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M. Raza Zaidi
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Gadd45 Stress Sensor Genes

Second Edition

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Introduction

The cellular stress response is complex, encompassing a myriad of molecular pathways with a plethora of regulators and effectors. Evidence has emerged that the Gadd45 family of proteins plays a unique and critical role as sensors of stress, including genotoxic, physiologic, and oncogenic stress.

The Gadd45 stress sensor family of genes (Gadd45a, Gadd45b, and Gadd45g), discovered in our laboratory and by other investigators, encode for small (18 kd) nuclear/cytoplasmic proteins. These genes are rapidly induced by a wide variety of endogenous and exogenous stress stimuli. Despite marked similarities, Gadd45 genes are regulated differentially and exhibit functional diversity. Gadd45 proteins are implicated in cell cycle arrest, DNA demethylation and repair, apoptosis, cell survival, genomic stability, inflammation, immunity, and response to physiological and oncogenic stress.

The functions of Gadd45 proteins are mediated by protein–protein interactions that modulate structure/function of other cellular proteins implicated in cell cycle regulation and the response of cells to stress. These interactions vary depending upon the biological context, including cell type, developmental stage, and stress/stimulus. Their protein partners include cdc2/cyclinB1, p21, the p38/JNK stress-induced kinase pathways, and PCNA/histones.

The purpose of the second edition of the "Gadd45 Stress Senor Genes" book is to provide a comprehensive updated picture of the global role Gadd45 proteins play as stress sensors and the molecular pathways they are involved in.

Philadelphia, PA, USA — Dan A. Liebermann

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GADD45 in Stress Signaling, Cell Cycle Control, and Apoptosis

1

Arslon Humayun and Albert J. Fornace Jr

Abstract

GADD45 is a gene family consisting of *GADD45A*, *GADD45B*, and *GADD45G* that is often induced by DNA damage and other stress signals associated with growth arrest and apoptosis. Many of these roles are carried out via signaling mediated by p38 mitogen-activated protein kinases (MAPKs). The GADD45 proteins can contribute to p38 activation either by activation of upstream kinase(s) or by direct interaction, as well as suppression of p38 activity in certain cases. In vivo, there are important tissue and cell type specific differences in the roles for GADD45 in MAPK signaling. In addition to being p53-regulated, GADD45A has also been found to contribute to p53 activation via p38. Like other stress and signaling proteins, GADD45 proteins show complex regulation and numerous effectors. More recently, aberrant GADD45 expression has been found in several human cancers, but the mechanisms behind these findings largely remain to be understood.

Keywords

GADD45 · Cell cycle · Apoptosis · MAPK · UV radiation · p53 · Foxo3a · Oxidative stress · BRCA1 · NFkappaB · Tumorigenesis

1.1 Overview

GADD45 was first identified based on increased mRNA levels following stress-induced growth arrest and was therefore given the acronym *G*rowth *A*rrest and *D*NA *D*amage (*GADD*) as its name (Fornace et al. 1989). *GADD45*, now designated *GADD45A*, shows no sequence homology with the other original members of the *GADD* gene group (Kastan et al. 1992; Zhan et al. 1994), and was subsequently found to be a member of a highly conserved three-gene family consisting of *GADD45A* (GADD45A, DDIT1, GADD45α), *GADD45B* (GADD45β, MYD118), and *GADD45G* (GADD45γ, cytokine responsive 6 or CR6). The *GADD* genes were first cloned from Chinese hamster ovary (CHO) cells, which were

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subsequently found to be p53-deficient, as a subset of transcripts that were consistently upregulated after exposure to ultraviolet (UV) radiation and in many cases to other DNA-damaging agents, including methyl methanesulfonate (MMS), hydrogen peroxide, and N-acetoxy-2-acetylaminofluorene, as well as to other growth cessation signals, such as medium depletion/starvation or hydroxyurea (Fornace et al. 1988). *GADD45A* was the 45th member of this collection of over a hundred cDNA clones (Fornace et al. 1988). *GADD45A* is responsive to a myriad of agents implicated in DNA damage, apoptosis, cell cycle checkpoint control, cell injury, and other growth regulatory processes. The GADD45 proteins have likewise been implicated in a wide variety of cellular processes often associated with stress signaling and with other growth regulatory pathways (Gao et al. 2009; Zhang et al. 2014). Many GADD45 binding proteins have been identified using methods such as two-hybrid (Vinayagam et al. 2011) and affinity chromatography (Gao et al. 2013). Some of the prominent interactions of the GADD45 proteins are summarized in Fig. 1.1, which highlights regulatory pathways and downstream targets. As shown in this figure, GADD45 has a broad scope of potential roles in many cellular processes that will be covered in this and subsequent chapters, with emphasis in this chapter on growth control and apoptosis.

Among the radiation-response genes, *GADD45A* was unique at the time because it could be induced in an ATM-dependent and protein kinase C-independent manner following human cell exposure to ionizing radiation (IR) (Papathanasiou et al. 1991). This IR-responsiveness was subsequently found to be p53-regulated (Kastan et al. 1992); indeed, *GADD45A* was the first stress gene discovered that was regulated by p53 at the transcriptional level (Hollander and Fornace 2002). *GADD45B* was originally cloned as a gene expressed after terminal differentiation and growth arrest of M1D+ myeloid precursor cells induced by IL-6 (Selvakumaran et al. 1994). *GADD45G* was originally cloned as an early IL-2 response gene in T cells (Zhang et al. 1999). All three members show responsiveness to a variety of environmental cues associated with growth control. These three proteins are highly conserved among Metazoa although insects have only a single *GADD45* gene that is most similar to *GADD45G*, indicating this may be the ancestral gene. The proteins are all small (18 kDa), highly negatively charged (in the top two percentile of proteins in the ratio of negative charge to amino acids) (Zhan et al. 1994), and localized to the nucleus (Cretu et al. 2009). GADD45A is the best-characterized isoform and will be a major focus of this review although the other family members have important characteristics that will also be discussed.

Like most signaling proteins, the GADD45 proteins are small, highly regulated at both the transcriptional and post-transcriptional levels, and have multiple roles in mediating stress signaling and growth regulation. In addition to repair and apoptosis, cell injury, particularly in response to genotoxic stress, is known to trigger growth delays in prokaryotes and eukaryotes (Friedberg 2006). GADD45 proteins have been shown to play important roles in these processes. There is also a remarkable overlap between responses to genotoxic stress and aberrant growth signaling by oncogenes, referred to as oncogenic stress, which triggers a variety of responses involving GADD45. Many of these genotoxic and oncogenic stress responses are highlighted in Fig. 1.1. While they are discussed individually in more detail below, this overview diagram exemplifies the complexity of GADD45 regulation and function in these processes.

The stress mitogen-activated protein kinases (MAPK), namely the JNK and particularly the p38 MAPK, have complex regulatory roles involving GADD45. Other growth-arrest associated regulatory factors such as p53, BRCA1, FOXOA3, C/EBP, and ATF participate in transcriptional regulation of *GADD45A* and to some extent of the less-studied *GADD45B* and *GADD45G* genes, which in multiple cases can have roles distinct from GADD45A. The GADD45 proteins are involved, directly or as part of regulatory pathways, in cell cycle checkpoints and stimulation of DNA repair. They interact with a wide variety of cellular proteins and protein com-

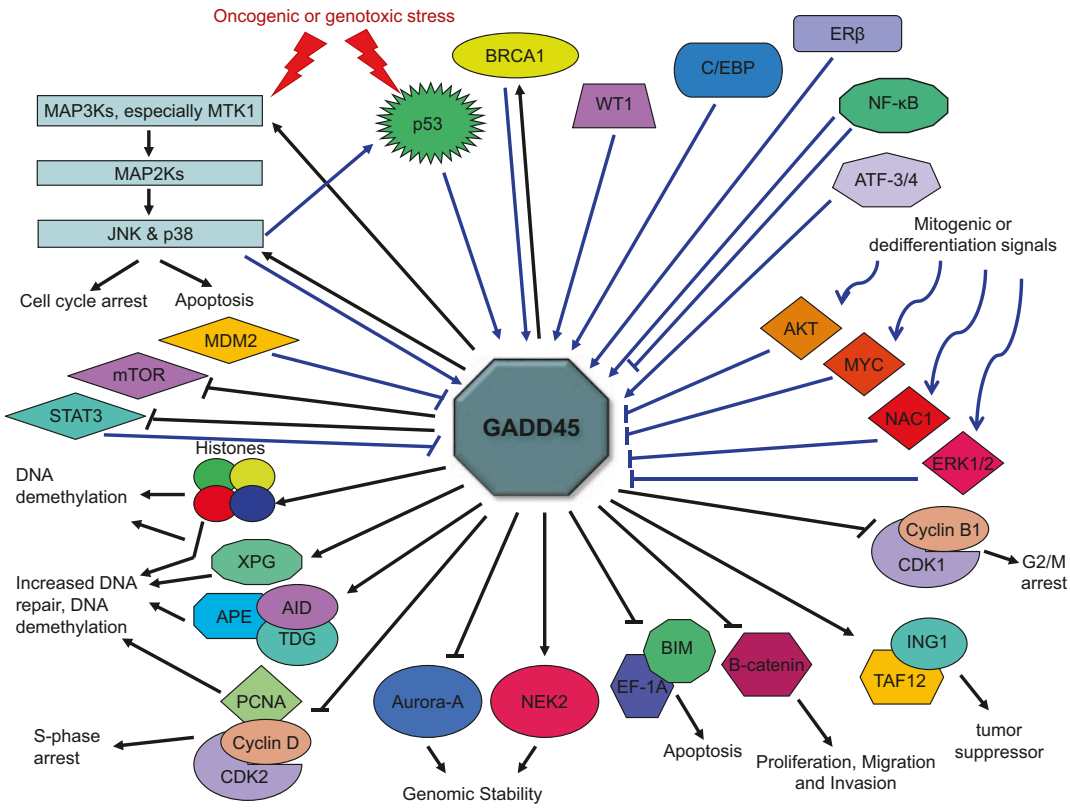

Fig. 1.1 Schematic representation of upstream regulators of *GADD45* and its downstream effects. Blue lines indicate upstream regulators, while black lines indicate downstream effects. Arrows indicate positive regulation, while blocked lines indicate negative regulation. Note that the interactions shown are primarily for GADD45A but may also occur for GADD45B and GADD45G. For example, all three proteins upregulate MTK1, but TGFβ (not shown in figure) is known to induce only GADD45B. Additionally, all GADD45 proteins are able to interact with each other and form homo- or hetero-dimers, which are crucial for GADD45 functions. Note that this is by no means a complete picture of all GADD45 interactions, but rather an overview of key interactions in stress signaling, cell cycle control, and apoptosis. For a discussion of GADD45 interactions involving methylation, please refer to Chap. 4: *GADD45 in DNA Demethylation and DNA Repair*

plexes, including cyclin-dependent kinase 1 (CDK1), for which it is a strong inhibitor of CDK1-Cyclin B1 activity both in vivo and in vitro and a component of certain G2 checkpoint events (Wang et al. 1999; Zhan et al. 1999; Vairapandi et al. 2002). Interestingly, like some other highly acidic proteins such as SET1, the GADD45 proteins bind directly to nucleosome histones and modify DNA accessibility, particularly on damaged chromatin (Carrier et al. 1999), which is one role reported for GADD45 in DNA repair (Smith et al. 2000). As shown in Fig. 1.1, the GADD45 proteins interact with and/or influence a variety of proteins involved in DNA repair, including APE (Jung et al. 2007), XPG (Barreto et al. 2007), PCNA (Smith et al. 1994), and p53. GADD45A in particular has been shown to play a role in heterochromatin relaxation (Chen et al. 2016). It has also been shown to bind to R-loops to promote DNA demethylation (Arab et al. 2019). These interactions will be discussed further in Chap. 4: *GADD45 in DNA Demethylation and DNA Repair*.

Although most of the interactions shown in Fig. 1.1 were initially discovered in cell culture systems, multiple functions of GADD45 have since been demonstrated using genetic approaches, both in vivo with mouse models and

in vitro with primary cells such as mouse embryo fibroblasts (MEFs). Among these findings, a consistent feature has been the prominent role of p38 MAPK signaling in vivo. For example, GADD45A-null mice lack the normal p53-mediated sunburn response in skin. As discussed in more detail later in this chapter, this is due to the requirement for p38 in p53 activation after stresses such as UV radiation (Hildesheim and Fornace 2004). Detailed studies in vivo and in MEF showed that GADD45 proteins can contribute to p38 activation either directly (Bulavin et al. 2003) or via MTK1, a MAPK kinase kinase (MAP3K) (Takekawa and Saito 1998) which is encoded by the *MAP3K4* gene. Additionally, p38 can directly phosphorylate regulatory sites in p53, such as Ser 46 (implicated in proapoptotic signaling) (Bulavin et al. 1999), and thus upregulate downstream effectors including GADD45A, which will then contribute to p38 activation. Thus, p38-p53-GADD45A defines a stress-activated regulatory loop, as shown in Fig. 1.2. While this positive feedback loop is transient during genotoxic-stress-induced growth arrest, it is necessary for oncogene-induced permanent growth arrest, i.e., premature senescence (Bulavin et al. 2003). Consistent with these findings, GADD45A-null mice show increased carcinogenesis after genotoxic stresses such as IR (Hollander et al. 1999) or UV radiation (Hildesheim et al. 2002).

1.2 GADD45 Regulation in Growth Arrest and Apoptosis

As outlined in Fig. 1.1 and Table 1.1, *GADD45* is regulated in response to genotoxic stress and other growth-arrest signals at both the transcriptional and post-transcriptional levels (Gao et al. 2009). *GADD45* plays an important role in stress-induced growth arrest, such that it is one of only a few genes that is upregulated consistently after IR in numerous conventional and gene expression profiling studies of p53 wild-type (wt) cells

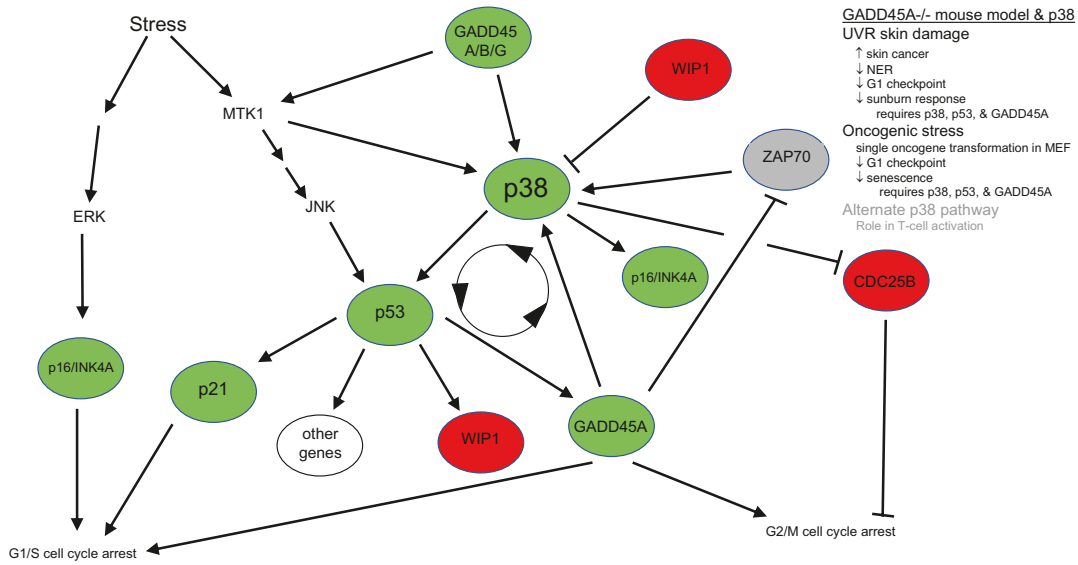

Fig. 1.2 Central role for p38 signaling in the GADD45A phenotype. Arrows indicate positive regulation, while blocked lines indicate negative regulation. Green circles indicate proteins typically considered tumor suppressors, while red circles indicate potential oncogenes. As described in the text, p38, p53, and GADD45A can function in a positive feedback loop (indicated by black circle with arrows) to maintain p53 signaling and growth arrest. GADD45 proteins are positive effectors for p38 activation after many stresses. As discussed in Chap. 5, GADD45A has a prominent role in the alternative p38 activation pathway in T cells and immunity by modulating ZAP70 activity

Table 1.1 GADD45 effectors with roles in growth control and apoptosis. Note that this is not a complete summary of all GADD45 interactions, but rather a list of key protein interactors involved in growth control and apoptosis. Protein interactors involved in methylation, such as ING1, will be discussed further in Chap. 4: *GADD45 in DNA Demethylation and DNA Repair*

p38	Cell cycle arrest, apoptosis, induction of senescence, negative regulation of T cell activation, full activation of innate immune cells
MTK1	Activation that signals to p38 and JNK branches of MAPK pathways
p53	p53 activation via p38 signaling, required for sunburn response in skin
CDK1	Inhibits CDK1/CLNB1 activity and contributes to G2 checkpoint activation
CDKN1A (p21)	Positive role in chondrocyte senescence (GADD45B); negative regulation of p21 in keratinocytes allowing nucleotide excision repair
APC	Destruction of ß-catenin via p38 signaling
ß-catenin	Inhibition of its pro-invasion program, increased ß-catenin plasma membrane localization and cell–cell adhesion
JNK	Cell cycle arrest and apoptosis; can be mediated by MTK1 signaling
EF-1A	Release of BIM, apoptosis
PCNA	S-phase arrest; DNA repair and demethylation
Aurora-A	Maintenance of genomic stability
NEK2	Maintenance of genomic stability
mTOR	Suppression of tumor angiogenesis by inhibition of mTOR signaling
STAT3	Inhibition of STAT3 promotion of tumor angiogenesis
ING1	Tumor suppression via DNA methylation

(Snyder and Morgan 2004). For example, in the NCI60 cell screen panel, only p53 wt human tumor lines showed appreciable *GADD45A* induction (Weinstein et al. 1997). Although ubiquitous, basal *GADD45* expression is usually very low and varies through the cell cycle, with highest levels during G_1 and lowest levels during S phase (Kearsey et al. 1995).

As highlighted in Fig. 1.1, GADD45A expression is induced by MAPK signaling via p38 and JNK kinases. These kinases activate c-Jun, which, similarly to p53, binds to the third intron of *GADD45A* and promotes transcription; this finding is not surprising since AP-1-binding sites have been identified both in the promoter region and the third intron of *GADD45A* (Gao et al. 2009). It is of interest that transient ERK signaling induces *GADD45A* expression, whereas sustained signaling represses it (Gao et al. 2009); this *GADD45A* induction might be due to transient activation of other MAPK pathways through crosstalk. Sustained or oncogene-driven ERK signaling also promotes upregulation of p16 (Bulavin et al. 2003), which plays an important role in G1/S cell cycle arrest, as shown in Fig. 1.2. Estrogen receptor β (ERβ) can bind to the *GADD45A* promoter in a ligand-independent manner and recruits c-Jun and NCOA2 to stimulate transcription and subsequent G_2/M arrest (Paruthiyil et al. 2011). Indeed, in a panel of human breast cancer samples, GADD45A expression was found to depend on estrogen receptor expression (Tront et al. 2013). BRCA1, a breast (and other) cancer tumor suppressor, has also been implicated in *GADD45A* gene regulation with binding sites in both the first and third exon of this gene (Harkin et al. 1999; Pietrasik et al. 2020).

1.2.1 Transcriptional Regulation of GADD45

At the transcriptional level, there are several tumor suppressor genes that induce *GADD45A* expression. As mentioned earlier, one well-characterized mechanism of *GADD45A* induction involves the binding of p53 to a conserved site within the third intron of the *GADD45A* gene (Kastan et al. 1992). This binding is induced by genotoxic stress but is necessary only in the case of IR exposure and not in the *GADD45A* response to UV radiation or MMS although loss of p53 does attenuate subsequent *GADD45A* induction. WT1, a transcription factor that is mutated in various tumors and congenital defects, can bind to the *GADD45A* promoter and induce transcription in a p53-dependent manner (You et al. 2019) but in the absence of direct p53-DNA binding in the response to non-ionizing radiation (Zhan

et al. 1998). BRCA1 induces *GADD45A* expression indirectly by interacting with the transcription factors OCT-1 and NF-YA. The CCAAT/enhancer-binding protein-α (C/EBPα) and other C/EBP proteins can induce *GADD45G* expression as well (Gao et al. 2009; Jung et al. 2000).

GADD45A has been identified as a direct target gene of FOXO3A, a tumor suppressor that is a member of the mammalian family of forkhead transcription factors. FOXO3A binds to *GADD45A* at the promoter region and promotes transcription in response to treatment with phosphoinositol-3 kinase inhibitor (Tran et al. 2002) or oxidative stress (Sengupta et al. 2011). However, FOXO3A has been observed to suppress the induction of GADD45B (Lee et al. 2008), suggesting a different possible role of GADD45B in response to stress (Tran et al. 2002). As shown in Fig. 1.1, activating transcription factor-4 (ATF-4) has a central role in cellular stress responses and induces *GADD45A* transcription in response to arsenite exposure, leucine deprivation, inhibition of the proteasome, and endoplasmic reticulum stress; GADD45A protein levels rise after arsenite exposure or proteasome inhibition, showing a sophisticated regulation of *GADD45A*, which responds differentially to various cellular stressors (Gao et al. 2009; Chang et al. 2007; Song et al. 2006). The TNF superfamily ligand APRIL also induces *GADD45* transcription. Binding of APRIL to the receptor BCMA triggers JNK2 phosphorylation, FOXO3A activation, and *GADD45* transcription, inhibiting cell proliferation in hepatocellular carcinoma cells through cell cycle arrest at the G_2/M checkpoint (Notas et al. 2012).

The interaction of *GADD45* with BRCA1, a key breast cancer tumor suppressor, plays an important role in cell cycle control and DNA repair (Pietrasik et al. 2020). BRCA1 has been shown to induce *GADD45* transcription after γ-radiation treatment of cells (Li et al. 2000; Park et al. 2008). Similarly, overexpression of BRCA1 resulted in increased GADD45 expression and also stimulation of nucleotide excision repair (NER) in a GADD45-dependent manner (Hartman and Ford 2002). Since BRCA1-deficient cells are hypersensitive to cisplatin, this suggests a defect in NER of cisplatin adducts (Husain et al. 1998). Additionally, in response to hypoxic shock or anisomycin treatment, ATF2 binds to BRCA1, NF-1, and OCT-1 to stimulate transcription of *GADD45A* (Maekawa et al. 2008), such that BRCA1 indirectly and directly (Park et al. 2008) activates transcription of *GADD45A*. The importance of BRCA1 in the DNA damage response (DDR) is well known (Wu et al. 2010), and these findings highlight the importance of GADD45 as a downstream effector of BRCA1. This will be discussed further in Chap. 10: *GADD45 in Breast Cancer*.

As shown in Fig. 1.1, there are also several growth stimulatory factors that are involved in negative regulation of *GADD45A*. Transcriptional repression by c-MYC and AKT proto-oncogenes expression highlights the frequent association of *GADD45* with cell growth suppression (Gao et al. 2009; Bulavin and Fornace 2004; Brown-Clay and Fornace Jr 2018). MYC regulates *GADD45A* gene expression by inhibiting FOXO3A-dependent transcription of *GADD45A* (Amente et al. 2011). AKT inhibition of *GADD45A* is also mediated by FOXO3A inactivation (Amente et al. 2011).

More recently, clinical studies have demonstrated the role of miRNA in regulating GADD45A expression. In Sertoli cells of patients with Sertoli-cell-only syndrome, miR-4270 has been found to inhibit *GADD45A* mRNA expression by binding to its 3′-UTR (Wang et al. 2020). In blood samples from patients with chronic myeloid leukemia, increased miR-362-5p levels were associated with decreased GADD45A levels (Yang et al. 2015).

1.2.2 Post-transcriptional Regulation of GADD45

Early on, it became evident that *GADD45A* regulation at the post-transcriptional level is complex and can be regulated based on the mRNA stability of *GADD45A* and other *GADD* genes (Jackman et al. 1994). In unstressed cells, AUF1 destabilized *GADD45A* mRNA and TIAR1 hindered its translation, potently inhibiting expres-

sion of the GADD45A protein. After cell exposure to MMS or UV radiation, these proteins dissociate rapidly from *GADD45A* mRNA and allow robust expression of the protein. Conversely, the mRNA stabilizing protein, nucleolin, binds *GADD45A* mRNA after cellular stimulation with arsenic chloride or NF-κB inhibition and potently increases both mRNA and protein levels (Lal and Gorospe 2006). MAPK kinases (MAP2Ks) upstream of p38 have been shown to phosphorylate three proteins involved in RNA regulation, HNRNPA0, TIAR, and PARN, resulting in stabilization of *GADD45A* mRNA (Reinhardt et al. 2010). In the same report, p38/MK2 complex was found to relocalize from the nucleus to the cytoplasm, where MK2 phosphorylated hnRNPA0, and stabilized GADD45A mRNA, while p38 was found to phosphorylate and release the translational inhibitor TIAR. At the post-translational level, arsenite stimulation of cells induces formation of an IκB-kinase-β (IKKβ)/NF-κB p50 subunit complex that reduces ubiquitinated GADD45A levels and its subsequent proteasomal degradation (Yang et al. 2009).

1.2.3 GADD45 and NF-κB

The role of NF-κB in the regulation of *GADD45* is complicated and appears to depend on cellular context. NF-κB signaling is often considered a pro-survival response and was reported to reduce GADD45A and GADD45G expression and escape from apoptosis in cancer cells (Zerbini et al. 2004). NF-κB activation of EGR-1 leads to direct EGR-1-mediated transcriptional activation of *GADD45A*. The NF-κB-activating kinases, IKKα and IKKβ, are also able to induce *GADD45* expression through a NF-κB-independent mechanism. The p65 (RelA) subunit of NF-κB binds directly to three κB elements in the *GADD45B* promoter and activates its transcription. However, NF-κB also inhibits *GADD45A* and *GADD45G* expression by activating c-MYC (Zhang et al. 2014). This differential regulation of *GADD45A* might therefore contribute to the observed pro- and anti-oncogenic actions of NF-κB although the mechanisms that govern this switch are not well understood (Yang et al. 2009). In the case of *GADD45B*- and *GADD45G*-specific mechanisms of transcriptional regulation, the p65 (RelA) subunit of NF-κB binds directly to three κB elements in the promoter of *GADD45B* and activates its transcription (Yang et al. 2009). Nucleus accumbens-1 (NAC1) is a transcription factor associated with embryonic stem cell self-renewal and pluripotency that is also upregulated in several cancer types, particularly chemoresistant, recurring ovarian carcinomas. NAC1-mediated GADD45G downregulation has been shown to contribute to paclitaxel resistance in ovarian cancer cells (Jinawath et al. 2009).

1.2.4 GADD45A Reporter as an Assessor of Genotoxicity

GADD45A mRNA and proteins are frequently induced by a plethora of stresses and types of injury, and this responsiveness can be used to monitor for such events. As discussed earlier, GADD45A regulation is complex and involves multiple regulatory factors that contribute to stress responsiveness. In addition to a classic p53-binding site in its third intron (Kastan et al. 1992) and a WT1 site in its promoter that can also contribute to p53 signaling (Zhan et al. 1998; Johnson et al. 2013), there are a variety of regulatory elements, such as OCT-1, AP-1, C/EBP, GRE, and EGR-1 in the *GADD45A* gene that can contribute to stress responsiveness (Zhang et al. 2014; Takahashi et al. 2001); for a complete listing, see https://www.genecards.org/cgi-bin/carddisp.pl?gene=GADD45A. There are numerous reports of GADD45A responsiveness to various types of injury in vivo. In TK6 cells, a human lymphoblastoid line used in many toxicology assays, *GADD45A* mRNA levels were rapidly increased following exposure to a variety of genotoxic agents such as heavy metals, resulting in the unfolded protein response (UPR), oxidative stress, medium (nutrient) depletion, and inhibition of glycolysis and certain other pathways of energy metabolism (Amundson et al. 2005; Li et al. 2017). While many such stresses can rapidly induce *GADD45A* mRNA expression, geno-

toxic stress agents typically trigger stronger responses (Li et al. 2015, 2017), such that *GADD45A* induction may have utility in monitoring for genotoxic stress that is triggered either directly by DNA damage or indirectly by agents such as topoisomerase poisons and DNA synthesis inhibitors. Importantly, there is a need for newer assays to assess for genotoxic stress because the current in vitro testing battery, especially mammalian cell assays, has high sensitivity but suffers from low specificity, leading to high rates of false or irrelevant positive findings (Li et al. 2007, 2017; Snyder and Green 2001; Kirkland et al. 2005; Goodsaid et al. 2010; Krewski et al. 2020). *GADD45A* promoter reporter constructs have been employed by a variety of laboratories to assess for genotoxicity since first reported (Todd et al. 1995). Using Green Fluorescent Protein (GFP) reporter, a study of 75 genotoxic and non-genotoxic compounds demonstrated that the assay could respond positively to various classes of genotoxic damage with high specificity and high sensitivity (Hastwell et al. 2006). This and other groups (Xin et al. 2015; Simpson et al. 2013; Luzy et al. 2013; Walmsley and Tate 2012; Röckner et al. 1989) have developed high-throughput screening approaches to apply *GADD45A* reporter constructs to assess for genotoxicity with rapid in vitro methodology.

While the *GADD45A* reporter construct approach has merit, concern may arise because a variety of non-genotoxic stress stimuli are known to induce *GADD45A* as discussed above. To complement these approaches, a variety of laboratories have proposed toxicogenomics approaches to assess for genotoxicity (Amundson et al. 2005; Li et al. 2007, 2015; Liu et al. 2019; Ellinger-Ziegelbauer et al. 2009; Cui and Paules 2010; Herwig et al. 2016; Moffat et al. 2015; Chepelev et al. 2015). Many of these reports include assessment of *GADD45A* mRNA levels. The advantage here is that bioinformatic approaches can be implemented to develop a more accurate prediction of genotoxicity rather than reliance on a single gene alone. As an example, a panel of 64 genes including *GADD45A* was developed to assess genotoxicity in TK6 cells, and prediction of genotoxicity was high using a panel of genotoxic and non-genotoxic agents (Li et al. 2015, 2017). Notably, 90% of non-genotoxic agents that were positive in the traditional mammalian cell genotoxicity assays were classified as non-genotoxic with this gene expression approach (Li et al. 2017). This toxicogenomic approach also has the capability for high-throughput screening (Li et al. 2017; Cho et al. 2019a) and offers an exciting strategy to complement classic in vitro toxicology in the assessment of genotoxicity (Krewski et al. 2020).

1.3 GADD45A Effectors in Growth Arrest and Apoptosis

GADD45A, GADD45B, and GADD45G share quite a bit in common when it comes to downstream effectors. However, the literature for GADD45A is much larger, so it will be discussed first. As can be anticipated for a protein that is predominantly stress-induced, many of the well-characterized GADD45A functions are associated with growth arrest and apoptosis. Although limited direct biochemical mechanisms have been shown for GADD45A, it has been found repeatedly to form complexes with a variety of proteins and even with chromatin. It thus seems likely that its biologic effects are due to its ability to facilitate protein–protein interactions as well as to directly affect protein conformation, as in the case of MTK1. These interactions and their effects are highlighted for selected proteins in Fig. 1.1 and Table 1.1.

1.3.1 GADD45A Effectors in Growth Arrest

As shown in Fig. 1.1, GADD45A has important roles in both S phase and G_2/M arrest (Hollander and Fornace 2002; Smith et al. 1994). *GADD45A* knockdown is associated with G_2/M checkpoint abrogation following endoplasmic reticulum stress (Lee et al. 2019). It can displace PCNA from the cyclin D1 complex, possibly inhibiting

DNA replication during S phase (Smith et al. 1994). Likewise, GADD45A can inhibit CDK1 activity by promoting dissociation of CDK1/Cyclin B1, arresting the cell cycle at the G_2/M checkpoint (Zhang et al. 2014; Wang et al. 1999; Zhan et al. 1999). GADD45A can directly inhibit purified CDK1/Cyclin B1 activity in vitro (Zhan et al. 1999). In the case of control of S phase progression, loss of GADD45A results in centrosome amplification, particularly when S phase progression is chemically inhibited; in normal cells, initiation of S phase and centrosome activity are tightly coordinated by GADD45A (Hollander and Fornace 2002). GADD45A interacts with the tumor suppressor cyclin-dependent kinase inhibitor 1a (encoded by *CDKN1A*), also known as p21, CIP1, or WAF1, such that deletion of both GADD45A and p21 is associated with attenuated S-phase arrest (Hollander et al. 2005a). The two protein products compete for interaction with PCNA, and GADD45A seems to negatively regulate CDKN1A expression in keratinocytes, allowing nucleotide excision repair (NER) after UV radiation (Gao et al. 2009).

GADD45A has been found to play a role in the inhibition of β-catenin signaling, a pro-growth pathway (Hildesheim et al. 2004, 2005). Following exposure to UV radiation, GADD45A stimulates p38 in the dephosphorylation of glycogen synthase kinase 3β (GSK3 β). This activates the adenomatous polyposis coli (APC) destruction complex, which increases β-catenin phosphorylation and degradation. GADD45A also increases p38 positive regulation of APC translocation to the nucleus, an important step in β-catenin degradation, as well as localization of β-catenin at the plasma membrane. This prevents activation of the pro-invasion transcriptional program and increases its interaction with caveolin-1, strengthening cell–cell adhesion (Gao et al. 2009). Consistent with its tumor suppressor-like properties, GADD45A inhibits tumor cell invasion and migration induced by high β-catenin levels (Hildesheim and Fornace 2004).

As mentioned above, GADD45A is often required in oncogene-induced senescence (Bulavin et al. 2003) and DNA damage-induced establishment of the senescent phenotype (Passos et al. 2010). In both cases, GADD45A signaling via p38 is essential for induction of this phenotype and for full transactivation of p53, whose activity is essential for cell entry into a senescent state. In senescent human fibroblasts, p53 preferentially occupies the promoters, resulting in a unique combination of phosphorylated p53 sites (Gao et al. 2009). The positive feedback loop between GADD45A, p38, and p53 (Fig. 1.2) is thus essential for induction and maintenance of the senescent phenotype after oncogene overexpression or severe DNA damage in fibroblasts and keratinocytes, and likely in other cell types as well. This will be discussed further in Chap. 8: *GADD45 in Senescence*. In addition to premature senescence, differentiation can be used to remove damaged or potentially tumorigenic cells from the growth compartment. GADD45A upregulation in response to genotoxic conditions is associated with increased terminal differentiation of hematopoietic stem cells (Wingert and Rieger 2016; Wingert et al. 2016).

1.3.2 GADD45A Effectors in Apoptosis

GADD45A has been repeatedly associated with apoptosis after oncogenic and genotoxic stresses. Its level rises notably in mammalian apoptotic cells, and inhibition of GADD45A expression reduces apoptosis in response to DNA damage. p38 and JNK often mediate the proapoptotic effects of GADD45A. All three GADD45 proteins bind the N-terminus of MTK1, which activates p38 and JNK signaling, inducing a conformational change that results in its autophosphorylation, activation, and a strong apoptotic response (Takekawa and Saito 1998; Mita et al. 2002). As shown in Fig. 1.2, GADD45A activation of p38 and JNK signaling, which are upstream activators of GADD45A (as well as of p53, which also induces GADD45A expression), forms the basis of a positive feedback loop that raises levels of these tumor suppressive signaling molecules in the event of genotoxic stress and unresolved DNA damage. Furthermore, GADD45A expression is necessary for sustained

p38 and JNK signaling and consequent growth arrest or apoptosis in keratinocytes after UV radiation (Hildesheim et al. 2002). The sunburn response, which has a prominent apoptotic component, requires p53, p38, and GADD45A (Hildesheim and Fornace 2004), whereas GADD45A is necessary for normal p53 activation after UV radiation of keratinocytes in vivo and in primary culture, it is not needed in dermal fibroblasts (Hildesheim et al. 2002). How p53 signaling compensates in GADD45A-null dermal fibroblasts is uncertain, but it has been shown that the other GADD45 proteins are expressed more abundantly in this cell type. This observation thus highlights the cell specificity for some in vivo roles of GADD45.

GADD45A has also been suggested to be involved in early events of the apoptotic cascade through interactions with the cytoskeleton. Elongation factor 1α (EF-1α) is a microtubule-severing protein that plays a key role in cytoskeletal stability by binding, bundling, and promoting microtubule assembly. Increased GADD45A expression results in interactions with EF-1α that inhibit microtubule bundling and destabilize the cytoskeleton (Tong et al. 2005). This causes release of BIM, a BCL-2 family proapoptotic protein, from microtubule-associated complexes and allows for BIM translocation to the mitochondria, triggering cytochrome C release into the cytoplasm and initiation of apoptosis (Gao et al. 2009).

At the same time, there are other features of GADD45A that can have an opposing effect on apoptosis potential. This is not surprising, as checkpoint activation and DNA repair can also enhance cell survival. For example, GADD45A deficiency sensitizes cells to cisplatin and UV radiation, implying subtleties to the proapoptotic effects of this protein that likely result in reduced DNA repair in the absence of GADD45A. In hematopoietic cells exposed to UV radiation, GADD45A is implicated in a NF-κB-p38 survival pathway (Cretu et al. 2009). GADD45A also protects neurons from apoptotic cell death after withdrawal of nerve growth factor in spinal cord ligation (Lin et al. 2011). The first two examples can be explained as GADD45A enhancing survival by mitigating the effects of genotoxic stress, that is, arresting cell replication and stimulating DNA repair. The last example is clearer evidence of a GADD45A pro-survival function and of pronounced tissue specificity in GADD45A action.

1.3.3 Other Notable GADD45A Effectors

GADD45A, through its involvement in cell cycle control, DNA repair, apoptosis, and p53 signaling, thus, has a key role in maintaining genomic stability. This is particularly evident in GADD45A-null cells and mice that exhibit centrosome amplification and incomplete chromosome condensation during mitosis. Mitotic abnormalities lead to defective chromosome segregation, which likely leads to the chromosome and chromatid aberrations often seen in this genotype (Hollander and Fornace 2002). The genomic instability phenotype resembles that of p53-null mice although GADD45A-null mice do not show the marked spontaneous tumorigenesis seen in p53-null mice. In the case of centrosome instability, GADD45A physically associates with Aurora-A protein kinase, whose deregulated expression produces centrosome abnormality and strongly inhibits its activity (Shao et al. 2006). Conversely, *GADD45A* and *BRCA1* are both needed for full, physiological transcriptional upregulation of NEK2 (Wang et al. 2004), the correct concentration of which is essential for timely centrosome separation (Gao et al. 2009).

GADD45A also has the ability to stimulate DNA repair, as discussed in detail in Chap. 4: *GADD45 in DNA Demethylation and DNA Repair.* In vitro and cell culture assays show that recombinant GADD45A can stimulate NER in chromatin-bound DNA (Smith et al. 1994; Tran et al. 2002), whereas loss of GADD45A expression in ex vivo assays of lymphoblasts results in substantially reduced NER (Gao et al. 2009). The ability of GADD45A to interact with acetylated or UV radiation-exposed mononucleosomes and increase local DNA accessibility might facilitate stimulation of DNA repair (Ma et al. 2009).

Also discussed in more detail in Chap. 4 is the role of GADD45A-related excision repair events in the removal of DNA methylation, which is an epigenetic marker associated with repression of transcriptional initiation. GADD45A interacts directly with the four core histones and increases DNase accessibility to DNA with hyperacetylated mononucleosomes in vitro, perhaps allowing access of demethylation and DNA repair complexes to DNA in chromatin. TATA-binding protein-associated factor 12 (TAF12) was found to recruit GADD45A and the nucleotide excision repair complex to the ribosomal DNA promoter and induce its transcription in a demethylation-dependent manner (Schmitz et al. 2009). GADD45 interacts directly with various nuclear hormone receptors, including constitutive active/androstane receptor (CAR) (Yamamoto et al. 2010), RXRα, RARα, ERα, PPARα, PPARβ, and PPARγ2, perhaps mediating or facilitating transcriptional initiation of their target genes (Ma et al. 2009). GADD45A- and GADD45B-mediated DNA demethylations are also necessary for full expression of epidermal differentiation-inducing genes during calcium-triggered differentiation of epidermal stem cells (Sen et al. 2010).

Although p38 is typically discussed in the context of growth arrest, it also has key stimulatory roles in lymphocytes. GADD45A has been shown to have an important regulatory role in the case of T cell activation via p38 signaling (Salvador et al. 2005a, b; Ashwell 2006). Surprisingly, GADD45A is a negative regulator of p38 signaling during T cell activation and subsequent proliferation, as discussed in Chap. 5: *GADD45 in Immunity*. Briefly, p38 is activated by an alternate pathway involving autophosphorylation of p38 at Tyr323, and it is this pathway that is inhibited by GADD45A (Ashwell 2006). Interestingly, inhibition of the p38 alternative activation pathway in infiltrating T cells inhibits pancreatic cancer progression (Alam et al. 2015). This was demonstrated with a plasma membrane–permeable GADD45A peptide, so in this case, GADD45A may well have a tumor suppressor effect by inhibiting tumor-promoting inflammation (Alam et al. 2015).

1.4 Roles for GADD45B and GADD45G

As mentioned earlier, less is known about GADD45B and GADD45G compared to GADD45A. However, GADD45B and GADD45G are clearly defined as proapoptotic, growth-arrest proteins that share several similarities with GADD45A. Both proteins inhibit CDK1 activity and have a role in S and G_2/M checkpoints. Loss of GADD45B is associated with G_2/M checkpoint arrest and premature senescence in mouse embryo fibroblasts (MEFs) (Magimaidas et al. 2016). Like GADD45A, GADD45B promotes dissociatio of CDK1/Cyclin B1 (Zhang et al. 2014). Both GADD45B and GADD45G activate MTK1 to trigger JNK signaling (Takekawa and Saito 1998; Yang et al. 2009). They also interact with p21, and GADD45B positively regulates its expression in senescing chondrocytes (Ijiri et al. 2005) although the result of this interaction is unclear in other tissues and contexts (Gao et al. 2009). GADD45B facilitates p38-mediated activation of retinoblastoma tumor suppressor protein (Rb) by enhancing their interaction after Fas stimulation in murine hepatocytes (Cho et al. 2010). It also mediates TGF-induced apoptosis in murine hepatic cells in a p38- and SMAD-dependent manner, as well as both GADD45B and GADD45G overexpression-induced apoptosis in HeLa cells. GADD45G is associated with neuronal cell death and GADD45B with the apoptotic response in neural ischemia (Cretu et al. 2009; Cho et al. 2019b). GADD45G levels are significantly lower in anaplastic thyroid cancer cells compared to primary cultured thyrocytes, and its reintroduction by viral expression has been shown to inhibit proliferation (Yang et al. 2009).

Both GADD45B and GADD45G have been suggested to have roles in the growth and development of specific tissues in the embryo, such that they are differentially expressed during embryonic development. For example, *GADD45B* is expressed in the chorion, whereas *GADD45G* is expressed in the mouse brain (Kaufmann et al. 2011). At the cellular level,

GADD45 genes are expressed in cells undergoing differentiation, including forming somites and neuronal precursors, and their expression pattern is consistent with a potential role in cell cycle arrest.

1.4.1 GADD45B and GADD45G in p38 and JNK Signaling

GADD45B has been reported to mediate TNFα-induced NF-κB suppression of JNK-induced apoptosis by directly binding to MKK7 and inhibiting its catalytic activity (Karin 2014). However, as discussed previously, the role for GADD45B in NF-κB signaling was somewhat uncertain since *GADD45B*-null mice do not show a clear phenotype, as might be expected for deletion of an upstream inhibitor of NF-κB. Still, NF-κB is frequently over-expressed in tumor cells, and suppression of JNK-induced apoptosis has been shown to be mediated by direct binding of GADD45B to MKK7. Additionally, development of a specific inhibitor that blocks GADD45B inhibition of MKK7 has been shown to trigger cell death in a panel of multiple myeloma cell lines with high constitutive levels of GADD45B (Tornatore et al. 2014a). The GADD45B-MKK7 complex has thus been suggested as a therapeutic target in the treatment of multiple myeloma (Tornatore et al. 2014b). GADD45B has also been described to suppress JNK signaling in hematopoietic cells in response to UV treatment (Yang et al. 2009). In mouse hepatocytes, stimulation of CAR also induces its interaction with GADD45B, leading to GADD45B-mediated repression of JNK signaling and subsequent cell death (Yamamoto et al. 2010). The role of GADD45B in TGFβ-mediated apoptosis was shown using a genetic approach in GADD45B-null hepatocytes, confirming the need for GADD45B in p38 activation (Yoo et al. 2003). GADD45B promotes liver regeneration in vivo (Papa et al. 2008) and protects retinal ganglion cells in response to neuronal injury, oxidative stress, TNFα, and glutamate cytotoxicity (Liu et al. 2009).

GADD45B and GADD45G show both similarities and differences to GADD45A in immune cells. Unlike GADD45A, they potentiate p38 signaling in Th1 and $CD8^+$ cytotoxic T cells in order to promote full effector function; like GADD45A, they are negative regulators of T cell activation and proliferation (Lu 2006; Ju et al. 2009). In addition, GADD45B is necessary for full expression of the Th1 lineage-inducing proteins, T-bet, and Eomes (Ju et al. 2009). The GADD45 family members thus seem to work together to promote full maturation and function of Th1 and $CD8^+$ cells, but they also prevent inappropriate overexpression, except under certain pathological conditions.

These results highlight the complex roles for the GADD45 proteins in MAPK signaling. As shown in Fig. 1.2, the GADD45 proteins clearly stimulate the stress-mediated activation of MTK1, which is upstream of p38 and JNK, as well as more directly for p38. However, GADD45B has an opposing effect on JNK signaling by inhibition of upstream MKK7, and GADD45A has a specialized role in dampening p38's role in T cell activation, as discussed in Chap. 5: *GADD45 in Immunity*. Taken together, one can conclude that the GADD45 proteins are important components of MAPK signaling and can have either stimulatory or inhibitory effects depending on the cellular context.

1.4.2 Notable Roles of GADD45G Only

With primarily genetic approaches, GADD45 has been found to have several features distinct from other GADD45 proteins. Recently, GADD45G has been suggested to play a role in cardiomyocytes following stress. GADD45G expression is elevated following myocardial infarction in murine cardiomyocytes, and it is associated with increased p38 MAPK-dependent apoptosis and heart failure (Lucas et al. 2015). Additionally, miR-128-1-5p has been shown to decrease GADD45G expression and apoptosis in cardiomyocytes following myocardial ischemia/reperfusion injury (Wan et al. 2020).

GADD45G has also been shown to have a specific role in gonad development, male fertility, and sex determination (Gierl et al. 2012; Warr et al. 2012; Johnen et al. 2013). Notably, mice deficient in GADD45G show an unexpected male-to-female sex reversal phenotype. GADD45G-deficient XY mice on a mixed 129/C57BL/6 background have varying degrees of disorders of sexual development, ranging from male infertility to complete gonadal dysgenesis (Johnen et al. 2013). On a pure C57BL/6 background, all GADD45G$^{-/-}$ XY mice were born as completely sex-reversed XY-females (Gierl et al. 2012; Warr et al. 2012; Johnen et al. 2013). The GADD45G expression pattern is not sexually dimorphic. GADD45G levels are similar in wt XY and XX gonads during the sex determination period, and peak at the time of primary sex differentiation, when SRY is also present. GADD45A and GADD45B are not expressed in purified somatic supporting precursor cells. Only GADD45G expression is induced robustly in embryonic gonads and in somatic precursor cells (Johnen et al. 2013).

In male gonads, SRY plays a key role in the male developmental pathway by promoting differentiation of a somatic supporting cell lineage into Sertoli cells. In the absence of SRY in XX gonads, SOX9 is downregulated, and a female-specific gene expression program is activated, leading to differentiation of the somatic supporting lineage into granulosa cells, which support oocyte development. Surprisingly, GADD45G, but not GADD45A or GADD45B, is necessary for activation of the male sex-determining pathway in mice, such that its absence leads to the development of female gonads. Lack of GADD45G decreases SRY expression and blocks SOX9 expression, resulting in ovary and Müllerian duct development, whereas lack of GADD45A and/or GADD45B has no effect on testis development (Johnen et al. 2013). Although it remains to be determined how GADD45G regulates SRY expression, it is proposed that GADD45G is needed to promote MAP3K4-mediated activation of p38 signaling in murine embryonic gonadal somatic cells. p38 can phosphorylate GATA4 and then phospho-GATA4 might bind and activate the *SRY* promoter to induce the male program (Gierl et al. 2012; Warr et al. 2012). In utero exposure to Di (2-ethylhexyl) phthalate (DEHP) has been shown to inhibit the GADD45G-dependent sex determination pathway in mice (Wang et al. 2015).

