Advances in Experimental Medicine and Biology 793

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Gadd45 Stress Sensor Genes

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ISSN 0065-2598
ISBN 978-1-4614-8288-8 ISBN 978-1-4614-8289-5 (eBook)
DOI 10.1007/978-1-4614-8289-5
Springer New York Heidelberg Dordrecht London

Library of Congress Control Number: 2013948607

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Introduction: Gadd45 Stress Sensors

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

The stress response Gadd45 family of genes (Gadd45a, Gadd45b, and Gadd45g), discovered in this lab and by other investigators (see Wikipedia), encode for small (18 kDa) nuclear/cytoplasmic proteins. These genes are rapidly induced by a wide variety of endogenous and exogenous stress stimuli. In spite of marked similarities, Gadd45 genes are regulated differently and exhibit functional diversity. Gadd45 proteins are implicated in cell cycle arrest, DNA demethylation and repair, apoptosis, cell survival, genomic stability, inflammation, and in response to physiological and oncogenic stress.

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 setting including cell type, developmental stage, and stress/stimulus. Protein partners include cdc2/cyclinB1, p21, the p38/JNK stress-induced kinase pathways, and PCNA/histones.

The purpose of this book is to provide a comprehensive picture of the unique global role Gadd45 proteins play as stress sensors and the molecular pathways involved.

Philadelphia, PA, USA — Dan A. Liebermann

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Chapter 1
Gadd45 in Stress Signaling, Cell Cycle Control, and Apoptosis

Jesús M. Salvador, Joshua D. Brown-Clay, and Albert J. Fornace Jr.

Abstract The first identified Gadd45 gene, *Gadd45a*, encodes a ubiquitously expressed protein that is often induced by DNA damage and other stress signals associated with growth arrest and apoptosis. This protein and the other two members of this small gene family, Gadd45b and Gadd45g, have been implicated in a variety of the responses to cell injury including cell cycle checkpoints, apoptosis, and DNA repair. In vivo, many of the prominent roles for the Gadd45 proteins are associated with signaling mediated by p38 mitogen-activated protein kinases (MAPK). Gadd45 proteins can contribute to p38 activation either by activation of upstream kinase(s) or by direct interaction. 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 been found to contribute to p53 activation via p38. Like other stress and signaling proteins, Gadd45 proteins show complex regulation and numerous effectors.

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D.A. Liebermann and B. Hoffman (eds.), *Gadd45 Stress Sensor Genes*, Advances in Experimental Medicine and Biology 793, DOI 10.1007/978-1-4614-8289-5_1,

1.1 Overview

The first *Gadd45* gene was identified on the basis of induction, i.e., increased mRNA levels, after a variety of stresses associated with growth arrest, hence the designation *g*rowth *a*rrest and *D*NA-*d*amage (*gadd*) inducible (Fornace et al. 1989). Gadd45, now designated Gadd45a, shows no sequence homology with the original *gadd* gene group and was subsequently found to be a member of a highly conserved three-gene family consisting of *Gadd45a* (Gadd45α, DDIT1), *Gadd45b* (Gadd45β, Myd118), and *Gadd45g* (Gad45γ, cytokine responsive 6, CR6). The gadd genes were first cloned from Chinese hamster ovary (CHO) cells 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. *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). Some of the prominent interactions of the Gadd45 proteins are summarized in Fig. 1.1 which highlights regulatory pathways and targets. As seen in this figure, Gadd45 has a broad scope of potential roles in many cellular processes that will be covered here with emphasis on growth control and apoptosis; many of these signaling pathways have a variety of roles covered in other chapters.

When it was first reported, *Gadd45a* was unique among the radiation-response genes in that it could be induced in an ATM-dependent and protein kinase C-independent manner after exposure of human cells to ionizing radiation (IR) (Papathanasiou et al. 1991). This induction is p53-regulated (Kastan et al. 1992); indeed, *Gadd45a* was the first stress gene discovered that was transcriptionally regulated by p53 (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. *Gadd45g* was originally cloned as an early IL-2 response gene in T cells. 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, which is 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), and localize 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.

