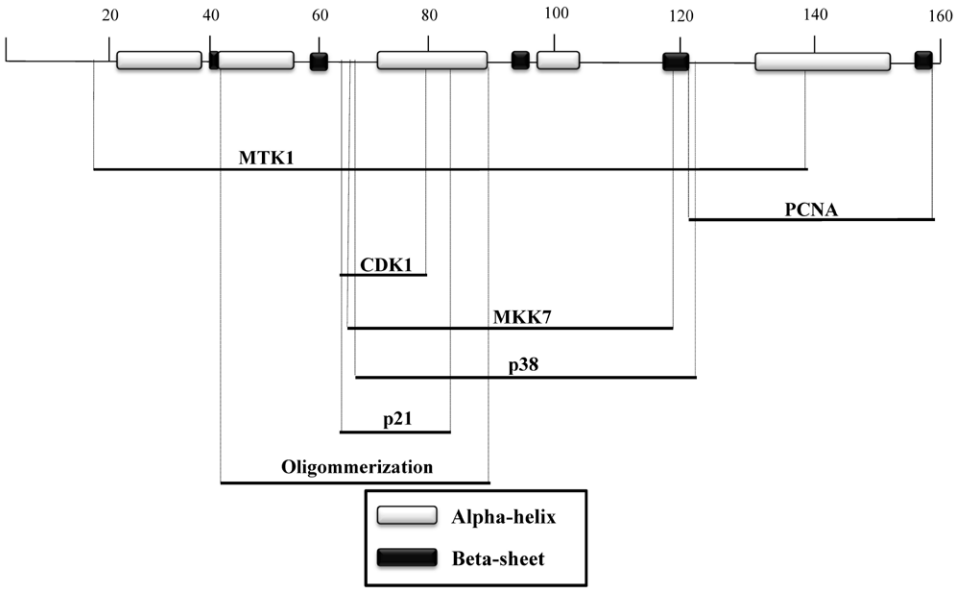

Figure 2.

Secondary structure of GADD45 proteins and interaction domains. The structures of GADD45α and GADD45γ proteins were determined by nuclear magnetic resonance and crystallography. α-helices and β-sheet structures are indicated in white and black, respectively. Cross-reference of mutational analysis shows the putative interaction domains of GADD45 proteins with PCNA, CDK1, MTK1, p38, PCNA, MKK7, $p21^{waf/cip/mda-6}$ and the dimerization domain.

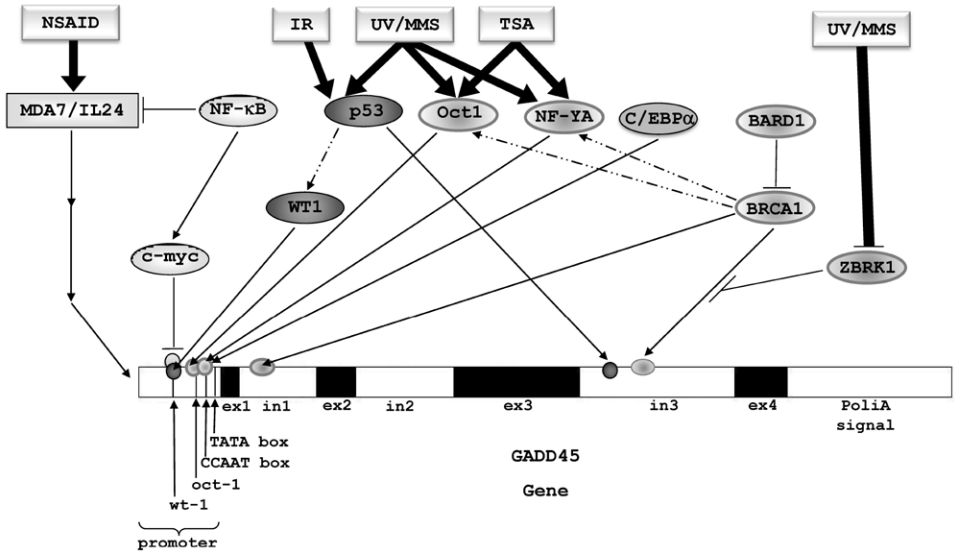

Figure 3.