1.5 Involvement of GADD45 in Tumorigenesis

Loss of GADD45A has been shown to confer a tumor-prone phenotype after genotoxic stress. GADD45A has been shown to inhibit autophagy in tumors, which likely provides a nutrient advantage to tumor cells, by inhibiting BECN1-PIK3C3 interactions (Zhang et al. 2015). Studies in GADD45A-null mice illustrate that GADD45A-dependent protection against UV irradiation-induced skin tumors requires functional p38 (Hildesheim et al. 2002). Abolition of either GADD45A or p38 activity results in compromised negative regulation of β-catenin via the APC destruction complex (Gao et al. 2009). p53-signaling in the sunburn response requires GADD45A for effective p38 activation, which then signals p53 (Hildesheim et al. 2002), as shown in Fig. 1.2. GADD45A-null mice also show increased rates of IR- or dimethylbenzanthracene-induced tumors, with a shorter latency period than controls (Hollander et al. 1999, 2001). Deletion of *GADD45A* in an XPC$^{-/-}$ mouse model of lung cancer led to an increase in lung tumor malignancy, and allelic deletion of *GADD45A* is associated with multiple tumor types, including lung (Hollander et al. 2005b) and mammary tissue (Pietrasik et al. 2020). Loss of GADD45A is also associated with worse outcomes in chronic myeloid leukemia in mice (Mukherjee et al. 2017), and similar findings have been demonstrated with loss of GADD45B as well (Sha et al. 2018). Increased expression of lncRNA NEAT1 and binding to BRG1 are associated with decreased

GADD45A expression and reduced survival for gastric cancer in mice (Ma et al. 2020). Sustained ERK1/2 signaling in an acute myeloid leukemia model cell line downregulates *GADD45A*, and the reintroduction of expression induces S phase arrest and apoptosis (Cretu et al. 2009). Simultaneous H-RAS overexpression and *GADD45A* knockout are sufficient to transform cells, indicating that *GADD45A* knockout can function as one of the "two hits" in oncogenic transformation (Bulavin et al. 2003).

GADD45 has been shown to play a role in the inhibition of angiogenesis, which is an important component of tumorigenesis. GADD45A is central to suppression of tumor angiogenesis by blocking the mTOR/STAT3 pathway. Lack of GADD45A increases STAT3 phosphorylation at Ser727 and elevates STAT3 transcriptional activity. This process induces the expression and secretion of vascular endothelial growth factor (VEGF-A) and promotes formation of tumor blood vessels. Moreover, GADD45A can interact with mTOR and suppress STAT3 phosphorylation, leading to downregulated expression of VEGF-A (Yang et al. 2013).

1.5.1 GADD45 Expression in Clinical Studies

Aberrant GADD45 expression has been found in an increasing number of clinical studies. These findings are summarized in Table 1.2 and in the text below. The *GADD45A* promoter is methylated in a majority of breast cancers and a significant fraction of prostate cancers, whereas the *GADD45G* promoter is likewise hypermethylated in several human hepatocellular carcinomas, in both cases with subsequent downregulation of expression (Cretu et al. 2009). However, the pregnane X receptor can activate GADD45B/p38 MAPK signaling to induce a change in morphology and migration in a hepatocellular carcinoma cell line (Kodama and Negishi 2011). Increased GADD45A expression is associated with improved prognosis in patients with ovarian cancer (Yuan et al. 2015). Decreased expression of GADD45A and GADD45G is associated with worse prognosis in patients with gastric cardia adenocarcinoma (Guo et al. 2013a). Loss of GADD45A in acute myeloid leukemia (Wang et al. 2012; Perugini et al. 2013) similarly carries a worse prognosis. Increased GADD45B expression is associated with worse prognosis in

Table 1.2 Examples of aberrant GADD45 protein expression in various human cancers. Based on mouse model studies, reduced expression (↓) of GADD45 proteins in human cancers would be expected, but there are also a limited number of examples where increased expression (↑) has also been found

Tumor type	Expression	Prognosis
Breast	↓ GADD45A	Worse
Prostate	↓ GADD45A	Worse
Hepatocellular carcinoma	↓ GADD45G	Worse
Ovarian	↑ GADD45A	Improved
Gastric cardia adenocarcinoma	↓ GADD45A+G	Worse
Acute myeloid leukemia	↓ GADD45A	Worse
Papillary thyroid carcinoma	↑ GADD45B	Worse
Colorectal	↑ GADD45B, ↓ GADD45G	Worse
Esophageal squamous carcinoma	↓ GADD45A&G	Worse
Pancreatic	↑ GADD45A, ↓ GADD45G	Worse
Thyroid	↑ GADD45A	Worse
Lung	↓ GADD45G	Worse
Lymphoma	↓ GADD45G	Worse
Nasopharyngeal carcinoma	↓ GADD45G	Worse
Cervical carcinoma	↓ GADD45G	Worse
Pituitary adenoma	↓ GADD45G	Worse

patients with papillary thyroid carcinoma (Barros-Filho et al. 2020) and colorectal cancer (Wang et al. 2012; Zhao et al. 2018). Decreased expression of GADD45A and GADD45G is associated with worse prognosis in patients with esophageal squamous cell carcinoma (ESCC) (Ishiguro et al. 2016; Guo et al. 2013b). More recently, GADD45G has been suggested to inhibit ESCC migration and invasion through its interactions with E-cadherin (Li et al. 2020).

Although GADD45 has clear tumor suppressor features, it might also offer pro-growth advantages to certain malignant cells, in line with its roles in cell growth arrest and DNA repair. In one study, point mutations were found in exon four of the *GADD45A* gene in 14% of pancreatic cancer samples, and GADD45A expression in p53-positive tumors was associated with a lower patient survival rate (Yamasawa et al. 2002). GADD45A induction can protect melanoma cells from UV radiation-induced death (Jean et al. 2001). Lack of GADD45A induction in cervical carcinomas correlates with a good clinical response to radiotherapy (Gao et al. 2009). In addition, despite decreased FOXO3A transcriptional activity, GADD45A expression is upregulated in thyroid cancers (Karger et al. 2009).

In cancer, given the higher reported rate of promoter hypermethylation or upregulation of GADD45-repressed transcription of a multitude of different proteins, multiple *GADD45* functions could be important as alteration of a single function might be insufficient to induce or intensify the tumor phenotype. GADD45G is also deficient in several tumors. Its gene promoter region is hypermethylated and its transcription is repressed in a significant number of non-small cell lung cancers (Na et al. 2010), lymphomas, nasopharyngeal carcinomas, cervical carcinomas, esophageal carcinomas, pituitary adenomas, and gastric, colorectal, and pancreatic cancers (Yang et al. 2009; Zhang et al. 2010); however, genetic mutation and inactivation are rare. Exogenous reintroduction of GADD45G results in G_2/M arrest in a number of tumor cell lines, including prostate carcinoma and pituitary adenoma (Yang et al. 2009).

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Roles for GADD45 in Development and Cancer

2

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Abstract

The Growth Arrest and DNA Damage-inducible 45 (GADD45) family of proteins are critical stress sensors that mediate various cellular responses, including DNA repair, cell cycle arrest, and apoptosis. Here, we review current literature investigating GADD45 family members as they relate to normal development and carcinogenesis. We first describe how modulation of GADD45 in model organisms has facilitated our understanding of roles for GADD45 family members in development and homeostasis. We then review current literature exploring roles for GADD45 in human cancer, describing cancer-associated alterations in expression of GADD45 family members; tumor suppressive and tumor promoting functions attributed to GADD5; and roles for GADD45 in cancer therapy. In exploring roles for GADD45 in development, homeostasis, and carcinogenesis, we aim to provide an informational resource that both highlighst current knowledge on this topic while also noting key gaps in our understanding of the biology of GADD45 that may be filled in order to best guide the development of novel approaches to improve diagnosis, monitoring, and therapy of human malignancies.

Keywords

GADD45 · Development · Cancer · Homeostasis · Drosophila · Zebrafish · Xenopus · Oryctolagus · Mouse · Tumor suppressor · Therapy

2.1 Introduction

The Growth Arrest and DNA Damage-inducible 45 (GADD45) family of proteins plays a critical role in integration of cellular responses to various stressors. In mammals, the GADD45 family consists of three genes, *GADD45A* (*GADD45/GADD45α*), *GADD45B* (GADD45β/*MyD118*), and *GADD45G* (GADD45χ/*CR6*), encoded on chromosomes 1, 19, and 9, respectively. The products of the GADD45 genes are small (18 kd), highly acidic proteins exhibiting a high degree of homology (Liebermann and Hoffman 2008). GADD45 family members localize to both the

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cytoplasm and the nucleus, and are ubiquitously expressed in normal human adult and fetal tissues, albeit typically at low abundance (Tamura et al. 2012). GADD45 expression is induced in response to stressors and contributes to regulation of diverse cellular functions, including DNA demethylation and repair, cell cycle, and apoptosis.

As GADD45 proteins lack enzymatic activity, their functions are mediated via interactions with partner proteins (Fig. 2.1). GADD45A has been shown to promote DNA demethylation via interactions with histones (Carrier et al. 1999). Additionally, GADD45A and GADD45B binding to proliferating cell nuclear antigen (PCNA) facilitates DNA repair, which in turn, erases methylation marks to relieve epigenetic gene silencing (Smith et al. 1994; Azam et al. 2001; Barreto et al. 2007). GADD45 family members induce G2/M arrest via interaction with CDC2 kinase, resulting in disruption of the cyclin B1/CDC2 complex which is required for the G2/M transition (Vairapandi et al. 2002; Wang et al. 1999). GADD45 proteins also bind to the cell cycle inhibitor p21 to promote G1 arrest (Azam et al. 2001; Kearsey et al. 1995; Fan et al. 1999). Intersection of GADD45 and stress-activated mitogen-activated protein kinase (MAPK) signaling has also been demonstrated. Each of the three GADD45 family proteins has the ability to bind to MTK1 (MEKK4) (Takekawa and Saito 1998), a critical regulator of stress-activated MAPK signaling. GADD45/MTK1 binding disrupts the auto-inhibitory domain of MTK1, promoting its activation followed by downstream induction of c-Jun N-terminal kinase (JNK) and p38, two members of the stress-activated MAPK pathway whose persistent activation promotes apoptosis (Chen et al. 1996). GADD45A may also bind directly to p38, promoting p38-MAPK activation (Bulavin et al. 2003). The kinase MKK7 is another activator of JNK. GADD45B-mediated association with MKK7 has been shown to block the catalytic activity of this kinase, thereby indirectly suppressing JNK activation (Papa et al. 2007). GADD45 interaction with these partner proteins may be regulated at the level of expression, subcellular localization, or posttranslational modification, all of which may occur on GADD45 proteins as well as on their interacting partners. This exquisite level of regulation serves as a rheostat, acting to ensure that GADD45-mediated cellular responses are accurately tuned to stress stimuli.

Here we will explore the diverse functions and molecular pathways regulated by GADD45 family members in the context of normal development and carcinogenesis. We will discuss (1) how modulation of GADD45 expression in model organisms has facilitated our understanding of roles for GADD45 family members in development and homeostasis; and (2) how studies in human patient tissues as well as in vitro and in vivo model systems have uncovered roles GADD45 in cancer biology.

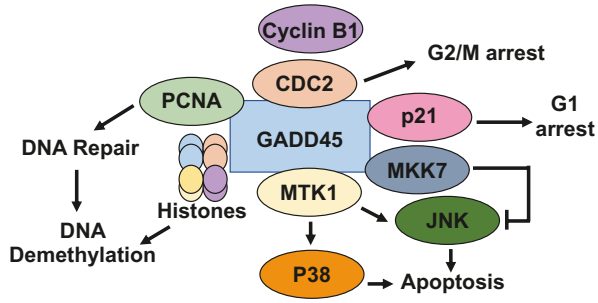

Fig. 2.1 GADD45 binding to interacting proteins mediates diverse cellular responses. A summary of proteins that GADD45 family members have been demonstrated to bind to and the impact of this binding on the cellular outputs. Although note shown, GADD45 binding to p38 has been demonstrated to promote p38-MAPK activation

2.2 GADD45 in Development and Homeostasis: Insights from Model Organisms

Effects of knockdown and overexpression of GADD45 family members have been evaluated in various model organisms, revealing both unique and overlapping functions for these proteins in development and homeostatic maintenance. Here, we provide an overview of studies exploring GADD45 modulation and the effects of such modulation at the whole body and tissue level in model organisms spanning the evolutionary scale from fly to mouse.

2.2.1 *Drosophila melanogaster* (Fly)

The *Drosophila melanogaster* genome encodes for only one member of the GADD45 family, *D-GADD45*. While ubiquitous overexpression of *D-GADD45* is lethal in *Drosophila* (Peretz et al. 2007), tissue-specific overexpression revealed several non-lethal phenotypes. With regard to somatic cells, apoptosis was observed upon *D-GADD45* overexpression in *Drosophila* follicle cells; however, this effect appeared to be tissue-specific as overexpression in the imaginal wing disc or compound eye failed to induce cell death (Peretz et al. 2007). Notably, *D-GADD45* expression level and duration may influence observed phenotypes as an independent study confirmed a lack of apoptosis in the imaginal disc upon transient *D-GADD45* overexpression; but identified robust induction of apoptosis as well as wing defects with sustained *D-GADD45* overexpression (Camilleri-Robles et al. 2019). In addition to playing a role wing development in *Drosophila,* genetic depletion of *D-GADD45* impaired regeneration of wing imaginal discs (Camilleri-Robles et al. 2019). In the germline, *D-GADD45* overexpression impacted dorsal-ventral polarity in the oocyte, resulting in a broad range of eggshell phenotypes and disrupting localization of anterior-posteriority determinants (Peretz et al. 2007). Activation of MAPK-JNK signaling was linked to defects in both polarity and wins formation in the context of sustained *D-GADD45* overexpression (Peretz et al. 2007; Camilleri-Robles et al. 2019). Indeed, *Drosophila* represents an ideal model system for investigating the GADD45/MAPK signaling axis, as in addition to the presence of only one GADD45 gene, *Drosophila* possess all of the MAPK families encoded in the mammalian genome, although MAPK pathways are typically encoded by fewer genes in the latter.

In the *Drosophila* brain, overexpression of *D-GADD45* failed to induce alterations in fertility or locomotor activity of young flies; however, increased lifespan was noted in flies with *D-GADD45* overexpression in the nervous system (Plyusnina et al. 2011). Neuroblasts of *D-GADD45*-overexpressing flies additionally demonstrated a decreased in the level of spontaneous single-strand DNA breaks, indicating enhanced efficiency in recognition and repair of DNA damage. Consistent with D-GADD45 acting to limit cellular stress, ultrastructural evaluation revealed that neuronal *D-GADD45* overexpression suppressed phenotypes associated with neurodegeneration, including cytoplasmic vacuolization and defects in mitochondrial cristae in aged *Drosophila* (Bgatova et al. 2015). The link between GADD45 and neurodegeneration is of particular interest as elevated GADD45 expression has been demonstrated in neurons of human Alzheimer's disease patients and overexpression of GADD45 in vitro protects against neuronal apoptosis (Torp et al. 1998). These findings make it tempting to speculate that GADD45 family members may act in a cytoprotective manner in the brains of Alzheimer's patients, which could be clinically exploited for therapy development. However, roles for individual GADD45 proteins as they relate to Alzheimer's disease should be studied further, particularly in light of a recent study indicating that aging reduces hippocampal expression of *Gadd45g* in mice and that hippocampal depletion of *Gadd45g* in young mice impairs cognitive function (Brito et al. 2020).

2.2.2 *Danio rerio* (Zebrafish)

In zebrafish, two homologs of human *GADD45*B were identified by in situ-based screening, *gadd45β1* (also known as *gadd45bb*), and *gadd45β2* (also known as *gadd45ab*) (Kawahara et al. 2005). Functional homology to human GADD45β was suggested as in vitro biochemical assays demonstrated that zebrafish GADD45β1 binds with human MTK1 and promotes p38 activation (Kawahara et al. 2005). During zebrafish embryogenesis, *gadd45β1* and *gadd45β2* are expressed in paired stripes adjacent to the neural tube in the anterior presomitic mesoderm (PSM), the predecessor of somites which will, in turn, give rise to vertebrae and muscles. A functional role for *gadd45β1* and *gadd45β2* in somitogenesis was indicated as either knockdown or overexpression of *gadd45β1* and *gadd45β2* impaired somite segmentation (Kawahara et al. 2005). In the context of knockdown, segmentation inhibition was only apparent upon injection of morpholinos targeting both *gadd45β1* and *gadd45β2* which further disrupted patterning of genes associated with somite development. The presence of normal segmentation with individual gene targeting suggests redundancy for *gadd45β* genes in regulation of zebrafish somite segmentation. Although overexpression of either *gadd45β1* or *gadd45β2* also resulted in impaired segmentation, this phenotype was associated with inhibition of segmentation-associated gene expression as opposed to the alterations in their patterning that were observed with *gadd45β1* and *gadd45β2* knockdown. These findings highlight the notion that *gadd45β* gene expression must be precisely regulated for proper somite segmentation in the context of zebrafish development.

2.2.3 *Xenopus laevis* (Frog)

Xenopus laevis expresses *Gadd45a*, *Gadd45b,* and *Gadd45g* with 62% sequence homology noted for *Gadd45b* in *Xenopus* and human (Barreto et al. 2007; de la Calle-Mustienes et al. 2002; Kaufmann and Niehrs 2011). Overexpression and knockdown studies have revealed that *Gadd45b* is dispensable during embryogenesis while *Gadd45a* and *Gadd45g* show functional redundancy (Kaufmann and Niehrs 2011). With regard to *Gadd45a* and *Gadd45g,* overexpression and morpholino-based depletion of either gene resulted in defects in embryonic axes, head formation, and gastrulation (de la Calle-Mustienes et al. 2002; Kaufmann and Niehrs 2011). These defects were more pronounced in *Gadd45a* and *Gadd45g* double morphants and were unaffected by injection of either human *Gadd45a* or *Gadd45g* mRNA alone; but could be rescued with a mixture of human *Gadd45a* and *Gadd45g* mRNA (Kaufmann and Niehrs 2011). Phenotypes observed upon knockdown and overexpression of *Gadd45a* and *Gadd45g* were associated with impaired cell proliferation, upregulation of cell cycle inhibitors, and induction of p53. Moreover, decreased expression of neural crest markers concurrent with upregulation of pluripotency markers in *Gadd45a* and *Gadd45g* double morphants suggests that *Gadd45a* and *Gadd45g* may be required for progression from multipotent precursors to differentiated cells during *Xenopus* embryogenesis (Kaufmann and Niehrs 2011). Morpholino-based depletion of *Gadd45b* failed to impact embryos even at high doses, perhaps owing to the noted low level of *Gadd45b* expression during *Xenopus* embryogenesis relative to that of *Gadd45a* and *Gadd45g* (Kaufmann and Niehrs 2011). *Gadd45b* mRNA injection also had little effect on embryogenesis underscoring the dispensability of this gene in embryonic development of *Xenopus* (de la Calle-Mustienes et al. 2002; Kaufmann and Niehrs 2011).

2.2.4 *Oryctolagus cuniculus* (Rabbit)

In rabbits, CRISPR/Cas9-mediated homozygous *GADD45G* knockout was recently found to induce death by postnatal day 3 associated with severe craniofacial defects (Lu et al. 2019). Specifically, impaired formation and fusion of the upper lip was identified in *GADD45G* knockout rabbits during embryonic development. At the cellular level, increased proliferation concur-

rent with decreased apoptosis and EMT was detected in the medial and lateral nasal processes in the context of *GADD45G* loss, resulting in persistence of the epithelial seam and a cleft lip/palate-like phenotype. These findings are not only interesting as they provide a mechanistic link for GADD45G to craniofacial development, but also because GWAS studies have identified GADD45G as a candidate cleft lip/palate-related gene in human subjects (Beaty et al. 2013; Yu et al. 2017).

2.2.5 *Mus musculus* (Mouse)

Expression of *Gadd45a*, *Gadd45b,* and *Gadd45g* has been mapped during murine embryogenesis with these genes exhibiting both differential and overlapping expression domains (Kaufmann et al. 2011). Specific expression domains include the tip of the closing neural tube for *Gadd45a,* the chorion for *Gadd45b,* and the mouse brain for *Gadd45g,* whereas the somites represent a common expression domain for the three genes. Genetically engineered mouse models with single knockout of *Gadd45a*, *Gadd45b,* or *Gadd45g* are viable and fertile (Hollander et al. 1999; Lu et al. 2001, 2004; Hoffmeyer et al. 2001). As these mice fail to display any overt abnormalities, it was initially proposed that GADD45 family members play redundant roles in the context of development; however, closer analysis of GADD45-deficient mice has revealed phenotypes in various tissues during development, under homeostasis, and in response to stressors.

Gadd45a-knockout mice display genomic instability, enhanced radiation-induced carcinogenesis, and low frequency of exencephaly (Hollander et al. 1999). In *Gadd45b*-knockout mice, embryonic defects in mineralization and decreased bone growth were identified (Ijiri et al. 2005). These phenotypes were associated with impaired expression of matrix metalloproteinase (MMP)-13, an essential mediator of chondrocyte differentiation with in vitro studies defining a mechanistic link between GADD45B and *Mmp13* gene expression via JNK-mediated phosphorylation of JunD. *Gadd45b*-knockout murine embryos also exhibit evidence of senescence in the skin with adult mice displaying increased DNA damage and premature aging (Magimaidas et al. 2016). In vitro studies further linked senescence in *Gadd45b*-knockout mouse embryonic fibroblasts (MEFs) to increased signaling through the p19ARF-p53-p21 axis as well as impaired CDC2 expression and defective G2/M cell cycle progression. In *Gadd45g*-deficient mice, examination of sexual development revealed a role for GADD45G in gender determination (Warr et al. 2012; Gierl et al. 2012). While XX *Gadd45g*-knockout mice were found to be fertile females as expected, XY *Gadd45g*-knockout mice displayed phenotypic traits of females, including identifiable ovaries as well as oviduct and uterine structures. Notably, although XY *Gadd45g*-knockout mice exhibited evidence of normal mating behavior, the animals failed to yield offspring. Sex reversal in *Gadd45g*-knockout mice was associated with reduced gonadal expression of sex-determining genes including *Sry*. Mechanistically, *Gadd45g* was proposed to genetically interact with *Map3k4*, resulting in p38-mediated phosphorylation of GATA4 and transcriptional upregulation of *Sry* in XY supporting cells (Warr et al. 2012; Gierl et al. 2012). Finally, alterations in immune cell biology were identified in *Gadd45a*-, *Gadd45b*-, and *Gadd45g*-deficient mice (Lu et al. 2001, 2004; Salvador et al. 2002). *Gadd45a*-knockout mice spontaneously develop a lupus-like syndrome that induced death in female animals starting at 7 months of age (Salvador et al. 2002). Additionally, T cells from *Gadd45a*$^{-/-}$ mice displayed enhanced proliferation following T cell receptor (TCR) stimulation while B cell stimulation and apoptosis were unaffected. In *Gadd45b*-deficient mice, naïve CD4+ T cells exhibited impaired MAPK activation and Interleukin (IL)-2 production. *Gadd45b* loss also resulted in diminished cytokine production in T helper (Th)1, Th2, and Th0 cells responding to TCR stimulation, as well as in dendritic cells responding to lipopolysaccharide. Impaired TCR-induced activation of p38 and JNK pathways as well Inteferon-γ production was also noted in Th1 cells from *Gadd45g*-deficient mice (Lu et al. 2001).

Although challenging to achieve in vivo, combined knockout of *Gadd45a*, *Gadd45b,* and *Gadd45g* in mouse embryonic stem (ES) cells was recently achieved using CRISPR/Cas9 technology (Schule et al. 2019). These triple knockout ES cells revealed that while GADD45 proteins are dispensable for ES cell pluripotency and self-renewal, DNA hypermethylation is detected at ~7000 sites with enrichment for loci undergoing TET-mediated methylation. DNA hypermethylation in triple knockout cells further correlated with downregulation of methylation-regulated genes with deregulated genes showing significant overlap with those identified in *Tet1* knockdown ES cells. Individual overexpression of GADD45A, GADD45B, or GADD45G in triple knockout ES cells was sufficient to rescue downregulation of methylation-regulated genes, indicating redundant roles ADD in regulation of ES cell gene expression. Triple knockout cells also displayed enhanced dysregulation of gene expression upon embryoid body and monolayer differentiation as well as impaired transition to the embryonic two-cell stage. *Gadd45a* and *Gadd45b* were further identified to show peak expression in the two-cell stage, and mice with combined knockout of *Gadd45a* and *Gadd45b* displayed decreased litter size and developmental arrest with phenotypes consistent with neural tube defects observed in surviving pups.

2.2.6 Summary

The described studies in model organisms provide evidence for GADD45 family members as critical regulators of development and homeostatic maintenance across tissue types, while also highlighting the diverse molecular pathways and cellular processes that are mediated by GADD45 family members. Given the evident intersection of GADD45 family members with tissue architecture establishment and maintenance, DNA damage, cell proliferation, apoptosis, differentiation, EMT, mitochondrial biology, and inflammatory signaling, it is not surprising that roles for GADD45 proteins have emerged in the context of cancer biology.

2.3 GADD45 in Cancer

Although GADD45 family members have been implicated in various aspects of carcinogenesis across tissue types, the precise roles for these proteins in cancer biology remain incompletely understood. Here, we review studies demonstrating altered expression of GADD45 family members in human cancer as well as mechanisms supporting these alterations. As both tumor suppressive and tumor promoting functions have been attributed to GADD45 family members, we review current literature exploring functional roles for GADD45 in tumorigenesis. Finally, we explore GADD45 family members as they relate to response to both established and experimental anti-cancer therapeutics.

2.3.1 Expression of GADD45 Family Members in Human Cancer

Alterations in expression of GADD45 family members have been noted in diverse tumor types (Table 2.1) with decreased expression detected in hepatocellular carcinoma (HCC) as well as ovarian, lung, breast, pituitary, and gastric cancers (Yuan et al. 2015; Higashi et al. 2006; Zhu et al. 2009; Qiu et al. 2003; Ou et al. 2015; Sun et al. 2003; Wang et al. 2005; Zhang et al. 2002; Guo et al. 2013a), and increased expression identified in glioblastoma, multiple myeloma, pancreatic ductal adenocarcinoma, and cholangiocarcinoma (Reddy et al. 2008; Tornatore et al. 2014; Schneider et al. 2006; Myint et al. 2018). In esophageal squamous cell carcinoma (ESCC), increased *GADD45A* RNA and decreased *GADD45G* RNA were demonstrated when comparing tumor tissue to matched normal esophageal mucosa while *GADD45B* RNA levels did not change (Guo et al. 2013b). Additionally, negativity for GADD45G at the protein level uniquely correlated with poor survival in esophageal cancer patients. Notably, protein evaluation revealed that while GADD45A positivity in the cytoplasm was evident in 39.1% of ESCC lesions and absent in normal tissues, GADD45A positivity in the cytoplasm was more frequently detected

Table 2.1 Summary of studies examining GADD45 expression in human cancers

Cancer type	GADD45 analysis method	Findings	Reference
Studies demonstrating decreased GADD45 expression			
Ovarian cancer	qRT-PCR	Decreased *GADD45A* in ovarian tumor tissues compared to ovarian tissues from normal subjects	Yuan et al. (2015)
Non-small cell lung cancer (NSLC)	qRT-PCR	Decreased *GADD45A* in NSLC tumor tissues compared to adjacent normal tissue	Higashi et al. (2006)
Hepatocellular carcinoma (HCC)	IHC	Decreased GADD45G, no change in GADD45A in HCC tumor tissues compared to adjacent normal liver tissue	Zhu et al. (2009)
	Microarray, Northern blot, qRT-PCR, IHC	Decreased GADD45B in HCC lesions compared to matched non-neoplastic liver	Qiu et al. (2003)
		Low GADD45B correlates with tumor differentiation and high nuclear grade	
	qRT-PCR, Western blot	Decreased GADD45G in HCC tumor tissues compared to adjacent normal liver tissue	Ou et al. (2015)
		Low GADD55G correlates with poor overall survival and vascular invasion	
	Northern blot	Decreased *GADD45G* in HCC tumor tissues compared to paired nontumor liver tissue	Sun et al. (2003)
Breast cancer	Northern blot	Decreased expression of G*ADD45A* in breast cancer tissues compared to normal breast cultures	Wang et al. (2005)
Pituitary adenomas	RT-PCR	Decreased expression of *GADD45G* in pituitary tumors compared to pituitary gland tissue from normal subjects	Zhang et al. (2002)
Gastric cardia adenocarcinoma (GCA)	RT-PCR, qRT-PCR, IHC	Decreased expression of GADD45A and GADD45G, no change in GADD45B in GCA tissue compared to adjacent normal gastric tissue	Guo et al. (2013a)
		Negative expression of GADD45A or GADD45G correlates with poor survival	
Studies demonstrating increased GADD45 expression			
Glioblastoma	qRT-PCR, IHC	Increased expression of GADD45A in glioblastoma tissues compared to normal brain tissue	Reddy et al. (2008)
Multiple myeloma (MM)	qRT-PCR	Increased expression of *GADD45B* in monoclonal CD138+ plasma cells from MM patients compared to healthy polyclonal plasma cells	Tornatore et al. (2014)
		High *GADD45B* correlates with shorter progession-free and overall survival	
Pancreatic ductal adenocarcinoma (PDAC)	qRT-PCR, IHC	Increased GADD45A in PDAC compared to normal pancreatic tissue	Schneider et al. (2006)
Choliangiocarcinoma (CCA)	IHC	Increased expression of GADD4B in CCA lesions as compared to surrounding stroma	Myint et al. (2018)
		High GADD45B correlates with metastasis	

(continued)

Table 2.1 (continued)

Cancer type	GADD45 analysis method	Findings	Reference
Studies demonstrating both decreased and increased GADD45 expression			
Esophageal squamous cell carcinoma (ESCC)	RT-PCR, qRT-PCR, IHC	Increased GADD45A, decreased GADD45G, no change in GADD45B in ESCC tissue compared to matched normal esophageal tissue	Guo et al. (2013b)
		Negative GADD45G expression correlates with poor survival	

IHC Immunohistochemistry, *qRT-PCR* quantitative real time reverse transcriptase-PCR, *RT-PCR* reverse transcriptase-PCR

in normal tissues (77.3%) compared to ESCC tissues (51.6%). Thus, GADD45 family members may play unique roles in carcinogenesis and should be evaluated individually with consideration for subcellular localization. Furthermore, such evaluations should be carried out not only across tumor types, but also within each subtype of cancer.

Studies across tumor types have largely failed to identify recurrent mutations in *GADD45* genes (Yuan et al. 2015; Zerbini and Libermann 2005; Yu et al. 2010). One notable exception is in pancreatic cancer, in which *GADD45A* mutations have been identified in 13.6% of resectable invasive pancreatic ductal carcinomas (Yamasawa et al. 2002). Presently, the functional consequences of these mutations in GADD45 remain to be determined. Single nuclear polymorphisms (SNPs) in *GADD45A* have also been detected in ovarian and breast cancer (Yu et al. 2010; Blaszyk et al. 1996; Desjardins et al. 2008) while SNPs in *GADD45B* have been found in lung cancer (Hou et al. 2017). Although the clinical significance of *GADD45* SNPs is largely unknown, the *GADD45A* 1506T > C polymorphism was shown to be significantly associated with cancer risk (Odds ratio = 1.71, 95% confidence interval [1.28–2.29]; $p < 0.001$) and poor prognosis in ovarian cancer. An association between *GADD45A* 1506T > C and *GADD45* RNA expression was also noted despite the SNP occurring in an intronic region. As GADD45A expression is regulated by multiple transcription factors, including p53 (Kastan et al. 1992), it is possible that the *GADD45A* 1506T > C polymorphism may impede transcription factor binding; however, additional studies are needed to assess the impact of cancer-associated SNPs on the expression and function of GADD45 family members.

Beyond changes in DNA sequence, cancer-associated methylation of all three *GADD45* genes has been demonstrated. In both primary breast cancer tissues and breast cancer cell lines, a contiguous series of four CpG residues ~700 bp upstream of the transcriptional start site of *GADD45A* have been shown to exhibit hypermethylation (Wang et al. 2005). Notably, methylation at these residues was absent in normal breast tissue and cell lines. Overall, *GADD45* RNA levels are diminished when comparing cell lines or tissues from breast cancer patients to those from normal human subjects (Wang et al. 2005). Moreover, treatment with the DNA methylation inhibitor 5-azacytidine induced *GADD45A* expression in several breast cancer cell lines, indicating that methylation actively contributes to *GADD45A* suppression. However, it must be noted that the relationship between methylation and *GADD45* induction by 5-azacytidine is likely to be complex. For instance, 5-azacytidine highly induced the expression of *GADD45* in several cell lines that failed to exhibit detectable methylation at the defined CpG residues (Wang et al. 2005). While these data are consistent with a model wherein *GADD45A* expression may be regulated by direct promoter methylation or by methylation of a trans-acting factor, additional studies are necessary to define the precise mechanics through which methylation contributes to GADD45 suppression in the context of breast cancer. Methylation of the same key CpG residues in

GADD45A has been demonstrated in tissues from patients with prostate cancer, gastric adenocarcinoma, and AML with a link to poor overall survival identified in AML (Guo et al. 2013a; Ramachandran et al. 2009; Perugini et al. 2013). Although methylation of *GADD45B* has only been characterized in HCC (Qiu et al. 2004), methylation of *GADD45G* has been shown in several types of cancer (Guo et al. 2013b; Ying et al. 2005; Bahar et al. 2004). In a study analyzing 75 cell lines, methylation of *GADD45G*, but not *GADD45A or GADD45B,* was detected in 85% of non-Hodgkin's lymphoma, 73% of nasopharyngeal carcinoma, 50% of Hodgkin's lymphoma, 50% of cervical carcinoma, 40% of lung carcinoma, and 29% of esophageal carcinoma cell lines (Ying et al. 2005). This study additionally evaluated a panel of primary human cancer specimens. Overall, *GADD45G* methylation was more frequently found in lymphomas, including 88% of Burkitt's lymphomas and 34% of Hodgkin's lymphomas, as compared to carcinomas; however, *GADD45G* methylation was noted in 16% of nasopharyngeal carcinomas, 11% of esophageal carcinoma, and 11% of gastric carcinomas. Methylation of *GADD45G* was neither detected in normal immortalized cell lines nor in normal tissue or blood specimens (Ying et al. 2005). Direct epigenetic silencing of *GADD45G* as a consequence of methylation was suggested as either inhibition of DNA methylation by treatment with 5-aza-2′-dideoxycytidine or double genetic knockout of DNA methyltransferases *DNMT1* and *DNMT3B* restored *GADD45G* expression in several cell lines (Ying et al. 2005). Evidence of potential prognostic utility for *GADD45* methylation was demonstrated in ESCC lesions where methylation of the proximal promoter of *GADD45G* was associated with decreased GADD45 protein levels and both GADD45G methylation status and protein expression were independently associated with patient survival (Guo et al. 2013b).

The described studies indicate that expression of GADD45 family members is altered in cancer biology with DNA methylation representing a common mechanism contributing to their downregulation. Consistent with both upregulation and downregulation of GADD45 in human cancer, evidence of GADD45 family members acting to both inhibit and promote tumorigenesis has been uncovered.

2.3.2 Tumor Suppressive Roles for GADD45 Family Members

As described above, enhanced radiation-induced carcinogenesis and genomic instability, including aneuploidy, are evident in *Gadd45a*-knockout mice (Hollander et al. 1999). *Gadd45a*-knockout mice have also been shown to exhibit increased susceptibility to the chemical carcinogen dimethylbenzanthracene with decreased DNA repair and increased mutation frequency noted in *Gadd45a*$^{-/-}$ tumors as compared to those from wild type controls (Hollander et al. 2001). MEFs from *Gadd45a*-knockout mice are capable of H-Ras-mediated single oncogene transformation, which is attributed to GADD45A binding to p38 to suppress p53 activation (Bulavin et al. 2003; Hollander et al. 1999), and provides additional support for GADD45A acting in a tumor suppressive fashion.

In vivo studies have also demonstrated that loss of either GADD45A or GADD45B accelerates BCR-ABL-driven leukemia in mice (Mukherjee et al. 2017; Sha et al. 2018). *GADD45A* loss in myeloid progenitors enhances proliferation and limits apoptosis while also increasing both leukemia stem cell content and bone marrow self-renewal (Mukherjee et al. 2017). GADD45A-loss in the presence of BCR-ABL was further associated with constitutive activation of PI3K/AKT, Stat5, and p38 as well as increased expression of the dominant negative transforming isoform of p30 C/EBPα (Mukherjee et al. 2017). Examination of GADD45A expression in relation to chronic myelogenous leukemia (CML) disease phase further revealed that *GADD45A* RNA levels are highest in patients in the chronic phase compared to those in the accelerated or blast crisis phase (Mukherjee et al. 2017). These data support *GADD45A* as a potential prognostic biomarker for CML disease staging with low *GADD45A* being associated with

poor patient outcomes. Unlike with GADD45A loss, differentiation was not impaired in GADD45B-depleted leukemic progenitors, which instead displayed increased proliferation and decreased apoptosis associated with hyperactivation of JNK and STAT5 signaling (Sha et al. 2018). Thus, distinct mechanisms support GADD45A- and GADD45B-mediated suppression of BCR-ABL-driven leukemia.

Several mechanisms through which GADD45 family members act to attenuate tumorigenesis have been identified. Overexpression of GADD45A, GADD45B, and GADD45G has been shown to limit proliferation in multiple cell lines with growth inhibition in response to GADD45A expression occurring regardless of p53 status (Jin et al. 2002; Zhan et al. 1994). As we have described, GADD45 family members induce G2/M arrest via disruption of the cyclin B1/CDC2 complex, and G1 arrest through direct interaction with p21 (Azam et al. 2001; Vairapandi et al. 2002; Wang et al. 1999; Kearsey et al. 1995; Fan et al. 1999). Human and murine cells with *GADD45A* depletion fail to display G2/M arrest in response to DNA damage induced by UV or methylmethane sulfonate (Wang et al. 1999). Additionally, senescence in response to oncogenic Ras is abolished in MEFs from *Gadd45a* knockout mice (Bulavin et al. 2003), highlighting GADD45 family members as essential mediators of cell cycle arrest in response to oncogenic stress. SMAD-interacting protein-1 (SIP1) has been identified as a downstream effector of GADD45G-induced senescence in HCC (Xu et al. 2015), a cancer type in which GADD45G downregulation was detected and shown to correlate with poor prognosis (Ou et al. 2015; Sun et al. 2003). SIP-1 was induced in a JNK-dependent manner in Sk-Hep1 and SMMC-7721 HCC cells undergoing GADD45G-mediated senescence in vitro. Genetic depletion of SIP-1 was further able to limit GADD45G-induced senescence in vitro and GADD45-mediated suppression of tumor growth in vivo. In human HCC lesions, a positive correlation was noted between expression GADD45G and SIP1, supporting the pathological relevance of the GADD45G/SIP-1 axis in HCC.

GADD45-mediated tumor suppression has also been linked to induction of apoptosis. In studies utilizing *Gadd45a*-deficient mice, introduction of GADD45A promoted skin keratinocyte apoptosis in response to UV (Maeda et al. 2002). Additionally, hepatocytes from *Gadd45b*-deficient exhibit resistance to cell death in response to transforming growth factor β (Yoo et al. 2003). A role for GADD45 in cancer cell apoptosis was indicated as ectopic expression of GADD45A or GADD45B in H1299 human lung carcinoma cells resulted in sensitization to various genotoxic agents (Zhang et al. 2001). Intersection has been demonstrated between GADD45 and NF-κB, a critical mediator of cancer survival. In breast and prostate cancer cell lines, nuclear factor-kappaB (NF-κB)-mediated suppression of GADD45A and GADD45G, but not GADD45B, is mediated by c-Myc and required for escape from cell death (Zerbini et al. 2004). As described below, GADD45B has shown to promote cancer cell survival, suggesting that individual GADD45 family members may differentially impact cell-survival decisions downstream of NF-κB.

In addition to induction of cell cycle arrest, senescence, and apoptosis, GADD45A-mediated suppression of migration and invasion has also been linked to tumor suppression (Tamura et al. 2016).

2.3.3 Tumor Promoting Roles for GADD45 Family Members

Increased GADD45A expression has been identified in human pancreatic ductal adenocarcinoma lesions as well as in a murine model of pancreatic cancer (Schneider et al. 2006). In the pancreatic cancer cell line MiaPaCa2, GADD45A knockdown limited proliferation and induced apoptosis. These studies support a tumor promoting role for GADD45A in pancreatic cancer; however, the direct molecular mechanisms through which GADD45A impacts proliferation and apoptosis in pancreatic cancer models have yet to be determined. In HuCCA-1 cholangiocarcinoma cells, GADD45B silencing suppressed AKT activation and induced apoptosis (Myint et al. 2018).

Additionally, genetic depletion of GADD45B limited migration and invasion in HuCCA-1 cells which further displayed downregulation of Vimentin and Slug concurrent with induction of E-cadherin, suggesting a negative impact on EMT. In patients with multiple myeloma, elevation of *GADD45B* expression has been demonstrated in CD138+ monoclonal plasma cells (Tornatore et al. 2014). Upon stratification at the time of diagnosis, multiple myeloma patients with high levels of *GADD45B* in CD138+ cells displayed poor progression-free and overall survival as compared to their *GADD45B* low counterparts. GADD45B was further identified as a pivotal survival factor downstream of NF-κB signaling in multiple myeloma cells in vitro. Specifically, GADD45B was shown to directly interact with MKK7, blocking the catalytic activity of MKK7 as well as subsequent induction of JNK-mediated apoptosis (Papa et al. 2007, 2008; Tornatore et al. 2014; De Smaele et al. 2001). In MEFs, apoptosis downstream of tumor necrosis factor (TNF) α has been shown to be antagonized by NF-κB via transcriptional upregulation of *Gadd45b* and subsequent JNK inhibition (De Smaele et al. 2001). This finding was challenged, however, by a subsequent study that failed to identify any difference in TNFα-mediated apoptosis when comparing *GADD45b*$^{-/-}$ MEFS to their wild type counterparts.

2.3.4 GADD45 Family Members in Cancer Therapy

Given that GADD45 family members are established mediators of cellular stress responses, it is not unexpected that roles for these proteins have been associated with response to chemo- and radiotherapy. In medulloblastoma cells, GADD45A expression is induced in response to, and increases sensitization to ionizing radiation (Asuthkar et al. 2011). In this context, the effects of GADD45 are multifactorial, limiting proliferation migration, invasion, and EMT. At the molecular level, GADD45A induced G2 arrest by binding to CDC2 and inhibiting its kinase activity, promoted membrane localization of β-catenin while limiting its nuclear translocation, and decreased MMP-9 expression and activity. In cervical cancer tissues, methylation of *GADD45A* was shown to be significantly higher in radioresistant specimens as compared to their radiosensitive counterparts (Lou et al. 2021). In vitro studies further revealed that *GADD45* methylation is associated with diminished AKT signaling and decreased sensitivity to ionizing radiation in cervical cancer cells. Moreover, treatment with 5-azacytidine, overexpression of GADD45A, or pharmacological AKT activation enhanced radiation-induced cell death in SiHa cervical cancer cells in vitro. GADD45A has also been shown to sensitize prostate cancer cells to the chemotherapeutic agent docetaxel in vitro. This sensitization was further augmented by pretreatment with 5-azacytidine, suggesting a role for DNA methylation in GADD45A-mediated apoptosis in response to docetaxel (Ramachandran et al. 2009). A prognostic role for GADD45A with regard to docetaxel response was indicated in a study of locally advanced non-metaplastic oral squamous cell carcinoma patients treated with neoadjuvant docetaxel in combination with carboplatin, in which GADD45 expression significantly correlated with 2-year overall and disease-free survival (Pandey et al. 2018). Resistance to the chemotherapeutic agent paclitaxel is a significant clinical challenge. Inhibition of heparin-binding epidermal growth factor receptor-like growth factor (HB-EGF) by cross-reactive material 197 (CRM 197) has shown promise in paclitaxel resistance in preclinical models of ovarian cancer with one study indicating a role for GADD45G (Tang et al. 2016). Specifically, CRM197 resulted in inhibition of nucleus accumbens-1 and downstream activation of GADD45-interacting protein 1, Gadd45G, and JNK/p38 signaling, ultimately inducing apoptosis in paclitaxel resistant A2780 and SKOV3 cells.

In contrast to these studies, protective roles for GADD45 family members have been described in cancer cells responding to chemo- and radiotherapy. Upon treatment with the chemotherapeutic agent temozolomide (TMZ), U87 glioblastoma cells exhibit G2/M arrest and apoptosis as well as induction of GADD45A in vitro

(Wang et al. 2017). Genetic depletion of GADD45A in U87 cells and several additional glioblastoma cell lines enhanced TMZ-mediated apoptosis and expression of p21. The effect of GADD45 knockdown on TMZ response was also seen in TMZ-resistant cells lines in which reduction of O^6-methylguanine-DNA methyltransferase, an indicator of TMZ resistance, was noted. Upregulation of both *GADD45A* and *GADD45G* was identified in melanoma cells responding to cisplatin (Liu et al. 2018). Additional studies revealed that genetic depletion of GADD45A enhanced DNA damage and apoptosis induced by cisplatin while also abrogating G2/M arrest. GADD45A induction in response to cisplatin was further shown to mediated by MAPK-ERK signaling and could be blocked by pharmacological MEK inhibition. Although this study did not assess the impact of MEK inhibition upon cisplatin-mediated cell death in melanoma, MEK inhibitors have been shown to enhance cisplatin sensitivity in ovarian cancer (Rowswell-Turner et al. 2019). As several MAPK inhibitors have been FDA approved and are in clinical trials for melanoma (Eroglu and Ribas 2016), it will be of interest to determine whether GADD45 family members play a role in response to these agents. As we have previously noted, loss of either GADD45A or GADD45B accelerates BCR-ABL-driven leukemia in mice (Mukherjee et al. 2017; Sha et al. 2018). Interestingly, bone marrow-derived myeloid cells derived from *Gadd45a*- or *Gadd45b*-knockout mice exhibit increased sensitivity to UV irradiation and the chemotherapeutic agents VP-16 and daunorubicin with unique protective roles for GADD45A and GADD45B identified in hematopoietic cells responding to UV radiation via respective activation p38-NFκB and inhibition of MTK1-JNK signaling (Gupta et al. 2005, 2006a). These findings raise the possibility that while diminished expression of GADD45 family members may promote carcinogenesis, it may also identify cells with enhanced susceptibility to therapy-induced genotoxic stress. However, loss of *Gadd45a* or *Gadd45b* has been shown to impair murine myelopoiesis in response to inflammatory stress or the chemotherapeutic agent 5-fluouracil (Gupta et al. 2006b), highlighting the need for additional studies to clarify the clinical and biological significance of GADD45 family members in leukemia.

As noted in Table 2.1, GADD45G expression is decreased in HCC where it correlates with poor prognosis. This downregulation has also been linked to response to the multi-kinase inhibitor sorafenib (Ou et al. 2015), a standard systemic therapy in advanced HCC (Bruix et al. 2011). In vitro and in vivo studies revealed that although basal expression of *GADD45G* did not differ when comparing sorafenib-sensitive and sorafenib-resistant HCC cell lines, *GADD45G* induction was more robust in sorafenib-sensitive cells upon treatment with sorafenib, potentially through CCAAT/enhancer binding protein-mediated transcriptional regulation (Ou et al. 2015). Additionally, GADD45G overexpression reversed sorafenib resistance, inducing cell death in vitro and xenograft tumor regression in vivo while genetic depletion of GADD45G partially abrogated sorafenib-mediated cell death in sorafenib-sensitive cells, with Survivin identified as a critical mediator of the pro-apoptotic effects of GADD45G. This study identifies a role for GADD45G in response to sorafenib and begs the question of whether GADD45 family members may play roles in response to other molecular targeted ant-cancer therapies.

GADD45 has also been implicated in response to several experimental therapeutics. Cucurbitacin E (CuE) is a natural compound that has been shown to have anti-cancer properties (Hung et al. 2013). CuE impaired cell proliferation in a panel of primary colorectal (CRC) cell lines and also induced expression of *GADD45A*, *GADD45B*, and *GADD45G* (Hsu et al. 2014a). G2/M arrest in CRC cells further promoted dissociation of the cyclin B1/CDC2 complex that was attributed to GADD45G binding to CDC2. GADD45G binding to CDC2 in the context of CuE-mediated G2/M arrest was also noted in human glioma cells as well as in pharyngeal and nasopharyngeal carcinoma cell lines (Hsu et al. 2014b). GADD45B binding to CDC2 was further associated with dissociation of cyclin B1/CDC2 and G2/M arrest in cervical cancer cells exposed to

sulforaphane (Cheng et al. 2016), a natural compound derived from dietary isothiocyanates. In addition to contributing to cytostatic effects mediated by CuE and sulforaphane, GADD45 has been shown to play a role in apoptosis induced in response to several natural agents. CIL-102 is a major active agent of the alkaloid derivative of *Camptotheca acuminata* that has been demonstrated to have anti-tumor activity (Zhao et al. 2005). In DLD-1 CRC cells, CIL-102 induces cell cycle arrest and apoptosis modulated by upregulation of GADD45 and p21 as well as activation of JNK1/2, NFκB p50, and p300 (Huang et al. 2017). Apoptosis induced by the polyphenol flavonoid myricetin has also been linked to GADD45 induction in T47D and MCF7 breast cancer cells (Sajedi et al. 2020; Soleimani and Sajedi 2020). These studies further implicate BRCA1 and p53 in myricetin-induced cell death; however, the detailed molecular mechanisms underlying these associations have yet to be explored in detail.