The Gadd45 proteins are typical signaling proteins in that they are small, rapidly regulated at both transcriptional and posttranscriptional levels, and have a variety of

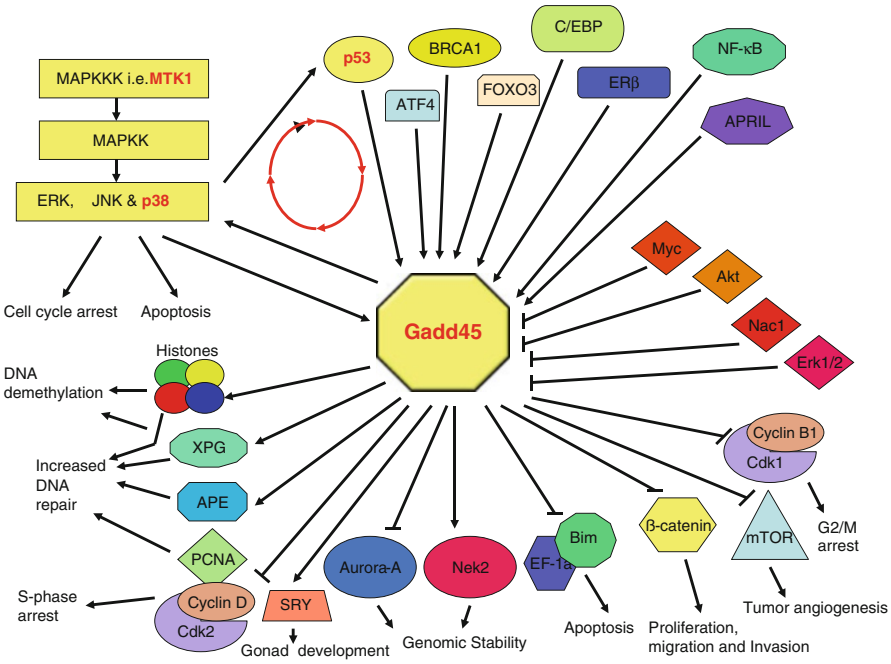

Fig. 1.1 Proteins with well-known roles in regulation of the Gadd45 genes and proteins are shown, as well as effector proteins. *Arrows* indicate positive regulation, while other *lines* indicate negative regulation. In some cases, regulation is complex such as for the p38 and JNK stress MAPK, which can contribute to Gadd45 induction and are important effectors of Gadd45 signaling. As described in the text, p38, p53, and Gadd45a can function in a positive feedback loop (indicated by *red circle* with *arrows*) to maintain p53 signaling and growth arrest. A variety of signaling events in addition to genotoxic and other stresses can influence Gadd45 expression

roles in mediating stress signaling and growth regulation. In addition to repair and apoptosis, cell injury, such as that induced by DNA damage (genotoxic stress), is known to trigger growth delays in prokaryotes and eukaryotes (Friedberg et al. 2006); Gadd45a and the other Gadd45 proteins have been implicated in many such 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 function in these processes. In regulation, the p38 and JNK stress mitogen-activated protein kinases (MAPK) have complex roles in the regulation of Gadd45. Other growth-arrest-associated regulatory factors such as p53, BRCA1, FOXOA3, C/EBP, and ATF4 participate in transcriptional regulation of *Gadd45a*, and to some extent of the less-studied *Gadd45b* and *Gadd45g*, genes. Gadd45 proteins are involved, directly or as part of regulatory pathways in cell cycle checkpoints

and stimulation of DNA repair. Gadd45 proteins interact with a wide variety of cellular proteins and protein complexes 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 for certain G2 checkpoint events (Wang et al. 1999; Zhan et al. 1999). Interestingly and 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, Gadd45 proteins interact and/or influence a variety of proteins involved in DNA repair including APE (Jung et al. 2007), XPG (Barreto et al. 2007), proliferating cell nuclear antigen (PCNA) (Smith et al. 1994), as well as p53 itself.