GADD45 modulation and activation. The GADD45 promoter region contains Oct-1, CCAAT-box, WT1, and Egr-1 elements, which are activated by direct interaction with Oct-1/NF-YA/CEBPα, or through indirect interaction of p53/WT1/BRCA1 with Oct-1/NF-YA. The first intron of GADD45 gene contains a BRCA1 element, while the third intron contains p53 and ZBRK1 elements. Using the third intron of the GADD45 gene, UV, MMS and IR agents may activate the expression of GADD45 gene by the induction of p53, through its direct interaction with the p53 element or ZBRK1 degradation through the proteasome pathway, resulting in the release of BRCA1 inhibition by ZBRK1. IR activation relies on p53; UV, MMS, TSA, and other drugs activate expression by means of other transcription factors. NSAIDs activate the expression of GADD45 genes through activation of the *mda*-7/IL-24 gene, which can be inhibited by NF-κB. NF-κB can also induce c-myc, which inhibits GADD45 genes expression through interaction with Oct-1 and NF-YA.

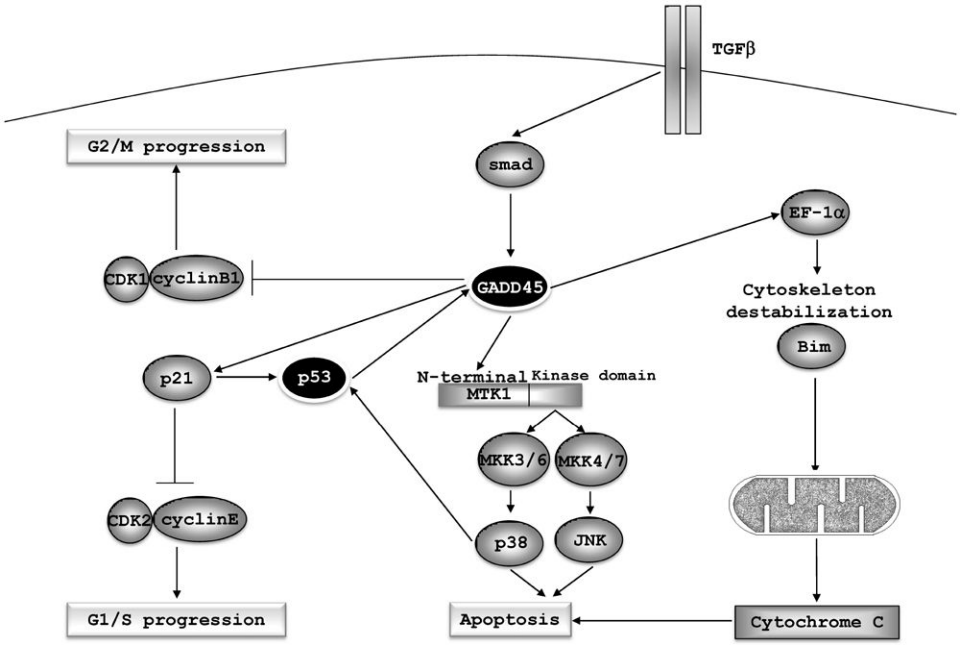

Figure 4.

GADD45-mediated cell cycle arrest and apoptosis. GADD45 inhibits the kinase activity of the CDK1/CyclinB1 complex and promotes G2/M arrest; interaction with $p21^{waf/cip/mda-6}$ contributes to G1/S arrest. Interaction of GADD45 proteins with the N-terminal domain of MTK1 leads to relieve of its auto-inhibitory domain and activation of the kinase domain, which activates both p38 and JNK pathways and induces apoptosis. GADD45 proteins also participate in mitochondria-mediated apoptosis by interaction with EF1-α, which leads to cytoskeleton destabilization and to the release of Bim. As a consequence, Bim translocates into the mitochondria promoting release of cytochrome-c, resulting in the induction of apoptosis. GADD45 proteins inhibition is an essential step in the NF-κB-mediated cell survival.