These studies supporting potential roles for GADD45 proteins in tumor cell response to therapy raise the question of whether GADD45 family members themselves may be targeted in the context of cancer. DTP3 is a tripeptide inhibitor of the GADD45B/MKK7 complex, an essential cancer-selective cell-survival module downstream of the NF-κB pathway (Tornatore et al. 2014). Although DTP3 binds directly to MKK7, this binding effectively disrupts the GADD45B/MKK7 interaction, inducing JNK-mediated apoptosis specifically in multiple myeloma cells that exhibit elevated *GADD45B* expression in vitro and in vivo (Tornatore et al. 2019a). In vitro studies using primary multiple myeloma cultures further revealed that DTP3 exhibited potent and cancer cell-selective activity that was superior to that of the proteasome inhibitor bortezomib, a well-established agent for clinical care of multiple myeloma patients. The effectiveness of DTP3 was confirmed in both subcutaneous and orthotopic models of multiple myeloma, neither of which revealed apparent side effects. Subsequent studies utilizing murine, rat, and canine models support DTP3 as a promising drug target in clinical oncology that combines on-target-selective pharmacology, therapeutic anti-cancer efficacy, and favorable drug-like properties with no off-target toxicity or adverse effects (Tornatore et al. 2019a). Indeed, the first-in-human Phase I study of DTP3 confirmed induction of JNK signaling and apoptosis specifically in multiple myeloma cells, but not normal cells with no evidence of toxicity or adverse effects (Tornatore et al. 2019b). Given the promise of DTP3 in multiple myeloma, it will be of great interest to determine if this agent may prove effective in other types of malignancy in which GADD45B has been implicated.

2.3.5 Summary

Alterations in the expression of GADD45 family members have been detected in various solid tumors as well as in supportive and inhibitory roles for these proteins in carcinogenesis and therapy response. A more comprehensive understanding of how expression of individual GADD45 family members changes in the context of carcinogenesis and in response to specific anti-cancer therapies as well as how these alterations in expression relate to clinicopathological data may facilitate the development of novel approaches to cancer diagnosis, monitoring, and therapy. Such studies should be performed with the understanding that heterogeneity is likely to exist with regard to roles for individual GADD45 family members both with a given type of cancer and when comparing different types of malignancy. Finally, approaches seeking to leverage our knowledge of GADD45 in cancer biology to improve cancer patient care must be guided by more in-depth functional investigations into roles for GADD45 family members in carcinogenesis and therapy response.

2.4 Concluding Remarks

The GADD45 family of genes clearly plays significant and diverse roles in both development and cancer. Thus, a more detailed understanding of the biological roles played by each member of

the GADD45 family in conditions of homeostasis and disease has great potential to improve human health in the context of cancer and beyond.

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Gadd45 in Normal Hematopoiesis and Leukemia

3

Dan A. Liebermann

Abstract

Gadd45a, Gadd45b, and Gadd45g have been implicated in cell cycle arrest, DNA repair, apoptosis, innate immunity, genomic stability, and modulation of normal blood cell development and leukemia. Each of the Gadd45 genes was shown to be regulated independently in myeloid cells in response to cytokine stimulation modulating blood cell survival and differentiation, including maintaining the quiescent stem cell pool. Gadd45a and Gadd45b were also shown to mediate the protective effects from UV in hematopoietic cells by separate signaling pathways involving either p38 activation or JNK inhibition. Furthermore, it was shown that gadd45a methylation in AML is predictive of poor survival. It was also shown that loss of Gadd45b accelerates the development of BCR-ABL driven CML in mice and leads to decreased median survival. The Gadd45b-deficient CML progenitors exhibited increased proliferation and decreased apoptosis, and this was associated with hyperactivation of c-Jun NH2-terminal kinase and Stat5. Moreover, loss of Gadd45a also accelerated the development of BCR-ABL driven CML, and this was associated with enhanced PI3K-AKT-mTOR-4E-BP1 signaling, upregulation of p30C/EBPα expression, and hyperactivation of p38 and Stat5. In human patients with chronic phase CML, gadd45a expression is up-regulated, whereas in accelerated and blast crisis phase patients, gadd45a is downregulated. Collectively, these results provide novel evidence that Gadd45a functions as a suppressor of BCR/ABL driven leukemia and may serve as a unique prognostic marker of CML progression. Thus Gadd45 proteins provide excellent targets for leukemia therapy.

Keywords

Gadd45 · Leukemia · Hematopoiesis

3.1 Gadd 45 Functions and Mode of Action

Each of the Gadd45 proteins has been shown to participate in cell cycle arrest, DNA repair, cell survival, and apoptosis in response to environmental and physiological stress, as well as having a role in development and carcinogenesis. Their physiological functions are mediated by interactions with partner proteins, including PCNA, cdk1, p21, MEKK4, and p38. This section will

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provide a brief overview of the Gadd45 proteins with regard to their function and mode of action.

Experiments in multiple cell types have demonstrated that Gadd45 proteins function in cell cycle regulation. In human endothelial and fibroblast cells, inhibiting endogenous gadd45 expression by antisense gadd45 impaired the G2/M checkpoint following exposure to either UV radiation or MMS (Vairapandi et al. 2002; Wang et al. 1999). In addition, microinjecting a gadd45a expression vector into primary human fibroblasts arrested the cells at the G2/M boundary of the cell cycle (Wang et al. 1999). Given that gadd45a is a transcriptional target of p53, these observations implicate Gadd45a in p53 mediated G2/M cell cycle arrest in response to certain genotoxic stress agents (Wang et al. 1999). The genomic instability observed in gadd45a null mice (Hollander et al. 1999) may reflect perturbations in G2/M cell cycle progression and/or impaired DNA repair. Additionally, it was observed that IPTG-induced ectopic expression of each of the gadd45 genes in the p53 null H1299 cell line retarded cell growth and increased accumulation of cells in the G1 phase of the cell cycle (Zhang et al. 2001).

Numerous observations also demonstrate a role for gadd45 in apoptosis. Blocking gadd45b expression, by either antisense technology in cell lines or using a KO mouse model, abolished TGF-b induced cell death, implicating gadd45b as a positive modulator of TGF-b induced apoptosis (Selvakumaran et al. 1994; Yoo et al. 2003). IPTG-induced ectopic expression of gadd45b accelerated TGF-b induced apoptosis in M1 cells (Liebermann and Hoffman 2003, 2007), and significantly enhanced apoptosis in H1299 lung carcinoma cells (Zhang et al. 2001). In addition, BRCA-1 induced gadd45a was implicated in apoptosis of breast cancer cells (Harkin et al. 1999), and gadd45a induced cell cycle arrest and apoptosis in UV-irradiated keratinocytes (Hildesheim et al. 2002).

Gadd45 proteins have also been shown to promote cell survival. As described in greater detail later on, Gadd45a and Gadd45b each protect hematopoietic cells from genotoxic stress (Gupta et al. 2005). In both gadd45a null MEFs and RKO cells expressing antisense gadd45 RNA, there is increased killing by UV irradiation and cisplatin compared to cells expressing gadd45 (Hollander et al. 1999; Smith et al. 1996). Gadd45b has been shown to mediate the protective effects of CD40 co-stimulation against Fas-induced apoptosis (Zazzeroni et al. 2003). Furthermore, the role Gadd45 proteins play in DNA repair is compatible with a survival function (Smith et al. 2000; Vairapandi et al. 2002).

The Gadd45 proteins also sense oncogenic stress, and the status of expression of each of these proteins can influence the initiation and progression of malignancies. Mice null for gadd45a showed accelerated Ras-driven mammary tumor formation (Tront et al. 2006). Consistent with these observations, cells null for gadd45a exhibit genomic instability and escape from senescence, and can be transformed by ras (Hollander et al. 1999).

The status of the different Gadd45 proteins also influences development. Mice null for gadd45a exhibit a low incidence of exencephaly, comparable to that of Trp53/mice (Hollander et al. 1999). Gadd45b is expressed in the embryonic growth plate and has been shown to be an essential mediator of matrix metalloproteinase-13 (MMP-13) expression during terminal chondrocyte differentiation (Ijiri et al. 2005). In Gadd45b null mouse embryos, defective mineralization and decreased bone growth accompanied deficient Mmp-13 and Col10a1 gene expression in the hypertrophic (Ijiri et al. 2005).

The gadd45 proteins display a complex array of physical interactions with other cellular proteins, and these protein–protein interactions are implicated in cell cycle regulation and the response of cells to stress (Fig. 3.1). Proteins that interact with Gadd45 include PCNA, cdk1, p21, MEKK4, MKK7, and p38. There is evidence that interaction of the Gadd45 proteins with PCNA promotes nucleotide excision repair (NER) of DNA (Azam et al. 2001; Liebermann and Hoffman 2007; Smith et al. 1996; Vairapandi et al. 2000). Interaction with cdk1 inhibits the kinase activity of the cdc2/cyclinB1 complex that plays a key role in transition of cells from the G2 to the M phase of the cell cycle (Vairapandi et al.

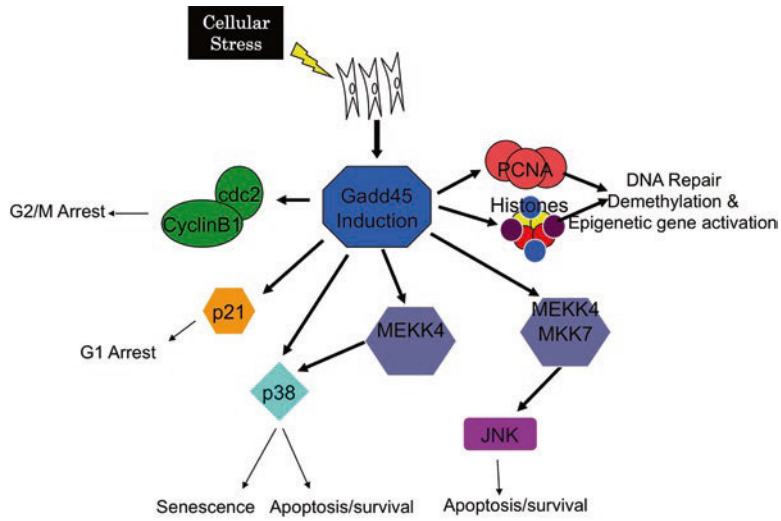

Fig. 3.1 The physiological functions of Gadd45 proteins are mediated by interactions with partner proteins, including PCNA, cdk1, p21, MEKK4, and p38

2002; Wang et al. 1999). It has been suggested that when Gadd45a interacts with p21 it serves to augment p21's CDKI activity, promoting G1 arrest (Fan et al. 1999; Liebermann and Hoffman 2007). Association of Gadd45 proteins with MEKK4 and p38 results in their activation (Liebermann and Hoffman 2007). It was reported that the MEKK4 N-terminus binds to its C-terminal segment, thereby inhibiting the C-terminal kinase domain, and that binding of Gadd45 to the MEKK4 N-terminal Gadd45-binding site disrupts this N–C binding, resulting in kinase activation (Miyake et al. 2007). Gadd45 binding can also induce dimerization of MEKK4 (Miyake et al. 2007).

Gadd45 proteins can also form homo- and hetero-oligomers with different family members although their role in mediating Gadd45 functions has not been established (Kovalsky et al. 2001; Liebermann and Hoffman 2003, 2007). That Gadd45 proteins do not necessarily associate as simple dimers can be seen with regard to MKK7 (Tornatore et al. 2008). Gadd45b is a major player in the endogenous NF-kB-mediated resistance to apoptosis in a variety of cell lines. In fibroblasts this mechanism involves inactivation of MKK7 (Papa et al. 2004), the upstream activator of JNK, by direct binding within the kinase ATP pocket. Evidence supports the existence of a large complex containing an MKK7-Gadd45b:Gadd45b-MKK7 tetrameric unit whose complexity could be further increased by the dimeric nature of the isolated MKK7 (Tornatore et al. 2008).

If protein–protein interactions govern the many functions of the Gadd45 family of proteins, it can be asked what determines these interactions. It is predicted that the interaction of Gadd45 with its partner protein is regulated by level of expression, cellular localization, and posttranslational modifications of both the Gadd45 proteins and their interacting partners.

3.2 Each of the Gadd45 Genes Is Regulated Independently in Myeloid Cells in Response to Cytokine Stimulation

Using the M1 myeloid leukemic cell line, where terminal myeloid differentiation is segregated from proliferation, this laboratory has shown that gadd45b is a differentiation primary response gene that is rapidly induced and then downregulated (Abdollahi et al. 1991). Gadd45g is also not dependent on de novo protein synthesis and induced immediately; however, its expression peaks at 1 day and it continues to be expressed. Finally, gadd45a is induced only at later times (Hoffman and Liebermann 2007; Zhang et al. 1999). Myeloid enriched BM treated with IL-3, GM-CSF,

G-CSF, or M-CSF results in rapid induction of all three gadd45 genes and their cognate proteins, yet the patterns of expression vary during the ensuing myeloid developmental program (Abdollahi et al. 1991; Gupta et al. 2006b; Zhang et al. 1999). The expression of gadd45b in gadd45a/BM and of gadd45a in gadd45b/BM was comparable to their expression in wild type BM cells. Gadd45g expression was the same for all genotypes. These studies indicate that gadd45 genes are independently regulated in response to hematopoietic cytokines.

Induction of gadd45 genes at the onset of myeloid differentiation suggested that Gadd45 protein(s) play a role in myelopoiesis, yet no apparent abnormalities were detected in the hematopoietic compartments of 2 months old mice deficient for either gadd45a or gadd45b (Gupta et al. 2006b). Since the gadd45 gene products are known stress sensors, myeloid cells from gadd45a/and gadd45b/mice were examined under different conditions of hematopoietic stress, both in vivo and in vitro.

3.3 Loss of Gadd45 Impairs Terminal Differentiation and Survival of Myeloid Cells in Response to Acute Stimulation with Cytokines

After 4 days of acute stimulation of myeloid enriched BM with hematopoietic cytokines GM-CSF, IL-3, M-CSF, or G-CSF, the percentage of mature macrophages and granulocytes was significantly reduced in gadd45a/and gadd45b/BM cells compared to wild type controls, using both morphological and immuno-phenotyping for assessment (Gupta et al. 2006b).

Comparing gadd45a/and gadd45b/BM cells to similarly treated wild type BM, there was no difference in survival using the cytokines IL-3 and GM-CSF; however, significantly increased apoptosis was observed following treatment with M-CSF and G-CSF (Gupta et al. 2006b). That both differentiated and undifferentiated myeloid cells were similarly affected, suggested that Gadd45a and Gadd45b modulate survival of both undifferentiated and differentiated cells.

Cytokine signaling activates Jak/STAT and MAPK pathways as well as inducing expression of gadd45 genes (Mangan and Reddy 2005; Platanias 2003). Since Gadd45 can modulate MEKK4, JNK, and p38 activity it is not surprising that Gadd45 modulates the effects of cytokines that control hematopoietic survival, proliferation, and differentiation.

Using colony assays gave further insight into the effect of loss of gadd45a and gadd45b on differentiation, proliferation, and survival. Gadd45 deficiency resulted in an initial decrease in colony formation by myeloid progenitors (CFU-GM), with fewer mature cells in the colonies compared to wild type, consistent with diminished differentiation and reduced survival (Gupta et al. 2006b). The decrease was less pronounced in first replating and by the second replating, gadd45a null and gadd45b null samples yielded more colonies than wild type (Gupta et al. 2006b). These observations are consistent with prolonged proliferation capacity due to the decreased ability to differentiate, reflecting the compromised cytokine induced myeloid differentiation.

Other studies have implicated an important role for the mixed lineage leukemia (mll) gene in hematopoiesis, mainly through maintaining Hox gene expression. Investigating the role of mll during zebrafish embryogenesis, particularly hematopoiesis, Wan et al. have shown that mll depletion caused severe defects in hematopoiesis as indicated by a lack of blood flow and mature blood cells as well as a significant reduction in expression of hematopoietic progenitor and mature blood cell markers. Moreover, mll depletion prevented differentiation of hematopoietic progenitors. In mll morphants, microarray analysis revealed a dramatic upregulation of gadd45αa. Multiple assays indicate that mll inhibited gadd45a expression and that overexpression of gadd45a mRNA led to a phenotype similar to the one seen in the mll morphants. These findings demonstrate that gadd45a serves as a downstream target for mediating the function of mll in hematopoiesis zebrafish (Wan et al. 2011).

3.4 Role of Gadd45 in Maintaining the Quiescent Stem Cell Pool

Administration of the anti-metabolite 5-FU eliminates many of the rapidly dividing, more committed progenitors in the bone marrow, providing a relatively enriched population of the most primitive hematopoietic progenitor cells. For these myelo-suppressed mice to recover, quiescent cells from the stem cell pool are activated, thereby replenishing the progenitor pool that subsequently differentiates along multiple cell lineages (Randall and Weissman 1997). Under these conditions of stress-driven myelopoiesis, the cellularity of the BM in gadd45a/and gadd45b/mice was significantly reduced and there was poor recovery of the mature myeloid compartment compared to wild type controls (Gupta et al. 2006b). Several explanations for these observations are available that merit further investigation, and it is very likely that more than one is in play to determine the ultimate outcome following myeloablation. First, as demonstrated, there is impaired terminal myeloid differentiation and survival following acute cytokine stimulation in culture, which may mimic events during recovery from 5-FU treatment. Furthermore, it can be asked if gadd45 gene products directly modulate the hematopoietic stem cell (HSC) compartment, co-operating in a stage specific function related to cell survival and/or self-renewal, so that loss of gadd45 diminishes the stem cell population of quiescent cells. Another possibility is that diminished survival of progenitors accelerates depletion of the stem cell compartment. It is of definite interest to assess the possible role of interaction of Gadd45 proteins with p21, one of its partner proteins (Vairapandi et al. 1996), in the above-described defect in myeloid recovery, since p21 has been implicated in the regulation of hematopoietic stem cell quiescence and cell survival (Cheng et al. 2000; Marone et al. 2002). Interestingly, it has been reported that the role of p21 in the HSC compartment may be more important under conditions of cellular stress rather than during steady state conditions (van Os et al. 2007). Gadd45b can be transcriptionally regulated by Egr-1 (Thyss et al. 2005), recently reported to regulate homeostasis of the HSC compartment (Min et al. 2008), and some of these effects of Egr-1 may be mediated through gadd45b.

Other studies have shown that Gadd45a induces differentiation in hematopoietic stem cells without inhibiting cell cycle or survival. The differentiation induction by Gadd45a was transmitted by activating p38 Mitogen-activated protein kinase (MAPK) signaling and allowed the generation of megakaryocytic-erythroid, myeloid, and lymphoid lineages (Wingert et al. 2016). Terminal differentiation induction by Gadd45a in hematopoietic stem cells has been implicated as a DNA-damage response (Wingert and Rieger 2016). Additionally, Gadd45a deletion was shown to aggravate hematopoietic stem cell dysfunction in ATM-deficient mice, resulting in high incidence of lymphoma. Other work has shown that C-terminal deletion mutant of RUNX1, RUNX1dC, attenuates DNA-damage repair responses in hematopoietic stem/progenitor cells. Expression profiling by real-time-PCR array revealed RUNX1dC represses the expression of Gadd45a, a sensor of DNA stress (Satoh et al. 2012).

Moreover, it was shown that Gadd45g induces differentiation and lineage selection in hematopoietic stem cells. Gadd45g was observed to induce and accelerate differentiation in LT-HSCs and override the self-renewal program by specifically activating MAP3K4-mediated MAPK p38. Conversely, absence of Gadd45g was observed to enhance the self-renewal potential of LT-HSCs (Thalheimer et al. 2014).

3.5 Loss of Gadd45 Reduces the Response of the Myeloid Compartment to Acute Inflammatory Stress

Intraperitoneal delivery of sodium caseinate causes rapid induction of myelopoiesis in murine BM as well as prompt migration of inflammatory cells from the BM and other hematopoietic reservoirs to the peritoneal cavity, where an increase

in mature myeloid cells and lymphocytes is observed by 3–4 days. Following sodium caseinate treatment WT BM cells consisted mostly of Gr-1 positive myeloid cells (98.6%) compared to 76.4% in untreated mice; however, there was no substantial increase in the proportion of Gr-1 positive BM cells in either gadd45a or gadd45b mice (Gupta et al. 2006b). In the peritoneal exudates from both gadd45a null and gadd45b null mice, there were significantly fewer cells and the percentage of both F4/80 and Gr-1 expressing cells was appreciably lower compared to similarly treated WT mice. These observations indicate that both Gadd45a and Gadd45b are important modulators of the myeloid cell response to acute inflammatory stress (Gupta et al. 2006b), since loss of either gene compromises the innate immune function of the myeloid compartment.

This defect can be due to impairment of differentiation, migration, and/or functions of leukocytes in response to inflammatory stress, among many possibilities. Whether this is the case, and how it integrates with the effect of loss of gadd45 function in the lymphoid (Lu et al. 2004) and non-hematopoietic cell compartments, including epithelial cells, needs to be explored. Evidence supports a role for Gadd45 proteins in T cell functions related to innate immunity (Lu et al. 2001, 2004; Salvador et al. 2005) and regulation of dendritic cell cytokine production (Jirmanova et al. 2007), where Gadd45 regulation of the stress-activated MAP kinase pathways has been implicated.

3.6 Loss of Gadd45a and Gadd45b Sensitizes Hematopoietic Cells to Genotoxic-Stress Induced Apoptosis

Myeloid enriched BM cells from gadd45a and gadd45b deficient mice were observed to be more sensitive to genotoxic stress, including UVC, VP-16, and DNR, compared to WT cells (Gupta et al. 2005). The increase in apoptosis, assessed by annexin, was accompanied by activation of caspase-3 and PARP cleavage and decreased expression of pro-survival cIAP-1, Bcl-2, and Bcl-xL compared to WT cells (Gupta et al. 2005). Both gadd45a and gadd45b deficient BM cells also displayed defective G2/M arrest following exposure to UVC and VP-16, but not to DNR, indicating the existence of G2/M checkpoints that are either dependent or independent of gadd45 (Gupta et al. 2005).

The pro-survival function of Gadd45a and Gadd45b was surprising in light of previous data that have identified Gadd45 as pro-apoptotic. Ectopic expression of either gadd45a or gadd45b sensitized H1299 human lung carcinoma cells to apoptosis induced by genotoxic stress (Zhang et al. 2001). Also, using the gadd45a/mice, it was reported that Gadd45a promoted UV induced apoptosis in skin keratinocytes (Maeda et al. 2002). Additional evidence for a pro-apoptotic function of Gadd45a and Gadd45g was documented using either over-expression of these proteins in prostate and breast cancer cell lines or siRNA-mediated knockdown that was observed to block IkB mediated apoptosis (Zerbini et al. 2004). As described previously, Gadd45b is required for TGF-b-mediated apoptosis in hepatocytes and myeloid cells. On the other hand, that Gadd45 can also be pro-survival was shown when it was observed that p53 null MEFs that lack gadd45a are sensitized to UV and cisplatin induced cell death (Smith et al. 2000). Also, a study on B cells showed that Gadd45b mediated the protective effects of CD40 co-stimulation against Fas-induced apoptosis (Zazzeroni et al. 2003). These experimental observations are consistent with the unifying hypothesis that the stress stimulus encountered, the cell type, and interaction with Gadd45-partner proteins determine whether the outcome will be either DNA repair and cell survival, or apoptotic cell death. This notion is also illustrated by our observations that gadd45b null BM cells, though sensitized to genotoxic stress induced apoptosis, are largely resistant to TGF-b induced apoptosis.

Consistent with studies demonstrating that Gadd45a and Gadd45b mediate the G2/M cell cycle checkpoint in both human and murine cells, both gadd45a null and gadd45b null BM cells are

defective in G2/M arrest following either UV or VP-16 treatment (Wang et al. 1999; Zhan et al. 1999). In contrast, when these BM cells were treated with DNR, they retained the G2/M checkpoint; thus, the DNR-mediated G2/M checkpoint pathway differs from the UV and VP-16 mediated pathways. That multiple G2/M checkpoints exist in response to specific types of DNA damage was shown for both human and murine cells treated with ultraviolet radiation and ionizing radiation (Wang et al. 1999; Zhan et al. 1999). Human cells expressing antisense gadd45a have an impaired G2/M checkpoint after exposure to either ultraviolet radiation or MMS but are able to undergo G2 arrest after ionizing radiation. Similarly, lymphocytes from gadd45 null mice also retained a G2/M checkpoint initiated by ionizing radiation and failed to arrest at G2/M after exposure to ultraviolet radiation (Wang et al. 1999). It is tempting to speculate that the increased apoptosis triggered by either UV or VP-16 in gadd45 deficient BM reflects, at least partially, the failure of these cells to arrest in G2/M to allow repair of damaged DNA.

3.7 Gadd45a and Gadd45b Mediate the Protective Effects from UV in Hematopoietic Cells by Separate Signaling Pathways Involving Either p38 Activation or JNK Inhibition

Work conducted in this laboratory has shown that Gadd45a and Gadd45b cooperate to promote cell survival in hematopoietic cells exposed to UV by two distinct and novel signaling pathways. These encompass activation of the Gadd45a-p38-NF-kB mediated survival pathway and Gadd45b mediated inhibition of the stress response apoptotic MKK4-JNK pathway (Gupta et al. 2006a; Fig. 3.2).

Gadd45a mediated activation of p38 resulted in phosphorylation and degradation of IkB, which in turn allowed nuclear localization of NF-kB and activation of its target genes (Gupta et al. 2006a), showing for the first time that there is crosstalk between Gadd45a-mediated activation of p38 and the NF-kB survival pathway (Fig. 3.2). Another documented crosstalk pathway between p38 and NF-kB involves p38 modulating the transcriptional activity of NF-kB via phosphorylation of RelA (Jijon et al. 2004). The Gadd45a-p38-NF-kB survival pathway in hematopoietic cells differs from previous work showing that p38 activation is linked to cell death in endothelial and epithelial cells (Harkin et al. 1999; Hildesheim et al. 2002; Jinlian et al. 2007), but is consistent with studies showing that activation of p38 is linked to survival in hematopoietic cells (Platanias 2003).

We have shown that Gadd45b mediated inhibition of UV-induced JNK activity cooperates with p38 activation to promote hematopoietic cell survival (Fig. 3.2). Gadd45b induction by NF-kB and subsequent inhibition of JNK activity has been implicated in MEF survival in response to TNFa (De Smaele et al. 2001), whereas, in contrast, our studies with Gadd45b/MEFs have shown that Gadd45b deficiency does not prolong JNK activity in response to TNFa (Amanullah et al. 2003). The reason for this discrepancy is not clear. Using BM cells null for gadd45b, we obtained data indicating that UV-induced Gadd45b blunts JNK activity, thereby promoting hematopoietic cell survival, and that gadd45b targets MKK4 rather than MKK7, the usual suspect activator of JNK (Wang et al. 2007), as the upstream regulator of JNK activity (Gupta et al. 2006a; Fig. 3.2). Our findings are consistent with the work of Liu and Lin, who suggested that although prolonged JNK activation is linked to cell death, transient activation plays a role in cell survival (Liu and Lin 2005).

3.8 Gadd45 in Leukemia

Induction of gadd45 genes at the onset of myeloid differentiation suggested that their cognate protein(s) have a role in hematopoiesis, yet no apparent abnormalities were observed in either the bone marrow (BM) or peripheral blood compartments of mice deficient for either gadd45a or gadd45b. However, under conditions of hemato-

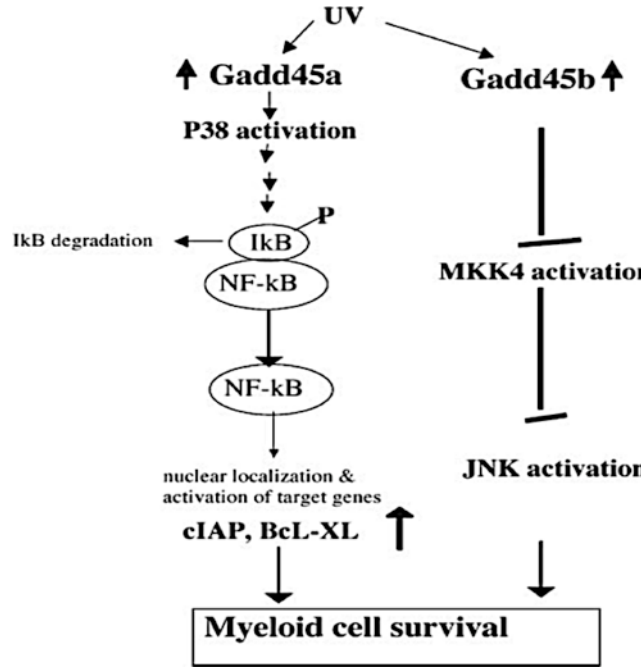

Fig. 3.2 Gadd45a and Gadd45b cooperate to promote cell survival in hematopoietic cells exposed to UV by two distinct and novel signaling pathways. These encompass activation of the Gadd45a-p38-NF-kB mediated survival pathway and Gadd45b mediated inhibition of the stress response apoptotic MKK4-JNK pathway

logical stress, including acute stimulation with cytokines, myeloablation, and inflammation, both gadd45a-deficient and gadd45b-deficient mice exhibited deficiencies (Gupta et al. 2005, 2006a, b; Hoffman and Liebermann 2009).

Since Gadd45 proteins play an important role in cell cycle control, cell survival, apoptosis, maintenance of genomic stability, DNA repair, and active DNA demethylation in response to environmental and physiological stress, including oncogenic stress (Hoffman and Liebermann 2009; Liebermann et al. 2011) suggest that it would also participate in the initiation and progression of leukemia and the response of leukemia to different therapeutics. This is supported by recent documented observations, as well as unpublished work currently conducted in this laboratory.

Activating mutations in FLT3 (Fms-like tyrosine kinase) is the most common genetic lesion in acute myeloid leukemia (AML); the aberrantly activated FLT3 pathway is found in 30% of AML cases (Parcells et al. 2006). In approximately 17–26% of AML cases, an internal tandem duplication (ITD) in the intracellular juxtamembrane (JM) domain (FLT3-ITD) is present. It was reported that gadd45a expression levels were significantly reduced in FLT3-ITD+ AML compared to FLT3-ITD−AML, consistent with FLT3-ITD-induced downregulation of gadd45a in human AML (Perugini et al. 2009). Furthermore, gadd45a expression data for primary AML samples, extracted from a published microarray study (Valk et al. 2004), indicate significant lower expression of gadd45a relative to normal controls in a number of AML clusters defined by their gene expression signature, including a cluster consisting only of patients with FLT3-ITD mutations (Liebermann et al. 2011). Downregulation in a cluster characterized by a t(11q23) rearrangement (predominantly MLL-AF9) may be by a related mechanism, since 11q23 events have been associated with increased FLT3 expression in several studies. Lower expression of gadd45a was also observed in a cluster characterized by the presence of t(8;21) translocations involving the AML–ETO fusion oncoprotein. Consistent with these observations, a report showed that gadd45a methylation in AML is predictive of poor survival (Perugini et al. 2013).

Since altering gadd45a expression revealed a role for this stress response gene in breast cancer,

behaving as a tumor suppressor in response to H-RAS (Tront et al. 2006, 2010), this laboratory initiated studies to assess how Gadd45 proteins modulate leukemia associated with constitutive RAS and BCR/ABL signaling.

RAS mutations occur at a frequency of 25% in AML, 30% in myeloma, and 6–20% in ALL; the highest incidence is found in MDS (30–40%), with 50–70% in the CMML subset. The most common mutations are found in N-RAS (~30%) and less frequently in K-RAS (~15%), and H-RAS) mutations are the most rare (Ikeda et al. 2006; Miyauchi et al. 1994; Reimann et al. 2006). Oncogenic N-RAS, K-RAS, and H-RAS each exhibit different leukemogenic potentials in mouse models (Parikh et al. 2007), with N-RAS inducing either an AML- or CMML-like disease and H-RAS an AML-like disease, whereas K-RAS invariably induces a CMML-like disease. This laboratory has data (Liebermann et al. 2011, unpublished), indicating that loss of gadd45a impeded N-RAS-driven leukemia, using the murine bone marrow transplantation (BMT) model. This is in contrast to the tumor suppressor role for gadd45a in H-RAS-driven breast cancer (Tront et al. 2006, 2010). On the other hand, knockout of the RAS endoprotease RCE1 was shown to accelerate myeloid leukemia by down-regulating GADD45b (Karlsson et al. 2020). These observations further support the hypothesis that the gadd45 oncogenic stress function depends upon cell type, developmental stage, and nature of stress/stimulus. Clearly, further studies need to be done on the role of Gadd45 proteins in different RAS-driven leukemias, using both mouse models and human patent samples.

Chronic Myelogenous Leukemia (CML) has been causally linked to the Philadelphia chromosome (Ph), which represents a balanced translocation involving chromosomes 9 and 22 that forms the BCR-ABL fusion oncoprotein, an active tyrosine kinase. CML is characterized by progression from an indolent "chronic phase" (CML-CP), characterized by hyperproliferation of mature granulocytes, to the aggressive and fatal "blast crisis" (CML-BC) phase, marked by the clonal expansion of differentiation-arrested immature blasts. Imatinib is a small molecule ABL kinase inhibitor that is highly effective in treating CML-CP patients; however, a substantial number of patients relapse due to development of resistance to imatinib therapy, which leads to CML-BC and death within weeks to months. Thus, identification of additional genetic aberrations that play a role in CML progression is of the utmost importance, as they may serve not only as prognostic markers, but also as novel therapeutic entry points.

Recently, Sha et al. reported that loss of Gadd45b accelerates the development of BCR-ABL driven CML in mice and leads to decreased median survival (Sha et al. 2018). The Gadd45b-deficient CML progenitors exhibited increased proliferation and decreased apoptosis, and this was associated with hyper-activation of c-Jun NH2-terminal kinase and Stat5. The same group previously reported similar findings for loss of another family member, Gadd45a. Loss of Gadd45a also accelerated the development of BCR-ABL driven CML, and this was associated with enhanced PI3K-AKT-mTOR-4E-BP1 signaling, upregulation of p30C/EBPα expression, and hyper-activation of p38 and Stat5. Similarly, Mukherjee et al. reported that in human patients with chronic phase CML, gadd45a expression is up-regulated, whereas in accelerated and blast crisis phase patients, gadd45a is downregulated (Mukherjee et al. 2017). Importantly, these findings are highly clinically relevant, since Gadd45a expression in human CML patients is high in the indolent, chronic phase of CML, but is markedly downregulated in the aggressive, accelerated phase of CML and blast crisis CML. Collectively, these results provide novel evidence that Gadd45a functions as a suppressor of BCR/ABL driven leukemia and may serve as a unique prognostic marker of CML progression. Also, these findings provide novel evidence that Gadd45b, like Gadd45a, functions as a suppressor of BCR-ABL driven leukemia, albeit via a different mechanism.

Furthermore, bone marrow cells from MDS/AML patients harboring the RUNX1-C-terminal mutation showed significantly lower levels of Gadd45a expression compared with those from MDS/AML patients with wild-type RUNX1. It

was found that RUNX1 directly regulates the transcription of gadd45a and that RUNX1 and p53 synergistically activate the gadd45a transcription. Together, these results suggest that Gadd45a dysfunction due to RUNX1 mutations can cause additional mutation(s) required for multi-step leukemogenesis (Satoh et al. 2012).

3.9 Gadd45 as a Target for Therapy

Due to the prevalence of gadd45 mutations or epigenetic modifications in leukemia and other types of cancer, it makes a tempting target for therapeutic drug development. For example, since both gadd45a and gadd45b have been shown to accelerate the development of BCR-ABL driven CML, it is possible that increasing expression of gadd45a and/or gadd45b would slow development of the disease. Furthermore, gadd45a methylation in AML, reducing expression of gadd45a, is predictive of poor survival. Using demethylating agents to increase expression of gadd45a could counter this effect and lead to increased survival in AML. More research needs to be done on these topics to see if a potential therapeutic benefit exists in these cases. However, multiple myeloma has already been shown to be a potential target for gadd45 related therapy. This occurs through gadd45's role as a downstream transcriptional target of NF-κB.

NF-κB is known to be aberrant in multiple myeloma cells, with studies indicating that at least 17% of multiple myeloma patients and ~40% of multiple myeloma cell lines may exhibit constitutive activation of the NF-κB pathway (Demchenko et al. 2010). Consequently, more than 80% of all primary multiple myeloma cell lines display nuclear accumulation of NF-κB, underscoring the importance of the protein complex to multiple myeloma (Tornatore et al. 2014). In these cell lines, NF-κB may inhibit apoptosis through suppressing activation of the JNK MAPK pathway. If NF-κB is functioning normally, the JNK MAPK pathway would lead to activation of other downstream effectors, such as gadd45 (Pearson et al. 2001). Further, gadd45 is upregulated in multiple myeloma cells by constitutive NF-κB activation and promotes malignant cell survival by binding to and inhibiting MKK7, thereby suppressing proapoptotic MKK7/JNK signaling (Bennett et al. 2018).

Initially, it was thought that directly inhibiting NF-κB would prevent the activation of the JNK MAPK pathway and thus provide a therapeutic benefit, however the side effects of global NF-κB inhibition were severe, including systemic inflammation (Greten et al. 2007). As such, attention was then focused on inhibiting downstream effectors of NF-κB, with the hope being to eliminate the anti-apoptotic effects of NF-κB constitutive activation while avoiding systemic side effects. Gadd45 was an attractive target, given its role in multiple myeloma. For example, patients expressing higher levels of gadd45β have dramatically shorter progression-free survival and significantly shorter overall survival than patients with low levels of GADD45β, even with the same treatment (Tornatore et al. 2014).

As such, Tornatore et al. developed DTP3, a D tripeptide which inhibits the GADD45β/MKK7 complex. Importantly, gadd45β expression is largely restricted to cancer cells. Thus, by blocking the GADD45β/MKK7 complex, DTP3 is able to selectively block NF-κB function in multiple myeloma cells, leading to apoptosis of the cancer cells with relative sparing of normal tissue (Rega et al. 2018). This theory has been borne out in in vivo and ex vivo studies, and the drug is currently in clinical trials (Tornatore et al. 2019a, b).

Relevant to this paper, the development of DTP3 shows that gadd45 is not merely of academic value, but as a drug target has proven real world applications as well. Knowledge of gadd45's role in NF-κB signaling could lead to development of treatments not just of multiple myeloma but of other kinds of NF-κB based pathologies as well (Tornatore et al. 2019b).

3.10 Concluding Remarks

Gadd45 proteins clearly influence the response of myeloid cells to acute stimulation with cytokines, myeloablation, and acute inflammatory stress,

and play a role in survival of myeloid cells in response to genotoxic stress. Further investigations on myeloid cells need to be done with gadd45g null mice as well as mice concomitantly deficient for two gadd45 genes.

A unifying hypothesis for the biological role of Gadd45 proteins is that their interactions with other proteins determine function, and that these protein–protein interactions are regulated by level of expression, cellular localization, and posttranslational modifications for both Gadd45 and the interacting partners. Defining these parameters and what influences them is a high priority to fully understand the biology of the gadd45 gene family, and is an area of ongoing investigations. However, the stress stimulus encountered, either intrinsic or extrinsic and its intensity, and the cell type and its physiological state appear to determine the function of Gadd45 proteins, and, therefore, must modulate the parameters.

Genetic lesions altering expression or function of any of the gadd45 gene products may impinge on the ability of an organism to respond to any challenge to the myeloid compartment. Therefore, these organisms may have altered responses to infection and/or toxic shock, which may be further exacerbated in elderly individuals since older mice deficient in either gadd45a or gadd45b exhibit cytopenia in their peripheral blood.

Similarly, individuals with defects in gadd45 may be more adversely affected compared to normal individuals by treatments that stress the hematopoietic stem cell compartment, including anti-cancer chemotherapeutic agents. Gadd45a status was shown to influence oncogenic processes, where loss of gadd45a increased ras-driven mammary tumorigenesis. What effect the status of gadd45 genes has on myeloid leukemias is currently under investigation. Integrating molecular, cellular, and genetic studies with animal models should further clarify the involvement of the gadd45 gene family in the different hematopoietic compartments following intrinsic and extrinsic stress.

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Gadd45 in DNA Demethylation and DNA Repair

4

Gurushankar Chandramouly

Abstract

Growth arrest and DNA damage 45 (Gadd45) family genes, Gadd45A, Gadd45B, and GADD45 G are implicated as stress sensors that are rapidly induced upon genotoxic/physiological stress. They are involved in regulation of various cellular functions such as DNA repair, senescence, and cell cycle control. Gadd45 family of genes serve as tumor suppressors in response to different stimuli and defects in Gadd45 pathway can give rise to oncogenesis. More recently, Gadd45 has been shown to promote gene activation by demethylation and this function is important for transcriptional regulation and differentiation during development. Gadd45 serves as an adaptor for DNA repair factors to promote removal of 5-methylcytosine from DNA at gene specific loci. Therefore, Gadd45 serves as a powerful link between DNA repair and epigenetic gene regulation.

Keywords

GADD45 · DNA methylation · DNA repair · Demethylation · CpG islands · Chromatin

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4.1 Introduction and Overview

The growth arrest and DNA damage-inducible 45 (Gadd45) gene family encodes three related Gadd45 proteins, Gadd45a, b, and g. They were originally identified as growth suppressing genes implicated in stress signaling in response to physiological or environmental stressors which results in apoptosis, cell cycle arrest, DNA repair, cell survival, and senescence (Abdollahi et al. 1991; Beadling et al. 1993; Zhan et al. 1994; Zhang et al. 1999). Gadd45 exerts these various functions via interaction with different effector proteins of apoptosis, cell cycle, or DNA repair (Fornace Jr. et al. 1988, 1992; Smith et al. 1996, 2000; Hollander and Fornace Jr. 2002; Barreto et al. 2007) (Fig. 4.1).

Among the Gadd45 family, Gadd45a was the first member identified while screening a cDNA library of increased transcripts after ultraviolet (UV) irradiation of Chinese hamster (CHO) cells (Fornace Jr. et al. 1988, 1989). Gadd45b was identified as a primary response gene transiently induced by IL-6 in myeloid leukemia cell lines (Abdollahi et al. 1991). Gadd45g was first described as an IL-2-inducible gene (Beadling et al. 1993). Gadd45a was surprisingly found as a top hit of a screen for an active DNA demethylase activity, that can actively remove the epigenetic mark 5-methylycytosine (5mC) from DNA (Barreto et al. 2007). With this identification of Gadd45 as promoter of active DNA demethyl-

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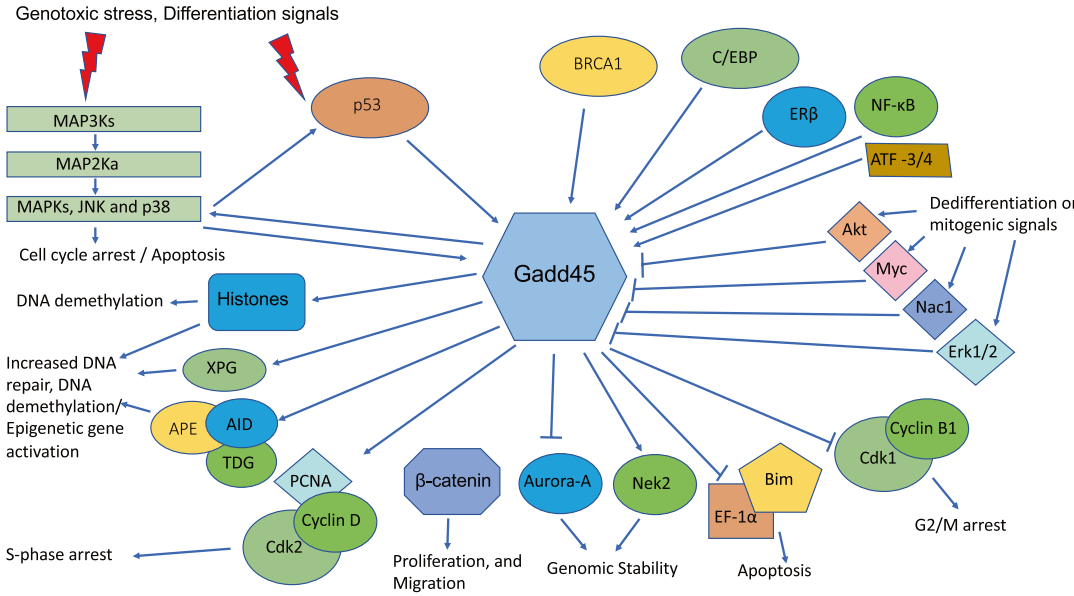

Fig. 4.1 Gadd45 interaction partners. Figure shows proteins that have well-known and defined roles in regulation of the Gadd45, as well as effector proteins and their effect on cell cycle regulation, senescence, apoptosis, DNA repair, and DNA demethylation. Upregulation is indicated by positive arrows, while other arrows indicate downregulation. Several interacting proteins of Gadd45 are depicted in the figure. MAPK kinase kinase MAPKKK (Takekawa and Saito 1998), p38-p53 (Bulavin et al. 2003), Histones (Carrier et al. 1999), Xeroderma Pigmentosum group G protein (XPG) (Barreto et al. 2007), activation-induced (cytidine) deaminase (AID) (Rai et al. 2008), thymine DNA glycosylase (TDG) (Cortellino et al. 2011), Proliferating Cell Nuclear Antigen (PCNA) (Smith et al. 1994; Azam et al. 2001), Cdk2/Cyclin D (Smith et al. 1994), APE (Jung et al. 2007), β-catenin (Hildesheim et al. 2004, 2005), Aurora-A (Shao et al. 2006), Nek-2 (Wang et al. 2004), EF-1α (Tong et al. 2005), Bim (Gao et al. 2009), Gao Cdk1/Cyclin B1 (Zhan et al. 1999: Vairapandi et al. 2002), Akt/Myc (Gao et al. 2009; Bulavin and Fornace Jr. 2004), Nac1 (Jinawath et al. 2009), ERK1/2 (Cretu et al. 2009), ATF (Gao et al. 2009; Chang et al. 2007), NF-κB (Zerbini et al. 2004; Lal and Gorospe 2006), C/EBP and BRCA1 (Jung et al. 2007; Gao et al. 2009)

ation, there were a lot of questions in the field on the implication of this particular function. Meanwhile, the crucial role of Gadd45 mediated DNA demethylation has been recognized in many different contexts.

Gadd45 proteins are small in size (~18 kD), with high homology among the multiple members with low abundance in normal cells and localize to both the nucleus and cytoplasm (Abdollahi et al. 1991; Zhang et al. 1999; Vairapandi et al. 2002). They belong to the L7Ae/L30e/S12e RNA binding protein superfamily. A comparison of the human Gadd45 amino acid sequences demonstrates that they have around 55% similarity to each other and are evolutionary highly related. The functions of Gadd45 proteins are similar, but not identical, and vary under diverse physiological conditions or in different cell types. The members—Gadd45a, b, and g are implicated in diverse cellular processes including apoptosis, cell cycle control, senescence, and DNA repair (Fornace Jr. et al. 1988, 1992; Smith et al. 1996, 2000; Hollander and Fornace Jr. 2002; Barreto et al. 2007; Magimaidas et al. 2016) (Fig. 4.1).

Since they lack enzymatic activity, they exert their function via interaction with effectors of apoptosis, cell cycle, and DNA repair. The roles of Gadd45 proteins in tumor and autoimmune suppression are also well documented. They are

highly expressed during the G1 phase of the cell cycle, with reduced expression in S phase and are ubiquitously detected in normal adult and fetal tissues, especially in quiescent cellular populations (Kearsey et al. 1995). Upon DNA damage all the Gadd45 protein family members are rapidly induced, resulting either in cell cycle arrest and/or apoptosis, or they actively participate in DNA repair mechanisms. In terms of mechanism, Gadd45 engages cellular DNA repair enzymes to remove 5mC from the DNA. Hence, Gadd45, including other DNA repair proteins have an unexpected epigenetic function. This will be the focus of this chapter.

4.2 DNA Methylation and Demethylation

Methylation of cytosine is the most prevalent covalent modification in DNA in many eukaryotes including fungi, plants, animals and is predominantly confined to CpG dinucleotides. Typically, DNA methylation serves as an epigenetic mark in the regulatory or promoter regions of the gene and confers transcriptional silencing. DNA methylation occurs predominantly at CpG dinucleotides in animals and can also be found in non-CpG dinucleotides in embryonic stem cells (Lister et al. 2009; Stadler et al. 2011). There are multiple biological processes where DNA methylation is required and it includes X chromosome inactivation, imprinting, tissue-specific gene expression, embryonic development, and cancer (Bird 2002; Deaton and Bird 2011; Hackett and Surani 2013). Several oxidized derivatives of 5mC, including hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine have been recognized in a variety of mammalian cells.