Many of the examples illustrated in Fig. 1.1 were discovered in cell culture systems, but major roles for Gadd45 have since been highlighted using genetic approaches in vivo with mouse models and in vitro with primary cells such as mouse embryo fibroblasts (MEF) and lymphocytes. A consistent feature has been a prominent role for p38 MAPK (p38) 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 stress 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 MAPK kinase kinase (MAPKKK) (Takekawa and Saito 1998). p38 can directly phosphorylate regulatory sites in p53, such as Ser 46 (implicated in proapoptotic signaling), 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. 1.1. 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 ionizing radiation (IR) (Hollander et al. 1999) or UV radiation (Hildesheim et al. 2002).

1.2 Gadd45 Regulation in Growth Arrest and Apoptosis

Gadd45a expression is regulated by many factors at the transcriptional, posttranscriptional, and posttranslational levels (Gao et al. 2009), in response to genotoxic stress as well as other growth-arrest signals (some illustrative examples are shown in Fig. 1.1). *Gadd45* is one of the very few genes that is upregulated consistently after IR in numerous conventional and gene expression profiling studies of p53 wild-type (wt) cells (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 very low and varies through the cell cycle, with highest levels during G_1 and lowest during S phase (Kearsey et al. 1995).

MAPK signaling, via p38 and JNK kinases, induces Gadd45a expression, as highlighted in Fig. 1.1. Specifically, these kinases activate c-Jun, which, similarly to p53, binds to the third intron of *Gadd45a* and activates its transcription. 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. 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).

Several tumor suppressor genes induce *Gadd45a* expression at the transcriptional level. A well-characterized mechanism of *Gadd45a* induction is p53 binding to a conserved site within the third intron of the *Gadd45a* gene, which stimulates its transcription (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 also induce *Gadd45* transcription in a p53-dependent manner 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 (Jung et al. 2000; Gao et al. 2009).

Gadd45a is a direct target gene of the tumor suppressor FOXO3A, a member of mammalian family of forkhead transcription factors. FOXO3Abinds directly to the *Gadd45a* promoter and induces its 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 for Gadd45b in the stress response (Tran et al. 2002). 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 in the G_2/M (Notas et al. 2012).

The key breast cancer tumor suppressor, BRCA1, also stimulates transcription of Gadd45 after γ-radiation treatment of cells (Li et al. 2000; Park et al. 2008). Overexpression of BRCA1 similarly resulted in higher Gadd45 expression and also stimulation of nucleotide excision repair (NER) in a Gadd45-dependent manner (Hartman and Ford 2002) and BRCA1-deficient cells are hypersensitive to

cisplatin, suggesting 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-I and Oct-1 to stimulate transcription of Gadd45 (Maekawa et al. 2008); so BRCA1 indirectly and directly (Park et al. 2008) activates transcription of Gadd45. The importance of BRCA1 in the DDR is well known (Wu et al. 2010), and these findings highlight the importance of Gadd45 as a downstream effector of BRCA1.

A variety of growth stimulatory factors can negatively regulate the Gadd45 genes (Fig. 1.1). 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 2012). 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).

It was found early on that *Gadd45a* regulation is complex and can be regulated at the posttranscriptional level with markedly increased 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 expression of the Gadd45a protein. After cell exposure to MMS or UV radiation, these proteins dissociated rapidly from *Gadd45a* mRNA through an unknown mechanism and allowed 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 upstream of p38 were recently shown to phosphorylate three proteins involved in RNA regulation, hnRNPA0, TIAR, and PARN, stabilizing *Gadd45a* mRNA (Reinhardt et al. 2010). At the posttranslational level, arsenite stimulation of cells induces formation of an IκB-kinase-β (IKKβ)/NF-κB p50 subunit complex that reduces ubiquinated Gadd45a levels and its subsequent proteasomal degradation (Yang et al. 2009).