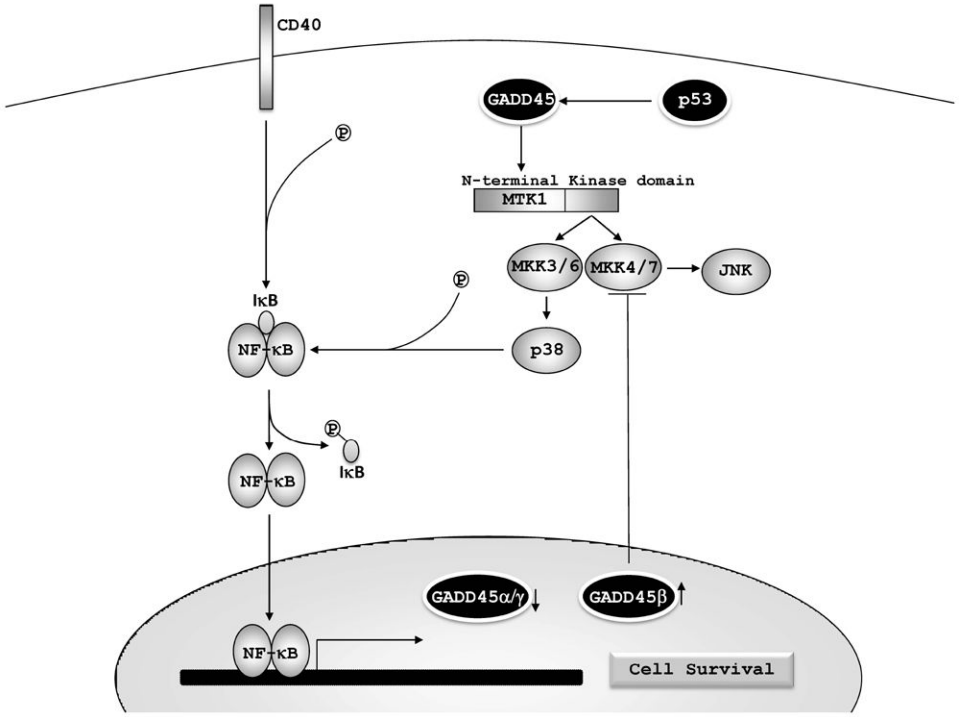

Figure 5.

GADD45 proteins and cell survival. NF-κB represses GADD45α and GADD45γ, while it induces GADD45β expression. NF-κB mediates cell survival and GADD45β activation leading to inhibition of MKK4/7 and JNK-mediated apoptosis. GADD45 proteins may also play a role in cell survival by induction of p38, which promotes degradation of IκB and activation of the NF-κB pathway.

Table 1

GADD45 mutations in human cancer cells.

Cancer cell type	GADD45 mutations
Atypical fibroxanthoma	No mutations in GADD45α [116]
Breast cancer	No mutations in GADD45α [113, 114]
Breast, bladder, cervix, epidermis, leukemia, lung, ovarian, sarcoma and testis	No mutations in GADD45α [115]
Pancreas invasive ductal carcinomas	Point mutations in exon 4 of GADD45α in 13% of the patients analyzed [117]
Pancreatic adenocarcinoma	GADD45α mutations in 9.1% of the Egyptian patients and 4.5% of the American patients analyzed [118]

Table 2

GADD45 gene expression deregulation and promoter methylation in human cancer cells.