DNA methylation is a very stable epigenetic mark since DNA methyltransferase 1 (DNMT1) not only copies the methylation pattern to the newly synthesized DNA strand during replication but also inherits the existing methylation pattern to the next cell generation. However, DNA methylation is also a dynamic modification that can be erased via DNA demethylation. DNA demethylation is the process of removal of a methyl group from cytosines. Interestingly, a key mechanism for removal of 5mC is via DNA repair, thus indicating that cellular DNA repair is not just involved in protecting the genome from exogenous, endogenous DNA lesions, and genome instability, but also contributes to epigenetic gene regulation. DNA demethylation can be either passive or active (Fig. 4.2). Passive methylation takes place in the absence of methylation of newly synthesized DNA strands by DNMT1 during several replication rounds. When DNMT1 or its essential cofactor Uhrf1 (Np95) is degraded or inhibited from the nucleus, it can lead to 50% reduction of 5mC in every round of replication and thus lead to progressive loss of DNA methylation. Since replication is required for passive DNA demethylation, it can operate only in dividing cells and not in terminally differentiated cells. Multiple mechanisms can be involved in passive DNA demethylation including down regulation of DNMT enzymes (Oda et al. 2013; Zampieri et al. 2009), impairment of DNMT recruitment on DNA (Bostick et al. 2007; Oda et al. 2013), decrease of DNMT substrate SAM (Ulrey et al. 2005), inhibition of DNMT enzymatic activity (Caiafa et al. 2009) (Fig. 4.2). Some examples of passive DNA demethylation include replication dependent removal of 5mC from the maternal pronucleus of mouse zygote, hypomethylation in primordial germ cells (Mayer et al. 2000; Oswald et al. 2000).

On the other hand, active DNA demethylation occurs via direct enzymatic removal of methyl group (Fig. 4.2) independently of DNA replication and has been reported to occur in non-dividing cells and non-replicating plasmids. Many studies show an involvement of active enzymatic process in demethylation of oligonucleotides or plasmid DNA in vitro using cell extracts (Gjerset and Martin Jr. 1982; Jost 1993; Weiss et al. 1996; Agius et al. 2006). Active DNA demethylation is mainly associated with epigenetic reprogramming, cellular differentiation, or stress response in animals and plants. Some examples include repro-

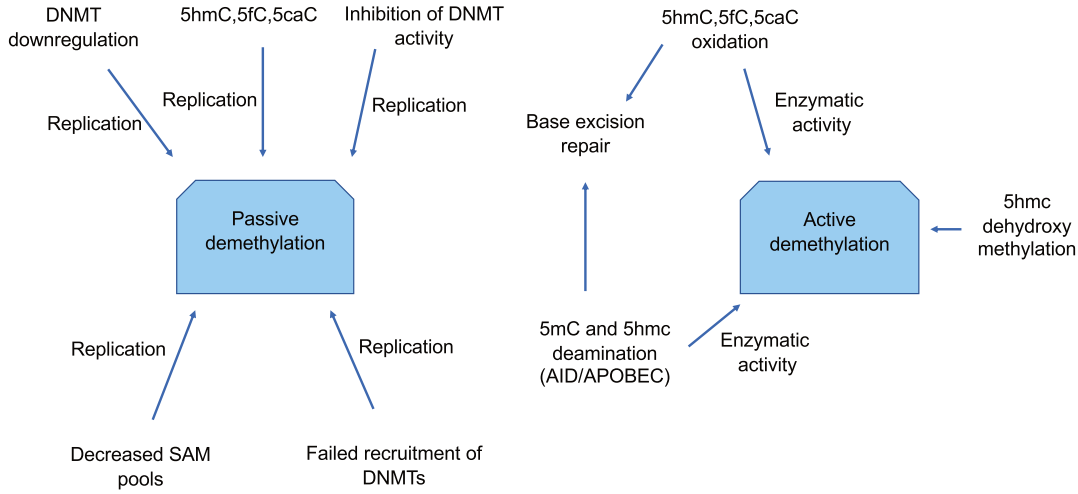

Fig. 4.2 Passive versus Active DNA demethylation. During passive DNA demethylation, multiple mechanisms that affect Dnmt1 activity prevent daughter strands to undergo re-methylation. With every round of replication, the methylation level is reduced and results in 50% completely unmethylated DNA molecules after the second replication. In contrast to the passive mechanism, active DNA demethylation is DNA replication independent, is involved in the active removal of 5mC via enzymatic modifications, activation of DNA repair pathways and is targeted. Key: *DNMT* DNA methyl transferase, *5mC* 5-methyl cytosine, *5hmC* 5-hydroxymethyl cytosine, *5fc* 5-fluorocytosine, *5caC* 5-carboxyl cytosine, *SAM* *S*-adenosyl methionine

gramming of the paternal pronucleus in the mouse zygote (Mayer et al. 2000; Oswald et al. 2000; Iqbal et al. 2011; Wossidlo et al. 2011), reprogramming in developing germ cells (Hajkova et al. 2010; Hackett et al. 2013), and activation of key pluripotency genes (Zhang et al. 2007; Mikkelsen et al. 2008; Bhutani et al. 2010). Demethylation and transcriptional activation of reelin in the hippocampus are crucial for neural memory during fear conditioning in rats. Differentiation stimuli can also induce demethylation, such as during T-cell activation that leads to demethylation of the *interleukin* 2 promoter (Bruniquel and Schwartz 2003). Moreover, during fear conditioning, DNA demethylation is essential for transcriptional activation of memory promoting genes in the hippocampus in rats (Miller and Sweatt 2007) as well as in the regulation of immediate early gene expression (IEG) in adult male mice (Li et al. 2019).

Altogether, these examples illustrate that DNA demethylation crucially affects cellular methylation pattern in many different contexts and thereby significantly contributes to the epigenetic mark of a cell (reviewed in Niehrs 2009; Wu and Zhang 2010).

4.3 Mechanisms of Active DNA Demethylation

There are multiple mechanisms in place that are involved in the removal of active 5mC. However, it is not clearly known how a specific mode of demethylation is engaged in a specific context, but one possible mechanism may be through tissue-specific expression of demethylating factors and regulators. For example, the three *Gadd45* isoforms are differentially expressed (Kaufmann et al. 2011) and induced upon differentiation cues to promote demethylation of some key genes that are involved in differentiation (Ma et al. 2009; Le May et al. 2010; Sen et al. 2010; Guo et al. 2011a; Zhang et al. 2011). Broadly, there are two mechanisms of demethylation, 5mC oxidation independent or repair based and 5mC oxidation dependent mechanisms. In oxidative DNA demethylation, 5mC is oxidized to 5-hydroxymethylcytosine (5hmC) by the ten-eleven-translocation (TET) proteins, Fe(II) and 2-oxoglutarate-dependent oxygenases (Tahiliani et al. 2009). TET proteins are Fe(II)-dependent dioxygenases that oxidize DNA using molecular

oxygen and α-ketoglutarate as co-substrates, generating oxidized DNA, succinate, and carbon dioxide (CO_2) as the co-products. TETs act on modified cytosines. Some examples include Tet1/Tet2 or Tet3 converting 5 mC to 5hmC in primordial germ cells (Hackett et al. 2013) and in the paternal pronucleus in the mouse zygote (Inoue and Zhang 2011; Iqbal et al. 2011; Wossidlo et al. 2011). TET proteins further oxidize 5hmC to 5-formyl- and 5-carboxylcytosine (Ito et al. 2011), which can be efficiently excised by Thymine DNA glycosylase (TDG) and repaired by base excision repair (BER) pathway (Guo et al. 2011b; He et al. 2011; Ito et al. 2011; Maiti and Drohat 2011). One possibility that has not been proven yet is that the 5-carboxylcytosine can be decarboxylated to yield unmethylated cytosine, or the oxidized cytosine residues can be excised from DNA to complete demethylation. AID/APOBEC mediated deamination of 5hmc followed by BER (Guo et al. 2011b) and direct removal of the oxidized 5-position substitute dehydroxymethylation by DNMTs are other examples of oxidative dependent demethylation. In contrast to oxidative DNA demethylation, canonical cellular DNA repair machineries are involved in repair-based 5mC removal in 5mC oxidation independent active DNA demethylation. Most evidence points to the BER pathway (Maiti and Drohat 2011; Xue et al. 2016; Muller et al. 2014; Cortellino et al. 2011; Hajkova et al. 2010), but its role in global demethylation has been challenged (Jin et al. 2015). Alternative pathways include nucleotide excision repair (NER) (Niehrs and Schäfer 2012; Barreto et al. 2007) and non-canonical mismatch repair (Grin and Ischenko 2016).

This strongly suggests that DNA repair proteins not only maintain genomic stability, but also critically influence epigenetic gene regulation. Overall, here are three major modes of repair that could be in place: (1) BER for excision of the methylated cytosine base or its oxidized derivative; (2) Conversion of 5mC to thymine after deamination by BER; (3) NER for excision of nucleotides encompassing the 5mC. At the end, in a common final step, DNA demethylation is completed by incorporation of unmethylated cytosine. Gadd45 proteins play a key role in the latter two processes and this will be the focus of the following sections.

4.4 DNA Demethylation Mediated by Gadd45

Apart from maintenance of genomic stability, DNA repair and suppression of cell growth, Gadd45 family proteins are multifaceted nuclear factors implicated in active DNA demethylation. Originally, Gadd45a was identified based on its ability to reactivate and demethylate methylation-silenced reporters (Barreto et al. 2007). This property of Gadd45a is conserved in frog, zebrafish, mouse, and man. This is also a characteristic for Gadd45b and Gadd45g (Barreto et al. 2007; Rai et al. 2008; Schmitz et al. 2009; Hu et al. 2010; Schäfer et al. 2013). Therefore, Gadd45 proteins might act redundantly in DNA demethylation in vivo and loss of one isoform might be compensated by the residual genes. Indeed, in zebrafish, DNA demethylation can be impaired only by combined knockdown of all Gadd45 isoforms (Rai et al. 2008).

Gadd45 mediated DNA demethylation is an active form of demethylation that operates on non-replicating plasmid DNA. Numerous target genes and various biological processes have been identified in Gadd45-mediated demethylation which suggests that the demethylation itself is gene specific and does not affect global DNA demethylation in mammalian cells. For example, Gadd45a through physical recruitment to *oct4* promoter mediates DNA demethylation of methylation-silenced mouse oct4 promoter in non-diving Xenopus oocytes (Barreto et al. 2007; Schäfer et al. 2013). There are numerous examples of Gadd45 demethylation targets found in various biological systems (Table 4.1). One recurrent feature of Gadd45 mediated DNA demethylation is that it is often initiated by cellular stress, nuclear hormones, or differentiation stimuli. Some examples include (a) induction of *Gadd45a* and *Gadd45b* upon calcium induced differentiation of epidermal progenitor cells to demethylate the differentiation marker S100P

Table 4.1 Examples of Gadd45 involvement in DNA demethylation

Gadd45 gene	Target	Model system	Cofactor	Reference
Gadd45a	*rDNA*	HEK293T NIH 3T3	XPG, XPA, XPF, TAF12	Schmitz et al. (2009)
Gadd45a	*RAR β2*	Hela cells, RA stimulated	XPC, XPA, XPG, XPF/ERCC1	Le May et al. (2010)
Gadd45a	*CD11a*, *CD70*	CD4+ T cells		Li et al. (2010)
Gadd45a	*oct4* promoter plasmid	*Xenopus* oocytes	XPG	Barreto et al. (2007)
Gadd45a	*Dlx5*, *Runx2*, *Osterix*	Osteogenic differentiation		Zhang et al. (2011)
Gadd45a	*MAGEB2, DHRS2, TAF7L,Hoxd8, Cxcl1*	HEK293T MEFs	Ing1	Schäfer et al. (2013)
Gadd45a	*Crabp2, Efs, Hoxa5, Rbp1 (demethylation is circumstantial)*	MEFs	AID, TDG	Cortellino et al. (2011)
Gadd45b	*BDNF IX*	Parietal cortex in psychosis		Gavin et al. (2012)
Gadd45a, -b	*S100*	Epidermal differentiation		Sen et al. (2010)
Gadd45b	*Bdnf IX*,*Fgf-1b* *Per2*, *Crebbp*	Mouse adult neurogenesis		Ma et al. (2009); Guo et al. (2011a)
Gadd45a, -b, -g	*Plasmid DNA (demethylation circumstantial in Neurod2, Sox2)*	Zebrafish embryos	Aid/Apobec, Mbd4	Rai et al. (2008)

(Sen et al. 2010), (b) induction of *Gadd45a* during osteogenic differentiation of mesenchymal stem cells and demethylation of key differentiation genes likes *Osterix* and *Runx2* (Zhang et al. 2011), (c) Gadd45 promoting neurogenesis by preventing epigenetic silencing of proneural markers like *Neurod2* during zebrafish development (Rai et al. 2008), (d) Reduced Gadd45b recruitment in psychotic disorders leading to *Bdnf IX* promoter hypermethylation (Gavin et al. 2012).

Overexpression of Gadd45a activates methylation-silenced reporter genes and promotes global DNA demethylation. However, despite the strong connection of Gadd45 proteins with DNA demethylation in numerous contexts, it is not clear if and how they promote DNA demethylation.

4.5 DNA Repair-Mediated DNA Demethylation by Gadd45

Although various examples point to a central role of Gadd45 proteins in active DNA demethylation, the lack of any obvious enzymatic activity of Gadd45 raises the question on the mechanism. Many recent studies have provided strong evidence on an attractive mechanism of repair-mediated demethylation by Gadd45. In an expression cloning screen for genes that activate methylated reporter plasmids, Gadd45a was identified as a non-enzymatic factor that promotes DNA demethylation (Barreto et al. 2007). Since Gadd45a interacts with the nucleotide excision repair (NER) endonuclease XPG, and the demethylation appears to require transcription-coupled NER, the mechanism of demethylation might be mediated by NER. In colon cancer cells, Gadd45a knockdown results in NER defects and thereby hypersensitivity to DNA damaging agents like cisplatin and UV irradiation (Smith et al. 1996). Likewise, complete genetic loss of Gadd45a leads to hypersensitivity to DNA damage and reduced global genomic NER (Smith et al. 2000; Hollander et al. 2001; Gupta et al. 2005). Also, Gadd45a knockout mice exhibit chromosomal abnormalities and increased radiation- and carcinogen induced tumorigenesis (Hollander et al. 1999, 2001; Hildesheim et al. 2002). Molecularly, Gadd45a recognizes UV-induced changes in the chromatin structure and modulates accessibility of the repair components to the DNA lesion (Carrier et al. 1999;

Smith et al. 2000) and influences NER via interaction with the proliferating cell nuclear antigen (PCNA), a NER component crucial for the DNA synthesis step (Smith et al. 1994; Vairapandi et al. 1996; Azam et al. 2001). Moreover, Gadd45a was shown to bind RNA, forming ribonucleoprotein particles (Sytnikova et al. 2011) and detected inside nuclear speckles that are sites of active transcription, RNA splicing, and processing. Therefore, Gadd45 could be involved in its epigenetic effects both through active DNA demethylation, chromatin remodeling, and post-transcriptional RNA regulation, which are closely linked to Gadd45-mediated DNA repair.

In the proposed mechanism that involves NER, 5-methylcytosine containing nucleotides are recognized and removed by Gadd45-XPG complex, and DNA polymerase δ/ε and ligase fill the resulting gap in DNA leaving CpGs unmethylated (Barreto et al. 2007). Since NER can be initiated either by transcription-coupled repair with an arrested RNA polymerase or by distortion of the DNA double-helical structure, it is not clear how NER is initiated in the actively demethylated genomic regions. Typical NER characteristics are detailed below (1) the excision and replacement of a stretch of nucleotides by endonucleases and (2) the involvement of Xeroderma pigmentosum (XP) group proteins. The 3′ NER endonuclease XPG binds Gadd45a and is required for demethylation in mammalian cells as well as in *Xenopus* oocytes. *oct4* demethylation in *Xenopus* oocytes is accompanied by incorporation of nucleotide, indicative of DNA synthesis and DNA repair (Barreto et al. 2007; Schmitz et al. 2009).

Many data point to a model whereby Gadd45a is targeted to specific sites of demethylation, recruits the NER machinery which subsequently excises the methylated cytosine. The resulting gap is later filled with unmethylated nucleotides by unscheduled DNA synthesis, completing DNA demethylation. This model is based on the rDNA locus in mammalian cells, where rDNA locus incorporates BrdU, a nucleotide analog following Gadd45a mediated DNA demethylation, in arrested NIH3T3 cells indicating unscheduled DNA repair synthesis (Schmitz et al. 2009). Furthermore, knockdown of NER components XPA, XPG, or XPF induces *rDNA* promoter hypermethylation associated with heterochromatic histone marks and transcriptional silencing and is essential for this demethylation. Other studies also confirm that Gadd45a and NER components co-associate at the *RARb2* promoter to demethylate the locus upon retinoic acid stimulation (Schmitz et al. 2009). Given, many NER factors are linked to transcriptional activation (Ito et al. 2011; Le May et al. 2010), it raises the question if they are required as true repair enzymes or transcription factors in Gadd45a mediated demethylation. One observation that suggests that indeed the enzymatic repair role of XPG is required for Gadd45a mediated demethylation is the requirement of NER endonuclease activity of XPG in rDNA demethylation (Schmitz et al. 2009).

Since Gadd45 and XPG have also been implicated in BER, one other possible mechanism that could explain the demethylation process is a BER related mechanism. Gadd45a is induced in response to base damaging agent methyl methanesulfonate (MMS) and is required for efficient repair of the base lesions (Fan et al. 1996; Jung et al. 2007). Furthermore, in zebrafish, Gadd45 interacts with the methyl CpG-binding domain protein 4 (Mbd4), a DNA glycosylase and a central enzyme of BER that recognize the damage and excise the base by hydrolyzing the β-*N*-glycosidic bond. The data point to a model where first step in the process of BER is conversion of 5mC to thymine by deaminases, followed by excision of the resulting G:T mismatch by Mbd4 glycosylase. Demethylation is accomplished by filling of gap with unmethylated cytosine. According to this model, Gadd45 targets Aid/Mbd4 along with another thymine DNA glycosylase, Tdg to the DNA demethylation site of target genes (Cortellino et al. 2011; Li et al. 2015). At the site, Tdg excises thymine, the product of 5mC deamination. It can also excise 5-carboxylcytosine or 5-formylcytosine generated by the Tet proteins that are oxidative DNA demethylation intermediates (Maiti and Drohat 2011). Here, Tdg might create a functional link between base excision repair and oxidative DNA

demethylation by interconnecting oxidized 5mC derivatives (Maiti and Drohat 2011) and Gadd45a (Cortellino et al. 2011). It is not clear if Gadd45 is involved in the removal of oxidized bases, however it is a tempting hypothesis given the overlap between Tet1 and Gadd45b demethylation targets in the brain. Since active DNA demethylation by DNA repair-based mechanism is not just restricted to 5mC, but can also occur in oxidized derivatives of 5mC, it may be generally referred as DNA demodification rather than demethylation.

In summary, data on DNA demethylation mediated by Gadd45 supports two discrete models for BER- and NER that may either be interconnected or independent. The two repair models are employed sequentially thus creating a hemimethylated intermediate: NER aiding in Gadd45 mediated DNA demethylation of the first strand followed by BER-mediated DNA demethylation of the second strand to accomplish full demethylation (Fig. 4.3). However, at least Gadd45 mediated demethylation of rDNA is not affected by knockdown of the BER enzyme TDG or by BER inhibitors, but only by the NER inhibitor gemcitabine. This suggests that BER and NER mediated demethylation is employed independently. However, further studies are required to know what factors are in play in pathway choice between BER and NER.

4.6 Determinants of Gadd45 Mediated Target Specificity and DNA Demethylation

Gadd45 mediated demethylation is a highly selective process and is not only gene specific, but even within a given gene, it affects only discrete CpGs (Barreto et al. 2007; Schmitz et al. 2009; Schäfer et al. 2013). It is however not

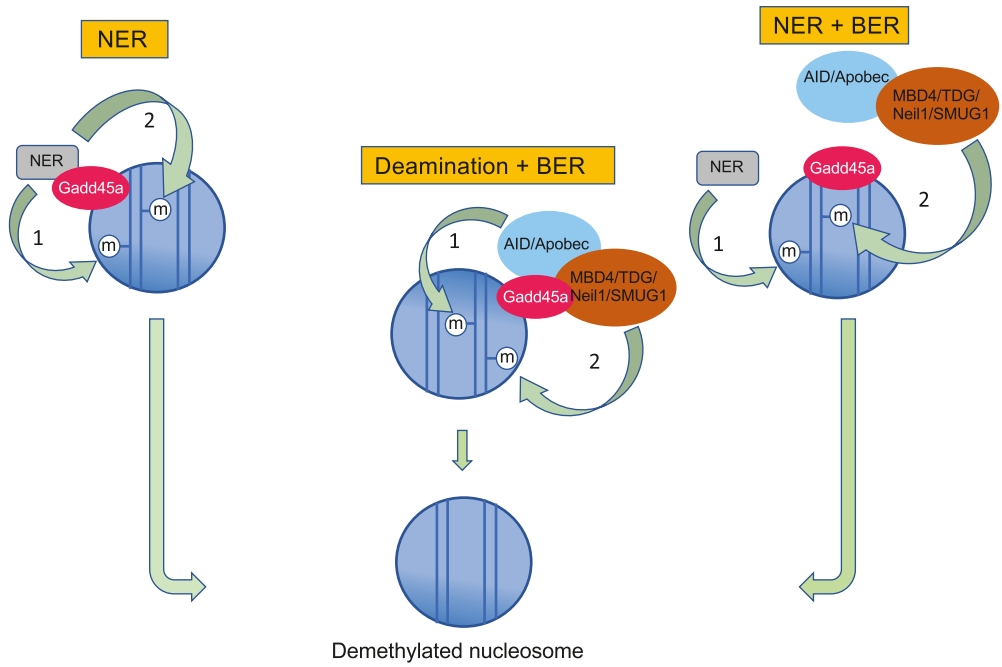

Fig. 4.3 Alternative models for Gadd45 mediated DNA demethylation by BER and NER. Figure depicts alternative models for demethylation by Gadd45 and DNA repair. It shows three proposed models for how methylated DNA strands within a nucleosome may be demethylated. This could be either by nucleotide excision repair (NER), or deamination followed by base excision repair (BER), or in a combined process involving consecutive NER and BER engagement. Key: *AID* activation-induced (cytidine) deaminase, *MBD4* methyl-CpG-binding domain protein 4, *TDG* thymine DNA glycosylase. The figure is modified from Niehrs and Schäfer (2012)

clearly known how targeting specificity of Gadd45 demethylation complex is achieved.

One of the key determining factors to achieve target specificity is a distinct chromatin pattern for each given target gene promoter. In the case of Gadd45 mediated DNA demethylation, CpGs proximal to the promoter are affected while the distal regions remain unaffected (Barreto et al. 2007; Schmitz et al. 2009; Le May et al. 2010; Cortellino et al. 2011; Schäfer et al. 2013). The histone modifications that are often present in proximal regions are H3 lysine 4 trimethylation (H3K4me) (Santos-Rosa et al. 2002; Schneider et al. 2004). Interestingly, Gadd45a interacts with Ing1, a sensor of histone mark H3K4me3 to promote demethylation (Schäfer et al. 2013). Gadd45a recruitment is dependent on the interaction between Ing1 and H3K4me3 via its PHD domain. Impairment of H3K4 methylation significantly reduces Gadd45a/Ing1 recruitment and target gene demethylation. Hence, Ing1 is considered an adaptor protein between chromatin and Gadd45a mediated DNA demethylation. Gadd45a is found at many more loci that are not regulated by DNA methylation, suggesting that H3K4me3 is essential, but not sufficient for Gadd45a recruitment. Therefore, there are several factors that are required for Gadd45a recruitment that are yet unidentified.

One other determinant for Gadd45 targeting is the presence of 5mC. This is exemplified in the recruitment of Mbd4/AID recruitment by Gadd45 to methylated but not unmethylated substrates (Rai et al. 2008). Additionally, Gadd45a binds Dnmt1 and impairs re-methylation of the hemi-methylated substrate (Lee et al. 2011), thus the function of Gadd45a may not be restricted to recruitment of DNA repair factors in DNA demethylation but might extend to protect a hemi-methylated DNA demethylation intermediate from re-methylation.

Noncoding RNAs (ncRNAs) are also involved on Gadd45 targeting. There is evidence for ncRNA-targeted DNA demethylation (Imamura et al. 2004; Zheng et al. 2008). Intriguingly, Gadd45 binds RNA and is part of a ribonucleoprotein complex. However, the physiological relevance and the role of this complex in DNA demethylation remain unknown. Nuclear hormone receptors (NRs) could also mediate target specificity. Gadd45 proteins bind to several NRs such as RXRa, RARa, ERa, PPARa, -b and –g to get recruited to hormone response elements and co-activate nuclear hormone reporters (Yi et al. 2000).

Some examples include dexamethasone, (Zhang et al. 2011) retinoic acid (Le May et al. 2010), or constitutive androstane receptor ligand treatment (Tian et al. 2011) leading to induction of Gadd45 binding to the hormone responsive target genes followed by DNA demethylation and activation. One other prerequisite for Gadd45 targeting and demethylation is low level of transcription. Gadd45 interacts with TAF12, a component of the PolI transcription complex and for Gadd45 mediated rDNA demethylation, transcription is required. Particularly, transcription is also required for histone acetylation-induced DNA demethylation.

4.7 Concluding Remarks

In recent years, Gadd45 has emerged as a key protein in DNA demethylation and it is indeed surprising that canonical DNA repair proteins form part of majority of DNA demethylation models. Despite several studies improving the understanding of this complex process of demethylation, there are several questions unanswered. It is not clearly known if NER- and BER-mediated DNA demethylation by Gadd45 are is interconnected or acts independently and this area requires more investigation. Further studies are needed to know if repair-mediated DNA demethylation plays a role in the removal of oxidized cytosine by Gadd45 during oxidative demethylation. Also, more studies are needed to understand the role of Gadd45-mediated DNA demethylation in defining cellular methylation patterns, development, and tumorigenesis. In summary, further in-depth studies can pave way in identification of more connections in demethylation as well as provide insights on the big picture of active DNA demethylation in specific chromatin contexts.

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Gadd45 Proteins in Immunity 2.0

5

Ingo Schmitz

Abstract

To protect the host against invading pathogens such as viruses, bacteria, and parasites as well as against tumor cells, the immune system of vertebrates has to distinguish between self and non-self, but also between harmless and dangerous. To do so, the immune system developed an innate and an adaptive branch that provide immediate and long-lasting protection, respectively. Furthermore, the immune system is composed of different cell types, which are specialized to combat different threats, and it is located at strategic locations to manage surveillance of the whole body. Therefore, immune cells need to communicate with each other. Growth arrest and DNA damage-inducible 45 (Gadd45) proteins are important components of intracellular signaling networks that convey messages from other cells in the receiving cell into biological responses. Within the 7 years that have passed since my first overview about Gadd45 proteins in the immune system, substantial progress has been made in our understanding of the immune system and with respect to Gadd45 proteins. Therefore, it is time for an update—Gadd45 proteins in immunity 2.0. In order to put Gadd45 proteins into the context of the immune system, I will first give a brief introduction into the immune system. Afterwards, I will give a brief introduction into Gadd45 proteins. For deeper insight, I refer to the other chapters of this book. The third section discusses the role of Gadd45 proteins in myeloid and lymphoid cells. The last two sections will be on the function of Gadd45 proteins in infection, autoimmunity, and tumor immunology.

Keywords

GADD45 · Immunity · Autoimmunity · Tumor immunology · Myeloid cells · Lymphoid cells · Infection · Autophagy

Abbreviations

AID	Activation-induced cytidine deaminase
ALV-J	J subgroup avian leukemia virus
AML	Acute myeloid leukemia
APC	Antigen presenting cell
ASK1	Apoptosis signaling kinase 1
ATG	Autophagy-related
BAC	Bacterial artificial chromosome
Bcl-x_L	B cell lymphoma x large
Cbl-b	Casitas B-lineage lymphoma proto-oncogene b

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CD	Cluster of differentiation
$CD4^+$	Cluster of differentiation 4-positive
CD40L	Cluster of differentiation 40 ligand
$CD8^+$	Cluster of differentiation 8-positive
c-FLIP	Cellular FLICE inhibitory protein
CLR	C-type lectin receptor
CpG	Cytosine phosphate guanosine dinucleotide motif
CR6	Cytokine response gene 6
CTL	Cytotoxic T lymphocyte
DISC	Death-inducing signaling complex
EAE	Experimental autoimmune encephalomyelitis
Egr	Early growth response
fMLP	*N*-formyl-methionine-leucine-phenylalanine
Gadd45	Growth arrest and DNA damage 45
GRAIL	Gene related to anergy in lymphocytes protein
HIV-1	Human immunodeficiency virus 1
IFN	Interferon
IL	Interleukin
IRF4	Interferon responsive factor 4
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
MAP2K	Mitogen-activated protein kinase kinase
MAP3K	Mitogen-activated protein kinase kinase kinase
MAPK	Mitogen-activated protein kinase
MEKK4	MAPK/ERK kinase kinase 4
MHC	Major histocompatibility complex
MKK	Mitogen-activated protein kinase kinase
MOG	Myelin oligodendrocyte glycoprotein
MPN	Myeloproliferative neoplasm
MTK1	MAP Three Kinase 1
Myd118	Myeloid differentiation primary response protein 118
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor κB
NKT	Natural killer T cell
NLR	Nod-like receptor
PAMP	Pathogen-associated molecular pattern
PCNA	Proliferating cell nuclear antigen
PDAC	Pancreatic ductal adenocarcinoma
PRR	Pattern recognition receptor
RCE1	RAS converting enzyme 1
RLR	Retinoic acid-inducible gene (RIG)-I-like receptor
RNP	Ribonucleoprotein
ROS	Reactive oxygen species
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TDG	Thymine DNA glycosylase
TET	Ten eleven translocator
TGF-β	Transforming growth factor beta
Th	T helper
TLR	Toll-like receptor
TNFR1	Tumor necrosis factor receptor 1
TNFα	Tumor necrosis factor alpha
vMIA	Viral mitochondrial-localized inhibitor of apoptosis
XPG	Xeroderma pigmentosum complementation group G; a.k.a. RECC5
ZAP-70	Zeta-chain associated protein of 70 kDa

5.1 Introduction into the Immune System

Our immune system protects us from all sorts of pathogens that threaten our body, e.g., viruses, bacteria, fungi, protozoa, and worms. Therefore, the immune system has to distinguish between self and non-self to attack invading pathogens but to leave the own body unharmed. Otherwise, infections or autoimmunity may arise. However, this is not enough. For instance, the microbiota and food also contain antigens, to which the immune systems might react. Yet, they are harmless and should not be attacked by the immune system to prevent constant inflammation. On the other hand, tumor cells are self, but due to their uncontrolled growth can harm the body. So, the immune system should react to this "altered self." Thus, the immune system has also distinguish between harmless and dangerous.

To accomplish these different distinctions, the immune system of vertebrates consists of an innate and an adaptive branch. Responses by the

innate immune system are immediate and activated by germ-line encoded pattern recognition receptors (PRRs), which recognize conserved pathogen-associated molecular patterns (PAMPs) (Janeway Jr. 1989). The PRRs include toll-like receptors (TLRs), C-type lectin receptors (CLRs), Nod-like receptors (NLRs), and cytosolic DNA sensors (RIG-I like receptors, RLRs) (Takeuchi and Akira 2010). Upon activation, these receptors initiate the NF-κB signaling cascade and type I interferons (IFNα and IFNβ) that in turn activate and recruit immune cells. Innate immune cells include granulocytes such as neutrophils, as well as macrophages and dendritic cells. The latter two cell types act as professional antigen presenting cells (APCs) that present peptide-antigens to T cells, which subsequently mount an adaptive immune response. T cells are lymphocytes that develop in the thymus (hence named T cell) and mediate cellular immunity. There are two major types of T cells, namely cytotoxic T cells and helper T cells. Cytotoxic T cells express the co-receptor CD8 (CD8+ CTLs), which interacts with MHC class I, and kill infected target cells via the induction of apoptosis. Helper T cells express the co-receptor CD4 (CD4+ Th cells), which interacts with MHC class II, and activate macrophages to digest intracellular pathogens. CD4+ Th cells also provide help to another type of lymphocyte called B cells, which develop in the bone marrow (hence named B cell), via co-stimulatory molecules such as the CD40/CD40L axis. Activated B cells then secrete antibodies (immunoglobulins) and thereby mediate humoral immunity. The adaptive immune system has two important characteristics, which make it essential for complex organisms such as vertebrates. First, cells of the adaptive immune system, i.e., lymphocytes, bare antigen receptors that are highly specific for a certain antigen. During the development of lymphocytes, gene fragments of the immunoglobulin and T cell receptor (TCR) genes are rearranged in a stochastic manner to generate up to 10^{15} receptor specificities (Davis and Bjorkman 1988). In contrast to innate immune cells, which react only to conserved pathogen structures such as lipopolysaccharide (LPS), a compound from the cell wall of gram-negative bacteria, lymphocytes are able to react to specific amino acid sequences and, thus, to a particular strain of a pathogen. The second important feature of the adaptive immune system is its ability to form memory, so that re-infection with the same pathogen results in a faster and stronger immune response. This is due to the fact that during the first encounter with a given pathogen some lymphocytes develop into long-lived memory cells that survive in lymphoid organs and other tissues for months and even years and have a lower threshold of activation (Woodland and Kohlmeier 2009). Therefore, they acquire effector functions much faster than naïve lymphocytes upon repeated antigen encounter. Consequently, the pathogens get cleared much faster and the infected host does not develop any symptoms of disease—the host is immune.

Cytotoxic T cells and B cells function as effector cells of the adaptive immune system by killing target cells and producing antibodies, respectively. Yet, CD4+ Th cells play a leading part during an adaptive immune response by providing co-stimulatory signals and secreting cytokines. Depending on the nature of the invading pathogen they are able to differentiate into various Th cell subsets, namely Th1, Th2, Th17 cells and others, each tailored to drive a type of immune response appropriate for the particular pathogen (Korn et al. 2009; Murphy and Reiner 2002; Reiner 2007). For instance, Th1 cells are induced by interleukin-12 (IL-12) and produce interferon-γ (IFN-γ), which activates infected macrophages to degrade microbes that persist in intracellular vesicles such as mycobacteria or *Listeria* (Murphy and Reiner 2002). Th2 cells are induced by IL-4 and are important for controlling infections caused by extracellular, multicellular parasites such as helminths (Murphy and Reiner 2002). Th2 cells produce IL-4, IL-5, and IL-13 to activate eosinophils, mast cells, and B cells, the latter differentiating into plasma cells to produce high amounts of specific antibodies. Th17 cells were named after the cytokine they secrete, IL-17 (Harrington et al. 2005; Park et al. 2005). Initially, it was shown in vitro that stimulation of naïve T cells with the immunosuppressive cytokine transforming growth factor beta (TGF-β) and the pro-

inflammatory cytokine IL-6 drives Th17 differentiation. Yet, also TGF-β independent differentiation pathways with a combination of IL-1β and IL-23 have been described (Kara et al. 2014; Korn et al. 2009). Th17 cells are instrumental in fighting extracellular bacteria and fungi by enhancing neutrophil responses.

Since the immune system is composed of many different cell types distributed over the whole body, it is compulsory that immune cells need to communicate with each other, either via direct cell-cell-contact or via cytokines over a distance. Inside the cells, complex signal transduction networks transmit and integrate these communication signals. Gadd45 proteins are part of the signaling networks in immune cells.

5.2 Some Background on Gadd45 Proteins

The Gadd45 proteins, namely Gadd45α (Gadd45), Gadd45β (Myd118), and Gadd45γ (CR6), are small proteins of 18–20 kDa with no enzymatic activity of their own. They execute their physiological functions by protein–protein interactions in the nucleus and cytoplasm of cells and are able to modulate cell proliferation, cell death, and cell survival. Due to their high homology (Takekawa and Saito 1998), Gadd45 family members are expected to have largely overlapping functions. Specificity might be brought about by different signals that drive the transcription of the various Gadd45 genes. For instance, Gadd45α is a p53 target gene (Kastan et al. 1992), the transcription of Gadd45β is induced by TGF-β, interleukins as well as the T cell receptor (Schmitz et al. 2003; Selvakumaran et al. 1994; Yang et al. 2001), and Gadd45γ transcription is stimulated by interleukin-2 (Beadling et al. 1993) (Fig. 5.1). Moreover, LPS, a cell wall component of gram-negative bacteria, induced Gadd45β in vivo (Zhang et al. 2005).

Gadd45 genes have been identified in many vertebrates. A NCBI search resulted in 241 nucleotide sequences for Gadd45α, 261 sequences for Gadd45β and 279 for Gadd45γ (as at of November 2020). Next to *Homo sapiens*, *Mus musculus,* and other mammals, also sequences in birds (e.g., *Gallus gallus*), reptiles (e.g., *Crocodylus porosus* (Australian saltwater crocodile)), amphibians (*Xenopus tropicalis*), and fish (e.g., the zebrafish *Danio rerio*) can be found. Interestingly, expression of CiGadd45aa and CiGADD45ab in the grass carp *Ctenopharyngodon Idella* is inhibited by microRNAs and regulates inflammation, apoptosis, and the immune response to bacterial infection in these fish (Fang et al. 2020a, b, c). Moreover, D-GADD45 regulates JNK-dependent apoptosis in *Drosophila melanogaster* indicating that Gadd45 genes are evolutionary conserved beyond vertebrates (Camilleri-Robles et al. 2019).

5.2.1 Functions of Gadd45 Proteins

As suggested by its name, growth arrest, and DNA damage (GADD), Gadd45 proteins are involved in the control of the cell cycle. For instance, all three Gadd45 proteins interact with the proliferating cell nuclear antigen (PCNA), which however impairs their activity to suppress growth (Azam et al. 2001; Smith and Mocarski 2005; Vairapandi et al. 1996, 2000). Since PCNA also binds $p21^{WAF1/CIP}$ and Gadd45 proteins act synergistically with p21 in growth suppression, sequestration of these proteins by PCNA might explain in part the positive effect of PCNA on proliferation (Azam et al. 2001; Chen et al. 1995; Vairapandi et al. 1996). With respect to direct cell cycle control, Gadd45 proteins were shown to interact with cyclin-dependent kinase/cyclin complexes to inhibit cell proliferation (Vairapandi et al. 2002; Zhan et al. 1999).

A second important function of Gadd45 proteins is the regulation of mitogen-activated protein kinases (MAPKs). Here, Gadd45 proteins induce activation of the kinase MEKK4, which is also known as MTK1 (Gerwins et al. 1997; Takekawa et al. 1997). MEKK4 is a mitogen-activated protein kinase kinase kinase (MAP3K) that activates p38 and JNK mitogen-activated protein kinases and can serve as a redox sensor (Gerwins et al. 1997; Matsushita et al. 2020; Takekawa et al. 1997). In resting cells, the kinase

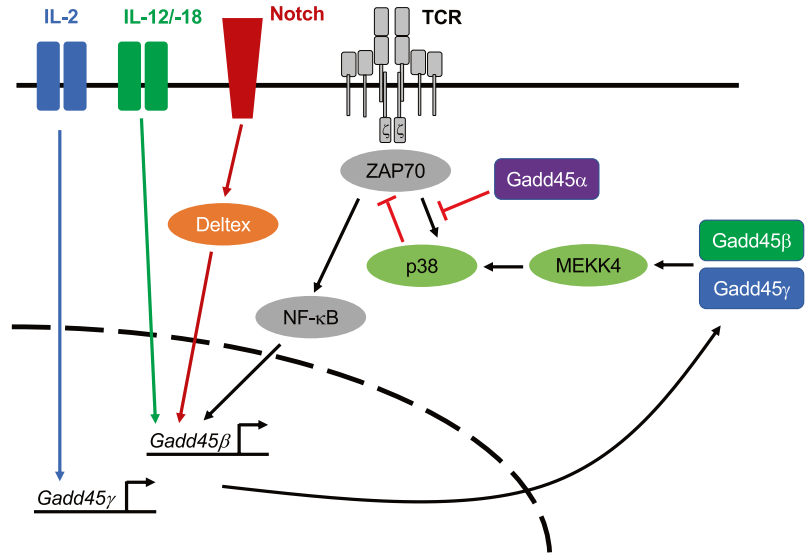

Fig. 5.1 Gadd45 proteins in T cells. Triggering of the T cell receptor (TCR) activates the zeta chain-associated protein of 70 kDa (ZAP-70). This tyrosine kinase leads to the activation of the transcription factor NF-κB and the p38 mitogen-activated protein kinase. NF-κB induces transcription of the *Gadd45b* gene. The same applies to the cytokines IL-12 and IL-18 as well as stimulation of the Notch receptor and its cytoplasmic effector Deltex. The cytokine IL-2 activates transcription of the *Gadd45g* gene. Both Gadd45β and Gadd45γ proteins interact with the kinase MEKK4, which leads to sustained p38 activation and, subsequently to interferon-γ (IFN-γ) production and Th1 differentiation (not depicted). Gadd45α is constitutively expressed in T cells and can inhibit the alternative activation of p38 by ZAP70-mediated tyrosine phosphorylation. In turn, active p38 acts in a negative feedback loop to inhibit ZAP70 activity. Activation of *Gadd45b* by Notch and Deltex may lead to T cell anergy by a yet unknown mechanism

is in a closed, inactive conformation. Upon binding of Gadd45 proteins MEKK4 adopts an open conformation that allows dimerization, autophosphorylation, and activation of downstream kinases (Mita et al. 2002; Miyake et al. 2007; Takekawa and Saito 1998). Downstream of MEKK4 are the MAP2Ks MKK3 and MKK6, which activate the p38 MAPK (Tanaka et al. 2002). Since most often Gadd45 proteins have to be induced by certain stimuli, e.g., by the cytokine TGF-β, the Gadd45-MEKK4-p38 axis is responsible for delayed and or sustained MAPK activation in cells (Takekawa et al. 2002). In T cells, Gadd45α has an additional role in the regulation of p38 MAPK activity. The TCR activates p38 by an alternative mechanism that does not involve a classical three-tire kinase cascade. Instead, TCR triggering activates the tyrosine kinase ZAP70 that phosphorylates p38 on tyrosine residue 323 leading to full p38 activation (Salvador et al. 2005b). Gadd45 proteins also interact with the MAP3K ASK1, which can activate the JNK MAPKs (Papa et al. 2004). However, the functional outcomes of this interaction have not been investigated in detail. In addition, Gadd45β interacts with the MAP2K MKK7, which induces JNK activation (Tournier et al. 1997). In contrast to the interaction with MEKK4, binding of Gadd45β to MKK7 inhibits the activity of the kinase and, thus, JNK activation is blocked (Papa et al. 2004). The inhibition of JNK signaling by Gadd45β is one way how NF-κB activity suppresses cell death and promotes cell survival (De Smaele et al. 2001).

A third function of Gadd45 proteins is related to epigenetic regulation of gene expression. Using *Danio rerio* (zebrafish) as a model organism, it was shown that Gadd45 together with the 5-methylcytosine deaminase AID and the G:T mismatch-specific thymine glycosylase Mbd4 forms an active DNA demethylation complex (Rai et al. 2008). In mammals, Gadd45α

interacts with thymine DNA glycosylase (TDG) and Ten-Eleven-Translocation (TET) enzymes to mediate DNA demethylation (Cortellino et al. 2011; Kienhofer et al. 2015; Li et al. 2015). In this regard it has been shown that Gadd45α recruits TET1 to CpG islands to induce demethylation allowing chromatin opening and gene expression (Arab et al. 2019). In the central nervous system, Gadd45β is required for activity-dependent demethylation in neural progenitors and Gadd45β deficiency resulted in growth defects in the hippocampus of mice (Ma et al. 2009). Furthermore, Gadd45-dependent demethylation has been shown in adipose-derived mesenchymal stem cells, embryonic stem cells, and hematopoietic cells (Schule et al. 2019; Suzuki et al. 2017; Zhang et al. 2011). In hematopoietic cells, Gadd45α might be recruited together with TDG and TET2 via RUNX1 to gene loci that are epigenetically regulated during hematopoiesis (Suzuki et al. 2017). However, while the RUNX1 TET2 interaction was demonstrated via co-immunoprecipitation in leukemic Jurkat T-lymphoblasts, the RUNX1 Gadd45α interaction was only shown by overexpression in non-hematopoietic HEK293T cells. Whether or not Gadd45 proteins are involved in locus-specific demethylation events during development and differentiation of immune cells awaits further studies.

5.3 Gadd45 Proteins in Immune Cells

5.3.1 Myeloid Cells

Within the innate immune system, Gadd45 proteins are crucial for the differentiation of myeloid cells as well as for the function of granulocytes and macrophages. For instance, in vitro differentiation of bone marrow cells into macrophage or granulocyte lineages resulted in reduced frequencies of these cell types in the absence of either Gadd45α or Gadd45β (Gupta et al. 2006). This correlated with increased apoptosis during differentiation and reduced clonogenicity of Gadd45α-deficient and Gadd45β-deficient cells. Reduced myeloid differentiation was also observed in vivo when myeloid cells were ablated by intraperitoneal injection of 5-Fluorouracil and recovery was observed 10 days post-injection (Gupta et al. 2006). In contrast, Gadd45γ is not required for myeloid differentiation (Hoffmeyer et al. 2001). Of note, Gadd45α- and Gadd45β-deficiency resulted in a higher proliferative capacity of immature myeloid cells (Gupta et al. 2006). Therefore, Gadd45 protein expression may support terminal differentiation of myeloid cells as well as inhibit the proliferation of these terminally differentiated cells.

In addition to myeloid differentiation, Gadd45α and Gadd45β appear to be important for the function of granulocytes and macrophages. In a mouse model of experimental sepsis, Gadd45α-deficient and Gadd45β-deficient mice exhibited impaired recruitment of myeloid cells into the peritoneal cavity upon LPS injection (Salerno et al. 2012). In vitro, macrophages and granulocytes of mice lacking either Gadd45α or Gadd45β were less efficient in migration as chemotactic assays using LPS, *N*-formylated peptides such as *N*-formyl-methionine-leucine-phenylalanine (fMLP) or the chemokine IL-8 as stimulus revealed. Both types of myeloid cells produced less reactive oxygen species (ROS) and cytokines. Moreover, the phagocytic capacity of Gadd45α-deficient and Gadd45β-deficient macrophages was strongly impaired (Salerno et al. 2012). Mechanistically, this was attributed to the regulation of p38 and JNK mitogen-activated protein kinase signaling by Gadd45 proteins. In summary, Gadd45 proteins play a crucial role in the differentiation, proliferation, and function of myeloid cells.

5.3.2 Lymphoid Cells

With respect to adaptive immunity, most of the work on Gadd45 proteins has concentrated on T cells. For instance, Gadd45β was shown to be important for IL-2 secretion in naïve T cells as well as in differentiated Th1 cells (Lu et al. 2004). Gadd45β-deficient naïve T cells showed reduced p38 MAPK activity suggesting reduced T cell

activation (Lu et al. 2004). In contrast, Gadd45γ was not required for hematopoiesis, T cell proliferation, or T cell responsiveness to IL-2 (Hoffmeyer et al. 2001; Lu et al. 2001).

Within the T cell compartment, Gadd45 proteins play a special role in the function of CD4+ Th1 cells. For instance, Gadd45γ was shown to be important for the IFN-γ production by Th1 cells (Lu et al. 2001). In contrast, Th2 polarized Gadd45γ-deficient cells showed similar IL-4 levels when compared to wildtype Th2 cells. Gadd45γ-deficient T cells also exhibited less p38 and JNK MAPK activity and were less prone to activation-induced cell death (Lu et al. 2001). Importantly, Gadd45γ-deficient mice showed reduced contact hypersensitivity demonstrating that Th1 responses were also impaired in vivo (Lu et al. 2001). Similar to Gadd45γ, Gadd45β is important for Th1 responses. Using retroviral overexpression and Gadd45β-deficient T cells, it was shown that Gadd45β promotes IFN-γ secretion upon TCR triggering or upon stimulation with IL-12 and IL-18, which drive Th1 differentiation (Lu et al. 2004; Yang et al. 2001). This was mediated via prolonged p38 MAPK activation (Lu et al. 2004) and inhibited via a dominant-negative version of MEKK4 (Yang et al. 2001), which is activated by GADD45 proteins (Takekawa and Saito 1998). In line with the data obtained with dominant-negative MEKK4, CD4+ T cells from MEKK4-deficient mice exhibited less IFN-γ secretion during Th1 differentiation and reduced p38 activation upon stimulation of the TCR or with IL-12 and IL-18 (Chi et al. 2004). Importantly, overexpression of Gadd45β or Gadd45γ in MEKK4-deficient T cells did not increase IFN-γ production while it did so in wildtype T cells demonstrating that the Gadd45 proteins together with MEKK4 comprise a common pathway that potentiates IFN-γ production and thereby Th1-mediated immunity (Chi et al. 2004). However, while one study described the expression of IFN-γ to be independent of STAT4 and its phosphorylation state (Chi et al. 2004), another study found that Gadd45β and Gadd45γ induced phosphorylation of STAT4 at serine residue 721 and that Ser721-phosphorylated STAT4 was crucial for IFN-γ expression and Th1 differentiation (Morinobu et al. 2002). Despite this controversy, it is clear that Gadd45 proteins are important regulators of IFN-γ expression in T cells and of Th1 differentiation.