Roles for NF-κB regulation of the *Gadd45* genes have been complicated with sometimes contradictory results (Amanullah et al. 2003). NF-κB signaling is often considered a pro-survival response and is reported to reduce Gadd45a and Gadd45g expression and escape 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* expression by activating c-Myc and downregulating nucleolin. 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; it is also upregulated in several cancer types, particularly chemoresistant, recurring ovarian carcinomas. Nac1-mediated Gadd45g downregulation contributes to paclitaxel resistance in ovarian cancer cells (Jinawath et al. 2009).

1.3 Gadd45a Effectors in Growth Arrest, Apoptosis, and DNA Repair

There is substantial overlap among downstream effectors of the Gadd45 proteins; as the literature for Gadd45a is much larger, 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 stimulation of DNA repair. Although only 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.

Gadd45a has roles in both S-phase and G_2/M arrest (Smith et al. 1994; Hollander and Fornace 2002) (summarized in Fig. 1.1). It can displace PCNA from the cyclin D1 complex, possibly inhibiting DNA replication during S phase (Smith et al. 1994). Likewise, Gadd45a can bind Cdk1, probably preventing its association with

Table 1.1 Gadd45 effectors with roles in growth control, apoptosis, and DNA repair

p38	Cell cycle arrest, apoptosis, negative regulation of T cell activation, full activation of innate immune cells, induction of senescence
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 β-catenin via p38 signaling
β-catenin	Inhibition of its pro-invasion program, increased β-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
XPG	Stimulates DNA nucleotide excision repair; potentially mediates DNA demethylation
APE	Stimulates DNA base excision repair
PCNA	DNA repair and demethylation; S-phase arrest
Aurora-A	Maintenance of genomic stability
Nek2	Maintenance of genomic stability
mTOR	Suppression of tumor angiogenesis

cyclin B1, inhibiting Cdk1 activity, and arresting the cell at the G_2/M checkpoint (Hollander and Fornace 2002). Gadd45a can directly inhibit purified Cdk1/cyclin B1 activity in vitro (Zhan et al. 1999). Gadd45a interacts with tumor suppressor cyclin-dependent kinase inhibitor 1a (encoded by *Cdkn1a*), also known as p21, Cip1, or Waft1. The exact nature of this interaction and its outcome nonetheless remains unclear. The two proteins 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 a variety of inhibitory effects on β-catenin signaling, which is a pro-growth pathway (Hildesheim et al. 2004, 2005). After UV radiation induction, Gadd45a stimulates p38 promotion of dephosphorylation of glycogen synthase kinase 3β (GSK3β), activating 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 its 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 et al. 2004).

As mentioned above, oncogene-induced senescence (Bulavin et al. 2003) and establishment of the senescent phenotype in response to DNA damage requires Gadd45a expression (Passos et al. 2010). In both cases, Gadd45a signaling through p38 is essential for induction of this phenotype and for full transactivation of p53, whose activity is essential for cells entry into a senescent state. In senescent human fibroblasts, p53 preferentially occupied the promoters, associated with a unique combination of phosphorylated p53 sites (Gao et al. 2009). The positive feedback loop between Gadd45a, p38, and p53 is thus essential for induction and maintenance of the senescent phenotype after oncogene overexpression or severe DNA damage in fibroblasts and keratinocytes, and probably in other cell types.

In addition to premature senescence, damaged or potentially tumorigenic cells can be removed from the growth compartment by apoptosis, and 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, exclusively, inducing a conformational change that results in its autophosphorylation, activation, and a strong apoptotic response (Takekawa and Saito 1998; Mita et al. 2002). As discussed, 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 (Fig. 1.1). 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. How p53 signaling compensates in Gadd45a-null dermal fibroblasts is uncertain, but other Gadd45 proteins are expressed more abundantly in this cell type. This observation highlights the cell specificity for some in vivo roles of Gadd45.

In early events in the apoptotic cascade, Gadd45a might also be involved through interaction with the cytoskeleton. Elongation factor 1α (EF-1α) is a microtubule-severing protein that binds, bundles, and promotes microtubule assembly, with a key role in cytoskeletal stability. Increased Gadd45a expression leads to its interaction with EF-1α and inhibits microtubule bundling and destabilizes the cytoskeleton (Tong et al. 2005). This causes release of Bim, a Bcl-2 family proapoptotic protein, from microtubule-associated complexes, and allows Bim translocation to the mitochondria triggering cytochrome C release into the cytoplasm and initiation of apoptosis (Gao et al. 2009).