Cancer cell type	Alterations in GADD45 gene
Carcinoma and lymphoma cell lines	GADD45γ promoter hypermethylation frequency of 75% [131]
Cervical carcinoma cell lines	GADD45γ promoter hypermethylation frequency of 50% [131]
Colon carcinomas clinical samples	Up-regulation of GADD45α gene in colon carcinoma clinical samples compared to normal tissue [10]
Esophageal carcinoma cell lines	GADD45γ promoter hypermethylation frequency of 29% [131]
Hepatocellular carcinoma clinical samples	Down-regulation of GADD45γ in 65% of the patients analyzed compared to their adjacent normal tissue [119]
Hodgkin's lymphoma cell lines	GADD45γ promoter hypermethylation frequency of 50% [131]
Lung carcinoma cell lines	GADD45γ promoter hypermethylation frequency of 40% [131]
Nasopharyngeal carcinoma cell lines	GADD45γ promoter hypermethylation frequency of 73% [131]
Non-Hodgkin's lymphoma cell lines	GADD45γ promoter hypermethylation frequency of 85% [131]
Non-small cell lung carcinoma clinical samples	GADD45α expression is 10 times lower compared to normal lung tissue [121]
	Methylation frequencies of 1.4% in GADD45α, 7.2% in GADD45β, and 31.6% in GADD45γ [124]
Pancreatic cancer cell lines and pancreatic ductal adenocarcinoma	GADD45α is overexpressed compared to normal pancreatic cell lines or tissues [117, 135, 136]
Pituitary tumor clinical samples	GADD45γ is down regulated compared to normal pituitary tissue [122]

Table 3

Effect of different drugs in different cancer cell lines on GADD45 expression.

Drug	Cancer cell type	Effect in GADD45 expression
Adenovirus TK/GCV	Human pancreatic adenocarcinoma	GADD45 and apoptosis induction [184]
Adenovirus GADD45α + Etoposide, Cisplatin and 5-Fluorouracil	Human pancreatic ductal adenocarcinoma	Increases sensitivity to chemotherapy [185]
Arsenic chloride	Human bronchial epithelial cell line	GADD45 mRNA stabilization through nucleolin [140]
		Promotes GADD45 mRNA translation through an internal ribosome entry site (IRES) [141]
CD437 (6-[3-adamantyl-4-hydroxyphenyl]-2-naphthalene)	Human fibrosarcoma, lung, mammary, bladder and colorectal carcinomas	GADD45 mRNA stabilization [139]
	Breast cancer	Increased GADD45 expression [11]
Curcumin	Human lung cancer cell line	Increased GADD45α expression, cell cycle arrest and apoptosis [188]
Fucoxanthin	Human hepatocarcinoma cell line	GADD45α and β activation, cancer cell growth inhibition and cell cycle arrest [186, 187]
	Human prostate cancer cell line	GADD45α up-regulation and G1 cell cycle arrest [186, 187]
Genistein	Human prostate cancer cell line	GADD45α induction and G2/M cell cycle arrest [50]
NSAIDs (Ibuprofen)	Human gastric cancer cells	GADD45α and G1 blockage [194]
NSAIDs (Sulindac and Indomethacin)	GADD45-/- B6129F1 mice or matching *w/t* mice. Human gastric carcinoma epithelial cell line	Up-regulation of GADD45α and apoptosis [167]
NSAIDs (Sulindac sulfide, Aspirin, Ibuprofen, Sulindac sulfone, Acetaminophen, Naproxen, NS-398, Celecoxib, Diclofenac, Finasteride, Flufenamic acid, Meloxicam, Ebselen, and Flurbiprofen)	Human prostate, renal, breast and stomach cancer cell lines	GADD45α and GADD45γ induction mediated by MDA-7/IL-24 [12]
Paclitaxel and Doxorubicin	Human osteosarcoma multidrug resistant cell line	Multidrug resistant cell line present defects in GADD45 expression and low levels of apoptosis mediated by the drugs [191]
Peptidylarginine deaminase 4 and histone deacetylase inhibitors	Human osteosarcoma cell line	GADD45α induction and cancer cell growth inhibition [148]
Trichostatin A	Human osteosarcoma cell line	GADD45α induction and G2/M cell cycle arrest [50]
Zinc	Human bronchial epithelial cell lines	GADD45α induction and G2/M cell cycle arrest [46, 47]
(-)-Xanthatin	FTI resistant breast cancer cell line	GADD45γ induction and reduced proliferation and induction of apoptosis [150]