Anergy is a state of unresponsiveness that is induced in T cells when they receive TCR stimulation in the absence of a co-stimulatory signal and a mechanism ensuring immunological tolerance next to clonal deletion by apoptosis or suppression of immune responses by regulatory T cells (Schwartz 2003). Among other mechanisms, anergy is mediated by the transcription factors nuclear factor of activated T cells (NFAT) as well as early growth response 2 (Egr2) and Egr3 that induce expression of E3 ubiquitin ligases such as Cbl-b and GRAIL (Fathman and Lineberry 2007). Surprisingly, a possible connection between T cell anergy and Gadd45β was reported. First, Gadd45β was identified by DNA array technology as a gene induced during induction of T cell anergy (Safford et al. 2005). Furthermore, analyzing the effect of the Notch target gene Deltex1 on T cell physiology, it was found that Deltex1 induced anergy of CD4+ Th cells (Hsiao et al. 2009). Importantly, Deltex1 induced transcriptional activation of the Gadd45β gene in addition to induction of the E3 ubiquitin ligase Cbl-b (Hsiao et al. 2009) (Fig. 5.1). It was suggested that the inhibitory effect of Gadd45β on JNK mitogen-activated protein kinase activity mediates T cell anergy in an E3 ubiquitin ligase independent manner. However, a direct role for Gadd45β in T cells anergy has not been directly tested, yet.

As described above, Gadd45 proteins, Gadd45β and Gadd45γ are involved in activation of the p38 mitogen-activated protein kinase via a classical kinase cascade. Gadd45β, Gadd45γ, and the mitogen-activated protein kinase kinase kinase MEKK4 are crucial for the differentiation of T cells into Th1 cells. In contrast, Gadd45α has a different role in the regulation of p38 MAPK activity in T cells. The TCR activates p38 by an alternative mechanism that does not involve a classical three-tire kinase cascade. Instead, TCR triggering activates the tyrosine kinase ZAP70 that phosphorylates p38 on tyrosine residue 323 leading to full p38 activation (Salvador

et al. 2005b). Interestingly, active p38 phosphorylates in turn ZAP-70 on threonine 293 reducing the activity of this tyrosine kinase and resulting in a negative feedback loop (Giardino Torchia et al. 2018). The importance of the alternative pathway was demonstrated by the generation of knock-in mice harboring a Tyr323Phe mutation, in which activation of p38α MAPK upon TCR stimulation with or without co-stimulation was abrogated (Jirmanova et al. 2009). Furthermore, T cells with mutated p38α MAPK exhibited reduced RNA synthesis upon T cell activation and secreted less IFN-γ, indicating impaired Th1 responses (Jirmanova et al. 2009). On the molecular level, the alternative p38 pathway induces NFAT and IRF4 for subsequent T cell activation (Alam et al. 2014, 2018). Importantly, Gadd45α inhibited this T cell-specific, alternative p38 activation pathway. Recombinant Gadd45α inhibited the activity of p38 in an in vitro kinase assay and this was specific for ZAP70-mediated but not MKK6-mediated p38 activation suggesting that Gadd45α prevents binding of ZAP-70 to p38 (Salvador et al. 2005a). In line with this notion, Gadd45α-deficient T cells displayed constitutive p38 activation (Salvador et al. 2005a). Therefore, the function of Gadd45α seems to be opposing that of Gadd45β and Gadd45γ regarding p38 MAPK activation in T cells. Physiological and clinical implications of this alternative p38 pathway will be discussed further below.

Next to T cells, there are also a few reports on other cell types of the adaptive immune system. For instance, NKT cells are a type of lymphocyte, possessing characteristics of NK cells and memory T cells. They express an invariant TCR with a TCRα chain containing a variable region encoded by the Vα14 gene and a joining region encoded by the Jα18 gene that restricts these cells to CD1d molecules (Kronenberg and Gapin 2002; Taniguchi et al. 2003). The latter are MHC-like molecules that present glycolipids as antigens such as α-galactosylceramide (Porcelli and Modlin 1999). NKT cells possess immunoregulatory functions and secrete large quantities of cytokines such as IFN-γ and IL-4 (Kronenberg and Gapin 2002; Taniguchi et al. 2003). Interestingly, NKT cells are more resistant to TCR-induced apoptosis than conventional T cells, which correlated with a higher induction of anti-apoptotic genes such as Gadd45β (Harada et al. 2004). However, no data has been provided so far that supports a functional role for Gadd45β in NKT cell survival. Therefore, the importance of Gadd45 proteins for NKT cell biology awaits further studies.

Moreover, it was shown that B cells, the lymphocytes responsible for the production of antibodies, strongly induce Gadd45β along with known anti-apoptotic proteins such as Bcl-xL and c-FLIP upon ligation of CD40, a TNF receptor superfamily member that provides co-stimulatory signals to B cells (Zazzeroni et al. 2003). Generation of cell lines stably overexpressing Gadd45β demonstrated that this protein inhibits CD95/Fas-induced (i.e., extrinsic) apoptosis, but has no effect on early events in the CD95 signaling cascade such as the formation of the death-inducing signaling complex (DISC). Instead, Gadd45β impaired the activation of the mitochondrial amplification loop although direct triggering of the mitochondrial (i.e., intrinsic) pathway was not affected (Zazzeroni et al. 2003). The latter discovery seems to contradict the finding that Gadd45 proteins are able to bind to cellular Bcl-xL as well as to the anti-apoptotic protein vMIA from the cytomegalovirus and thereby enhances the cell's resistance towards CD95-induced apoptosis (Smith and Mocarski 2005). Therefore, the exact mechanism of the influence of Gadd45β on apoptosis is unknown and it remains to be dissected whether Gadd45β targets the CD95 signaling cascade similar to the TNFR1 pathway (De Smaele et al. 2001), or whether it targets mitochondria (Smith and Mocarski 2005). Nevertheless, as in myeloid precursor cells (Gupta et al. 2005), Gadd45β appears to be an anti-apoptotic protein in B cells since it protected them from activation-induced cell death.

Antigen presenting cells are at the borderline of innate and adaptive immunity since they can be innate immune cells (macrophages, myeloid dendritic cells) or of lymphoid origin (B cells and lymphoid dendritic cells). Dendritic cells are the most potent professional antigen presenting cells

to initiate T cell responses (Shortman and Liu 2002) and are, thus, mentioned in this section. Dendritic cells capture antigens from the environment and present them via MHC class II to CD4+ Th cells. Depending on the nature of antigen and the route of antigen uptake, dendritic cells express cytokines that drive immune responses into a given direction, e.g., they secrete IL-12 and IFN-γ to promote a Th1 response (Murphy and Reiner 2002). Bone marrow-derived dendritic cells express all three Gadd45 proteins (Jirmanova et al. 2007). Interestingly, dendritic cells from Gadd45α-deficient mice exhibited less activation of the classical MKK3/6-p38 mitogen-activated protein kinase (MAPK) cascade, less production of the Th1 cytokines IL-12 and IFN-γ and reduced expression of the co-stimulatory molecule CD40 upon stimulation with soluble antigens from *Toxoplasma gondii* (Jirmanova et al. 2007). Similarly, Gadd45β-deficient dendritic cells produce less IFN-γ upon stimulation with LPS (Lu et al. 2004). Therefore, the activation of classical MAPK signaling by Gadd45 proteins is crucial for mounting a Th1 response via activation of dendritic cells. It is not known whether the Gadd45-p38-axis regulates cytokine expression in dendritic cells via transcriptional or post-transcriptional mechanisms. However, since cytokine expression is often regulated by mRNA stability, it is tempting to speculate that p38 activation by Gadd45 proteins is required for stabilization of cytokine mRNAs in dendritic cells, as has been shown for TNFα mRNA (Lu et al. 2004).

5.4 Gadd45 Proteins in Infection and Autoimmune Disease

5.4.1 Autoimmunity

Gadd45 proteins have been linked to diseases, in which the immune system plays a pivotal role. For instance, Gadd45 proteins have been associated with autoimmunity. As stated in the previous section, Gadd45α-deficient T cells exhibit constitutive p38 MAPK activity (Salvador et al. 2005a), which could indicate an aberrant activation of T cells in these mice. In line with this hypothesis, Gadd45α-deficient mice spontaneously develop an autoimmune disease that is characterized by the presence of autoantibodies against double-stranded and single-stranded DNA, as well as against histones (Salvador et al. 2002). At 9 months of age Gadd45α-deficient mice showed signs of proteinuria and glomerulonephritis. Furthermore, these mice had reduced numbers of leukocytes and lymphocytes in their peripheral blood (Salvador et al. 2002). Interestingly, female rather than male mice were affected, which is similar to systemic lupus erythematosus (SLE) in humans (Tsokos 2011). Of note, this phenotype was reverted when Gadd45α-deficient mice were crossed to mice harboring a Tyr323Phe mutation in both p38α and p38β, the two isoforms of p38 MAPK expressed in T cells (Jirmanova et al. 2011). This strongly supports the notion that the alternative p38 activation pathway in T cells is regulated by Gadd45α and accounts for the development of autoimmune disease in Gadd45α-deficient mice. In addition, p38 Tyr323Phe double knock-in mice were less susceptible towards the induction of collagen-induced arthritis and experimental autoimmune encephalomyelitis (EAE), which are mouse models for rheumatoid arthritis and multiple sclerosis, respectively (Jirmanova et al. 2011). Therefore, the Gadd45α-regulated alternative p38 activation pathway in T cells might contribute to several autoimmune disorders.

Not only Gadd45α, but also Gadd45β and Gadd45γ have been connected to autoimmunity. For instance, Gadd45β-deficient mice showed exacerbated and prolonged clinical symptoms in myelin oligodendrocyte glycoprotein (MOG) peptide-induced EAE (Liu et al. 2005). The differences to wildtype T cells became even more obvious in a transfer EAE model, in which naïve T cells of either wildtype or the knock-out genotype were transferred into immunodeficient recipients. At later time points, Gadd45β-deficient animals showed severe signs of inflammation as shown by IFN-γ expression of CD4+ Th cells and the activation status of microglia cells (Liu et al. 2005). In vitro, Gadd45β-deficient

T cells proliferated more than wildtype cells and were more resistant towards the induction of apoptosis, which may provide a mechanistic basis for the observed autoimmune phenotype (Liu et al. 2005). Further supporting the notion that Gadd45 proteins regulate autoimmunity is the fact that Gadd45β and Gadd45γ double-deficient mice develop a spontaneous lymphoproliferative disease and splenomegaly (Liu et al. 2005). This was also associated with increased immunoglobulin levels in the serum and deposition of immunoglobulins in glomeruli suggesting a lupus-like autoimmune phenotype.

With respect to humans, high Gadd45β expression was detected in T cells from the synovial fluid of rheumatoid arthritis patients, which correlated with reduced T cell apoptosis (Du et al. 2008). In contrast, reduced expression of Gadd45β was found in synovial fibroblasts of rheumatoid arthritis patients (Svensson et al. 2009). Overexpression of Gadd45β in these cells resulted in reduced JNK mitogen-activated protein kinase activation and expression of matrix metalloprotease 3, which plays an important role in joint destruction during rheumatoid arthritis. Of note, Gadd45β-deficient mice exhibited increased JNK activity, expression of matrix metalloprotease 3 and 13 as well as joint inflammation, which resulted in a higher clinical score in this murine model of serum-induced arthritis (Svensson et al. 2009). Lower expression of Gadd45β in rheumatoid arthritis human patients was confirmed in an independent study performed in Taiwan (Li et al. 2019). Furthermore, two single nucleotide polymorphisms (SNPs) were identified in the Taiwanese cohort, GADD45a -589CC (rs581000) and GADD45b -712CT (rs3795024), that correlated with susceptibility to rheumatoid arthritis. Here, the GADD45a -589CC allele and the T allele of GADD45b -712CT were associated with protection from rheumatoid arthritis in HLA-DR4 negative individuals (Li et al. 2019). In contrast, 11 out of 14 patients with systemic lupus erythematosus (SLE) having anti-ribonucleoprotein (RNP) antibodies harbored the GADD45b -712CT genotype (Li et al. 2019). Similarly, the -589GG and -589GC alleles of GADD45a associated with the presence of rheumatoid factor in SLE patients. Therefore, genetics and expression levels of Gadd45 genes are connected to autoimmune diseases.

5.4.2 Infection and Autophagy

Autophagy is an essential catabolic pathway for maintaining protein homeostasis and for energy production within a cell (Mizushima and Komatsu 2011). Importantly, an additional function of macroautophagy is the degradation of intracellular pathogens (Levine et al. 2011) and, consequently, TLRs are able to induce macroautophagy (Delgado et al. 2008; Sanjuan et al. 2007; Xu et al. 2007). Macroautophagy starts with de novo synthesis of a membrane structure called the phagophore that encloses a part of the cytosol. Upon elongation and closure of the phagophore, a double-membrane enclosed vesicle is formed, which is called the autophagosome. Finally, the autophagosome fuses with a lysosome and its content is degraded by hydrolases. Important mediators of macroautophagy are the autophagy-related (ATG) proteins (Mizushima et al. 2011). Central to the elongation phase of autophagosome formation is a protein complex comprised of ATG5, ATG12, and ATG16L1, without which canonical macroautophagy cannot take place (Mizushima et al. 2001). Consequently, ATG5-deficient mice die postnatally since they are not able to survive the starvation period newborns experience between nutrient provision via the umbilical cord and milk feeding (Kuma et al. 2004).

Interestingly, the ATG5-12-16L1 complex is targeted by a Gadd45β-MEKK4-p38 signaling pathway since Gadd45β and MEKK4 direct p38 to the autophagosome (Keil et al. 2013). When localized to the autophagosome, p38 phosphorylates ATG5 at threonine residue 75, an event that prevents fusion of autophagosomes with lysosomes (Keil et al. 2013). Thus, Gadd45β-activated p38 inhibits macroautophagy (Fig. 5.2). Of note, Gadd45β-deficient fibroblasts and macrophages exhibited enhanced macroautophagy upon LPS

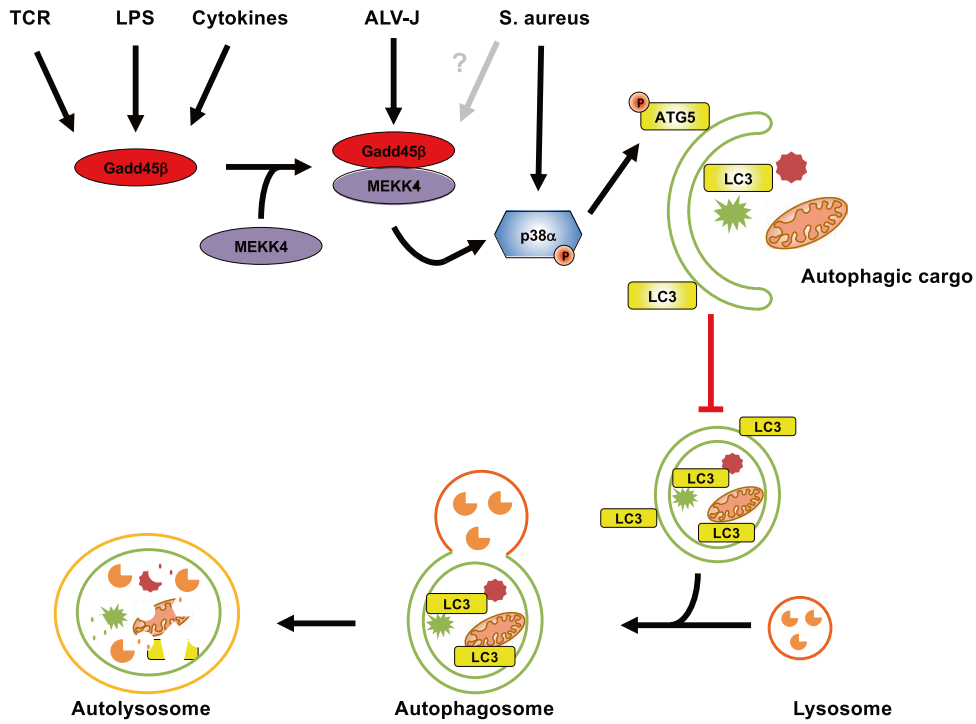

Fig. 5.2 Gadd45β and autophagy. Upon starvation or infection with intracellular pathogens, the cell mounts an autophagic response. The autophagosomal membrane (green) forms de novo and elongates in an LC3-II- (yellow) and ATG5-dependent (yellow) manner to engulf protein aggregates, organelles, or intracellular pathogens. Subsequently, the autophagosome (green) fuses with a lysosome (orange) leading to vesicle acidification and subsequent cargo degradation. Gadd45β (red) and MEKK4 (gray) together direct the p38 mitogen-activated protein kinase (blue) to the autophagosomal membrane, where it phosphorylates ATG5. This event inhibits maturation of the autophagosome and, thus, blocks autophagy. *Gadd45b* expression is induced by TCR triggering, by Toll-like receptors 4 or by certain cytokines. The Gadd45β/MEKK4 pathway is also activated by the ALV-J retrovirus to inhibit autophagy. *S. aureus* inhibits autophagy by activating p38, probably independent of Gadd45β

treatment (Keil et al. 2013). This suggests that LPS as a pathogen-associated molecular pattern (PAMP) activates autophagy via its receptor to fight the infection and that autophagy induction is inhibited by the Gadd45β-induced signaling pathway. In line, it was shown that infection with the Gram-positive bacterium *Staphylococcus aureus* induces autophagy in mouse fibroblasts and human keratinocytes and that *S. aureus* inhibits its degradation by autophagy via induction of p38 (Neumann et al. 2016). However, no connection to Gadd45β was reported for this bacterial infection.

In contrast, Gadd45β was shown to modulate autophagy upon infection with the retrovirus J subgroup avian leukemia virus (ALV-J). Infection with ALV-J inhibited autophagy and this effect was mimicked by overexpression of Gadd45β (Liao et al. 2020). Of note, ALV-J resulted in activation of the Gadd45β-MEKK4-p38 signaling pathway and RNA interference-mediated knockdown of Gadd45β or MEKK4 reverted autophagy inhibition by ALV-J infection (Liao et al. 2020). Surprisingly, Gadd45β expression inhibits replication of ALV-J (Zhang et al. 2016), which might suggest that autophagy promotes virus propagation. However, this might also be explained by different cell types used in the two studies (fibroblasts versus liver cells) since it has been shown for HIV-1, another retrovirus, that depending on the cell type, autophagy can promote or inhibit virus replication (Richetta and Faure 2013; Sir and Ou 2010). Of note, all three Gadd45 proteins have been demonstrated to inhibit HIV-1 replica-

tion. This was mediated by affecting transcription from the HIV-1 long terminal repeat (LTR) and did not involve transcription factors known to bind to viral LTR nor known Gadd45 interacting proteins from the host such as MEKK4, PCNA, XPG, and others (Liang et al. 2016). Thus, the exact mechanisms of action of Gadd45 proteins on HIV-1 replication remain elusive. Nevertheless, RNA interference-mediated knockdown of Gadd45 gene expression resulted in reactivation of latent HIV-1 in a Jurkat cell model (Liang et al. 2016). Therefore, Gadd45 proteins may be involved in the regulation of HIV-1 latency.

Analyzing transcriptomic changes in bone marrow-derived macrophages infected with either a hypo- or a hypervirulent strain of *Mycobacterium tuberculosis*, cell cycle control, and DNA mismatch repair pathways was identified as common responses (Leisching et al. 2017). Importantly, the Gadd45 pathway was specifically activated in response to hypervirulent *M. tuberculosis* infection. Whether Gadd45 proteins induce growth arrest, induce cytokine expression in a MAP kinase-dependent manner, or modulate autophagic activity in response to hypervirulent *M. tuberculosis* infection was not investigated and remains to be elucidated.

5.5 Gadd45 Proteins and Tumor Immunity

Next to the regulation of autoimmunity and infection, Gadd45 proteins seem to promote antitumor responses. In a study that aimed to increase such responses by immunization with inactivated autoreactive T cells that are thought to promote depletion of such autoreactive cells, it was shown that T cells from immunized mice were more resistant to activation-induced cell death and that this correlated with Gadd45β expression (Wang et al. 2006). Importantly, the growth of the tumor T cell line was inhibited in immunized mice compared to non-immunized mice. Supporting the idea of a Gadd45 protein-aided anti-tumor immune response, tumor growth was enhanced in Gadd45β-deficient mice using a mouse B16 melanoma model (Ju et al. 2009). CD8+ T cells from Gadd45β-deficient mice produced less IFN-γ in vivo upon tumor challenge and upon stimulation with IL-12 and IL-18 or via TCR triggering in vitro. Moreover, Gadd45β-deficient CD8+ T cells expressed less T-bet and Eomes upon activation, two transcription factors that are crucial for the development of CD8+ memory T cells (Intlekofer et al. 2005). Most importantly, tumor vaccination failed in mice double deficient for Gadd45β and Gadd45γ (Ju et al. 2009). Taken together, Gadd45 proteins have important functions in tumor immunosurveillance.

Gadd45 proteins also play an important role in the control of hematopoietic malignancies. For instance, loss of either Gadd45α or Gadd45β in myeloid progenitors that were transduced with the BCR-ABL oncogene resulted in accelerated development of chronic myeloid leukemia (CML) (Mukherjee et al. 2017; Sha et al. 2018). Common alterations were enhanced proliferation, reduced apoptosis and hyperactivation of STAT5. While Gadd45α deficiency resulted in constitutive p38 activation, Gadd45β deficient CML cells exhibited higher JNK activity (Mukherjee et al. 2017; Sha et al. 2018). These effects may be related to alternative p38 activation regulated by Gadd45α (Salvador et al. 2005a) and Gadd45β inhibiting JNK via MKK7 binding (Papa et al. 2004), respectively. Next to BCR-ABL, a deregulated RAS pathway can result in myeloid leukemia. KRAS-induced progressive myeloproliferative neoplasm (MPN) transforms into acute myeloid leukemia (AML) upon deletion of the RAS converting enzyme 1 (RCE1) that is important for proper localization of RAS. Transcriptomic analysis of RCE1-deficient $KRAS^{G12D}$-mutant cells revealed a string downregulation in Gadd45β expression (Karlsson et al. 2021). Re-expression of Gadd45β via a retroviral vector resulted in longer survival, delayed anemia, and reduced spleen and liver weights of mice receiving RCE1-deficient $KRAS^{G12D}$ cells.

Although the results described above are based on mouse models and their relevance for tumor formation in humans remains to be elucidated, Gadd45α was shown to serve as a biomarker in peripheral blood cells after radiotherapy of cancer patients (Balazs et al. 2019; Tichy et al. 2018). Furthermore, Gadd45β is highly expressed

in plasma cells from patients with multiple myeloma and Gadd45β expression levels correlate with patient survival (Tornatore et al. 2014). Since it was shown that Gadd45β mediated NF-κB-dependent survival of multiple myeloma cells by inhibiting JNK activity, a peptide-based inhibitor was developed that interferes with Gadd45β binding to MKK7, the MAP2K that activates JNK. This peptide was called DTP3 since It is a tetra-peptide composed of D-amino acids (Tornatore et al. 2014). DTP3 interacts with two amino acid stretches on MKK7 preventing Gadd45β binding (Rega et al. 2018) and exhibits favorable pharmacokinetics and pharmacodynamics with no detectable off-target toxicity (Tornatore et al. 2019). DTP3 showed potent anti-tumor activity against multiple myeloma cells in vitro and in xenograft models (Tornatore et al. 2014). In a similar vein, a Gadd45α-derived cell-permeable peptide was developed to treat cancer. In pancreatic ductal adenocarcinoma (PDAC) it was found that tumor infiltrating T cells expressed pro-inflammatory cytokines such as TNFα and IL-17A and this correlated with a strong signal for tyrosine Y323 phosphorylated p38 MAP kinase (Alam et al. 2015). Y323 phosphorylation marks the alternative p38 activation pathway that is operative in T cells (described above; Fig. 5.1). Accordingly, PDAC growth was inhibited in mice with a targeted mutation that does not allow alternative p38 activation (Alam et al. 2015). Subsequently, a cell-permeable peptide containing amino acids 71–85 of Gadd45α was shown to bind p38 and inhibit T cell proliferation and cytokine expression. Importantly, the Gadd45α peptide inhibited tumor growth in vivo and enhanced survival of tumor bearing mice (Alam et al. 2015). In conclusion, Gadd45 protein-derived peptide-based therapeutics are promising candidates for novel treatment options for human cancer.

5.6 Outlook

In the past couple of years, we have seen tremendous progress in the field of tumor immunology and related therapies. In this respect, the therapeutic peptides described above that are derived from Gadd45 proteins might develop into important additions to the arsenal of immune checkpoint inhibitors and CAR T cells. Furthermore, novel techniques like CRISPR/Cas-mediated gene editing may allow a comprehensive look at GADD45 proteins in different cellular models due to combined deletion and/or mutation of the respective genes. Gene editing tools may also allow to address questions in cell types and organisms that could not be analyzed before. In this regard, immunology has witnessed the identification of novel cell type, e.g., innate lymphoid cells or tissue resident immune cells, in which the function of Gadd45 proteins is unknown, yet. Here, novel mouse models such as the recently described Gadd45γ-mCherry bacterial artificial chromosome (BAC) reporter mouse might be valuable tools to explore novel function of Gadd45 proteins in the immune system (Warr et al. 2018).

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Gadd45 in the Liver: Signal Transduction and Transcriptional Mechanisms

6

Jianmin Tian and Joseph Locker

Abstract

Injury and growth stimulation both remarkably increase the hepatic expression of Gadd45β. This contrasts with expression in liver cancer, where promoter methylation frequently silences Gadd45β, due to a suppressive function that is often proapoptotic. In normal hepatocytes, Gadd45β facilitates cell survival, growth, and proliferation. Gadd45β binds MKK7—downstream of TNFα and its receptors—to prevent this kinase from activating JNK2. Hence, the *Gadd45β−/−* genotype increases cell injury and decreases cell proliferation during liver regeneration (compensatory growth and proliferation). Liver hyperplasia (de novo growth and proliferation) is an alternate form of growth, caused by drugs that activate the nuclear receptor, CAR. As in regeneration, the *Gadd45β−/−* genotype considerably slows growth during hyperplasia. However, there is no injury and the slowing occurs because Gadd45β normally binds to CAR and activates its transcriptional stimulation. Thus, Gadd45β protects the liver through two entirely different processes: Binding MKK7 to block damaging signal transduction, or binding CAR to coactivate anabolic transcription.

Keywords

CAR/*Nr1i3* · PPARα · NFκB · STAT3

Abbreviations

AHR	Aryl hydrocarbon receptor
APAP	Acetaminophen
CAR	Constitutive androstane receptor; *Nr1i3*
FOXO1, FOXO3	Forkhead box proteins O1 and O3
Gadd45	Growth arrest and DNA damage inducible 45 proteins
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
IL1	Interleukin 1
IL6	Interleukin 6
JNK	c-Jun N-terminal kinase
MKK4/JNKK	MAPK kinase 4/JNK kinase 1
MKK7/JNKK2	MAPK kinase 7/JNK kinase 2
NAQPI	*N*-acetyl-*ρ*-benzoquinone imine
NFκB:	Nuclear factor kappa B

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p65	p65 subunit of NFκB; *Rela*
PCN	Pregnenolone-16α-carbonitrile
PH	Partial hepatectomy
PXR	Pregnane X receptor; *Nr1i2*
SHP	Small heterodimeric partner; *Nr0b2*
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD); dioxin
TCPOBOP	1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene
TNFR1, TNFR2	Tumor necrosis factor α receptor 1 and 2
TNFα	Tumor necrosis factor α

6.1 Introduction

The growth arrest and DNA damage 45 (Gadd45) family consists of three homologous acidic proteins (α, β, and γ). They are cellular responders to physiological and environmental stress (Liebermann and Hoffman 2008), and despite their small size (17–18 kD), each protein has a surprising number of functions and binding partners. In adapting to different kinds of stress, the liver stimulates expression of individual Gadd45 protein in various responses to injury, inflammation, chemicals, and drugs. The diverse processes of the liver include xenobiotic detoxification, bile metabolism, adipogenesis, carbohydrate metabolism, and serum protein synthesis. Each process must modulate in response to metabolic resources and specific stimuli, persist during liver injury and inflammation, and coordinate with cell growth and proliferation.

Because they are so similar, the distinct biological effects of each Gadd45 protein probably reflect the context of specific inducing signals, or the specific cell type that expresses the protein. Indeed, the liver consists of hepatocytes, Kupffer cells (fixed macrophages), stellate cells, biliary epithelium, endothelial cells, and a variable number of inflammatory cells. It is often unclear which of these cells express a particular Gadd45 protein and this review will highlight the hepatocyte because it is the dominant liver cell. Gadd45 proteins are also associated with hepatocytic neoplasia, both carcinogenesis and established hepatocellular carcinoma (HCC). Nevertheless, the most striking changes occur in normal hepatocytes. These cells induce an exceptional increase Gadd45β in response to either xenobiotic compounds or cell loss, with critical effects on cell survival and liver growth (Su et al. 2002; Locker et al. 2003; Papa et al. 2008; Tian et al. 2011). The survival effects act through MKK4 and MKK7, which activate the p38 and JNK pathways in response to stress and cytokines like TNFα (Papa et al. 2009). The growth effects act through a different process, transcriptional coactivation, an important activity of Gadd45 proteins. Because these transcriptional functions have received limited attention in liver or any other tissue, the last section of this review describes these mechanisms in detail.

6.2 Gadd45α and γ

Normal liver expresses moderate levels of Gadd45α, with four- to sevenfold induction by ischemia, partial hepatectomy, chemical induction of hyperplasia with TCPOBOP (1,4-bis[2-(3,5)-dichoropyridyloxy] benzene), or treatment with 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), a "nongenotoxic" carcinogen (Fallsehr et al. 2005; Su et al. 2002; Locker et al. 2003; Fletcher et al. 2005). In contrast, hepatic Gadd45α is induced >20-fold by dimethylbenzanthracene (DMBA). The liver activates this carcinogen to produce DNA adducts that require nucleotide excision repair, and DMBA produces 3 times more mutations in the *Gadd45α*-null mouse (Hollander et al. 2001). Thus, as in other tissues, Gadd45α responds to DNA damage and facilitates DNA repair.

Transcriptional regulation of *Gadd45α* links to DNA damage through factors expressed in liver and most other tissues, including p53 (Kastan and Bartek 2004; Zhan et al. 1998), BRCA1 (Jin et al. 2000; Campanero et al. 2008), nuclear receptor TR4/NR2C2 (Yan et al. 2012), and Myc (Amente et al. 2011). Gadd45α is also regulated by the stress-responsive ATF/CREB family of transcription factors (Maekawa et al. 2008). Among these, CREBH and ATF5 are

abundant latent transcription factors in the liver. Unfolded proteins and ER stress activate CREBH (Luebke-Wheeler et al. 2008), and fasting, ER stress, and oxidative stress activate ATF5 (Shimizu et al. 2009; Zhou et al. 2008).

FOXO3, a transcriptional mediator of oxidative stress, also regulates Gadd45α via a pathway characterized in HCC cells. Binding of APRIL, a TNF superfamily ligand, to its receptor, BCMA, leads to JNK2 activation, which then phosphorylates and activates FOXO3 (Amente et al. 2011; Notas et al. 2012). These findings also suggest feedback inhibition among Gadd45 proteins. Their prior expression might block activation of FOXO3, since they are strong inhibitors of JNK2 (Papa et al. 2008); see below).

Gadd45α is also an important regulator of liver stellate cells, where it opposes TGFβ/Smad signaling (Hong et al. 2016). Significant downregulation of Gadd45α, but not Gadd45β or Gadd45γ, was observed in fibrotic liver tissues of mice following chronic treatment with CCl_4. Forced expression of Gadd45α inhibited activation of isolated stellate cells and expression of fibrotic genes while Gadd45α inhibition stimulated TGFβ/Smad signaling.

Gadd45γ has received little attention in the liver although expression profile studies have confirmed low level constitutive expression with modulation in response to growth stimulators or injury (Su et al. 2002; Jee et al. 2007). However, the main transcriptional regulators attributed to Gadd45γ, Oct1, and NF-Y (Campanero et al. 2008) have little relationship to phenotypic expression in the liver. The most notable liver effect was a 22-fold increase in mice observed 180 days after infection with a helminthic parasite, but in this case, expression was localized to infiltrating lymphocytes (Zhang et al. 2012).

6.3 Gadd45β in Liver

Two types of hepatocyte proliferative responses induce striking early and persistent expression of mouse Gadd45β: regeneration, compensatory proliferation after the loss of hepatocytes; and hyperplasia, de novo proliferation caused by drugs and xenobiotic activators of the Constitutive Androstane Receptor (CAR; *Nr1i3*). The induction is exceptionally high—100-fold following CAR stimulation by the hydrocarbon TCPOBOP and 70-fold after partial hepatectomy (PH)—making Gadd45β one of the most strongly induced genes in either process (Su et al. 2002; Locker et al. 2003; Tian et al. 2011, 2018). TCPOBOP treatment also moderately stimulates Gadd45α (~4-fold at 24 h), and strongly inhibits Gadd45γ (0.05-fold at 24 h). Phenobarbital, a non-ligand that activates CAR in all species, also strongly stimulates Gadd45β (Hori et al. 2016).

In addition to CAR and its dimeric partner, RXR, transcription of *Gadd45β* is stimulated by PPARα, STAT3, and NFκB p65, through extensive regulatory regions recently defined by ChIP-seq (Fig. 6.1). Other studies have demonstrated regulation by TGFβ-SMAD and PXR in human hepatocytes and cancer cells.

Besides CAR, Gadd45β is also regulated by two xenobiotic receptors that have limited effects on hepatocyte proliferation. First, the aryl hydrocarbon receptor (AHR), a bHLH/PAS family transcription factor, is activated by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Second, the Pregnane X Receptor (PXR; *Nr1i2*) is a nuclear receptor activated by rifampicin in humans and pregnenolone-16α-carbonitrile (PCN) in mice. TCDD stimulates Gadd45β expression and induces AHR binding to a region 1.3 kb from the promoter (Lu et al. 2019). PXR stimulation is species specific. Human PXR, activated by rifampicin, stimulates expression of Gadd45β, associated with induced binding of PXR near the promoter (Kodama and Negishi 2011). However, rodent responses and target genes are significantly divergent. In mice, PXR activated by PCN stimulates expression of many genes also stimulated by CAR, but not Gadd45β (Cui and Klaassen 2016). Nor does PXR induce binding near the Gadd45 promoter (Cui et al. 2010). Indeed, CAR stimulation in humans is also questionable, since phenobarbital does not increase Gadd45β observed in mice with livers repopulated by human hepatocytes (Yamada et al. 2014).

An additional nuclear receptor, the peroxisome proliferator-activated receptor alpha

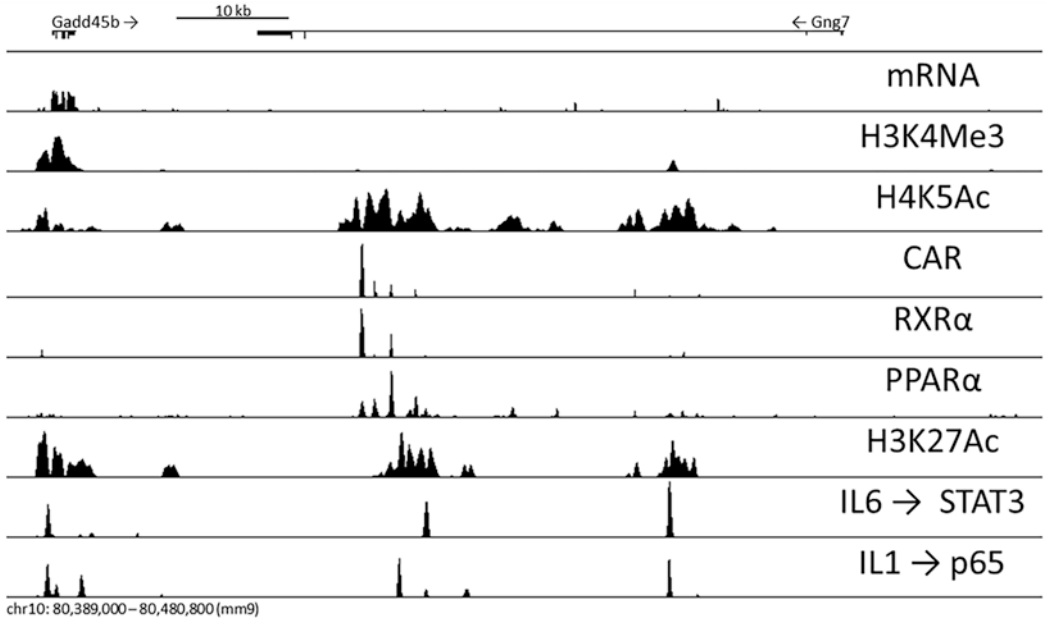

Fig. 6.1 *Gadd45β* transcription controls in mouse liver. The 2.1 kb *Gadd45β* gene has proximal regulatory regions at the 5′- and 3′-end and an extended downstream region of distant enhancers marked by acetylated histones H4K5Ac and H3K27Ac, extending from +26 to +58 kb within the non-expressed *Gng7* gene. Enhancers are marked by transcription factor binding sites surrounded by regions of acetylation. The main enhancers are CAR-RXR, +27.9 kb; PPARα, +30.6 kb; STAT3, −0.4, +33.8, and +55.7 kb; and NFκB p65, −0.5, +2.7, +31.6, and +55.6 kb. The mRNA, CAR, RXR, and H4K5Ac tracks were compiled from datasets of liver treated with TCPOBOP for 3 h (Tian et al. 2018); the PPARα track was compiled from a dataset of untreated liver (Boergesen et al. 2012); and the STAT3, p65, and H3K27Ac tracks were compiled from datasets of cultured hepatocytes treated with IL1 or IL6 for 3 h (Goldstein et al. 2017)

(PPARα) also induces Gadd45β in mice, following treatment with the strong activator, Wy-14,643 (Kim et al. 2014). This treatment also induces degradation of STAT3, and additional experiments showed that PPARα stimulation of *Gadd45b* was stronger in mice with hepatocyte-specific knockout of STAT3. Nuclear receptors activate transcription by common mechanisms, so the data suggest antagonism between STAT3 and nuclear receptor stimulation. Even if their mechanisms are antagonistic, however, PPARα and STAT3 both stimulate transcription of *Gadd45b* in appropriate regulatory contexts.

Regeneration and hyperplasia stimulate *Gadd45b* through separate transcriptional mechanisms. PH, or liver damage from toxic agents like CCl_4, activates two known signaling pathways that stimulate *Gadd45b* transcription, TNFα-NFκB, and TGFβ-SMAD. PH leads to rapid activation of NFκB (Ohmura et al. 1996), which specifically binds to upstream sites near the Gadd45β promoter and strongly activates transcription (Jin et al. 2002). PH-mediated induction of Gadd45β is impaired in the *Tnfr1*−/− mouse, which confirms this relationship (Papa et al. 2008). NFκB also accounts for the induction of *Gadd45b* by *S*-adenosylmethionine (Seewoo et al. 2012). A recent study analyzed IL1-NFκB and IL6-STAT3 responses in cultured hepatocytes (Goldstein et al. 2017). Data from this paper showed that each induces *Gadd45b*, and their effects are additive.

TGFβ, another inducer of *Gadd45b* transcription (Yoo et al. 2003), is an important mediator of early liver regeneration that is released into the local circulation within 1 h after PH (Michalopoulos 2007). TGFβ activates Smad3, which stimulates *Gadd45b* transcription through a downstream enhancer (Major and Jones 2004). Two other agents induce hepatic transcription of

Gadd45b, but through undefined transcriptional regulators and binding sites. The multikinase inhibitor, sorafenib strongly induces *Gadd45b* in sensitive but not resistant to HCC cell lines, acting through a 72-bp upstream regulatory region (Ou et al. 2010). Oxaliplatin, a DNA-damaging drug, and insulin also induce *Gadd45b* in HCC cell lines by undefined mechanisms (Seewoo et al. 2012; Bortoff et al. 2010).

Induction of *Gadd45b* by CAR is independent of the TNFα and TGFβ pathways, since TCPOBOP treatment activates neither. The full induction by TCPOBOP in *Tnfr1−/−* and *Tnfr1−/− Tnfr2−/−* knockout mice confirms this independence. In contrast, the *CAR−/−* genotype prevents induction by TCPOBOP (Columbano et al. 2005).

CAR-mediated induction of *Gadd45b* is independent of signaling by tyrosine kinase growth factor receptors. Two recent studies have combined an EGFR inhibitor with hepatocyte-specific knockout of MET, to ablate the two receptors that regulate the hepatocyte in liver regeneration. This combination completely blocks liver regeneration after PH, but allows significant but attenuated growth stimulation following treatment with TCPOBOP (Paranjpe et al. 2016; Bhushan et al. 2019). The treatment still strongly induces Gadd45β, about 60% of the wild type response at 1 day, later exceeding the full wild type response. The response is a little slower—not surprisingly considering the great alterations caused by elimination of both receptors—but CAR stimulation of *Gadd45b* transcription remains intact.

Liver *Gadd45b* is induced by fasting, a change that links to both lipid metabolism and diabetes (Fuhrmeister et al. 2016). Fasting alters lipid metabolism, and Gadd45β has a direct effect on this response since lipid uptake was significantly higher in *Gadd45b−/−* mice. The increased uptake correlated with direct binding of Gadd45β to FABP1, which relocates from membrane to cytoplasm. Reduced Gadd45β, increased lipid uptake, and membrane localization of FABP1 were also observed in obese/diabetic *db/db* mice. These changes were reversed in both models by overexpression following transduction of a Gadd45β-AAV construct.

Relationships of CAR and Gadd45β to lipid metabolism and diabetes are substantiated by studies of mice fed a high-fat diet, where CAR activation was anti-diabetic, reducing fat and improving insulin sensitivity (Gao et al. 2009). A new extension of these findings showed that absence of *Gadd45β* almost completely blocked the metabolic benefits of CAR in this model (Cai et al. 2021).

6.4 Gadd45 Proteins in HCC

The *Gadd45b* promoter is hypermethylated in many HCC (Qiu et al. 2003, 2004; Higgs et al. 2010; Hou et al. 2017), a change that correlates with low or absent expression. Treatment with 5-azacytidine induces re-expression of Gadd45β and growth inhibition, suggesting a suppressive impact on cancer cells, the opposite of its growth effects in non-neoplastic liver. However, the mechanism of tumor suppression is unresolved. In contrast, studies of Gadd45γ in HCC do not indicate suppressive effects. One paper reported that increased Gadd45γ expression was part of a profile that correlates with the most aggressive HCC in rats and humans (Frau et al. 2012). In another study, increased Gadd45γ mRNA was part of a liver profile that discriminated the responses to genotoxic and nongenotoxic carcinogens (Suenaga et al. 2013). The association of Gadd45γ expression with the response to genotoxic carcinogens suggests DNA repair functions similar to those reported for Gadd45α (Hollander et al. 2001).

6.5 Contradictory Effects on Hepatocyte Proliferation

The *Gadd45b−/−* mouse had significantly reduced proliferation during liver regeneration, showing that the protein is essential for the full adaptive response to loss of liver mass (Papa et al. 2008). In contrast, the *Gadd45b−/−* genotype caused an increase in proliferation following treatment with TCPOBOP along with doubling of Cyclin D1 expression (Tian et al. 2011).

Proliferation in these two models has numerous differences, so it remains possible that Gadd45β functions in a pathway that activates compensatory proliferation. The increased hyperplasia in the knockout, however, indicates that Gadd45β does not have an intrinsic role in direct cell cycle control or DNA replication. The effects of Gadd45β on hepatocyte proliferation therefore depend on the specific inducing process and the context of concurrent changes. Indeed, loss of Gadd45β has different effects on proliferation induced by TCPOBOP and phenobarbital, both CAR activators. The Gadd45β−/− genotype increased TCPOBOP-induced proliferation (Tian et al. 2011), but reduced phenobarbital-induced proliferation (Hori et al. 2018). The difference between the two effects is observed because phenobarbital activates CAR indirectly through p38 MAPK signaling, which is significantly reduced without Gadd45β. Thus, Gadd45β affects phenobarbital but not TCPOBOP-mediated activation and nuclear translocation of CAR. Transcriptional coactivation by Gadd45β is a later step that is independent of the mechanism of CAR activation.

6.6 Gadd45β Mutation Impairs Liver Regeneration

Following PH, the absence of Gadd45β causes liver injury with significant mortality due to the unopposed activation of JNK (Papa et al. 2008). Notably, the knockout mouse is unable to compensate for Gadd45β deficiency via increased synthesis of Gadd45α or Gadd45γ. This confirms their separate transcriptional regulation and demonstrates that the PH effects are entirely dependent on Gadd45β deficiency.

By binding and inhibiting the Jun kinase kinase MKK7/JNKK2, Gadd45β prevents activation of JNK and thus ameliorates the potential damage mediated by TNFα signaling (De Smaele et al. 2001; Papa et al. 2004). This is a critical pathway because TNFα initiates liver regeneration and the *Tnfr1*−/− mouse fails to regenerate its liver following PH (Yamada et al. 1998). The *Gadd45β*−/− genotype does not completely abolish regeneration, but 56% of these mice die after PH because of severe cell injury and inflammation (Papa et al. 2008). Compensatory proliferation is also lower than normal. PH in wild type mice causes rapid phosphorylation of JNK2 and MKK7 with significant reduction by 8 h, while the *Gadd45β*−/− mouse has much greater JNK2 and MKK7 phosphorylation with persistent high levels through 72 h. The effect is JNK specific because two other MAPK pathways—ERK and p38—showed no differences between wild type and *Gadd45β*−/−. A further experiment confirmed this relationship. JNK2 knockout introduced into the *Gadd45β*−/− background fully restored liver regeneration (Papa et al. 2008). The experiments also confirmed an antiproliferative effect of JNK2 in isolated hepatocytes (Sabapathy et al. 2004). The effects also contrast the functions of JNK1 and JNK2. JNK1 activates proliferation via phosphorylation of Jun. JNK2 instead reduces cellular levels of Jun and reduces its activation of cell proliferation (Sabapathy and Wagner 2004). The critical function of Gadd45β liver regeneration is therefore to moderate the damaging effects of TNFα signaling, because dampening the activation of JNK2 shifts the balance towards protective growth-stimulatory responses.

6.7 Gadd45β Impairs Rapid Growth During Hyperplasia

Because inflammatory mediators initiate and guide liver regeneration, similar effects seemed likely for hyperplasia. Indeed, several papers have shown that CAR activation—and CAR-induced Gadd45β—can protect liver cells from apoptosis (Baskin-Bey et al. 2006, 2007; Yamamoto et al. 2010). These studies combined a CAR inducer with liver damage via Fas ligand, TNFα, or a methionine-choline deficient diet. Pure hyperplasia, however, is the response of an intact liver without inflammation, cell injury, or activation of TNFα signaling (Tian et al. 2011; Ledda-Columbano et al. 2000; Columbano and Shinozuka 1996). Nevertheless, CAR activation produces extremely rapid liver growth (Tian et al. 2011).

Following treatment with TCPOBOP, liver mass increases 30% in the first 3 h and doubles by 18 h. Growth pauses at the beginning of S phase (24 h) and then resumes at 40 h after cell division. Hyperplasia is part of an adaptive response to xenobiotic and toxic exposure and growth results from synthesis of drug-metabolizing enzymes, transport molecules, and membrane scaffolds for these proteins. Cell division presumably makes hepatocyte detoxification even more efficient by increasing hepatocyte surface area. In the *Gadd45β−/−* mouse, proliferation increased but liver growth slowed. This slowing reflected blunting of mRNA synthesis induced by TCPOBOP. By 48 h, however, growth and induced gene expression of the mutant liver were equivalent to wild type. Thus Gadd45β critically accelerates the program of induced transcription to enable rapid adaptation.

6.8 Gadd45β Is a Transcriptional Coactivator

The effect on early mRNA synthesis suggested a transcriptional mechanism, but such effects could be the indirect consequence of a signal transduction pathway. However, an earlier study showed that Gadd45 proteins can act as direct transcriptional coactivators of nuclear receptors (Yi et al. 2000). These authors noted the characteristic coactivator motifs, LXXLL, in all three Gadd45 proteins, and then demonstrated critical properties of coactivators. (1) Each Gadd45 protein bound directly to nuclear receptor RXRα in yeast two-hybrid assays. (2) GST-fusions of each Gadd45 protein, synthesized in vitro, bound directly to nuclear receptors RXRα, RARα, ERα, PPARα, PPARβ, and PPARγ in pull-down assays. (3) Gadd45α and γ coactivated nuclear receptors RXRα, PPARα, and PPARγ in assays of transfected reporter plasmids. Similarly, a more recent study characterized direct binding of Gadd45β to CAR (Yamamoto et al. 2010).