Some other Gadd45a features can have an opposite effect on apoptosis potential. This is not surprising since both checkpoint activation and DNA repair can enhance cell survival. For example, Gadd45a deficiency sensitizes cells to cisplatin and UV radiation, implying subtleties to the proapoptotic effects of this protein or more likely 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 enhances 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.

Its functions in cell cycle control, DNA repair, apoptosis, and p53 signaling confer several roles on Gadd45a in maintaining genomic stability. This is particular 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 probably 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 animals. 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 has the ability to stimulate DNA repair, seemingly through its ability to interact with PCNA and DNA repair complexes. In vitro and cell culture assays showed that recombinant Gadd45a can stimulate NER (Smith et al. 1994; Tran et al.

2002), whereas loss of Gadd45a expression in ex vivo assays of lymphoblasts resulted in a substantially reduced NER (Gao et al. 2009). More recently, Gadd45a deficiency has been linked to reduced base excision repair (BER), cytoplasmic localization of apurinic endonuclease (APE, a key enzyme in the BER pathway), and decreased APE interaction with PCNA, as well as delayed removal of apurinic sites. The ability of Gadd45a to interact with acetylated or UV radiation-exposed mononucleosomes and to increase local DNA accessibility might facilitate stimulation of DNA repair (Ma et al. 2009).

As discussed elsewhere in this book, Gadd45a-related excision repair events are implicated in removal of DNA methylation, an epigenetic marker associated with repression of transcriptional initiation. Acetylation is a requisite step for DNA demethylation, as is also general RNA transcription (indicating that transcription of additional factors not normally present at high levels, such as Gadd45, is necessary). Gadd45a interacts directly with the four core histones and increases DNase accessibility to DNA with hyperacetylated mononucleosomes in vitro, perhaps allowing access of demethylation/DNA repair complexes to DNA in the cell. In vivo studies showed notable specificity of Gadd45b-mediated DNA demethylation and Gadd45a- and Gadd45b-null mice have conserved global genomic methylation patterns, indicating that Gadd45 is likely to be involved in demethylation and transcriptional activation of specific genes. Two early studies did not find a role for Gadd45 in DNA demethylation but a number of recent reports do emphasize the highly cell-type and context-specific nature of this mechanism; this finding, together with differing experimental conditions, could explain the discrepancies observed (Ma et al. 2009). TATA-binding protein-associated factor 12 (TAF12) was found to recruit Gadd45a and the nucleotide excision repair complex to the ribosomal DNA promoter and induces its transcription in a demethylation-dependent manner (Schmitz et al. 2009). The Gadd45 protein 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). Lastly, Gadd45a is markedly overexpressed in $CD4^+$ T cells from systemic lupus erythematosus patients and mediates demethylation, with subsequent increased transcription of the CD11a and CD70 promoter regions. Both of these contribute to autoimmunity and thus to disease progression or maintenance (Li et al. 2010), although Gadd45a is a negative regulator in T cells. Gadd45b is needed for specific DNA demethylation of factors critical for activity-induced adult neurogenesis (Ma et al. 2009).

Whereas p38 and Gadd45a are typically associated with growth arrest in most cell types, p38 activation has key stimulatory roles in lymphocytes. In the case of T cell activation, p38 is necessary and Gadd45a has a central role in regulating this process (Salvador et al. 2005a, b; Ashwell 2006). Surprisingly, Gadd45a is a negative regulator of p38 signaling during T cell activation and subsequent proliferation. Gadd45a-null mice develop an autoimmune disease similar to human systemic lupus erythematosus. The clinical signs of autoimmunity are more severe in female

mice and it is characterized by high titers of anti-DNA and anti-histone autoantibodies, leukopenia, lymphopenia, proteinuria, immune complex deposition, and glomerulonephritis (Salvador et al. 2002). Lymphoid organ cell subsets show a 50 % increase in total numbers of $CD3^+$ T cells ($CD4^+$ and $CD8^+$ subsets), but not of B cells in Gadd45a$^{-/-}$ mice. Splenocytes and lymph node cells have a three- to tenfold lower activation threshold and proliferate more vigorously in response to anti-CD3-mediated TCR activation. This increased proliferative rate is not due to a reduction in apoptosis by TCR-activated T cells; cell cycle analysis showed a decrease in the proportion of cells in G_1 phase and an increase in S phase in Gadd45a$^{-/-}$ compared to wt T cells. These findings showed that Gadd45a acts as an autoimmune suppressor in vivo by negatively regulating T cell proliferation in response to TCR activation (Salvador et al. 2002, 2005a).

Notably, in resting T cells from Gadd45$^{-/-}$ mice, p38 is constitutively active as a result of constitutive Tyr323 p38 phosphorylation, and recombinant Gadd45a inhibits the activity of p38 isolated from resting Gadd45$^{-/-}$ T cells (Salvador et al. 2005a). The capacity of Gadd45a to inhibit Tyr323 p38 phosphorylation and p38 kinase activity was mapped to a central part of the124-amino-acid Gadd45a protein. An in vitro kinase assay showed that recombinant Gadd45a inhibits p38 enzyme activity and that the Gadd45a–p38 interaction depended on Gadd45a residues 71–96 (Salvador et al. 2005a). In T cells, therefore, Gadd45a has an important role as a p38 inhibitor both in vivo and in vitro. However, in dendritic cells, Gadd45a is necessary for efficient soluble *T. gondii* tachyzoite antigen (STAg)- or lipopolysaccharide (LPS)-induced production of the Th1 cytokine IL-12 through p38 activation via the stress pathway (Jirmanova et al. 2007). The failure of Gadd45a$^{-/-}$ mice to generate Th1 responses is not T-cell intrinsic, but is due to reduced p38 activation in dendritic cells. This demonstrates that Gadd45a has opposing tissue specific and functions in p38 activity; in dendritic cells, it enhances p38 activity, a critical event in Th1 cell polarization, whereas in T cells it reduces TCR activation-induced proliferation and Th1 effector functions through inhibition of p38.

1.4 Roles for Gadd45b and Gadd45g

Although information for Gadd45b and Gadd45g is more limited than for Gadd45a, they are clearly defined as proapoptotic, growth-arrest proteins and are thus similar to Gadd45a. Both Gadd45b and Gadd45g inhibit Cdk1 activity and have a role in S and G_2/M checkpoints. Gadd45b and Gadd45g activate MTK1 to trigger JNK signaling (Yang et al. 2009). Both Gadd45b and Gadd45g 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 and both Gadd45b and Gadd45g overexpression-induced apoptosis in HeLa cells. Gadd45g is associated

with neuron cell death and Gadd45b with the apoptotic response in neural ischemia (Cretu et al. 2009). Gadd45g levels are significantly lower in anaplastic thyroid cancer cells compared to primary cultured thyrocytes and its reintroduction by viral expression inhibited proliferation (Yang et al. 2009).

Gadd45b is reported to mediate TNFα-induced NF-κB suppression of JNK-induced apoptosis by direct binding to MKK7 and inhibition of its catalytic activity, although this finding is debated. Gadd45b was also described to suppress JNK signaling in hematopoietic cells in response to UV treatment (although this was later challenged) (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 which confirmed the need for Gadd45b protein for p38 activation (Yoo et al. 2003). Gadd45b promotes liver regeneration in vivo (Papa et al. 2008) and protects retinal ganglion cells in the response to neuronal injuries, such as oxidative stress, TNFα, and glutamate cytotoxicity (Liu et al. 2009).

In immune cells, Gadd45b and Gadd45g show similarities and differences to Gadd45a. Unlike Gadd45a, Gadd45b and Gadd45g potentiate p38 signaling in Th1 and $CD8^+$ cytotoxic T cells, which is necessary for 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). 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.