Essential coactivator functions are intrinsic to different domains of Gadd45β (Fig. 6.2) (Tian et al. 2011). First, the Gadd45β coactivation of CAR is strong, comparable to coactivation by the p160 coactivator, Src1/Ncoa1. Second, Gadd45β contains an intrinsic domain that has direct activation function when bound to a reporter gene via fusion to a heterologous DNA-binding domain. Activation localized to the N-terminal region from aa 125–160. Third, Gadd45β bound directly to CAR, demonstrated with cell-free translated protein and with native protein synthesized in 293T cells. The latter analysis localized the CAR-binding domain to a central region from aa 69–92. Similarly, an internal deletion without aa 41–92 was not coprecipitated by CAR (Hori et al. 2018). Fourth, the two LXXLL domains—in a region between the binding and activation domains—are essential for coactivation, because mutation of either converts Gadd45β from a coactivator to a dominant negative inhibitor of CAR. Fifth, chromatin immunoprecipitation assays of TCPOBOP-treated liver show that Gadd45β and CAR bind together at a characteristic response element upstream of a major target gene, *Cyp2b10*.

In addition to CAR, Gadd45β also interacts with AHR and coactivates AHR target genes. Like CAR, AHR binds Src1 and other p160 coactivators, and also binds a large C-terminal region of Gadd45β although the strongest binding coincides with the activation domain (Tian et al. 2011; Lu et al. 2019).

Gadd45β also regulates gluconeogenesis—closely linked to diabetes— by coactivation of FOXO1, an activating transcription factor that binds the Insulin Response Element (IRE) to stimulate genes like PCK1 and G6PC. Several observations related Gadd45β to FOXO1. First, gluconeogenesis was reduced in *Gadd45b−/−* mice. Second, Gadd45β bound and stabilized FOXO1. Finally, Gadd45β specifically coactivated FOXO1 on an IRE-reporter plasmid in cultured hepatocytes (Kim et al. 2021).

Gadd45β has strong coactivator function comparable to 160-kdA Src1/Ncoa1 and there is significant homology between their α-helical LXXLL-containing segments (Fig. 6.2). Mutation of these motifs in Src1 blocks transcriptional activation (Heery et al. 1997). Similar mutations convert Gadd45β to a dominant negative inhibitor although they do not block binding by a more

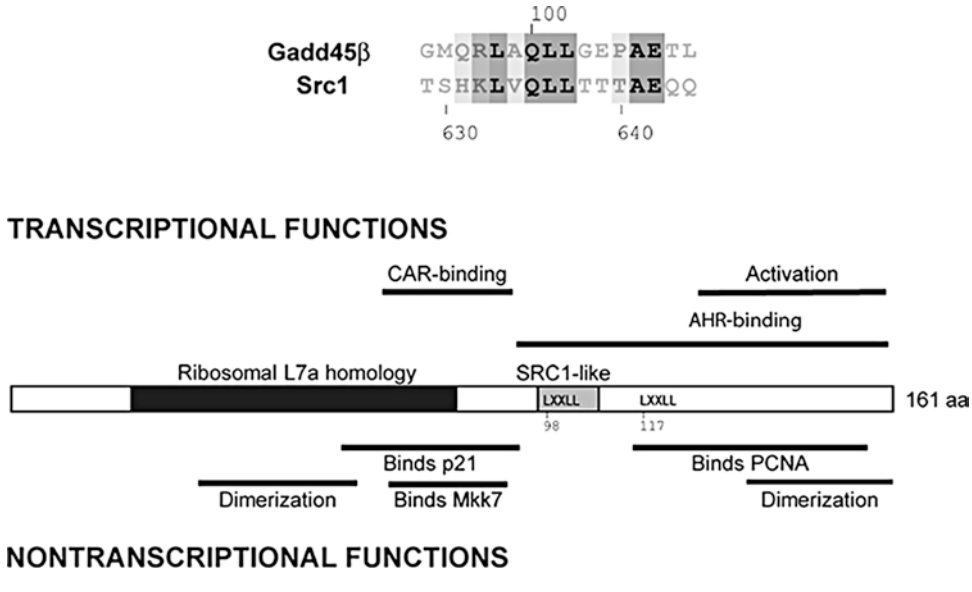

Fig. 6.2 Gadd45β domain structure. **Above, alignment of LXXLL regions of Gadd45β and Src1/*Ncoa1***. There is strong sequence homology between the LXXLL motifs of Gadd45β at aa 88, and Src1 at aa 633, and each is within a short amphipathic α-helical segment, an essential property for binding and activation of nuclear receptors (Tornatore et al. 2008; Torchia et al. 1997). **Below, mapped domains of Gadd45β**. Gadd45 proteins contain a domain homologous to several RNA binding proteins, including ribosomal protein L7a. RNA binding has been demonstrated in vivo and in vitro for Gadd45α although the binding function has not been mapped (Sytnikova et al. 2011). Mapped transcriptional functions include binding to CAR, independent transcriptional activation, and mutation of either LXXLL motif converts Gadd45β to a dominant negative inhibitor of CAR (Tian et al. 2011). Binding of AHR has also been mapped to the C-terminal region (Lu et al. 2019). Study of peptide functions has also localized domains that bind Mkk7 (Papa et al. 2007), p21 (Zhao et al. 2000), and PCNA (Vairapandi et al. 2000). Gadd45β dimerizes by interaction of proximal and C-terminal domains. There is significant overlap of function. CAR, p21, and Mkk7 all bind to the same region, which includes the distal part of the L7a homology domain. PCNA binding, AHR binding, transcriptional activation, and one of dimerization domains overlap at the C-terminal. The proximal dimerization domain overlaps with the proximal part of the L7a homology domain

proximal domain. LXXLL mutations have a similar effect on another coactivator, Nrbf2 (Nuclear receptor binding factor 2) (Yasumo et al. 2000; Flores et al. 2004). When bound, an LXXLL domain of Src1 simultaneously aligns with Helix 3 and Helix 12 of the nuclear receptor ligand-binding domain (Shiau et al. 1998; Pike 2006). Ketoconazole—an agent that binds this region of CAR and blocks binding of Src1—also blocks Gadd45β binding (Huang et al. 2007; Tian et al. 2011). The mutant forms of Gadd45β presumably bind to the same region but cannot align correctly with Helices 3 and 12. Thus, mutated Gadd45β not only fails as a coactivator, it acts as a dominant negative by blocking access of other coactivators.

Gadd45β also has an important role in toxic liver injury by acetaminophen (APAP). In this injury, APAP activates CAR, which induces Gadd45β and p450 genes. The latter metabolize APAP to *N*-acetyl-*ρ*-benzoquinone imine (NAQPI), a toxic product. NAQPI then activates JNK1/2 to cause centrilobular necrosis. In this injury model, investigation of SHP—a repressing nuclear receptor without a DNA-binding domain—showed a unique relationship to Gadd45β (Kim et al. 2018). When SHP−/− mice were treated with APAP, the centrilobular injury was greatly reduced. However, when SHP−/− and Gadd45β−/− genotypes were combined, the full injury was restored. Thus, SHP has an important role in generating the injury. The high level of Gadd45β induced by CAR, however, is suffi-

cient to block the injury in the absence of SHP. SHP lacks a DNA-binding domain but does have a coactivator-binding domain (Lee et al. 2000). SHP may therefore bind Gadd45β and prevent its inhibition of JNK activation.

6.9 Conclusions

The three Gadd45 proteins are so similar that they are likely to share almost all functions. Their different biological roles in liver reflect many inducing processes, but Gadd45β is most prominent. In various liver models, Gadd45β has contradictory roles. Its positive effects promote proliferation, growth, and cell survival. Its negative effects inhibit proliferation and stimulate apoptosis. The positive effects dominate in hepatocytes and the negative functions are most apparent in HCC. Each role clearly depends on context and interacting partners.

The studies of Gadd45 proteins in hepatocytes exemplify the diverse mechanisms attributed to these proteins, ranging from signal transduction to transcriptional coactivation. Surprisingly, the domains that mediate transcriptional functions also mediate binding to MKK7, p21, and PCNA (Fig. 6.2). This functional dichotomy seems to reflect two biological circumstances in liver. During injury, especially with inflammation, the signal transduction mechanisms are dominant. In the absence of injury, the transcriptional mechanisms dominate. The striking high-level induction of Gadd45β suggests simultaneous participation in multiple processes. Alternatively, it is possible that some effects attributed to direct interaction in signal transduction pathways are actually mediated by transcription, a mechanism in which upregulation of transcriptional targets stimulates or inhibits responses. Despite the functional dichotomy, Gadd45β responses in hepatocytes do have a unifying feature. The robust early synthesis reflects the importance of Gadd45β for rapid adaptations.

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Gadd45 in Preeclampsia

7

Ossie Geifman-Holtzman, Yali Xiong, and Eliezer J. Holtzman

Abstract

Preeclampsia is a pregnancy-induced complex of multiple pathological changes. Numerous stresses during pregnancy, including hypoxia, immune activation, inflammatory cytokines, and oxidative stress were reported as contributing factors to the preeclamptic pathology. Seeking common sensors of various stressors in preeclampsia is of new interest and can potentially benefit in disease prevention and treatment. Recent studies have highlighted the role of the Gadd45a protein as a stress sensor in preeclampsia. In response to various pathophysiological stressors, notably hypoxia, oxidative stress, inflammatory cytokines, and AT1-AAs, Gadd45a activates Mkk3-p38 and or JNK signaling. This, in turn, results in immunological and inflammatory changes as well as triggering the production of circulating factors such as sFlt-1, which are believed to account for many of the pathophysiological-related symptoms of preeclampsia. Activation of inflammatory/immune responses in preeclampsia may function in a feedback loop to maintain elevated expression of Gadd45a protein.

Keywords

Gadd45a · Preeclampsia · Circulating factors · sFlt-1 · MAPK pathway · GADD45 · Stress · Hypoxia · Inflammatory cytokines · TNF-alpha · Interleukins

7.1 Stress and Preeclampsia

Preeclampsia, which affects approximately 5–8% of all pregnancies, is one of the leading causes of maternal and fetal morbidity and mortality (Turner 2010; MacKay et al. 2001). It is a pregnancy-induced complex of multiple pathological changes, which are manifested as elevated blood pressure, proteinuria, and edema in the mid-late term of gestation (ACOG Committee on Practice Bulletins—Obstetrics 2002). Multiple stresses were found contributing to the pre-

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eclamptic condition (Hubel 1999; Benyo et al. 2001; Teran et al. 2001).

7.1.1 Hypoxia

Hypoxia (i.e., placental ischemia) is essential in the pathogenesis of preeclampsia and is caused through a variety of mechanisms involved with abnormal placentation. Inadequate trophoblast invasion that results in deficient remodeling of the uterine spiral arteries is regarded as a primary cause of placental ischemia (Conrad and Benyo 1997). Poor placentation impairs the development of the early placenta and the maternal blood supply (Redman and Sargent 2005). This process starts from the sixth week of gestation and is prolonged to the latter two trimesters, eventually resulting in typical clinical presentations of preeclampsia, including intrauterine growth retardation (IUGR) (Redman and Sargent 2005).

Hypoperfusion can be both a cause and a consequence of abnormal placental development. A causal connection between poor placental perfusion, abnormal placental development, and preeclampsia is supported by the following evidences: medical conditions associated with vascular insufficiency (e.g., hypertension, diabetes, systemic lupus erythematosus, renal disease, acquired and inherited thrombophilias) increase the risk of abnormal placentation and preeclampsia (ACOG Committee on Practice Bulletins—Obstetrics 2002; Dekker 1999; Tal 2012). Reducing utero-placental blood flow in pregnant rat model reproduced characteristic preeclamptic manifestations (Li et al. 2012; Makris et al. 2007). Recent updates, on the other hand, showed 2.5-fold upregulation of hypoxia-inducible factor-1α (HIF-1α) protein (a surrogate of hypoxia) in placentas from preeclampsia when compared to non-hypertensive controls (Korkes et al. 2017).

One remarkable consequence of hypoxia is the endothelial cell dysfunction, which subsequently increases circulating factors such as fms-like tyrosine kinase receptor-1 (sFlt-1) and soluble endoglin (sENG) from the placenta, and triggers preeclamptic pathology (Maynard et al. 2003). Both sFlt-1 and sENG were found elevated in the serum of preeclamptic patients as well as in their placentas. sFlt-1 is a splicing variant of the VEGF receptor, and acts as a VEGF antagonist due to the absence of transmembrane and cytoplasmic domains, resulting in vessel constriction and high blood pressure (Maynard et al. 2005). Injecting sFlt-1 into pregnant rats generated systemic preeclamptic changes such as hypertension, proteinuria, and renal pathology (Maynard et al. 2003). sENG, a soluble TGF-β co-receptor, induces vascular permeability and hypertension in vivo, correlated with disease severity. Injection of sFlt-1 in combination with sEng into pregnant rats produced nephrotic-range proteinuria, severe hypertension, and biochemical evidence of HELLP syndrome (Venkatesha et al. 2006). Recent study of a screen of >7 million genetic variants in 2658 offspring from preeclamptic women and 308,292 population controls identified a single association signal close to the sFlt-1 gene, on chromosome 13, suggesting that dysregulation at the sFlt-1 gene locus in the fetal genome (likely in the placenta) is a fundamental molecular defect in preeclampsia (Gray et al. 2018).

7.1.2 Immune Activation

Multiple factors trigger immune activation in preeclampsia.

Paternal Antigen: retrospective studies have shown that preeclampsia occurs mostly in the first pregnancy. Likewise, partner change is correlated with increased risks of preeclamptic or hypertension in pregnancy (Zhang and Patel 2007). The prevailing hypothesis is that after the first pregnancy, the maternal immune system has "recognized" the paternal antigens and could tolerate the same antigens in subsequent pregnancies. Changing partner introduces new paternal antigens and with it there is a new risk for preeclampsia. The maternal immune system, therefore, has to re-establish an immune tolerance (Zhang and Patel 2007). Failure of this tolerance to occur may contribute to preeclampsia.

HLA system: human trophoblast has a limited expression of strong transplantation antigens. These include nonpolymorphic HLA-E, F, and G (without signal paternal specificity) and HLA-C, on extravillous cytotrophoblast in Interface II (with signal paternal specificity). It is reported that this interface regresses in the second half of pregnancy (Choudhury and Knapp 2001a, b). Since it is devoid of HLA expression at the third trimester, alloantigen-provoked pathological change occurs in the first half of pregnancy with the clinical presentation of preeclampsia in the late second or third trimester of the pregnancy.

Autoimmune antibodies: autoimmune antibodies were highlighted recently by numerous researches of their role in preeclampsia. Agonistic Angiotensin II type 1 (AT1) receptor autoantibodies (AT-1 AAs) share the same AT-1 receptor with Angiotensin II (Wallukat et al. 1999; Zhou et al. 2008) and were found exclusively in peripheral blood of preeclamptic patients (Wallukat et al. 1999), are stressors that elicit preeclamptic symptoms (hypertension, proteinuria, renal damage, and sFlt-1 elevation) in vivo (Wallukat et al. 1999). Therefore, triggering AT-1 receptor signaling by circulating autoimmune antibodies (AT1-AAs) is notable evidence how immune activation is involved in preeclamptic pathology (Zhou et al. 2008; Tal 2012).

7.1.3 Inflammatory Cytokines

Although normal pregnancy evokes systemic inflammatory including innate immune responses which mainly take place in the third trimester (Redman et al. 1999), preeclampsia is associated with a more extreme maternal systemic inflammatory response (Redman and Sargent 2004).

7.1.3.1 TNF-α

Tumor necrosis factor (TNF-α) is a multifunctional pro-inflammatory cytokine. It is produced chiefly by activated macrophages (Carswell et al. 1975), and can also be produced by other cells/tissues including human placentas (Wang and Walsh 1996; Kirwan et al. 2002). The primary role of TNF-α is regulating immune cells. TNF, as an endogenous pyrogen, induces fever. It elicits apoptotic cell death, sepsis, cachexia, inflammation, and inhibits tumorigenesis and viral replication (Idriss and Naismith 2000). It was reported that TNF-α was abnormally elevated in the peripheral blood of preeclamptic patients (Wang and Walsh 1996). Chronic infusion of TNF-α into normal pregnant rats resulted in significant increase in arterial pressure and a decrease in renal hemodynamics. TNF-α infusion in pregnant rats also triggered AT1-AAs production (LaMarca et al. 2007), suggesting that TNF-α can cause both inflammatory and immune activation in preeclampsia. Recent meta-analysis disclosed that TNF-α −308 GA and GA/AA genotypes were associated with increased risks of preeclampsia in Asians (Lin et al. 2019).

7.1.3.2 IL-1

IL-1, including IL-1α, IL-1β, is also an important inflammatory and immune regulator. Both IL-1α and IL-1β are produced by macrophages, monocytes, fibroblasts, and dendritic cells (Dinarello 2011). They play an important role against infection. IL-1 is also an endogenous pyrogen and regulates hematopoiesis. Increased IL-1 levels were found in the peripheral blood of preeclamptic patients with other inflammatory cytokines (Greer et al. 1994; Southcombe et al. 2015). Intracisternal or intravenous infusion of IL-1 beta increases blood pressure in a prostaglandin dependent manner in rats (Takahashi et al. 1992).

7.1.3.3 IL-6

IL-6 is secreted by T cells and macrophages (Kishimoto 2010). It is one of the most important mediators of fever and the main regulator of acute phase response. Increased IL-6 levels were found in the serum of severe preeclamptic patients (Greer et al. 1994). Both IL-6 receptor and TNFSF11 (tumor necrosis factor ligand superfamily member 11) polymorphisms were found associated with increased risk of early-onset preeclampsia (Fan et al. 2017). Chronic infusion of IL-6 into normal pregnant rats resulted in similar effect as TNF-α, causing significant increases in arterial pressure and a decrease in

renal hemodynamics (LaMarca et al. 2007). However, TNF-α activates the endothelin system in placenta, renal and vascular tissues, whereas IL-6 stimulates the renin–angiotensin system (Gadonski et al. 2006). In addition, these inflammatory cytokines may activate the sympathetic nervous system. They may also play an important role in causing hypertension in response to chronic reductions in uterine perfusion during pregnancy, by activating multiple neuro-humoral and endothelial factors (LaMarca et al. 2007).

7.1.4 Oxidative Stress

Free radicals are atoms with an unpaired number of electrons that can be formed when oxygen interacts with certain molecules. Once formed these highly reactive radicals can start a chain reaction. They react with and thus damage cellular components such as DNA or the cell membrane. The most common physiological radical is the superoxide anion. Sources of superoxide under physiological conditions include the enzymes, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, 5 cytochrome P450, and other oxido-reductases (Muller et al. 2007).

Oxidative stress (i.e., NADPH oxidase) is generated substantially at the maternal–fetal interface during pregnancy, particularly in the early trimester. It functions in the normal development of the placenta and contributes to the pathophysiology of pregnancy complications such as miscarriage, pre-eclampsia, IUGR, and premature rupture of the membranes (Burton and Jauniaux 2004; Jauniaux et al. 2006). Unlike in normal pregnancy, oxidative stress and the systemic inflammatory response are more critical in preeclampsia (Redman et al. 1999; Redman and Sargent 2004, 2005). Preeclampsia, particularly early onset preeclampsia, was associated with placental oxidative stress including increased concentrations of protein carbonyls, lipid peroxides, nitrotyrosine residues, and DNA oxidation (Myatt and Cui 2004; Burton et al. 2009). Preeclamptic umbilical cord tissues exhibited increased oxidatively modified low density lipoprotein (Ox-LDL) and decreased SOD levels (Li et al. 2018). Moreover, early onset pre-eclampsia, which is frequently associated with IUGR, was reported with high levels of endoplasmic reticulum (ER) stress in the placenta (Burton et al. 2009).

Auto-antibodies AT1-AAs also trigger oxidative stress in pre-eclampsia. They stimulate NADPH oxidase, resulting in an increase in Reactive Oxygen Species (ROS) production (Dechend et al. 2003).

Cellular response to oxidative stress is via the mitogen-activated protein kinases (MAPK) pathway. For example: ROS-induced activation of extracellular regulated kinases (ERK1/2) generally promotes cell survival and proliferation, whereas stimulation of p38 and stress-activated protein kinase–c-Jun amino terminal kinases (SAPK–JNK) mostly induces apoptosis (Trachootham et al. 2008; Liebermann and Hoffman 2008).

7.2 The Role of Gadd45 Stress Sensors in Preeclampsia

Evidence accumulating in recent years has highlighted the role of the Growth Arrest and DNA Damage-inducible 45 (Gadd45) family of genes as important sensors of environmental and physiological stress, including genotoxic damage (UV, X-ray), hypoxia, oxidative stress, and pro-inflammatory cytokines (Fornace et al. 1992; Liebermann and Hoffman 2002). Gadd45 proteins are, in essence, signal transducers that convert environmental and physiological stresses into various cellular stress responses including inflammation (Gupta et al. 2006), innate immunity (Gupta et al. 2006), and autoimmune diseases (Salvador et al. 2005). Gadd45 proteins bind to and regulate the activity of several downstream stress response proteins (Liebermann and Hoffman 2002) such as MTK1/MEKK4, an upstream activator of MKK3 and MKK6 that ultimately mediates activation of both p38 and JNK stress response kinases (Takekawa and Saito 1998; Gupta et al. 2005).

The first direct evidence showing Gadd45 as a stress sensor contributing to preeclampsia was

via the placenta examination. Placental tissue from both preeclamptic and normotensive (control) patients was examined for the mRNA levels of the Gadd45 family genes (a, b, and g). Although the expression of all three genes was elevated in preeclamptic placentas, the difference was statistically significant only for Gadd45a mRNA. In addition, Gadd45a protein was readily detectable only in preeclamptic placentas, and this elevation was independent of different Body Mass Index (BMI) or race between the preeclamptic and control groups. Further, via immunohistochemical detection, Gadd45a protein was found localized in preeclamptic placentas, particularly in endothelial and trophoblast cells with the increased expression of Gadd45a downstream effector p-38 protein. With dual double immunofluorescence staining for both Gadd45a and sFlt-1(circulating factor and a key player in preeclampsia), the co-expression of these two proteins was targeted at the preeclamptic placental endothelial cells (Xiong et al. 2009).

7.2.1 Hypoxia and Gadd45a in Preeclampsia

As previously discussed, hypoxia is essential in the pathogenesis of preeclampsia. In-vitro culture of both endothelial cells and placental explants showed that Gadd45a protein was induced with the downstream p-38 protein phosphorylation under hypoxic circumstances. The activation of Gadd45a signaling caused elevation of sFlt-1 in the supernatant of cultured endothelial cells of placental explants. When Gadd45a expression was knocked down by specific Gadd45a RNAi, the elevation of sFlt-1 was depleted. The regulation of sFlt-1 secretion by Gaddd45a occurred via the p-38 activation (Xiong et al. 2009, 2013).

7.2.2 Oxidative Stress and Gadd45a in Preeclampsia

Silencing of Gadd45a gene triggered by siRNA could inhibit the p38 MAPK signaling pathway, reduce the levels of 8-isoprostane and Ox-LDL, increase SOD level, and promote cell migration, invasion, and blood vessel formation in order to alleviate oxidative stress (Li et al. 2018).

7.2.3 AT-1 AAs and Gadd45a in Preeclampsia

Angiotensin II is a vessel constrictor which causes increasing blood pressure and shares the same AT-1 receptor with AT-1 AAs. In order to study the interaction of Gadd45a and AT-1 AAs in preeclampsia, Angiotensin II was introduced to cultured placental explants. Treatment of placental explants with Angiotensin II resulted in Gadd45a induction, p-38 phosphorylation (i.e., activation) and elevation of sFlt-1 in the supernatant (Xiong et al. 2013).To establish a causal link between Gadd45a induction, p38 activation, and elevated secretion of sFlt-1, Gadd45a expression was knocked down with Gadd45a RNAi in the placental explants. RNAi mediated knockdown of Gadd45a, abolished Angiotensin II induced p38 activation and significantly reduced sFlt-1 levels in culture. Furthermore, blocking p38 activation with the specific chemical inhibitor also resulted in attenuated levels of sFlt-1 in the culture medium. On the other hand, blocking the activation of JNK, which is also a downstream effector of Gadd45a, did not attenuate sFlt-1 secretion (Xiong et al. 2013).

7.2.4 Inflammatory Cytokines and Gadd45a in Preeclampsia

Two important preeclampsia associated inflammatory cytokines IL-6 and TNF-α were examined with Gadd45a stress response cascade.

Incubation with IL-6 induced Gadd45a in placental explants is associated with activation of the downstream effectors p38 and phospho-JNK, as well as elevated levels of sFlt-1 in the culture medium. RNAi mediated knockdown of Gadd45a abolished p38 activation and significantly reduced sFlt-1 levels in the culture medium following IL-6 treatment. Blocking p38 also attenu-

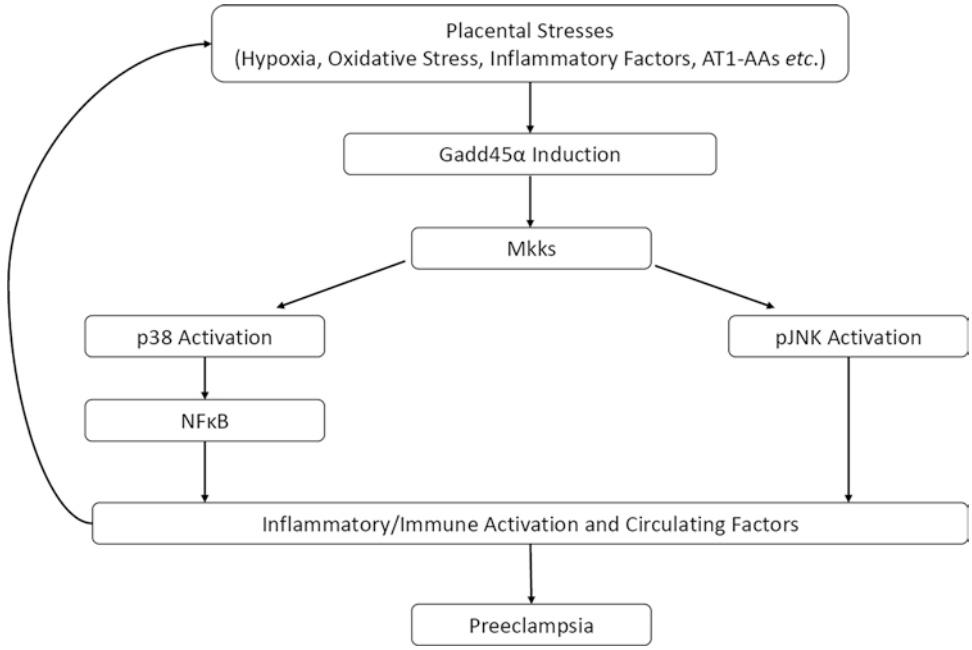

Fig. 7.1 Gadd45a in Preeclampsia

ated sFlt-1 secretion in the culture medium, whereas blocking JNK activation had no effect on sFlt-1 levels (Xiong et al. 2013).

Induction of Gadd45a in response to TNF-α was prompt (peak time at 10 or 20 min), compared to the other stressors discussed above. In addition, it was associated with both p38 and JNK activation, and increased sFlt-1 levels in the culture medium. However unlike other pre-inflammatory stressors, it was the inhibition of JNK activation, but not p38 activation that attenuated sFlt-1 secretion (Xiong et al. 2013).

7.3 Conclusions

Gadd45a protein works as a stressor sensor in preeclampsia. In response to various pathophysiological stressors, notably hypoxia, inflammatory cytokines, and AT1-AAs, Gadd45a activates Mkk3-p38 and JNK signaling. This, in turn, results in immunological and inflammatory changes, as well as triggering the production of circulating factors such as sFlt-1, which are believed to account for many of the pathophysiological related symptoms of preeclampsia (Maynard et al. 2003). Inflammatory/immune activation in preeclampsia may function in a feedback loop to maintain elevated expression of Gadd45a protein (Fig. 7.1).

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Gadd45 in Senescence

8

M. Raza Zaidi and Dan A. Liebermann

Abstract

Gadd45a, Gadd45b, and *Gadd45g* have been implicated in cell cycle arrest, DNA repair, apoptosis, innate immunity, genomic stability, and more recently in senescence. Evidence has accumulated that Gadd45a deficiency results in escape of mouse embryo fibroblasts from senescence, whereas Gadd45b deficiency promotes premature senescence and skin aging. Moreover, recently Gadd45b deficiency was found to promote senescence and attenuate liver fibrosis, whereas Gadd45a was observed to exert a protective effect against hepatic fibrosis. These findings indicate that the Gadd45 stress response proteins play important roles in modulating cellular responses to senescence. Thus, exploring how Gadd45 proteins modulate cellular senescence has the potential to provide new and innovative tools to treat cancer as well as liver disease.

Keywords

Gadd45 · Gadd45a · Gadd45b · Gadd45g · Senescence · Liver · Fibrosis · Hepatic fibrosis · Carbon tetrachloride · CCl_4 · Collagen

8.1 Gadd45 Family of Stress Response Genes

Gadd45a, Gadd45b, and Gadd45g constitute a family of genes that encode small (18 kDa) evolutionarily conserved proteins, which are highly homologous to each other (Liebermann and Hoffman 2013). Despite marked similarities, these genes are regulated in a differential manner and exhibit functional diversity. They play a pivotal role in regulating diverse cellular functions such as cell cycle control, survival, and apoptosis and are regulated by the nature of the encountered stress stimulus, its magnitude, and the cell type. *Gadd45a, Gadd45b,* and *Gadd45g* have been implicated in cell cycle arrest, DNA repair, apoptosis, innate immunity, genomic stability, and more recently in senescence (Liebermann and Hoffman 2013).

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8.2 Cellular Senescence

Senescence is a cellular state of irreversible growth arrest in response to various types of stresses. Senescence can be triggered within several days in somatic cells that are undergoing stress stimuli (He and Sharpless 2017). It was shown that in normal primary cells, overexpression of an oncogene, such as activated H-Ras, can trigger growth arrest, with features of cellular senescence, as well as apoptosis in fibroblasts and epithelial cells (Bringold and Serrano 2000; Bulavin et al. 2002; Ferbeyre et al. 2002; Lin and Lowe 2001; Serrano et al. 1997). These responses have been implicated as protective by removing cycling cells when an oncogene has become active. The mechanism for senescence involves multiple pathways, including those involving p53 and RB. To a large extent, Ras-induced cell cycle arrest is dependent on p53 signaling (Serrano et al. 1997). In mouse cells, growth arrest after oncogenic stimulation is dependent on the p19/ARF pathway-mediated stabilization of p53 (Ferbeyre et al. 2002), which is not the case for some human cells in which p14/ARF is not induced (Wei et al. 2001). Although p53 accumulation is an important feature, full activation of p53 involves other events including posttranslational modifications, which involve a variety of regulatory kinases, such as the mitogen-activated protein kinases (MAPK) (Appella and Anderson 2001). In addition to activation of the ERK1/2 pathway by oncogenic Ras, which regulates p16/Ink4a levels (Lin et al. 1998; Zhu et al. 1998), increasing evidence indicates that two other major MAPK pathways, p38 MAPK (p38) and c-Jun N-terminal kinase (JNK), have important roles in the cellular response to oncogenic stress (Pruitt et al. 2002). In the case of H-Ras activation, all three (ERK, p38, and JNK) major branches of MAPK signaling are activated. Sequential activation of the ERK pathway and then the p38 pathway has been reported to contribute to the induction of senescence by H-RasG12V (Wang et al. 2002).

8.3 Gadd45 Proteins in Cellular Senescence

8.3.1 Gadd45a Deficiency Results in Escape of Mouse Embryonic Fibroblasts from Senescence, Whereas Gadd45b Deficiency Promotes Premature Senescence and Skin Aging

A role implicating Gadd45a in senescence was first documented in 2003 (Bulavin et al. 2003), which showed that in *Gadd45a*$^{-/-}$ mouse embryonic fibroblasts (MEF), overexpression of H-Ras activates extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) but not p38 kinase, and this correlates with the loss of H-Ras-induced senescence. Inhibition of p38 mitogen-activated protein kinase (MAPK) activation correlated with the deregulation of p53 activation, and both a p38 MAPK chemical inhibitor and the expression of a dominant-negative p38α inhibited p53 activation in the presence of H-Ras in wild-type MEF. p38, but not ERK or JNK, was found in a complex with Gadd45 proteins. The region of interaction was mapped to amino acids 71–96, and the central portion (amino acids 71–124) of Gadd45a was required for p38 MAPK activation in the presence of H-Ras. An increase in Gadd45a expression has been correlated to H_2O_2 stress-induced senescence as well (Furukawa-Hibi et al. 2002). The first evidence to suggest a role of Gadd45b in senescence was obtained in the senescence-accelerated mouse (SAMP1) model, revealing that Gadd45b exhibits a higher expression in the aging articular cartilage of SAMP1 mice as compared to the control mice (Shimada et al. 2011).

Research conducted in our laboratory has shown that Gadd45a-null MEFs escape senescence, whereas Gadd45b-null MEFs stop proliferating and undergo premature senescence (Fig. 8.1 and Magimaidas et al. 2016). The impaired proliferation and increased senescence in Gadd45b-null MEFs are partially reversed by

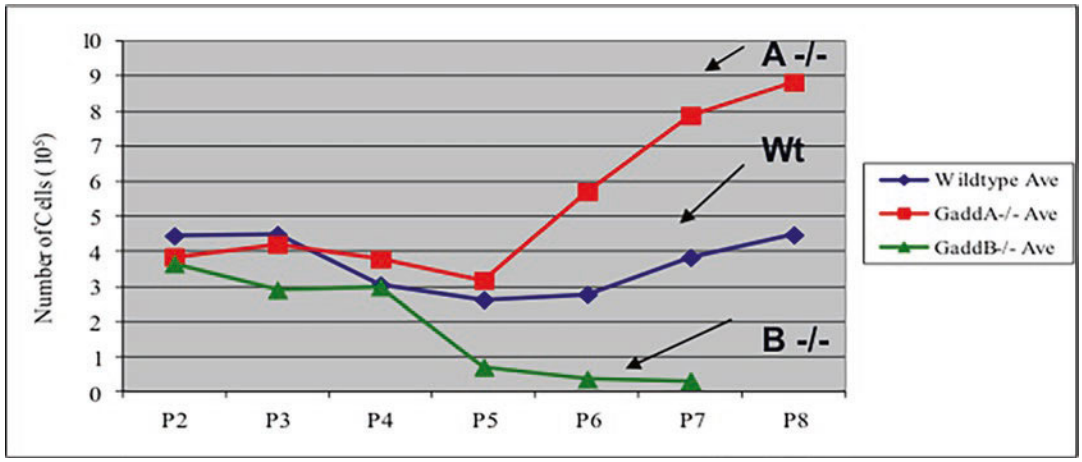

Fig. 8.1 Gadd45a-null MEFs escape senescence, whereas Gadd45b-null MEFs stop proliferating prematurely and undergo premature senescence

culturing at physiological oxygen levels, indicating that Gadd45b deficiency leads to decreased ability to cope with oxidative stress. Interestingly, Gadd45b-null MEFs arrest at the G2/M phase of cell cycle, in contrast to other senescent MEFs, which arrest at G1. FACS analysis of phosphohistone H3 staining showed that Gadd45b-null MEFs are arrested in G2 phase rather than M phase. H_2O_2 and UV irradiation, known to increase oxidative stress, also triggered increased senescence in Gadd45b-null MEFs as compared to wild-type MEFs. In vivo evidence for increased senescence in Gadd45b-null mice includes the observation that embryos from Gadd45b-null mice exhibit increased senescence staining compared to wild-type embryos (Fig. 8.2). Furthermore, it has been shown that Gadd45b deficiency promotes senescence and aging phenotypes in mouse skin (Magimaidas et al. 2016). Together, these results highlight a novel role for Gadd45b in stress-induced senescence and in tissue aging (Fig. 8.3 and Magimaidas et al. 2016). Together, these results highlight a novel role for Gadd45b in stress-induced senescence and in tissue aging.

8.3.2 Gadd45b Deficiency Promotes Senescence and Attenuates Hepatic Fibrosis, Whereas Gadd45a Exerts a Protective Effect Against It

Fibrosis is a wound healing process characterized by deposition of extracellular matrix components including collagens, leading to encapsulation of the injury site. Liver fibrosis, a pathological feature that is a precursor of cirrhosis, is characterized by the accumulation of fibrotic tissue and the concomitant loss of liver function. It can be triggered by chronic liver damage associated with hepatitis virus infection, alcohol abuse, or liver steatosis (fatty liver disease; Friedman 2003; Bataller and Brenner 2005). It has been shown that during chronic damage, hepatic stellate cells (HSCs) become activated and abnormally proliferate as myofibroblasts (damage-activated fibroblasts) (Gäbele et al. 2003; Moreira 2007). Recent studies have shown that myofibroblasts become senescent and produce a stable fibrotic scar with abundant collagen and other extracellular matrix components (Kis et al. 2011). In human patients, SA β-gal-positive cells accumu-

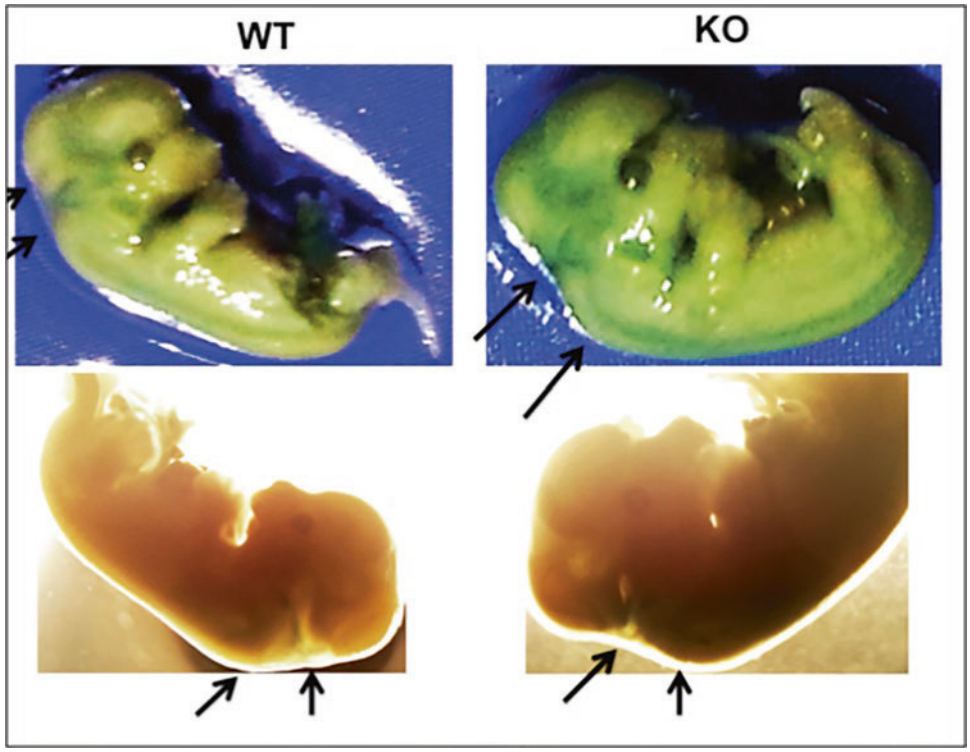

Fig. 8.2 Photographs of SA-β-gal staining of *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ E14 embryos. Arrows indicate embryonic regions with strong senescence staining. Strong staining was found in the head and neck regions of all mutant embryos tested ($n = 6$). To investigate the role of *Gadd45b* in liver fibrosis, *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ mice were injected intraperitoneally twice weekly for 6 weeks with sub-lethal doses of CCl_4 in olive oil or an equal volume of olive oil alone as vehicle control. Three days after the final injection, livers were harvested and processed for sectioning and histopathological evaluation

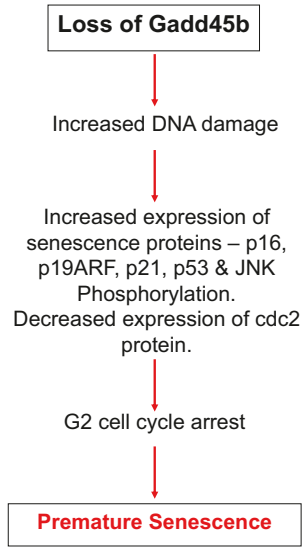

Fig. 8.3 Proposed role of Gadd45b in premature senescence

late in the periphery of the fibrotic scar. In rodents, chronic treatment with carbon tetrachloride (CCl_4, a liver-damaging agent) produces liver fibrosis, which is characterized by positive SA β-gal-positive cells that are derived from activated HSCs and show increased p53, p21, and p16 and other senescence markers (Wiemann et al. 2002). Mice deficient in these genes have demonstrated the beneficial role of senescence in restricting liver fibrosis. In response to liver damage, mice lacking *Trp53* and/or *Cdkn2a* present senescence negative fibrotic areas that are larger than those in senescence-competent mice. Similarly, the extracellular matrix protein CCN1 produced by damaged hepatocytes has been shown to be a key mediator of senescence induction in HSCs. Accordingly, mice with *Ccn1* deficient hepatocytes do not execute HSC senescence

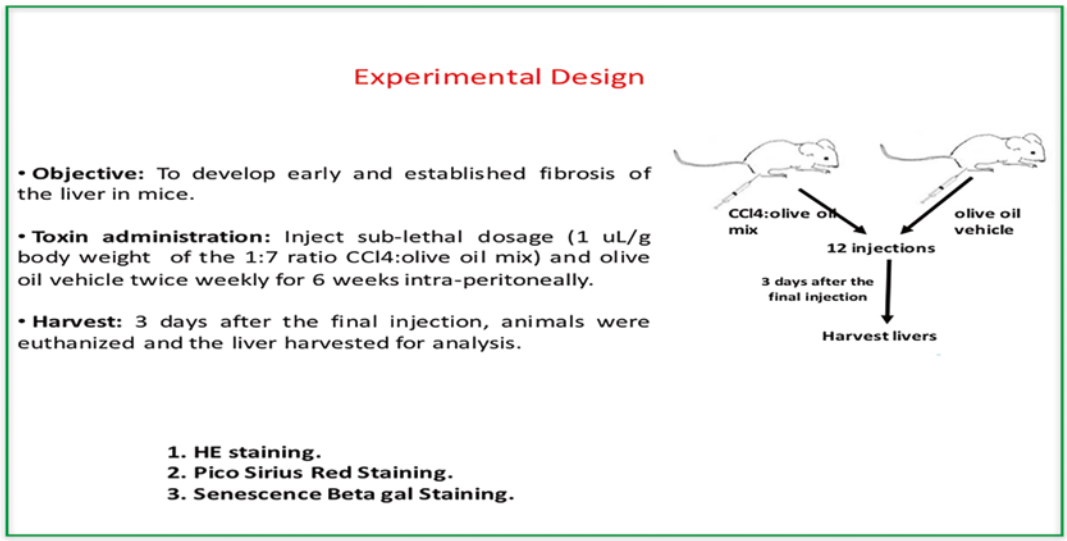

Fig. 8.4 Experimental design for the liver fibrosis analysis in mice

leading to an exacerbated fibrotic response (Kim et al. 2013). Taken together, these observations suggest that the Gadd45a-mediated promotion of HSC senescence plays a role in protecting liver cells from fibrosis and could be a potential therapeutic strategy to limit liver fibrosis.

Mouse models of liver fibrosis have proven invaluable to the investigation of liver fibrogenesis and chronic hepatic injury(Krizhanovsky et al 2008). Thus, to investigate the role of *Gadd45b* in liver fibrosis, *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ mice were injected intraperitoneally twice weekly for 6 weeks with sub-lethal doses of CCl_4 in olive oil or an equal volume of olive oil alone as vehicle control. Three days after the final injection, livers were harvested and processed for sectioning and histopathological evaluation (Fig. 8.4). Interestingly, histopathological analysis using H&E staining revealed reduced cyto-architectural damage (fibrotic scars) in *Gadd45b*$^{-/-}$ mice compared with *Gadd45b*$^{+/+}$ mice. In order to further characterize this fibrotic phenotype and analyze the collagen deposition, Sirius red staining was carried out. *Gadd45b*$^{-/-}$ mice showed reduced hepatic collagen accumulation as compared with *Gadd45b*$^{+/+}$ mice after the chronic CCl_4 challenge. These data indicate that loss of *Gadd45b* protected against CCl_4-induced chronic hepatic injury (Fig. 8.5).

Given that senescence has been shown to be a protective mechanism against liver fibrosis, and that *Gadd45b*$^{-/-}$ MEFs show increased senescence as compared to *Gadd45b*$^{+/+}$ MEFs, it was of interest therefore to investigate the level of cellular senescence within the liver samples. To identify senescent cells in situ, liver sections from CCl_4 and vehicle-treated mice were subjected to senescence associated β-galactosidase staining. As predicted, *Gadd45b*$^{-/-}$ mice showed increased senescence staining in livers of CCl_4-treated mice compared with *Gadd45b*$^{+/+}$ mice. These data indicate that loss of *Gadd45b* leads to increased senescence, which is protective against CCl_4-induced liver fibrosis, leading to reduced fibrotic scars and reduced collagen deposition.

In conclusion, the results obtained highlight a novel and significant role for *Gadd45b* in the senescence response to CCl_4-induced liver injury providing the impetus to further investigate the role of Gadd45 proteins in physiological and pathological conditions that trigger senescence in liver.

In contrast, data have been obtained to indicate that Gadd45a exerts a protective effect against hepatic fibrosis induced by CCl_4, via the inhibition of canonical transforming growth factor-β/Smad signaling and fibrogenic gene expression, as well as by exerting ROS scaveng-

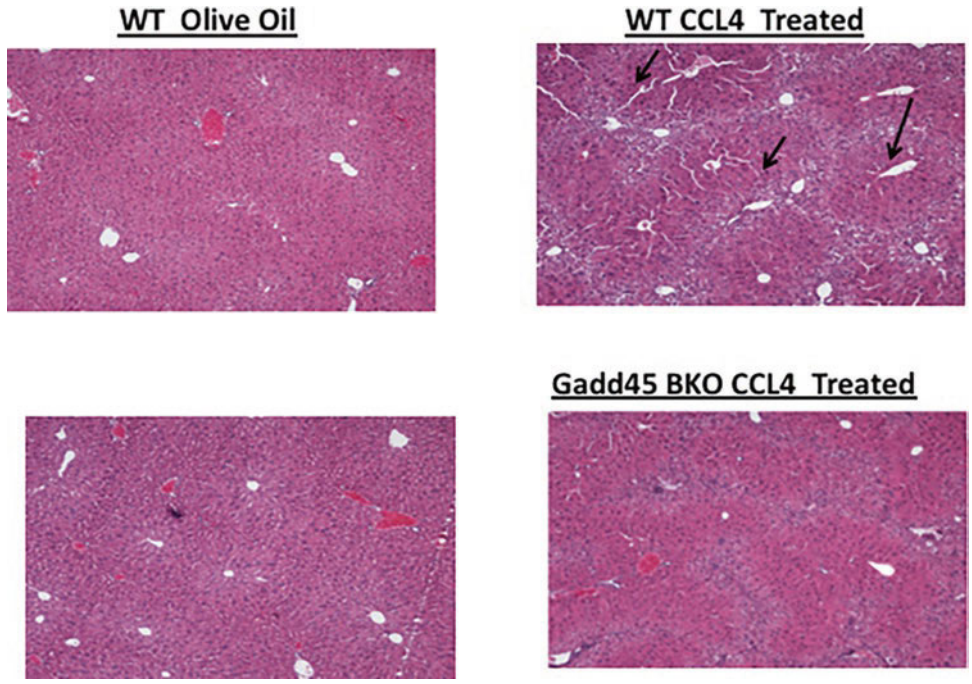

Fig. 8.5 *Gadd45b* deficiency attenuates CCl_4-induced collagen accumulation in liver. Histopathological analysis using Pico-Sirius Red staining of liver sections from *Gadd45b*$^{+/+}$ and *Gadd45b*$^{-/-}$ mice treated with sub-lethal doses of CCl_4 in olive oil or olive oil control showed a significant decrease in collagen accumulation in CCl_4 treated *Gadd45b*$^{-/-}$ mice as compared with their wild-type counterparts. Arrows indicate liver sections with collagen accumulation

ing effects via upregulation of expression of antioxidant enzymes (Hong et al. 2016).

Moreover, recent data have shown that Gadd45a-null mice have more severe hepatic inflammation and fibrosis, higher levels of mRNAs encoding pro-inflammatory, pro-fibrotic, and pro-apoptotic proteins, and greater oxidative and endoplasmic reticulum (ER) stress compared with WT mice, where Gadd45a was induced in response to ER stress in primary hepatocytes, indicating that Gadd45a plays protective roles against methionine and choline-deficient diet (MCD)-induced nonalcoholic steatohepatitis (NASH) (Tanaka et al. 2017).

Taken together, these data suggest that Gadd45a and Gadd45b play opposing roles in modulating liver senescence and fibrosis. It is plausible that Gadd45a exerts a protective effect against hepatic fibrosis, whereas Gadd45b plays a role in sensitizing the liver to hepatic injury and fibrosis. It would be important to elucidate how this novel nexus of Gadd45 stress sensors cooperate in modulating liver homoeostasis upon injury and fibrosis (Fig. 8.6).

Finally, Gadd45g expression was shown to directly induce senescence in HCC Sk-Hep1, SMMC-7721, and Hep3B cells. Notably, knockdown of GADD45G in Sk-Hep1 tumor cells by small interfere RNA (siRNA) attenuated MG132-induced senescence (Xu et al. 2015).

8.4 Summary and Future Prospects

The stress response Gadd45 proteins appear to play important roles in modulating cellular responses to senescence. How do they function in this capacity has been largely unexplored. Thus, exploring how Gadd45 proteins modulate cellular senescence is important and novel. Furthermore, it has the potential to provide new innovative tools to treat cancer as well as liver disease.

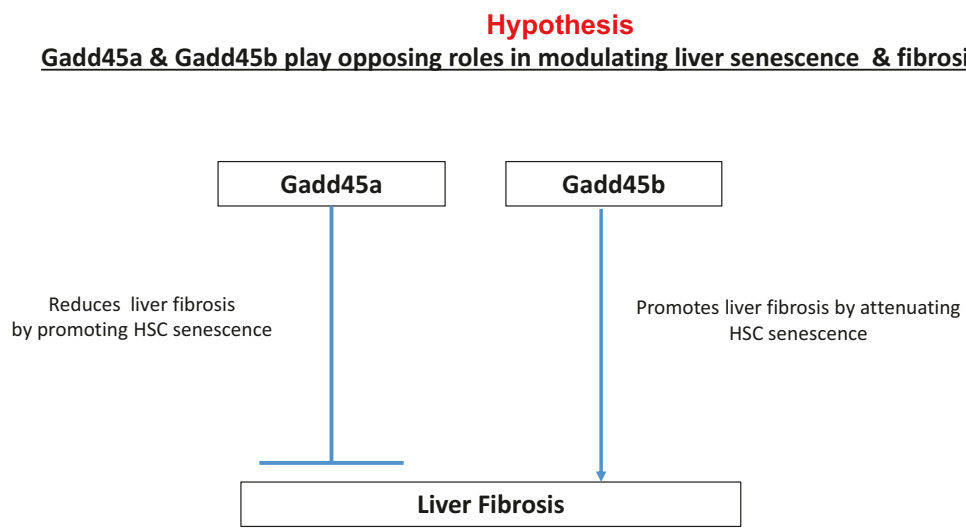

Fig. 8.6 Hypothetical model for the proposed role of Gadd45a and Gadd45b in liver fibrosis

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Gadd45 in Neuronal Development, Function, and Injury

9

Faraz A. Sultan and Bassel E. Sawaya

Abstract

The growth arrest and DNA damage-inducible (Gadd) 45 proteins have been associated with numerous cellular mechanisms including cell cycle control, DNA damage sensation and repair, genotoxic stress, neoplasia, and molecular epigenetics. The genes were originally identified in in vitro screens of irradiation- and interleukin-induced transcription and have since been implicated in a host of normal and aberrant central nervous system processes. These include early and postnatal development, injury, cancer, memory, aging, and neurodegenerative and psychiatric disease states. The proteins act through a variety of molecular signaling cascades including the MAPK cascade, cell cycle control mechanisms, histone regulation, and epigenetic DNA demethylation. In this review, we provide a comprehensive discussion of the literature implicating each of the three members of the Gadd45 family in these processes.

Keywords

GADD45 · Neuronal development · Central nervous system · CNS development · Neurodevelopment · MAPK · microRNA · Ischemia · Neuronal injury · Neoplasia · Seizures · Neuroepigenetics · Memory · Autism · Alzheimer's · Aging · Psychosis

9.1 Introduction

The growth arrest and DNA damage-inducible (Gadd)45 family includes the related Gadd45α, Gadd45β/Myd118, and Gadd45γ/CR6 proteins, referred to here as Gadd45a, Gadd45b, and Gadd45g, respectively. The corresponding genes were identified in different cell lines following irradiation stress and interleukin treatment (Fornace et al. 1988; Abdollahi et al. 1991; Beadling et al. 1993). These evolutionarily conserved proteins are small (~18 kD), acidic homologs with both nuclear and cytoplasmic expression

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(Tamura et al. 2012). Expression of the *gadd45* genes was confirmed in a variety of tissues including skeletal muscle, heart, kidney, lungs, brain, and testis (Zhang et al. 1999) as well as in Drosophila (Bgatova et al. 2015). Consistent with their discovery, the *gadd45* genes were shown to be sensitive to a wide variety of stressors in diverse cell lines (Tamura et al. 2012). In the central nervous system (CNS), the genes have been investigated as critical contributors to neuronal and glial stress responses, apoptosis, and mitosis. This is consistent with their long-established role in cell cycle checkpoint regulation (Kearsey et al. 1995; Hildesheim et al. 2002). More recently, Gadd45a and Gadd45b were implicated in epigenetic control of gene expression, and this discovery prompted a growing literature documenting their role as players in adult cognitive function and CNS diseases (Barreto et al. 2007; Ma et al. 2009; Day and Sweatt 2011). In this review, we summarize the body of evidence showing the Gadd45 proteins regulate nervous system development, injury responses, and cognitive neuroepigenetics.

9.2 Nervous System Development

9.2.1 Expression Patterns of the Gadd45 Genes in Neural Development

We begin with a discussion of the role of the Gadd45 family in the development of the central nervous system. One study in particular provides a comprehensive assessment of the expression patterns of the Gadd45 genes throughout murine embryonic development (Kaufmann et al. 2011). The authors began by assessing expression in mouse embryos. Gadd45a mRNA was found to be expressed at low levels in the gastrula stage, and this expression increased during neurulation, plateauing by embryonic day 9. In contrast, while strong *gadd45b* transcription was found during gastrulation, expression decreased rapidly during neurulation. Gadd45g mRNA expression begins at low levels and increases continuously following gastrulation. Relative quantification of the three transcripts at embryonic day 10.5 revealed very minute *gadd45b* expression and robust expression of *gadd45a* and *gadd45g*. These results suggest Gadd45b may play the least critical function among the three genes in embryogenesis. In *Xenopus* embryogenesis, similar relative expression patterns were found. *Gadd45a* expression peaks between embryonic days 10.5 and 12.5, *gadd45b* expression is minimal at embryonic day 10.5 but increases thereafter, and *gadd45g* expression peaks between embryonic days 12.5 and 18 (Kaufmann and Niehrs 2011).

Region-specific analysis of transcription in mice showed prominent *gadd45a* expression early in the mesoderm and primitive streak, the region through which cell migration gives rise to the three germ layers. *Gadd45b*, in contrast is strongly localized to the chorion but not epiblast cells at embryonic day 7.5. However, *gadd45b* is mildly expressed later in the posterior remnants of the primitive streak. *Gadd45g* expression is also excluded from the epiblast cells at this stage. It is instead found in the ectoplacental cone and the extraembryonic ectoderm.

Upon neurulation, Gadd45a mRNA is highly enriched in the neural folds, suggesting a significant role in initial formation of the neural tube. Supporting this hypothesis is the finding that a fraction of homozygous Gadd45a-null mutants exhibit exencephaly, a condition in which the brain grows outside of the skull due to improper neural tube closure (Hollander et al. 1999). However, this finding may not be due to Gadd45a-regulated apoptosis, as the requirement of apoptosis for neural tube closure has been challenged (Massa et al. 2009). At this stage, Gadd45b is not found in the lips of the neural folds but is expressed in the progenitors of the midbrain and hindbrain. Between embryonic days 8.5 and 8.75, *gadd45g* transcription continues in the extraembryonic allantois, and embryonic expression is found in the presomitic mesoderm. Notably, expression begins in neural fold precursors and then becomes prominent in dorsal midbrain. Milder expression is found in the facio-acoustic and dorsal root ganglia at this stage as well.

By embryonic day 9, *gadd45a* expression builds in placodes of the trigeminal and dorsal root ganglia (Kaufmann et al. 2011). By day 9.5, expression is found in the facio-acoustic, glossopharyngeal, and vagal ganglia as well as the olfactory epithelium. Transcripts in the neural tube, however, begin to fade but persist in the caudal neuropore and lamina terminalis, the final regions to undergo closure. After embryonic day 9.5, a ubiquitous basal expression pattern of *gadd45a* persists, and this includes the cranial ganglia VII-X. At day 10.5 expression appears in the epithelium of the telencephalic ventricles. Expression was not detected in the midbrain and hindbrain regions. In contrast, by embryonic day 9, expression of *gadd45b* builds in the dorsal midbrain and somites. In light of this pattern of embryonic Gadd45b transcriptional patterning, it is not surprising that prominent expression in the striatum was found in the adult brain (Sultan et al. 2012). Somitic expression of Gadd45b persists in the trunk somites by embryonic day 10.5, and additional expression occurs in the dorsal aorta, first branchial arches and forelimb bud. Relative to expression in these regions, Gadd45b mRNA was only weakly detected in the forebrain, suggesting it plays a less significant role in neural development. Finally, *gadd45g* transcription after embryonic day 9 was found to be the most pronounced of the three loci (Kaufmann et al. 2011). Highly pronounced staining was found in the dorsal midbrain, the cranial and dorsal root ganglia, and neural tube. At day 9.5, *gadd45g* was expressed robustly at the forebrain-midbrain junction, the trigeminal and facio-acoustic ganglia, the otic cup, and the latero-ventral hindbrain. However, expression in the neural tube was not found to be homogenous; instead, *gadd45g* is predominantly transcribed in the dorsal and ventral peripheral cells of the neural tube. By embryonic day 10.5, expression remains pronounced throughout the neural tube, extending from the most caudal tip to the midbrain rostrally. At this point, diffuse expression in the forebrain and dense expression in the dorsal root ganglia begin to appear as well. Murine expression of the *gadd45* genes is summarized in Fig. 9.1.

In situ hybridization analysis of expression patterns of the *gadd45* genes in *Xenopus* also revealed differential transcriptional profiles (Kaufmann and Niehrs 2011). *Gadd45a* transcripts are present homogenously in gastrulae in both ectoderm and mesoderm layers. Upon neurulation, significant expression continues in the ectoderm and neuroectoderm, but little expression is seen in the neural tube as in murine development. However, *gadd45b* expression was shown to be relatively concentrated in the neural tube as well as the initial ectoderm during gastrulation even though overall expression was very low compared to *gadd45a* and *gadd45g*. Similar to its murine homolog, the *Xenopus gadd45g* gene showed the most prominent neural expression, beginning in primary neuron precursors and later in the brain and eye. Although the expression of *Xenopus gadd45a* and *gadd45b* differ somewhat from their respective mouse homologs, it should be noted that the frog *gadd45b* gene shares under 60% homology with mouse *gadd45b*. The frog *gadd45a* and *gadd45g* genes, however, share over 70% homology with murine orthologs. Finally, neural expression of *gadd45g* was similarly confirmed in the medaka, *Oryzias latipes* (Candal et al. 2004). Expression was found in the neurula, and this spreads throughout the entire brain, lens, olfactory bulbs, and optic tectum.

Together, these results implicate each of the Gadd45 family genes in embryogenesis and neurulation but to differing extents. By far, *gadd45g* expression is the most robust in nervous tissue progenitors in both mice and frogs, suggesting it plays the strongest role in the ontogeny of the central and peripheral nervous system. In mice, *gadd45b* expression in nervous tissue is relatively weak, especially outside of the dorsal midbrain, and is instead more selectively localized to somites, mesodermal tissue that later gives rise to skeletal muscle, dermis, and vertebrae (Kawahara et al. 2005; Kaufmann et al. 2011). *Gadd45a* expression likely plays the strongest role in neural tube closure in mice. Beyond embryogenesis, *gadd45a* transcription and protein expression persist in the adult murine cerebral cortex, (Sarkisian and Siebzehnrubl 2012). Interestingly,

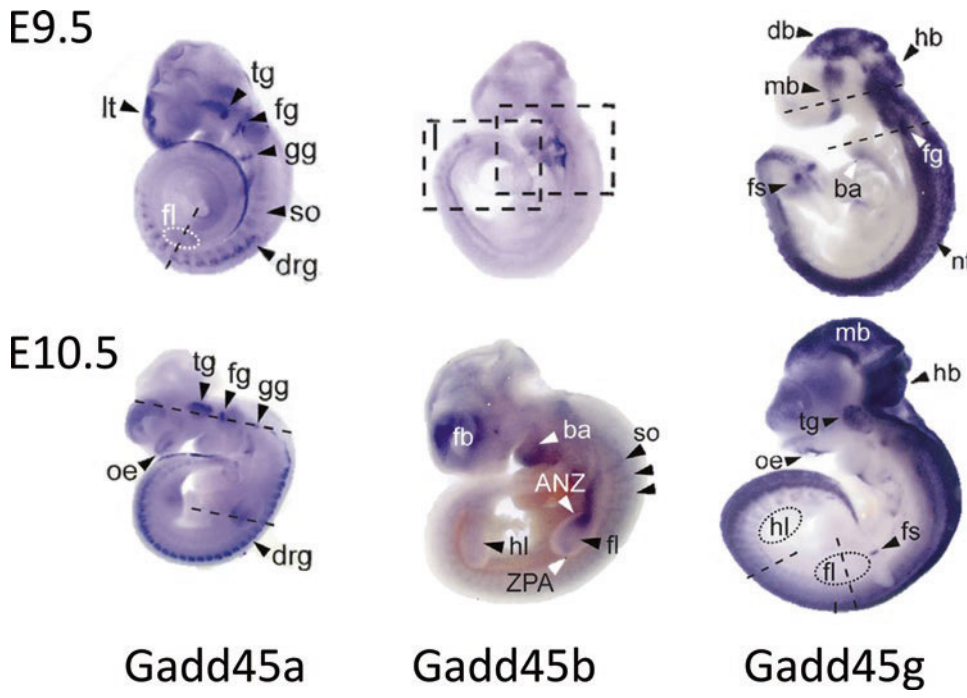

Fig. 9.1 Expression of the *gadd45* genes in mouse embryonic development. Whole mount in situ hybridization on E9.5 (top row) and E10.5 (bottom row) murine embryos was used to visualize expression of *gadd45a* (left), *gadd45b* (middle), and *gadd45g* (right). Lateral views are shown. Key: (ANZ) anterior necrotic zone, (ba) first branchial arch, (db) dorsal midbrain, (drg) dorsal root ganglia, (fb) forebrain, (fg) facio-acoustic ganglia, (fl) forelimb bud, (fs) forming somite, (gg) glossopharyngeal ganglia, (hb) hindbrain, (hl) hindlimb bud, (lt) lamina terminalis, (mb) midbrain, (nt) neural tube, (oe) olfactory epithelium, (so) somitic mesoderm, (tg) trigeminal ganglia, (ZPA) zone of polarizing activity. Reproduced with permission from Kaufmann et al. (2011)

cortical expression in the human fetus was similarly observed, suggesting a conserved developmental transcriptional profile for Gadd45a. Future studies are needed to assess the transcriptional profile of *gadd45b* and *gadd45g* beyond embryogenesis.

9.2.2 Regulation of Nervous System Development

There is also evidence from recent studies that both Gadd45a and Gadd45g play functional roles in neural development. In addition to the proclivity for exencephaly in Gadd45a-null mice, both knockdown and overexpression of *gadd45a* in *Xenopus* embryos, for instance, produced a range of developmental defects including gastrulation defects, reduced pigmentation, and head defects (Kaufmann and Niehrs 2011). Surprisingly, *gadd45b* manipulations failed to produce a developmental phenotype, suggesting that it is low but detectable expression in the embryo is less consequential. In contrast, increases or decreases in *gadd45g* gene product led to pleiotropic phenotypes similar to those of *gadd45a* manipulation (de la Calle-Mustienes et al. 2002; Kaufmann and Niehrs 2011). In medaka, overexpression of *gadd45g* attenuated embryonic development and cell number, and knockdown produced developmental and morphological abnormalities after the neurula stage (Candal et al. 2004).

The functionality of Gadd45a in neurodevelopment extends beyond gross anatomical features and pertains to cell differentiation as well. An in vitro assay of cortical neuron development found that both overexpression and knockdown of *gadd45a* transcription suppress the formation of distal neurite processes and often promotes aberrantly shaped and sized cell bodies (Sarkisian

and Siebzehnrubl 2012). The authors similarly found that reduced *gadd45a* expression in the cortex in vivo impairs dendritic arborization and neuronal migration to superficial cortical layers. Overexpression failed to affect migration but caused irregular and hypertrophied cell body development. Additionally, enhanced expression reduced survival and impaired development in a rat glioma cell line in vitro, suggesting Gadd45a regulates apoptosis. Likewise, overexpression of *gadd45g* in an embryonic carcinoma cell line produced a neuronal phenotype, suggesting Gadd45g regulates not only anatomical development but also differentiation of neurons (Huang et al. 2010). Together, these studies demonstrate a significant role of the Gadd45 genes, particularly Gadd45a and Gadd45g in the development of the nervous system at both a gross and cellular scale (Matsunaga et al. 2015).

9.2.3 Molecular Mechanisms in Neurodevelopment

The molecular pathways mediated by the Gadd45 family in neurodevelopment are under investigation, but some studies have uncovered a link with the mechanisms of cell cycle regulation and apoptosis (Fig. 9.7a, b). The low-penetrance exencephaly finding in Gadd45a-null homozygotes, for instance, has been linked to the XPC-associated pathway (Patterson et al. 2006). XPC functions as an oxidative DNA damage repair factor, acting through both nucleotide- and base-excision repair mechanisms at a genome-wide scale. It also modulates tumor formation and redox homeostasis (Hollander et al. 1999; Melis et al. 2011). Indeed, *gadd45a*/*XPC*-null double mutant mice displayed no further increase in exencephaly rate compared to single mutants, suggesting an overlapping mechanism. In contrast, homozygous deletion of *Trp53*, the gene encoding the tumor-suppressing p53 protein, caused a substantial increase in neural tube closure rate compared to *gadd45a* homozygous single mutants (Patterson et al. 2006). This finding suggests Gadd45a and p53 operate through separate pathways in development, a surprising result in light of the indirect induction of the Gadd45a promoter by p53 (Zhan et al. 1998). Additionally, both genes contribute to genome stability, apoptosis, G1 cell cycle checkpoint control through p21, and global genome repair. Concurrently, *gadd45a*/*Cdkn1a*-null mutants, those lacking both Gadd45a and the cyclin-dependent kinase inhibitor p21, also exhibited pronounced exencephaly rates versus single mutants (Patterson et al. 2006). At a subcellular level, Gadd45a-null mice exhibit genomic instability including aneuploidy, chromosomal aberrations, centrosome instability, abnormal growth, and mitosis. The lack of observed alterations in apoptosis rate in most cell lines and lack of a phenotype in induction of JNK and p38 kinase cascades upon cytotoxic stress in Gadd45a-null cells indicate that the mechanism for the observed phenotype may involve perturbed G2/M progression rather than p53-dependent cell death (Hollander et al. 1999). Accordingly, Gadd45a-null cells exhibit attenuated G2 checkpoint activation upon DNA damage (Wang et al. 1999). Future studies are needed to determine whether this mechanism underlies neural tube defects.

A second proposed mechanism for Gadd45a function in brain development involves the MAPK kinase kinase (mitogen-activated protein kinase kinase kinase), MEKK4, which is known to promote neuronal migration and maturation (Sarkisian et al. 2006; Yamauchi et al. 2007). Gadd45a directly binds and activates MEKK4 (Takekawa and Saito 1998). Furthermore, knockdown of MEKK4 reduced neurite arborization in cortical neurons in vitro, recapitulating the effect of Gadd45a reduction and suggesting a common functional pathway in neuron maturation (Sarkisian and Siebzehnrubl 2012). The Gadd45a-MEKK4 pathway furthermore has been shown to stimulate phosphorylation of JNK (c-Jun N-terminal kinase), a subfamily of MAPK proteins, and this pathway modulates neurite outgrowth in a neuroblastoma cell culture (Yamauchi et al. 2007). This study additionally identified this linear pathway as a target of the mood-stabilizer valproic acid (VPA). VPA is used to treat manic-depressive states by regulating a number of mechanisms

targeting neurotransmitter uptake and catabolism, postsynaptic receptors, and histone deacetylation. VPA has been shown to stimulate adult neurogenesis, neurite extension, and neuroprotective mechanisms (Coyle and Duman 2003). VPA directly induces *gadd45a* expression in fibroblast and neuroblastoma cultures and adult cortical neurons (Yamauchi et al. 2007; Sarkisian and Siebzehnrubl 2012). Furthermore, in neuroblastoma cells, VPA was shown to induce *gadd45a* expression in association with neurite extension. Gadd45a is necessary for the VPA-induced neurite extension effect, and overexpression of *gadd45a* is sufficient to recapitulate this phenotype. Both VPA and Gadd45a require MEKK4 expression to stimulate cell maturation, suggesting a conserved role of the Gadd45a-MEKK4 interaction in neuronal maturation. Additionally, this interaction promotes the canonical MAPK cascade upon VPA exposure: Both VPA and *gadd45a* overexpression were found to enhance phosphorylation of JNK and its focal adhesion protein effector, paxillin, and this is dependent on MEKK4. Finally, neurite extension induced by both VPA and Gadd45a requires the ability to phosphorylate both JNK and paxillin. Interestingly, the activity of ERK, a separate MAPK, is necessary for maturation by VPA but not Gadd45a, suggesting VPA requires a broader range of MAPK function to exert a morphological effect. In a separate paradigm in which neurite extension is promoted by depriving cells of serum, JNK and paxillin phosphorylation is necessary but only partially sensitive to Gadd45a knockdown (Yamauchi et al. 2006). Taken together, these findings reveal that Gadd45a mediates neuronal maturation to differing extents in a context-dependent manner. The in vivo developmental implications of this result, however, are not fully clear. Furthermore, the VPA- and serum deprivation-induced pathways may have a convergent point, but it is unclear where this lies.

Gadd45 has also been identified as a downstream target of miR-130a-3p (involved in neurons differentiation) and miR-152-3p (plays a role in cardiac fibrosis and several cancer) as obtained using PC-12 cells (Łuczkowska et al. 2020). Further, Gadd45a is a downstream target of Sonic hedgehog (Shh), a morphogen with diverse neurodevelopmental roles (Galvin et al. 2008). Shh is known to signal through the Gli family of transcription factors; in neural stem cells, Shh was found to induce Gli1 selectively, and overexpression of Gli1 strongly induced *gadd45a* transcription. *Gadd45a* overexpression in neural stem cells induces the G2/M phase arrest and the prevalence of the pro-apoptotic marker, activated caspase-3. Gli1 may bind the *gadd45a* promoter directly or may act indirectly, such as via the p53 pathway. It is also hypothesized that Gadd45a functions in neural stem cell mitosis may coordinate the activity of the Cdc2 kinase and cyclin B1 as it does in other cell lines (Jin et al. 2002; Maeda et al. 2002). In light of the potential role of the Shh-Gli1 pathway in adult neurogenesis within the hippocampus and subventricular zones of the brain, it is additionally possible that Gadd45a may influence this process. However, adult neurogenesis has not yet been assessed in *gadd45a* mutants. Taken together, these results show Gadd45a likely functions through at least two signaling pathways which may regulate different aspects of nervous system development at both an anatomical and cellular scale.

The mechanistic basis for Gadd45b and Gadd45g in nervous tissue development has been less thoroughly studied. However, both proteins are also involved in MEKK4 activation in human cells, suggesting possible roles in development that may also be redundant with those of Gadd45a (Takekawa and Saito 1998). In a murine embryonic carcinoma cell line, each of the *gadd45* genes was robustly up-regulated in response to retinoic acid (RA), a universal morphogen critical to mammalian embryonic patterning (Sheng et al. 2010). RA binds a heterodimeric nuclear complex and coordinates a host of transcriptional events in development. In neural development, RA was shown to antagonize signaling by bone morphogenetic protein (BMP), a crucial developmental player whose activation stimulates a cascade of phosphorylation of Smad cytoplasmic effectors. Activated Smad complexes translocate to the nucleus to

orchestrate a pattern of gene expression affecting the developmental trajectory of the tissue. In the mammalian dorsal–ventral axis, BMP signaling is restricted to the dorsal region, whereas RA signaling resides in the intermediate region. During neurulation, BMP naturally promotes proliferation of progenitor cells and inhibits neurogenesis. In the chick neural tube, it was confirmed that RA suppresses BMP signaling and promotes proliferation and differentiation of neural progenitor cells (Sheng et al. 2010). In the dorsal neural tube, the interplay between these factors also coordinates specification of neural progenitors in the primitive spinal cord. The induction of *gadd45b* and *gadd45g* in vitro appears to be functional in the RA-induced downregulation of phospho-Smad1. Indeed, reduction in Gadd45b and Gadd45g expression restores pSmad1 in the presence of RA and disrupts the RA-induced association between pSmad1 and Smurf2, an E3 ubiquitin ligase. Since MAPK signaling was similarly shown to mediate the reduction in pSmad1, it is a reasonable to hypothesize that RA-mediated induction of the *gadd45* genes triggers a MAPK cascade which leads to the reduction in pSmad1 levels and that the resulting alterations in transcriptional programming promote a neuronal cell phenotype. The mechanisms by which Gadd45b and Gadd45g regulate neuronal differentiation are also not fully clear. Indeed, it appears Gadd45g influences molecular pathways of cell cycle arrest and hence promotes neuronal development indirectly rather than directly (Candal et al. 2005).

The breadth of upstream regulators of transcription of the Gadd45 family in neurogenesis is complex and not fully understood. For instance, in a similar culture system, the proneurogenic protein Ascl1, a member of the basic helix-loop-helix family of transcription factors, was shown to induce *gadd45g* by binding two E-box elements in its promoter (Huang et al. 2010). Neurogenin-2 and Mash1, regulators of dorsal and ventral telencephalon development, respectively, similarly coordinate *gadd45g* transcription (Gohlke et al. 2008). Since these transcriptional factors regulate glutamatergic and GABAergic neuron development, respectively, additional studies are needed to address the possible function of Gadd45g in either or both of these classes. Additionally, the in vivo function of Ascl1 during neuronal differentiation in the embryo has not yet been confirmed. One additional pathway regulating Gadd45g expression in conjunction with *Xenopus* neuronal development involves the *iroquois* family of genes encoding repressors of neural differentiation (de la Calle-Mustienes et al. 2002). Indeed, *Xiro1* overexpression suppresses *gadd45g* transcription, but this may be an indirect consequence of its induction of neuronal repressors. In early *Xenopus* neural development, *gadd45g* expression prefigures many territories where cells will stop dividing, and it is hypothesized that it links cell cycle arrest, neuronal differentiation, and Notch signaling. In the neural plate, cells expressing the Notch receptor XDl1 escape lateral inhibition from nearby cells; concurrent expression of *gadd45g* in these cells permits them to exit the cell cycle and differentiate into neurons through a process likely dependent on cyclin and cyclin-dependent kinases. Indeed, knockdown of Gadd45a and Gadd45g produced growth arrest and upregulation of cell cycle inhibitors p21, p15 and cyclin G1 as well as p53 (Kaufmann and Niehrs 2011). In contrast, neighboring cells undergoing Notch signaling exhibit decreased expression of *gadd45g* and proneural genes, and these fail to undergo differentiation (Gohlke et al. 2008). Finally, it should be noted that the phenotypes observed from single gene manipulation studies may belie the full effect of the Gadd45 family because of compensatory autoregulation (Kaufmann and Niehrs 2011).

9.3 Neuronal Lesions

The majority of studies of the Gadd45 genes in nervous system focuses on their dynamic regulation and function in pathological states. Here, we review the role of the Gadd45 family in conditions of ischemia, physical and chemical injury, neoplasm, and seizure-associated excitotoxicity.

9.3.1 Ischemia

Early studies of Gadd45a in the brain argued for a key role in regulating neuronal response to damage by conditions of low perfusion. In cases of global or localized ischemia, oxygen and glucose deprivation results in ATP reduction and energy depletion, and this often triggers excitotoxicity and cell death due to excessive glutamate release and stimulation of intracellular calcium signaling (Taoufik and Probert 2008). This condition is most prevalent in human stroke, and ongoing studies aim to uncover the underlying aberrant signaling mechanisms and novel therapeutic avenues.

In a neonatal rodent model of focal ischemia involving unilateral, transient occlusion of the common carotid artery, a well-delineated cortical infarct showing characteristic molecular and morphological features of apoptosis is produced after reperfusion (Charriaut-Marlangue et al. 1999). Gadd45a protein levels were elevated in cortical layers II and III, but the most salient feature of this upregulation is its association with cells that did not experience DNA fragmentation as evidenced by the TUNEL stain. Furthermore, these cells were largely localized in the penumbra, the border of the infarct. In contrast, adjacent TUNEL-positive cells largely failed to show upregulation of Gadd45a (Fig. 9.2). The authors posed that Gadd45a plays a protective function in cells experiencing ischemic stress. However, this expression may decrease once double-stranded DNA breaks become prevalent and defy DNA repair mechanisms; these cells may then become committed to apoptosis. This model predicts that the growing infarct is always delineated by a Gadd45a-positive border which demarcates the extent of cerebral damage and that the border expands as damaged cells lose Gadd45a expression. Similarly, another study of transient focal ischemia confirmed that Gadd45a mRNA increases broadly throughout the ischemic cortex 4 h after the onset of damage but is restricted to the penumbra after 24 h (Jin et al. 1996). At both time points, Gadd45a protein is more selectively expressed in sublethally injured cells of the penumbra. This also suggests a pretranslational mechanism controls Gadd45a levels in association with sublethal degrees of ischemic damage. In similar models, increases in Gadd45a transcripts were found in pyramidal neurons, the principal excitatory units of the cortex, at the edge of infarct and in association with reversibly damaged cells (Hou et al. 1997; Li et al. 1997b). Expression of the Gadd45 family in relation to the survival rate of interneurons after ischemic damage, however, has not been investigated. The pattern of Gadd45a expression broadly during induction of neuronal damage and more focally during the recovery phase was replicated in a similar focal ischemia model (Schmidt-Kastner

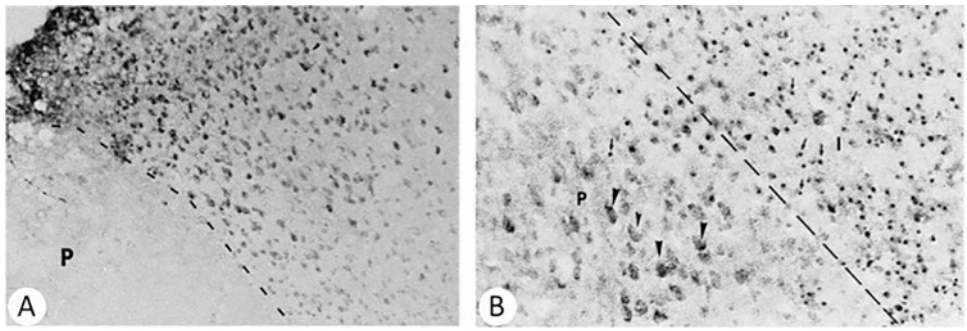

Fig. 9.2 Expression of Gadd45a protein in the cortex after ischemia induced by unilateral MCA occlusion in the neonatal rat. (**a**) After 24 h, Gadd45a was detected in layers II and III and largely absent from the penumbra (P). (**b**) Double staining for Gadd45a immunoreactivity and TUNEL assay performed at 48 h. Small arrows indicate TUNEL-positive nuclei, which are mostly localized to the infarct (I) rather than the penumbra. Arrowheads delineate Gadd45a-positive cells in the penumbra, suggesting a protective function of Gadd45a. Reproduced with permission from Charriaut-Marlangue et al. (1999)

et al. 1998). *Gadd45a* expression was most strongly associated with modestly damaged brain regions, suggesting a protective function. Even the dorsomedial cortex and dorsal hippocampus, regions spared from damage, exhibited enhanced Gadd45a levels. These responses could represent a prophylactic mechanism by cells sensing distant ischemic damage possibly because of spreading waves of perifocal depolarization and resultant gene induction in unaffected brain regions. Accordingly, in the hippocampus, forebrain ischemia produces strong Gadd45a induction in apoptotic granule cells of the dentate gyrus but only weak induction in largely necrotic CA1 pyramidal cells (Li et al. 1997a). Recent studies showed that Gadd45b and its target, BDNF expression levels increased during global ischemia suggesting that Gadd45b plays a protective against neuronal insults (Cho et al. 2019).

The hippocampus is still subject to ischemic damage in other rodent models and shows similar patterns of Gadd45a induction, particularly 24 h after ischemia (Wang et al. 2011). Additionally, in a model of transient global ischemia in the rodent brain, Gadd45a transcripts were rapidly induced in the dentate gyrus and, shortly afterwards, in the CA1 and CA3 subfields (Chen et al. 1998). Expression remained elevated 24–48 h after ischemia only in CA1. Interestingly, while protein expression was induced in all three regions, by 72 h expression was reduced below baseline in CA1. Similarly, neurons with double-stranded breaks failed to show Gadd45a expression in the striatum and thalamus. This finding correlates with strong evidence of DNA fragmentation at this time point, suggesting again that Gadd45a expression in global ischemia plays a protective role after sensing DNA damage but is no longer induced when cells, predominantly neurons, are largely damaged.

Little is known of the molecular mechanisms by which the Gadd45 genes potentially influence post-ischemia recovery. One study, however, points to NF-κB in a model of neonatal hypoxia-ischemia (Nijboer et al. 2009). This transcription factor regulates numerous target genes during inflammation and influences cell death and survival. In ischemia, NF-κB inhibition protects the brain from injury and blocks ischemia-induced upregulation of *gadd45b*. Since Gadd45b is known to inhibit JNK signaling and subsequent activation of the transcription factor AP-1, the authors speculate that Gadd45b mediates signaling between NF-κB and the JNK cascade in association with cell survival (De Smaele et al. 2001). Indeed, inhibition of JNK abrogated the neuroprotective effect of NF-κB inhibition. It should be added that NF-kB regulates the Gadd45b expression and DNA demethylation in hippocampal neurons during fear formation (Jarome et al. 2015). However, a causative role of Gadd45b or Gadd45a, which similarly mediates MAPK signaling, in cerebral ischemia has not yet been established.

Given this speculative role in recovery from ischemia, Gadd45b inhibition could serve as a novel therapeutic target in stroke. However, one study which showed cerebral ischemia induces *gadd45b* expression in the cortex contradicts this notion (Liu et al. 2012). Electrical stimulation of the fastigial nucleus of the cerebellum was found to induce further cortical Gadd45b expression, particularly in cell nuclei. This treatment improved recovery from the injury as assessed by a motor task 1 month after the injury. This suggests a positive association between Gadd45b expression and stroke recovery. Further studies are needed to delineate the precise role of the Gadd45 genes in ischemic recovery and to investigate the therapeutic potential of targeting their expression and function.

9.3.2 Neuronal Injury

Numerous studies have investigated expression patterns and functionality of the Gadd45 family after neuronal lesions. Here, we summarize results pertaining to both physical and chemical lesion models.

9.3.2.1 Physical Neuronal Lesions

A number of studies have assessed Gadd45 transcription in association with peripheral nerve injury in rodent models. Transection of the sciatic nerve (axotomy) has been shown to induce

gadd45a transcription robustly in dorsal root ganglion (DRG) cells, the primary afferent stream for peripheral sensory information, days after the injury (Costigan et al. 2002; Xiao et al. 2002; Befort et al. 2003; Lin et al. 2011). *Gadd45g* is induced to a lesser extent, whereas *gadd45b* is only modestly up-regulated in the DRG, suggesting all three Gadd45 genes may mediate the injury response but that Gadd45a plays the strongest role (Befort et al. 2003). Supporting this hypothesis is the co-localization of Gadd45a transcripts with c-jun and ATF3, markers of injury, in primary sensory neurons of the DRG as well as motor neurons in the ventral spinal cord after sciatic nerve transection (Fig. 9.3). This is a surprising finding because these motor neurons and DRG cells have cell bodies located in different regions, but both cell types have axons running in the periphery and the ability to survive and regenerate following injury. Similarly, Gadd45a is induced across all cell types broadly in the DRG after other peripheral nerve lesions such as the more proximal spinal segmental nerve lesion and sciatic nerve crush. Likewise, Gadd45a is up-regulated after more distal lesions including chronic constriction of the sciatic nerve and spared nerve injury in which the tibial and common peroneal nerves are ligated and sectioned.

In contrast, a dorsal rhizotomy lesion involving transection of dorsal roots proximal to the DRG cell bodies only modestly induced Gadd45a, suggesting Gadd45a plays different roles in transcriptional networks prompted by central and peripheral injuries (Befort et al. 2003). Indeed, the dorsal rhizotomy spares axonal contact between the periphery and DRG neurons and results in very little cell death and biochemical

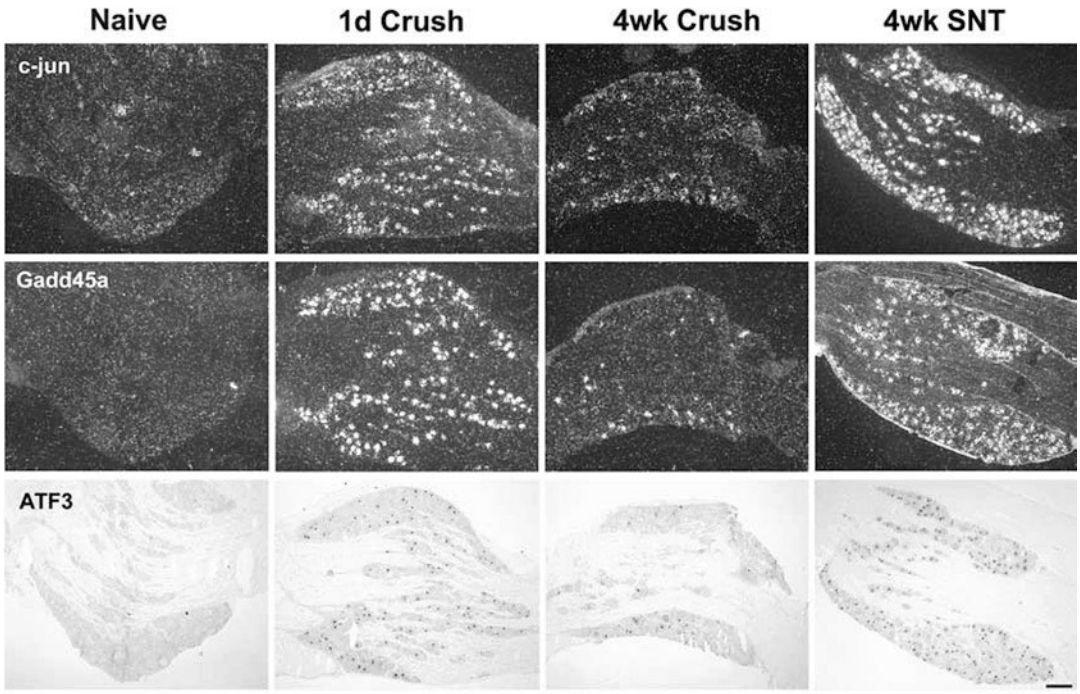

Fig. 9.3 Expression of *gadd45a* mRNA in the dorsal root ganglion (DRG) after sciatic nerve injury. Shown are sections of L4 DRGs from naïve, 1 day or 4 week after crush injury sections. Additionally, 4 week post-sciatic nerve transection (SNT) sections are displayed. Transcripts of c-jun (top row), Gadd45a (middle row), and ATF3 (bottom row) are presented. One day after crush injury, all markers are up-regulated, and Gadd45a expression remains elevated when regrowth is prevented (4 weeks transection with ligation). Gadd45a expression is down-regulated in the DRG when peripheral nerve regrowth after crush injury has completed (4 week crush). Scale bar, 200 μm. Reproduced with permission from Befort et al. (2003)

changes in comparison to those spurred by peripheral damage. It is interesting to note that injury associated *gadd45a* induction in the DRG reverts to its pre-injury, virtually undetectable baseline level when injured axons are allowed to regrow and reinnervate peripheral targets. This reduction is not seen when repair is physically impaired, and this suggests a retrograde signal from the target cells attenuates *gadd45a* expression when the injury is repaired. Similarly, direct injury to the spinal cord enhanced the expression of cell cycle regulators including *gadd45a*, particularly in neurons (Di Giovanni et al. 2003). We also note the unique role of Gadd45a expression after injury in that embryonic and postnatal DRG cells do not express the gene at detectable levels (Befort et al. 2003). Hence, the injury-induced response is truly a de novo phenomenon and not merely a recapitulation of expression patterns in early ontogeny as is the case with other regeneration- and survival-associated genes such as GAP-43 and Hsp27.

These models are clinically relevant in relation to molecular mechanisms of neuropathic pain, defined as pain initiated by primary lesions or dysfunction of the nervous system. Indeed, *gadd45a* is induced along with other cell cycle and apoptosis regulators in the DRG following spinal nerve transection or ligation (Wang et al. 2002; Xiao et al. 2002). However, one study challenges the notion that direct injury to afferent sensory nerves is required for pain-associated transcriptional programming; the authors examined the possibility that incisional pain induced by skin lesions that spare nerve injury could induce regeneration-associated transcription in the DRG (Hill et al. 2010). Indeed, DRG neurons innervating the affected skin region exhibited moderately enhanced *gadd45a* expression. This effect likely follows from sensitization of the neurons exposed to skin wound-related processes and inflammation and suggests tissue injury even in the absence of direct nerve injury can promote a state of enhanced growth capacity in sensory neurons. Gadd45a may regulate this process. However, induced acute local inflammation failed to up-regulate *gadd45a* in the DRG cells innervating the corresponding peripheral site (Befort et al. 2003). One explanation for this discrepancy is that inflammation alone is insufficient to induce *gadd45a*; this reinforces the notion that *gadd45a* induction reflects a highly regulated response to the presence and maintenance of peripheral nerve injury and not merely the presence of cellular stress.

It has become clear that Gadd45a expression in the DRG not only correlates with peripheral nerve injury but also mediates survival potential of the afferents. After spinal nerve ligation, adult DRG cells remain largely intact and correlate with strong Gadd45a mRNA and protein induction patterns, whereas neonatal DRG cells, which show undetectable *gadd45a* expression, are highly susceptible to cell death (Lin et al. 2011). Knockdown of Gadd45a in the DRG impairs survival and promotes apoptosis after injury. Concomitantly, overexpression of *gadd45a* in vitro protects DRG cells from nerve growth factor (NGF) withdrawal-induced apoptosis, and this effect may be mediated by maintenance of anti-apoptotic Bcl-x_L levels. Further, Gadd45 expression levels were shown to associated with attenuated emotional and cognitive manifestation of neuropathic pain (Martínez-Navarro et al. 2019).

These studies of peripheral nerve lesion-induced *gadd45a* regulation suggest that Gadd45a-regulated survival mechanisms may be a promising target of therapy in neuropathic pain and denervation conditions. The utility of such therapy may not be limited to neurons, though, as skeletal muscle similarly experiences a prolific enhancement in *gadd45a* transcription after denervation (lower motor neuron loss) or spinal cord injury (upper motor neuron loss) (Zeman et al. 2009). Accordingly, the finding of Gadd45a elevation extends to other nerve injury models as well. After optic nerve transection, both primary (initial lesion site) and secondary (regions beyond the initial lesion site) neurodegeneration exhibits enhanced *gadd45a* transcripts (Levkovitch-Verbin et al. 2011). Interestingly, elevated protein expression persists longer in the secondary region, a similar finding to the previously discussed reports of Gadd45a elevation in the penumbra of tissue damaged by ischemia. Elevation

and nuclear translocation of Gadd45a and known binding partner PCNA were also found in apoptotic cells, localized to the margins of the cortical contusion and hippocampus, in a rodent traumatic brain injury model (Kaya et al. 1999). These studies further support the protective role of Gadd45a after neuronal insult. It is possible, however, that injury-related *gadd45b* expression plays an opposing role. For instance, mice carrying a mutation that confers protection against Wallerian degeneration, which involves fragmentation of axon segments separated from their somas, myelin sheath segmentation, and removal of debris by Schwann and immune cells, show reduced expression of *gadd45b* and other plasticity-associated genes compared to wildtype mice after sciatic nerve lesion (Barrette et al. 2010). Still, a causative role of Gadd45b in injury-related processes has not been established, and altered expression is not necessarily functional. Collectively, these results strongly implicate Gadd45a in the protection of neurons after physical nerve injury both proximal and distal to the affected cells.

9.3.2.2 Neuronal Injury by Non-physical Insults

Nerve damage results not only from physical insults such as trauma and skin incisions discussed above but also from exposure to other environmental neurotoxins. A number of reports document damage-associated regulation of the Gadd45 family. Early studies, for instance, showed induction of *gadd45a* transcripts following gamma irradiation exposure in various tissue including the brain (Yoshida et al. 1994, 1996). This confirmed previous reports of similar *gadd45a* upregulation following ionizing radiation in vitro, including the original report identifying the *gadd45a* gene in irradiated Chinese hamster ovarian cells (Fornace et al. 1988; Papathanasiou et al. 1991). Irradiation-associated DNA damage is known to modulate p53, and it is postulated that this mechanism is responsible for Gadd45a signaling in response to cell damage (Yoshida et al. 1996).

Chemical injury associated with neuropathology produces similar induction patterns. Neurodegeneration characterized by ataxia, paralysis, and axonopathy follows from exposure to organophosphorus-ester chemicals, and a study of this condition in hens found enhanced *gadd45a* and *Bcl-2* expression in the cerebrum, cerebellum, brain stem, and spinal cord (Damodaran et al. 2011). Enhanced *gadd45g* expression was also associated with exposure to the environmental neurotoxin, carbonyl sulfide, in the posterior colliculi, an especially susceptible brain region that regulates auditory processing (Morrison et al. 2009). While few studies have investigated damage-induced Gadd45g induction in the brain, it is likely to play a similar role in cellular stress response as previously shown in chemical-, radiation-, and inflammation-associated induction in other cell lines (Zhang et al. 1999; Jung et al. 2000). Chemical damage to the auditory system is also associated with altered *gadd45a* expression. For example, salicylic acid, the main ingredient in Aspirin, impairs hearing at high concentrations; salicylic acid damaged spiral ganglion neurons and peripheral fibers in rodent cochlear organotypic cultures and induced *gadd45a* in association with apoptosis (Wei et al. 2010). Its temporal expression recapitulates the protective pattern found in ischemia and physical nerve lesion studies in the auditory system in a model of noise-induced apoptosis in the cochlear epithelium and lateral wall (Hu et al. 2009). Namely, *gadd45a* expression is enhanced shortly after noise exposure but is reduced even below baseline after 1 week during a broad pro-apoptotic response. Thus, *gadd45a* expression peaks when cells are trying to stay alive but falls once they "give up" and enter apoptosis.

An alternative mechanism of chemical injury associated with altered Gadd45 signaling involves toxicity resulting from excessive neuronal activity. This can be induced by pharmacological agents that boost excitatory, glutamatergic signaling or impair inhibitory tone. For instance, quinolinic acid, an NMDA receptor agonist and driver of neuronal activity, injected into the striatum produces DNA fragmentation, p53 activation, and enhanced Gadd45a transcript and protein expression in the striatum and cortex (Hughes et al. 1996). Gadd45a exhibited both

rapid and prolonged upregulation, and it is hypothesized to be regulated initially by immediate early gene (IEG) transcription factors such as Fos and Jun-B and later by nuclear p53. In a different model, enhancing glutamatergic signaling by chemical inhibition of glutamate reuptake in the striatum prompted *gadd45a* expression (Lievens et al. 2000). Enhanced transcripts were found in the periphery of the lesion after short-term lesion, reflecting both neuronal and glial expression, but after long-term quinolinic acid treatment, Gadd45a was only up-regulated within the lesion core, composed mostly of reactive astrocytes. These results suggest that Gadd45a is associated with neuroprotection and preventing the core lesion site from expanding and that its protective effects may not be limited to neurons.

9.3.2.3 Neuronal Injury by Drugs of Abuse

A recent study linked Gadd45b to drugs of abuse mainly cocaine. The authors found that absence of Gadd45b lowers the expression of genes involved in psychostimulant addiction (Zipperly et al. 2021). Further, they described that silencing Gadd45b blocks the induction of immediate early genes by dopamine receptor D1 (DRD1). These results prevent DRD-1 mediated changes in DNA methylation. Finally, the absence of Gadd45b decreases striatal neurons action potential burst duration in vitro suggesting that striatal Gadd45b is necessary for cocaine reward memory (Zipperly et al. 2021).

9.3.2.4 Neoplasia

An extensive literature characterizes the aberrant function and regulation of the Gadd45 family in various cancers including pancreatic, hepatocellular, lung, cervical and gastrointestinal carcinomas, and different lymphomas (Tamura et al. 2012). The *gadd45* genes contain genetic or epigenetic alterations in these conditions. Accordingly, the Gadd45 family has emerged as a potential target in anti-tumor therapy including drugs that promote expression by upstream signaling control and epigenetic regulation.

Studies examining the Gadd45 proteins in nervous system neoplasias have predominantly focused on pituitary adenoma, pilocytic astrocytoma, and medulloblastoma. Pituitary adenomas are monoclonal tumors and the most prevalent intracranial neoplasms and present clinically in relation to the specific subclass of cells undergoing growth; these include cells that secrete prolactin, growth hormone, ACTH, and gonadotropes. Postmortem analysis of human gonadotrope tumors revealed substantially reduced Gadd45b and Gadd45g mRNA and protein but normal Gadd45a expression (Zhang et al. 2002; Michaelis et al. 2011). Additionally, most nonfunctioning, growth hormone-secreting, and prolactin-secreting pituitary tumors failed to show detectable Gadd45g transcripts, whereas expression was found unanimously in normal pituitary tissue (Zhang et al. 2002). These reductions likely play a functional role in tumorigenesis because overexpression of *gadd45g* in various pituitary tumor lines and *gadd45b* in gonadotrope tumors attenuates colony formation in vitro. Gadd45b was also shown to promote apoptosis in gonadotrope cells. These proteins likely act through common G1/S and G2/M growth arrest mechanisms. However, their upstream regulation is unclear, as p53 gene mutations are not common and NF-κB is not up-regulated in pituitary tumors (Michaelis et al. 2011). Additionally, these mechanisms exhibit cell origin-specificity, as ACTH-releasing tumors demonstrated a slight upregulation, not reduction, of *gadd45b*.

Although Gadd45a has not been shown to modulate pituitary neoplasms, its expression is enhanced in pilocytic astrocytoma tissue (Jacob et al. 2011). These low grade, sporadic tumors predominate childhood CNS neoplasms and tend to affect the cerebellum and optic nerve pathways. Altered MAPK activation in pilocytic astrocytomas is thought to drive senescence, a hallmark of these tumor cells. Gadd45a and other senescence markers are speculated to drive this activity; of note, Gadd45a has been shown to promote MAPK-induced senescence in skin cancer (Hildesheim et al. 2002).

Altered Gadd45a signaling has also been implicated in cerebellum-originating medulloblastoma, the most common malignant CNS tumor in children (Chou et al. 2001; Asuthkar

et al. 2011). In tumor cells, NGF causes apoptosis by binding the receptor, TrkA, and this interaction is necessary for coincident NGF-induced *gadd45a* expression (Chou et al. 2001). Mutations in TrkA that block apoptosis also abolish *gadd45a* induction, providing correlational evidence that Gadd45a regulates NGF-induced apoptosis. This study intriguingly supports evidence for an entirely novel pathway for Gadd45a regulation by NGF; NGF-induced ERK, p38 and JNK activation (all MAPK proteins) is similar in medulloblastoma and pheochromocytoma lines in which NGF produces opposite effects on apoptosis. Additionally, p38 was shown to be unnecessary for NGF-induced apoptosis in medulloblastoma cells. This suggests some CNS tumor lines harbor different mechanisms of *gadd45a* regulation that do not necessary rely on p53 and MAPK signaling. One possibility is a pathway mediated by the medulloblastoma-associated tumor suppressor gene, PATCHED1, and Sonic hedgehog signaling (Kappler et al. 2004).

Medulloblastoma cells also appear to utilize novel Gadd45a-dependent mechanisms in association with radiation exposure (Asuthkar et al. 2011). Radiotherapy attenuates tumor growth but also activates sporadic recurrences in part by activating matrix metallopeptidase (MMP)-9, which helps dissolve extracellular matrix to aid in cell migration (Goc et al. 2013). IR-induced growth arrest in cultured medulloblastoma cells was associated with enhanced expression of Gadd45a and its binding partner, Cdc2, and Gadd45a was shown to promote arrest and apoptosis (Asuthkar et al. 2011). The Gadd45a-Cdc2 interaction is likely important for growth arrest in these cells as was in colon carcinoma cells (Jin et al. 2000). *Gadd45a* overexpression blocked invasion potential of irradiated medulloblastoma cells and reduced MMP9 expression in vitro and in vivo (Asuthkar et al. 2011). Gadd45a also promotes p53 activation and modulates the membrane expression of β-catenin and its binding partners E- and N-cadherin. This effect on transmembrane proteins likely affects invasion potential by regulating cell–cell contact. Together these findings implicate Gadd45a as a critical regulator of tumorigenesis that acts through diverse signaling pathways affecting cell growth, apoptosis, and cell–cell interaction.

9.3.2.5 Seizures

We previously discussed the involvement of the Gadd45 family in excitotoxicity induced by chemical lesions. Related to these findings are a number of studies documenting altered expression of these genes in association with seizures. Here we review these results, highlighting the neuroprotective role of these genes in excitotoxic conditions.

Seizures are defined as brief behavioral changes in response to abnormal, synchronized, and repetitive burst activity of neuron populations, and epilepsy is characterized as a syndrome of recurrent, spontaneous seizures (Shin and McNamara 1994). Partial seizures emanate from a localized brain region, whereas generalized seizures exhibit diverse, bilateral activity. Excessive glutamatergic signaling characterizes seizure activity and associated necrotica, apoptotic, and autophagic cell death (Wang and Qin 2010). Aberrant downstream mechanisms include intracellular calcium homeostasis, free radical production, kinase and protease activity, transcription factor activity, and IEG activation.

Human temporal lobe epilepsy, in which excitotoxic cell death appears in the limbic system and related structures, is commonly modeled in the rodent by kainate treatment (Zhu et al. 1997). Kainic acid (KA) activates a subclass of glutamate receptors and likely produces excitotoxicity by inducing sodium influx, depolarization, and subsequent activation of NMDA receptors and calcium influx. KA-toxicity is associated with apoptosis and autophagy more than necrosis which often follows NMDA receptor-mediated cell death (Wang and Qin 2010). Subcutaneous KA injection was shown to induce Gadd45a expression in the parietal and piriform cortex, hippocampus, striatum, and thalamus, and focal intra-amygdala KA similarly induced expression in the limbic system, thalamus, and cortex (Zhu et al. 1997; Henshall et al. 1999). Moreover, intracerebral KA-induced *gadd45a* expression in the hippocampus (Choi et al. 2011). Transcript

expression was largely localized to neurons, and enhanced protein expression was also confirmed (Zhu et al. 1997). Moreover, both studies showed evidence of DNA fragmentation and apoptosis, especially in the CA3 subfield. *Gadd45a* transcription after subcutaneous KA is initially widespread but later remains elevated only in vulnerable regions. However, these regions exhibit decreased Gadd45a protein levels that coincide with the extent of neurodegeneration, suggesting that dying cells experience a translational block of Gadd45a as with other cell survival genes (Zhu et al. 1997). Amygdala-evoked seizures produced cell death and DNA fragmentation only in CA3 even though *gadd45a* transcription was widespread (Henshall et al. 1999). Still, transcript expression was highest in CA3; these findings reinforce the conclusion that cells up-regulate *gadd45a* in response to insults to remain viable, but that translation may eventually be impaired as cells commit to apoptosis. KA-induced *gadd45a* induction may occur in part through p53 signaling, but p53 is only activated in vulnerable regions after systemic KA (Zhu et al. 1997). Alternatively, KA-induced DNA modification in the form of 8-hydoxyl-2-deoxyguanosine may spur *gadd45a* expression as previously shown (Henshall et al. 1999).

Nevertheless, as with other forms of neurotoxicity, glutamate-driven *gadd45a* expression plays an ambiguous role in cell survival. In murine hippocampal cultures, for instance, glutamate treatment induces Gadd45a mRNA and protein expression in association with cell death, but knockdown of Gadd45a or p53, which is also phosphorylated by the treatment, rescues cell viability (Fig. 9.4) (Choi et al. 2011). However, this cell line lacks ionotropic glutamate receptors but is still subject to oxidative neurotoxicity. Accordingly, inhibition of reactive oxidative species signaling prevented glutamate-induced *gadd45a* induction. This study also provides evidence for a cell death model in which glutamate signaling induces oxidative species which then activates the MKK4-JNK-p53 pathway, triggering *gadd45a* transcription and apoptosis. It appears, therefore, that Gadd45a promotes cell death under certain conditions such as oxidative stress but may prevent excitotoxic cell death in vivo, a scenario that likely drives its expression through multiple pathways including non-p53 signaling. At a molecular level, however, it is still unclear what characterizes the protective or death-inducing role of Gadd45a.

In contrast, Gadd45b and Gadd45g appear to play a less equivocal role in neuronal survival after excitotoxic insults. In hippocampal neurons, cAMP-response element-binding protein (CREB), a key mediator of synaptic activity-dependent gene expression, promotes expression of both of these genes and coincidently confers protection against excitotoxic cell death (Tan et al. 2012). Synaptic neuronal activity exerts a neuroprotective effect against future insults in part through regulation of a class of activity-regulated inhibitor of death (AID) genes including *gadd45b* and *gadd45g* (Zhang et al. 2009). Glutamate-driven calcium signaling activates nuclear calmodulin-dependent protein kinase IV (CAMKIV), a critical mediator of CREB-dependent transcription of AID genes after periods of action potential bursting. Indeed, manipulations of *gadd45b* and *gadd45g* confirmed that these genes confer protection against chemically- and growth factor withdrawal-induced cell death in vitro and mediate activity-dependent resistance to cell death. Moreover, these genes ameliorate KA-induced hippocampal cell death in vivo. However, expression of the Gadd45 proteins may not always change, as KA-induced seizure preconditioning, a protective paradigm against future insults, failed to induce Gadd45b protein in the hippocampus (Miller-Delaney et al. 2012).

In adult rodents, seizures are associated with enhanced neurogenesis in the dentate gyrus, a unique niche of postnatal mitosis in the CNS (Naegele 2009). However, a consequence of hyperactivity-driven neurogenesis is that newborn neurons fail to integrate normally into granule cell layer targets and instead migrate to ectopic locations in the hilus; improper hippocampal rewiring often spurs hyperexcitability and epileptogenesis. Seizures were found to upregulate *gadd45g* and, more so, *gadd45b* in the dentate gyrus granule cell layer (Ma et al. 2009).

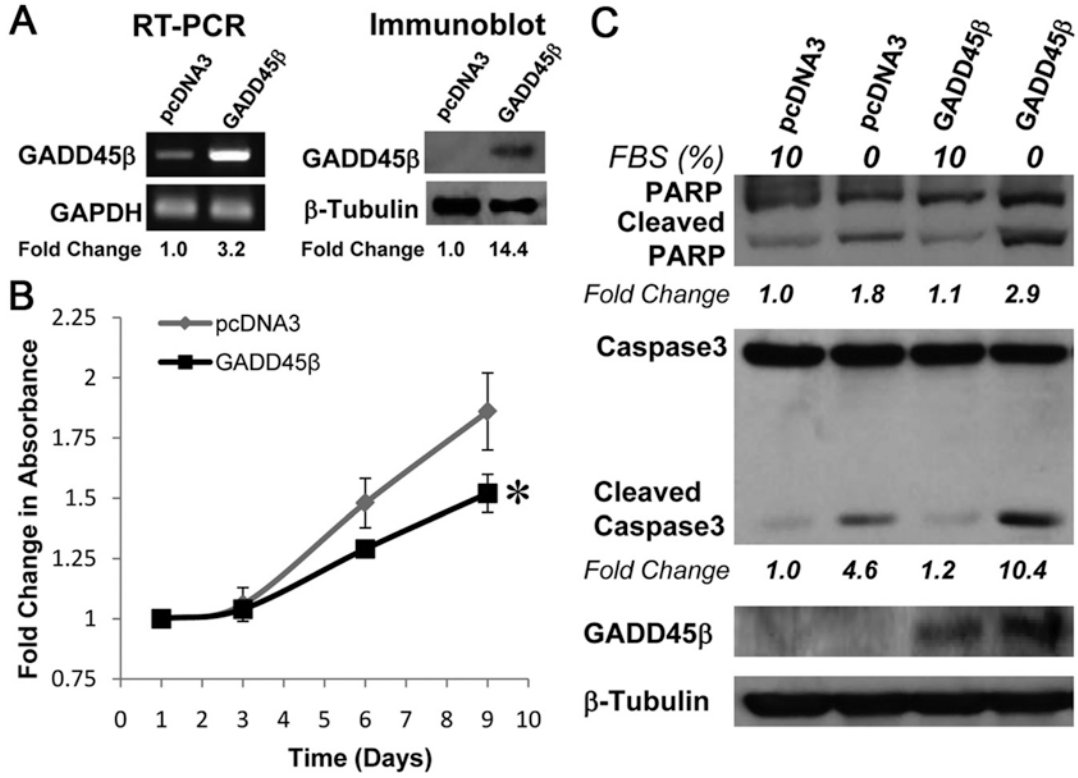

Fig. 9.4 Gadd45a mediates glutamate-induced oxidative cytotoxicity in HT22 hippocampal neuronal cell line. (**a**) *Gadd45a* transcripts and (**b**) Gadd45a protein levels were quantified after 5 mM glutamate treatment. Enhanced expression coincided with attenuated cell viability, assessed through MTT assay. (**c–e**) Cells were treated with 5 or 8 mM glutamate for 24 h after 24 h of pretreatment with Gadd45a siRNA. RT-PCR, MTT assay and phase contrast microscopy were used to confirm knockdown of *gadd45a* and rescue of cytotoxicity induced by 5 mM glutamate. Reproduced with permission from Choi et al. (2011)

Furthermore, Gadd45b knockdown or knockout impaired activity-driven proliferation of neural progenitors and dendritic development of newborn neurons (Fig. 9.5a–c). Together, these findings show Gadd45b and Gadd45g are especially critical in periods of hyperexcitability in guarding against cell death and, in the case of Gadd45b, postnatal mitosis and development.

9.4 Cognitive Neuroepigenetics

In the preceding discussion, we focused on mechanisms of the Gadd45 family in a host of conditions of cell proliferation and injury in the nervous system. Here we focus on the relatively recently appreciated contribution of the Gadd45 family to the emerging field of cognitive neuroepigenetics (Day and Sweatt 2010). The most salient difference between these and the previously discussed mechanisms is their function in normal, senescent adult neurons rather than those undergoing programmed cell death or proliferation. Neuroepigenetic mechanisms have instead been studied most exquisitely in relation to plasticity-associated mechanisms in neuronal function.

The term "epigenetics" was coined by Conrad Waddington who speculated that a landscape of pretranscriptional mechanisms mediates gene–environment interactions which control the phenotype of the cell. Epigenetic mechanisms were traditionally defined as heritable alterations in a chromosome that affect gene expression without affecting the underlying DNA sequence (Berger et al. 2009). A number of distinct molecular

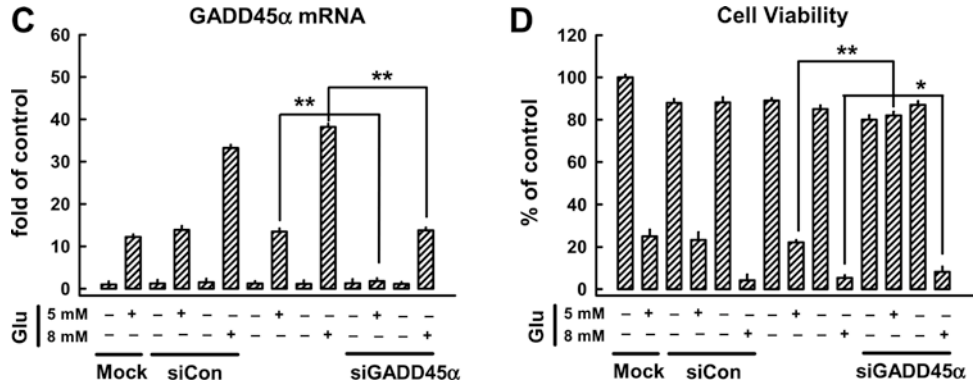

Fig. 9.5 Essential role of Gadd45b in activity-associated phenotypes in the adult CNS. (**a**–**c**) Wildtype and *gadd45b*-null mutant mice were injected with retroviruses expressing GFP to label adult-generated neural progenitors and their progeny. A single episode of electroconvulsive treatment (ECT) or sham treatment was given after 3 days, and dendritic morphology was assessed 14 days after virus injection. Mean dendritic length and dendritic complexity were impaired in knockout mutants after ECT but not in naïve mice. (**d**) Wildtype and *gadd45b*-null mutants were subjected to one of three background contextual fear conditioning paradigms of increasing robustness. Mutants exhibited heightened contextual fear memory 24 h after training, and this phenotype was most pronounced after mild conditioning. Reproduced with permission from Ma et al. (2009) (**a**–**c**) and Sultan et al. (2012) (**d**)

mechanisms fit this definition, but posttranslational modifications of histones and DNA methylation are considered the two canonical epigenetic phenomena.

In the nucleus, DNA is packaged in an elegant, hierarchical fashion. The first layer of compaction involves demarcation of nucleosomes, each of which is comprised of 147 bp of DNA wrapped around an octomer of histone proteins and a linker region with variable length in accordance with cell type and organism (Sadeh and Allis 2011). Nucleosomes are organized in nonrandom, regularly spaced arrays throughout the genome, and complex molecular regulators of nucleosome positioning and density are potent mediators of *cis*-acting transcriptional dynamics. Core histone units are composed of central globular domains and projecting N-terminal tails which bear several residues subject to modification by acetylation, phosphorylation, methylation, ubiquitination, and ADP-ribosylation (Sultan and Day 2011). A number of residue-specific "writer" and "eraser" enzymes corresponding to these marks have been characterized; likewise, "reader" proteins help translate the mark into an enhancement or suppression of transcription (Maze et al. 2013).

In addition to core histones and regulatory DNA-binding proteins, DNA itself also constitutes chromatin and can undergo covalent modification (Day and Sweatt 2011). Epigenetic DNA methylation usually refers to the addition of a methyl group to the 5′ position on a cytosine ring. Usually the target cytosine is followed by a guanine, termed a "CpG site." CpG sites occur at a lower frequency than statistically expected and tend to cluster in regions called "CpG islands," spans of DNA containing a high frequency of CpGs that are largely unmethylated. This is unsurprising, as CpG islands tend to exist at promoter regions of active genes, and DNA methylation usually (although not exclusively) represses transcription. DNA methylation is catalyzed by DNA methyltransferase enzymes, DNMT1, DNMT3a, and DNMT3b in adults (Grayson and Guidotti 2013). DNMT1 maintains DNA methylation in hemimethylated DNA strands after cell division so that complementary CpGs both carry the methyl mark. DNMT3a and DNMT3b catalyze de novo methylation by single carbon transfer from *S*-adenosylmethionine (SAM) to unmethylated cytosines. Methyl-cytosine readers include numerous methyl-binding domain (MBD) proteins that facilitate a transition of local

chromatin to a transcription-permissive state or repress it (Chahrour et al. 2008; Grayson and Guidotti 2013). Methylated cytosines can be passively demethylated after cell division if DNMT1 activity is suppressed, but reversing methylation in senescent cells, termed "active DNA demethylation," remains a vexing question and subject of future research (Wu and Sun 2009). Further, while Gadd45a, b, and g increase, the expression of DNMT3b decreases in the amygdala of adolescent after alcohol exposure, a phenomenon that contribute to adult anxiety later in life (Sakharkar et al. 2019).

9.4.1 Memory

Memory formation proceeds in a sequence of steps of increasing stability after the learning event (Miyashita et al. 2008). During consolidation, a hippocampus-dependent process for certain types of learning paradigms, information progresses beyond the initially labile phase into a long-term, stable trace. Both consolidation and maintenance, a cortical event, rely on a diverse array of orchestrated de novo gene transcription (Sweatt 2009). This well-replicated finding motivated a number of studies that implicate dynamic histone modifications in memory formation (Day and Sweatt 2011). Contextual fear conditioning, for instance, induces global changes in both permissive and repressive marks including H3K9 dimethylation, H3K4 trimethylation, H3S10 phosphorylation, and numerous H3 and H4 acetylation events (Chwang et al. 2006; Gupta et al. 2010; Peleg et al. 2010). Of particular interest clinical interest is the memory-boosting effect of inhibiting histone deacetylase complex (HDAC) 2 in hippocampus-dependent memory (Levenson et al. 2004; Guan et al. 2009).

Persisting molecular signatures are theoretically required for memory trace stabilization; the need to identify such mechanisms that defy erasure during normal neuronal metabolism prompted investigations into the role of DNA methylation, long thought to be a stable epigenetic mark, in memory formation (Day and Sweatt 2010). Active methylation in the hippocampus was indeed shown to regulate fear memory consolidation and synaptic potentiation, a cellular correlate of behavioral memory (Levenson et al. 2006; Miller and Sweatt 2007; Feng et al. 2010). Although gene-specific methylation and demethylation events were found after training, these marks reverted to baseline levels after 1 day, suggesting hippocampal methylation dynamics do not modulate memory persistence (Miller and Sweatt 2007). Additionally, active demethylation of genes including *reelin* and *bdnf*, active memory regulators, spurred the hunt for a demethylation signaling cascade (Miller and Sweatt 2007; Lubin et al. 2008).

Gadd45a was then implicated in active DNA demethylation of exogenous genes including the Oct4 promoter in an in vitro system, but this finding remains equivocal (Barreto et al. 2007; Jin et al. 2008). In the nervous system, robust induction of Gadd45b expression was similarly implicated in activity-induced DNA demethylation of the fibroblast growth factor (FGF)-1B and BDNF exon IXa promoters in the dentate gyrus (Fig. 9.6a) (Ma et al. 2009). Moreover, induction of the corresponding genes was impaired in Gadd45b-null mice, suggesting Gadd45b-mediated demethylation functionally regulates gene transcription. Importantly, seizure induction did not produce significant cell death or excitotoxicity in wildtype or mutant mice. Therefore, the effects of Gadd45b deletion are likely to reflect only plasticity-related induction of trophic factors by mature granule cells rather than seizure-associated reductions in cell viability.

The characterization of Gadd45b as a hippocampal IEG that facilitates epigenetic regulation of BDNF encouraged studies of Gadd45b in hippocampus-dependent memory. Indeed, *gadd45b* and *gadd45g* but not *gadd45a* transcripts were found to be up-regulated following fear memory learning in the hippocampus and amygdala (Leach et al. 2012; Sultan et al. 2012). Both transcripts were also induced by potassium depolarization and tetrodotoxin-withdrawal, a means of inducing quasi-synchronous activity, in neuron cultures (Saha et al. 2011; Sultan et al. 2012). Furthermore, Gadd45b-null mutants were found to exhibit enhanced hippocampus-

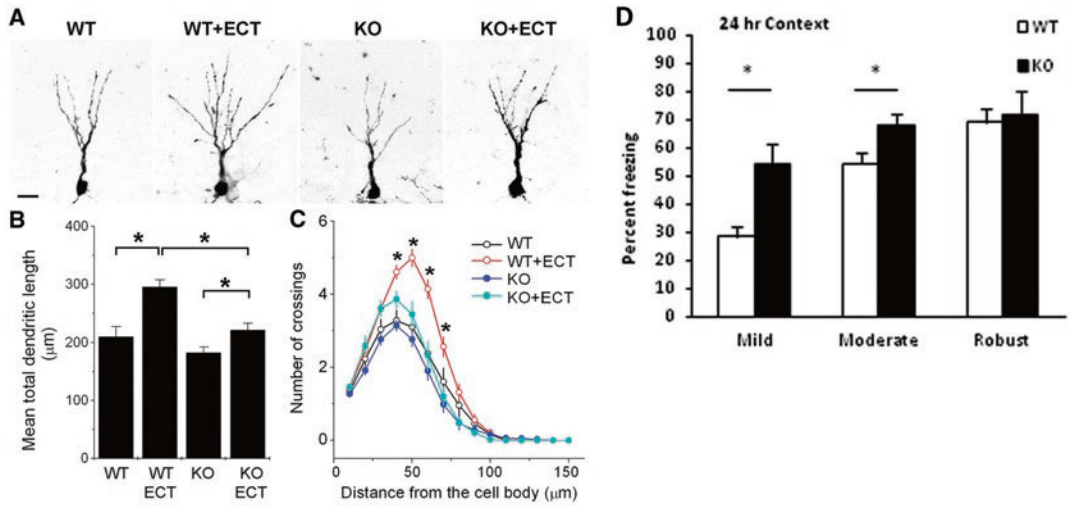

Fig. 9.6 Gadd45b regulates active DNA demethylation in an activity-associated manner. (**a**) Wildtype and *gadd45b*-null mutant mice were subjected to ECT or sham treatment. After 4 h, demethylation was found at the transcriptional start sites of *BDNF exon IX* and *FGF-1B* genes by sodium bisulfite sequencing of dentate gyrus tissue. Normal baseline DNA methylation was found between genotypes, but knockouts were impaired demethylation after ECT. (**b**, **c**) Schematics of the putative cytosine demethylation mechanism. DNMT enzymes catalyze single carbon transfer to produce 5-methylcytosine (5mC). The ten-eleven-translocase (TET) family of enzymes catalyze oxidation to 5-hydroxymethylcytosine (5hmC). TET1, TET3, and DNMT1 contain –CXXC– domains which bind clustered, unmethylated CpG dinucleotides. Oxidative deamination of 5hmC to 5-hydroxymethyluridine (5hmU) may occur through the AID (activity-induced cytidine deaminase)/APOBEC (apolipoprotein B mRNA-editing catalytic polypeptide) family of deaminases. Subsequently, the uracil-DNA glycosylase (UDG) family including thymine-DNA glycosylase (TDG, MBD4) and single-strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1) are thought to process 5hmU through a base-excision repair (BER) mechanism. Additional intermediates such as 5-formylcytosine and 5-carboxylcytosine may be generated as well. Gadd45b may facilitate this process although the mechanism is unknown. Reproduced with permission from Ma et al. (2009) (**a**) and Grayson and Guidotti (2013) (**b**, **c**)

dependent fear and spatial memory and long-term synaptic potentiation (Sultan et al. 2012). In particular, these enhancements were most prominent in conditions of mild behavioral and synaptic activity, suggesting Gadd45b regulates the threshold for memory formation but not capacity (Fig. 9.5d). Furthermore, mice performed normally in most baseline behavioral tasks and a test of amygdala-dependent fear memory, suggesting Gadd45b primarily functions in hippocampus-dependent tasks. One study, however, found contextual fear memory deficits in mutants (Leach et al. 2012). In addition to Gadd45b, the expression level of Gadd45γ was described to be elevated in the hippocampus of aged human, a phenomenon that could disrupt cAMP-response element-binding protein (CREB) signaling and its genes (Brito et al. 2020a, b). This increase and loss of function of CREB expression contributes to spatial memory impairments in young adult mice. These conflicting results may reflect differences in task parameters or epigenome–genome interactions during backcrossing. Still, these studies agree that Gadd45b selectively regulates hippocampus-dependent memory.

The breadth of loci targeted by Gadd45b for epigenetic regulated is largely unknown and a subject for future investigation (Fig. 9.7c). A large number of putative of targets exist, as synchronous neuronal activation was shown to modulate methylation in over 1% of CpGs assessed throughout the genome (Guo et al. 2011a). In this model, Gadd45b was found necessary for active demethylation for candidate sites including CREB-binding protein (CBP), a critical memory-associated transcription factor and epigenetic

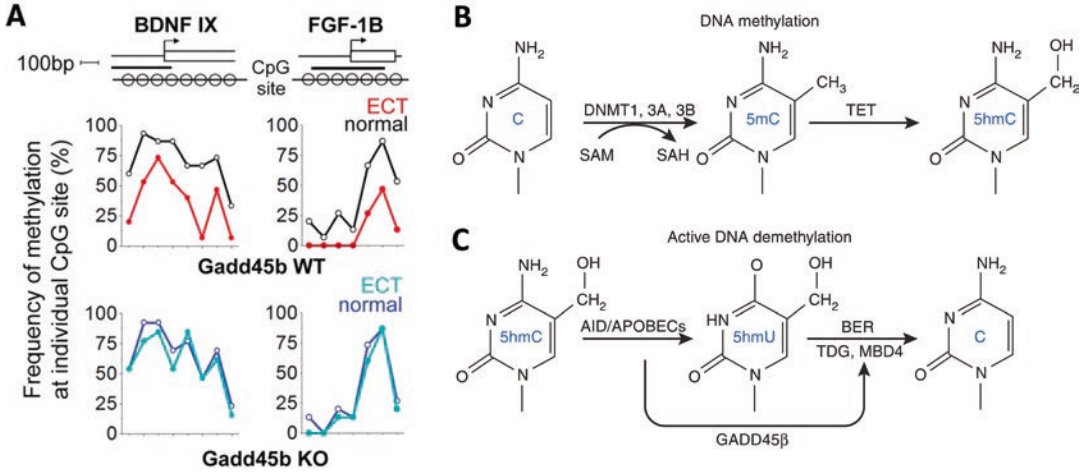

Fig. 9.7 Neuronal molecular signaling by the Gadd45 proteins. (**a**) Numerous upstream cascades impinge on *gadd45a* expression in neuronal development, injury, and tumor formation. Still, the function of Gadd45a in the balance between cell survival and death is largely dependent on the cell type and context. Green arrows indicate pathways in which Gadd45a generally plays a protective function. Red arrows indicate a predominant role of Gadd45a in promoting cell death or apoptosis. Black arrows preceding Gadd45a represent ambiguous, context-dependent signaling pathways. (**b**) Schematic similarly outlining potential regulation and function of Gadd45b and Gadd45g in neurodevelopment and injury. (**c**) Potential mechanisms for Gadd45b regulation and function in neuroepigenetic dynamics during activity-associated states such as seizures and memory formation. Gadd45b has been shown or hypothesized to regulate each of the genes listed at the bottom, illustrating a potentially diverse breadth of function in tuning experience-dependent neuronal output. See text for details and references

regulator itself, and Grip1, an AMPA receptor-binding protein necessary for synaptic depression. Further investigation is also needed to address the potential contributions of Gadd45a and Gadd45g to memory-related signaling.

An understanding of molecular epigenetic dynamics requires an appreciation for extensive cross-talk between canonical mechanisms, especially DNA methylation, histone acetylation, and histone methylation (Kondo 2009; Brinkman et al. 2012). These horizontal interactions along with complex, sequence-specific and activity-tuned functions of epigenetic writers and erasers engender unique permutations of epigenetic signatures at gene regulatory regions. The possibility that these permutations selectively drive changes in local transcription defines the "epigenetic code," a speculative concept that is the subject of current investigation (Day and Sweatt 2011). In memory consolidation, Gadd45b may play a significant role in neuronal epigenetic coding. For instance, mice treated with an HDAC inhibitor showed enhanced fear memory and a trend towards reduced *gadd45b* expression (Vecsey et al. 2007).

A finding of great interest is the discovery that 5-hydroxymethylcytosine (5hmC) is present at considerable frequency in the genome of principal cerebellar nuclei (Kriaucionis and Heintz 2009). Catalyzed by the ten-eleven translocase (TET) protein family, 5hmC is formed by hydroxylation of 5mC and is present at particularly high levels in the brain (Khare et al. 2012). Its function is not fully understood, but there is evidence that 5hmC represents an oxidated intermediate in active demethylation of 5mC in neurons (Guo et al. 2011b). The conversion of 5mC to 5hmC may be followed by base-excision and repair (BER) immediately or after conversion of 5hmC to 5-hydroxymethyluracil (5hmU) by the activation-induced deaminase (AID) and apolipoprotein B mRNA-editing enzyme complex (APOBEC) family of deaminases (Bhutani et al. 2010; Guo et al. 2011b). Gadd45a and Gadd45b are involved in BER of mutated cytosines and may act similarly in neurons (Liebermann and

Hoffman 2008; Rai et al. 2008). As evidence of this hypothesis, Tet1 and APOBEC regulate seizure-induced demethylation and upregulation of BDNF IX and Fgf-1B, known Gadd45b binding targets (Ma et al. 2009; Guo et al. 2011b). Gadd45b may therefore coordinate the conversion of 5hmC to unmethylated cytosine by recruiting deaminase and BER factors (Fig. 9.6b, c). Alternatively, at some loci, a direct mutation-BER route may bypass 5hmC. Finally, an interesting finding is that DNA methylation itself appears to control expression of the *gadd45* genes in cancer (Tamura et al. 2012). Should a similar mechanism mediate expression in the brain, this would imply the *gadd45* genes function in a complex, epigenetic positive or negative feedback loop in association with cognitive processing. Future studies are needed to investigate these possibilities in periods of high neuronal activity.

9.4.2 Autism

Spatial memory assessment of Gadd45b-null mice in the Morris water maze task revealed a surprising finding; in a control, pretraining phase, mice are taught to associate a visible flag with the escape platform. Although mutant and wildtype mice learned the task rapidly, mutants exhibited significant aversion to swim towards the flag in initial trials (Sultan et al. 2012). Since mutants did not show impairments in swim speed or other performance factors, we surmised that mutants bear a mild neophobia phenotype. As neophobia is associated with autism spectrum disorders, Gadd45b may plausibly regulate autistic features (Patterson 2011). Autism disorders are characterized by pathological neurodevelopment, and patients present with deficits in social interaction and cognition, aberrant communication and language skills, and stereotyped behaviors (Nguyen et al. 2010). A microarray study revealed elevated *gadd45b* transcripts in the superior temporal gyrus of autistic patients (Garbett et al. 2008). Studies have also uncovered dysregulation of DNA methylation in lymphoblastoid cells and histone methylation prefrontal cortex neurons in autistic patients (Nguyen et al. 2010; Shulha et al. 2012). The functionality of Gadd45b in autistic behavior and epigenetically targeted loci has yet to be uncovered. Potential autism-associated genes include SHANK3, which encodes a synaptic scaffolding protein, and neuroligin3, which encodes a neuronal transmembrane signaling protein; furthermore, both are sensitive to DNA methylation (Guo et al. 2011a; Uchino and Waga 2013). Additionally, serum BDNF is reduced in patients (Hashimoto et al. 2006; Abdallah et al. 2013).

9.4.3 Alzheimer's Disease and Aging

Aging is associated with oxidative stress, altered calcium homeostasis, chromosomal abnormalities, deficits in DNA repair, and nuclear and mitochondrial damage (Irier and Jin 2012). These features accelerate neurodegeneration and probably mediate age-related cognitive decline, most notably in Alzheimer's disease (AD). AD patients suffer from debilitating deficits in memory, decision-making, and language. Age is the strongest risk factor for developing AD, and it is likely that AD and aging are sensitive to disturbances in overlapping molecular pathways.

The deposition of extracellular plaques of amyloid β-peptide (Aβ) in the brain is a hallmark pathological feature of AD (Lambert et al. 1998). In a human preneuron cell line, Aβ exposure induced DNA damage and robust *gadd45a* expression, suggesting *gadd45a* is sensitive to genotoxic stress in AD and mediates a repair response as in the previously discussed injury models (Santiard-Baron et al. 1999, 2001). AD patients' brains also exhibit enhanced Gadd45a and Bcl-2 expression in DNA-damaged cells (Torp et al. 1998). Gadd45a also appears to confer protection against DNA damage-induced apoptosis. However, in a study of skin fibroblasts, which show impaired oxidative metabolism in AD, oxidative stress applied to patient cells produced less cell death than in control cells (Uberti et al. 2002). Additionally, while normal cells showed stress-induced Gadd45a and p53 protein

upregulation, AD cells showed a blunted response. These findings suggest Gadd45a regulates DNA damage-associated stress responses in AD, but its net effect on cell viability may vary by cell type and treatment paradigm.

Future studies of the Gadd45 proteins in AD may focus on epigenetic mechanisms. Indeed, global DNA methylation in the cortex is reduced in AD patients, and 5mC levels inversely correlate with neurofibrillary tangles, a canonical intracellular hallmark of AD pathology (Mastroeni et al. 2010). Similarly, 5hmC was shown to be enriched in pathways associated with age-related neurodegeneration (Song et al. 2011). Site-specific alterations in methylation of disease-related genes have also been documented in apolipoprotein E (APOEε4) in patients and presenilin 1 (PSEN1) in a mouse model (Wang et al. 2008; Fuso et al. 2012). Similarly, altered histone modifications are associated with AD, and enhancement in histone acetylation alleviates memory deficits (Francis et al. 2009; Kilgore et al. 2010; Stilling and Fischer 2011; Sultan and Day 2011; Gräff et al. 2012). In addition, HDAC inhibitors are speculated to boost cognitive function in a number of neurodegenerative disease states (Gräff and Tsai 2013). In light of the pro-cognitive and epigenetic roles of Gadd45b in memory and dysregulation of one-carbon transfer pathways in AD, the Gadd45 family and associated active demethylation regulators may emerge as a second class of targets in neuroepigenetic pharmacotherapy (Fuso and Scarpa 2011; Sultan et al. 2012). The protective function of the Gadd45 genes in excitotoxicity discussed above may also mediate broader protection against neurodegeneration; accordingly, aberrant neuronal excitation is present in numerous disease states including AD (Mehta et al. 2013).

The role of the Gadd45 proteins in stress responses spurred investigations into their role in aging. In fruit flies, expression of the Gadd45a ortholog (*D-GADD45*) is reduced in the nervous system with age, and overexpression of the gene in the nervous system prolongs life span (Plyusnina et al. 2011, 2012; Moskalev et al. 2012). The longevity phenotype is likely conferred by more efficient DNA repair as evidenced by reduced spontaneous DNA aberrations in overexpressing mutants. Importantly, overexpression of *gadd45a* does not affect fecundity or motor behavior, suggesting the gene selectively attenuates the metabolic effects of aging. Moreover, *gadd45a* is up-regulated in response to thermal, oxidative, and food deprivation stressors and regulates the effects of stress on longevity (Moskalev et al. 2012). Flies with *gadd45a* mutations also exhibited impaired hormesis, in which preexposure to low-dose radiation attenuates the life span reduction caused by subsequent high-dose radiation. Gadd45a likely acts through damage control mechanisms similar to those described previously, including the MAPK cascade, apoptosis pathways, and oxidative damage signaling. However, it is reasonable to speculate that the Gadd45 proteins also affect aging-related epigenetic changes. Indeed, a plethora of studies have investigated DNA methylation changes in human aging (Johnson et al. 2012). Methylation profiles of whole blood may even serve as a biomarker reflecting advancing age and age-related disease states (Hannum et al. 2013). Additional studies are needed to delineate the potential contribution of the *gadd45* genes to age-associated epigenetic drift.

9.4.4 Psychosis

Major psychosis refers predominantly to schizophrenia (SZ), a neurodevelopmental disorder characterized by multiple symptom types, and bipolar disorder (BP), a condition of bouts of mania and depression (Peedicayil 2011; Grayson and Guidotti 2013). SZ affects up to 1% of the global population and usually produces clinically notable impairments in late adolescence and early adulthood (Lewis and Sweet 2009). SZ patients present with three categories of symptoms. Positive symptoms include delusions, rigidly held false beliefs, hallucinations and other perceptual difficulties, aberrant thought, and psychomotor activity in the form of disorganized behavior, posturing, and catatonia, a condition of severely altered motor function (Lewis and Sweet 2009). Negative symptoms include social with-

drawal, impaired volition, disturbed affect, poverty in speech, and anhedonia (impaired ability to experience pleasure). Cognitive symptoms reflect dysfunction in selective attention, working and episodic memory, executive function, language, and social and emotional processing. These are considered the most significant clinical features of SZ, occurring for longer periods of time than positive symptoms in patients. The degree of cognitive impairment is also the most accurate indicator of prognosis. SZ comorbidities include depression, emotional disability, cardiovascular disease, substance abuse, and heightened risk of suicide. Affected individuals' families are likely to experience emotional distress, and the disease is associated with substantial economic effects due to lost productivity and medical expenses.

Studies of schizophrenia pathophysiology show that alterations in cortical circuit function largely underlie the clinical features of the disease. SZ is accordingly associated with diminished cortical neuropil and pyramidal neuron spine density and soma volume (Akbarian et al. 1995; Kolluri et al. 2005). Changes in the function of interneurons, the principal regulators of inhibition in the brain, have also been associated with SZ (Lewis 2012). Interneurons broadly modulate circuit tone and synchronized oscillations, which are thought to contribute to normal cognitive function and are altered in psychosis, by releasing the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) (Gonzalez-Burgos et al. 2010). A collection of studies has confirmed that transcription of glutamic acid decarboxylase (GAD67), the primary enzyme responsible for GABA production, is reduced in cortices from patients (Spencer et al. 2003; Ford et al. 2007; Gonzalez-Burgos et al. 2010; Lewis 2012). The consequent impairment in inhibitory tone is likely a factor in symptoms such as memory impairment and hallucinations. Further, Gadd45 family members were described as putative targets for unpredictable chronic mild stress (UCMS) treatments (Grassi et al. 2017).

A growing collection of studies have investigated alterations in epigenetic programming in association with major psychosis, and it is in this context that aberrant Gadd45 signaling has been hypothesized to modulate the disease phenotype (Peedicayil 2011). These have been predominantly assessed in cortex biopsies from patients. Utilizing a candidate gene approach, these have robustly uncovered alterations in DNA methylation in promoters of *reelin* and *gad1*, the gene encoding GAD67, in relation to psychosis. For example, one study found enhanced *DNMT1* and reduced *reelin* transcription in SZ cortex (Veldic et al. 2004). Notably, DNMT1 protein colocalizes with Reelin, but transcription of each respective gene was almost exclusively localized to interneurons. Enhanced DNA methylation in the *reelin* gene promoter was also found in SZ brains (Abdolmaleky et al. 2005; Grayson et al. 2005). Although it is unclear whether this epigenetic mark directly influences transcription in the SZ cortex, it is interesting to note that higher methylation was found in close association with a putative cycle AMP response element and stimulating protein-1 site in the *reelin* promoter. In light of the localization of Reelin to GABAergic interneurons and its role in the integrity of the extracellular matrix, the neuropil and synaptic plasticity, studies have also sought to examine GAD67 epigenetic regulation in order to dissect a broader function link between epigenetic dysfunction and GABAergic tone. For example, the fastest spiking interneurons in the cortex, those in layers I, II, and IV, also contain the highest DNMT1 transcripts, suggesting a positive association between DNA methylation and GABAergic tone (Veldic et al. 2004). Additionally, GAD67 transcription negatively correlates with DNMT1 transcription in psychotic patient cortices (Veldic et al. 2005). To the contrary, one finding suggests demethylation of *gad1* may be noncanonically associated with downregulation of GAD67 or that other gene regulatory elements outside the promoter may be more critical in modulating expression (Huang and Akbarian 2007).

Still, the finding of aberrant methylation in SZ suggests Gadd45 function may affect neural tone alterations in patients. Two studies in particular have examined this hypothesis. The first assessed DNA methylation dynamics in response to altered activity of metabotropic glutamate recep-

tors (mGlu), as activation of the group II subclass (composed of types mGlu2 and mGlu3) attenuates presynaptic glutamatergic activity, and agonists have been associated with antipsychotic effects (Matrisciano et al. 2011). The study first found enhanced *gadd45b* and *gadd45g* mRNA and Gadd45b protein expression in the frontal cortex of naïve mice in response to a single or repeated doses of systemic mGlu2/3 activation. Both valproic acid (VPA), a weak HDAC inhibitor, and the atypical antipsychotic clozapine but not the typical antipsychotic haloperidol similarly induce *gadd45b* transcription, suggesting Gadd45b may play a role in certain form of antipsychotic therapy. Because Gadd45b was shown to bind *bdnf exon IX* and *fgf-1B* promoters in association with activity-induced demethylation (Ma et al. 2009), the authors examined possible binding to *reelin*, *bdnf exon IX,* and *gad1* promoters after mGlu2/3 activation. Enhanced binding was confirmed along with active demethylation of each of these promoters following methionine-induced hypermethylation. VPA and clozapine similarly reduced *reelin* baseline methylation as well as the methionine-induced hypermethylated promoter.

The second study analyzed cortical tissue from psychotic subject from two brain banks (Gavin et al. 2012). The study found selectively reduced Gadd45b recruitment in the proximal promoter but not in a downstream region of *bdnf exon IXabcd* in psychotic patients. The authors then elegantly demonstrated strong hypermethylation of the same promoter region in affected subjects and a similar increase in 5-hmC signal. Concomitantly, reduced total BDNF expression was confirmed. If Gadd45b plays a causative role in demethylation in association with the psychosis phenotype, its reduced binding to target loci such as *bdnf exon IX* may have caused the demethylation cascade to become "stuck" in the hydroxymethylated state. According to this model of *bdnf* demethylation, Gadd45b would promote the conversion of 5-hmC to unmethylated cytosine, but this has not been confirmed (Guo et al. 2011b). Although (Gavin et al. 2012) dissected the epigenetics of BDNF expression, it is not clear whether attenuated BDNF signaling is a key mediator of the disease phenotype or whether it is only one of many epigenetically dysregulated genes. It is also not clear which cortical cells specifically harbor the alterations in Gadd45b-mediated signaling; indeed both excitatory and inhibitory cortical cells but not glia express Gadd45b in the normal prefrontal cortex (Gavin et al. 2012). Finally, the authors uncovered a counterintuitive finding that *gadd45b* transcripts and protein are both elevated in psychotic subjects' cortices. Of course, this conflicts with the finding of suppressed DNA binding and elevated methylation of *bdnf exon IX*, and this suggests that global expression differences in Gadd45b and possibly other potent modulators of the epigenome do not necessarily drive commensurate changes in site-specific binding patterns. It remains to be seen which specific characteristics of promoter sequences and associated chromatin influence the recruitment of the demethylation machinery. Alternatively, enhanced expression could represent a compensatory effect to other factors associated with a more restrictive chromatin state in SZ such as enhanced DNMT expression, DNA methylation, and repressive histone modifications (Veldic et al. 2004, 2005; Grayson et al. 2005; Gavin and Sharma 2010).

Together, these results provide evidence that Gadd45b influences altered neuronal signaling in SZ and mediates certain forms of antipsychotic therapy. Future studies, such as with Gadd45b-mutant mice, are needed, however, to confirm a functional effect of the protein. It should be noted, however, that Gadd45b-null mutants did not exhibit alterations in prepulse inhibition, an index of sensorimotor gating that is affected in SZ models (Sultan et al. 2012). Additionally, it remains to be seen that Gadd45b specifically drives demethylation and upregulation of *reelin*, *gad1,* and *bdnf* in psychosis. Of particular interest is the possible role of Gadd45b in epigenetically altered *gad1* expression and inhibitory tone. If the Gadd45 proteins do indeed influence cortical inhibition in this manner, demethylation mechanisms could become a novel molecular target in SZ treatment.

Few studies have sought to profile the span of methylation changes in psychosis, but (Mill

et al. 2008) utilized an epigenome-wide effort to address this problem. Genomic DNA from frontal cortex biopsies of schizophrenic and bipolar patients differed at a number of loci from control brains when global methylation was assessed. These epigenetic modifications corresponded with changes in steady-state transcripts encoding regulators of glutamatergic and GABAergic signaling and neurodevelopment and other transcripts highlighted in genetic linkage studies. For example, loci proximal to the NMDA receptor subunit gene *NR3B* and the AMPA receptor subunit gene *Gria2* were hypomethylated in patients, suggesting aberrantly high demethylation activity, possibly due to altered Gadd45 signaling could drive excessive excitatory activity. Likewise, genes encoding vesicular glutamate transporters VGLUT1 and VGLUT2 also showed altered methylation patterns associated with downregulation of the former and demethylation and upregulation of the latter. In hypothesizing a functional link between DNA methylation changes and psychosis etiology, the authors emphasize the complex, interactive effects of epigenomic marks in the global transcriptional network. It is important, therefore, not to place excessive weight on single transcriptional or epigenetic changes in psychosis or other disease states, and future studies will need to take this into account. In addition, a recent study found elevated *TET1* and suppressed *APOBEC* expression in psychosis patient cortices and associated increases in 5hmC throughout the genome and at *bdnf* and *gad1* promoters (Dong et al. 2012). The associated decreases in BDNF and GAD67 expression may result from similar reductions in the ability of neurons to convert 5hmC to unmethylated cytosine and hence accumulation of 5hmC or from a direct repressive function of TET1 independent of its enzymatic activity (Grayson and Guidotti 2013). Therefore, future studies of Gadd45 proteins in SZ will need to address potential alterations in 5hmC levels as well. Finally, the functional interactions between canonical epigenetic cascades suggest that the Gadd45 proteins may also mediate the contributions of histone modifications to psychosis pathology and treatment (Sharma et al. 2008; Guidotti et al. 2009; Kurita et al. 2012; Labrie et al. 2012).

9.5 Conclusions

We have summarized the key findings of Gadd45 protein function in the CNS. The Gadd45 family is expressed in distinct patterns during the development of the nervous system and likely mediates cell cycle control during mitosis. In a variety of nervous system stressors including physical and chemical injury to nerves and neoplasia, the Gadd45 family has been implicated in sensing DNA damage and controlling the balance between apoptosis and cell survival. More recently, an exciting literature has identified the Gadd45 family as regulators of active DNA demethylation, a still elusive molecular epigenetic mechanism that appears to control adult cognitive function and neuropsychiatric dysfunction. Future work is needed to delineate the breadth of mechanisms that stimulate Gadd45 expression and those by which the proteins mediate DNA repair, cell cycle control, and epigenetic regulation of transcription. Studies will also need to identify small molecule regulators of Gadd45 function and to investigate their potential uses in conditions including CNS cancers, nerve damage, and cognition.

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Competing InterestsThe authors declare no competing interests.

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