The Gadd45 family also has roles for growth and development of specific tissues in the embryo. In mice, the Gadd45 genes are differentially expressed during embryonic development; for instance, Gadd45b is expressed in the chorion, whereas Gadd45g is expressed in 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.

A specific role was recently identified for Gadd45g 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 expression triggers differentiation of a somatic supporting cell lineage into Sertoli cells, which direct the male developmental pathway. 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 and its absence leads to 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).

1.5 Involvement of Gadd45 in Tumorigenesis

Whereas Gadd45a-null mice do not typically show elevated tumorigenesis, loss of this gene confers a tumor-prone phenotype after genotoxic stress. Studies in Gadd45a-null mice illustrates 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) (see Fig. 1.1). 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 leads to an increase in lung tumor malignancy, and allelic deletion of Gadd45a is associated with multiple tumor types, including lung (Hollander et al. 2005). The Gadd45a promoter is methylated in a majority of breast cancers and a significant fraction of prostate cancers, whereas the Gadd45b promoter is likewise hypermethylated in several human hepatocellular carcinomas, in both cases with subsequent expression downregulation. Sustained ERK1/2 signaling in an acute myeloid leukemia model cell line downregulates Gadd45a, and the reintroduction of expression induced 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 might be one of the "two hits" in oncogenic transformation (Bulavin et al. 2003).

Angiogenesis is an important component of tumorigenesis, and Gadd45 is also implicated in inhibiting this process. 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 (VEGFa) and promotes formation of tumor blood vessels. Moreover, Gadd45a can interact with mTOR and suppress STAT3 phosphorylation, leading to down-regulated expression of VEGFa (Yang et al. 2013).

Aberrant Gadd45 expression has been found in a variety of human tumor studies. Whereas it has clear tumor suppressor features, Gadd45 might also offer survival advantages to certain malignant cells, in line with its roles in cell growth arrest and DNA repair among other functions. 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. Gadd45b expression was associated with increased relapse and patient death in human colorectal carcinoma (Wang et al. 2012) Gadd45a induction also protects melanoma cells from UV radiation-induced death. 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 was upregulated in thyroid cancers (Karger et al. 2009). The pregnane X receptor can activate Gadd45b/p38 MAPK signaling to induce change of morphology and migration in a hepatocellular carcinoma cell line (Kodama and Negishi 2011). Given the higher reported rate of promoter hypermethylation or upregulation of Gadd45 transcription proteins, many 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 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 (Yang et al. 2009), and gastric, colorectal, and pancreatic cancers (Zhang et al. 2010); however, genetic mutation and inactivation are very 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). Additionally, decreased Gadd45g expression predicts poor prognosis and tumor expression in esophageal squamous cell carcinoma (Guo et al. 2013b) and loss of Gadd45a in gastric cardia adenocarcinoma Guo et al. 2013a) and acute myeloid leukemia (Perugini et al. 2013) similarly carries a worse prognosis.

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Chapter 2
Gadd45 in Modulation of Solid Tumors and Leukemia

Barbara Hoffman and Dan A. Liebermann

Abstract The stress response gadd45 gene family participates 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. Given these diverse functions, it is anticipated that gadd45 genes can influence the initiation and progression of malignancy and the response to different treatments. This chapter will provide an overview of how the different members of the gadd45 gene family are expressed in different tumors and leukemia, how this may impact on progression of disease, and what happens when expression is manipulated. Studies from human tumor/leukemia samples, cell lines, and animal models are included in this review. An overriding theme is that each of the gadd45 genes has both tumor suppressor and tumor promoter functions, dependent on the tissue/cell type and transforming event.

2.1 Background on gadd45

The stress response gadd45 family of genes is comprised of three members: gadd45a, gadd45b, and gadd45g. Each gene encodes for small (18 kDa), evolutionarily conserved proteins that are highly homologous to each other (55–57 % overall identity at the amino acid level), highly acidic, and primarily, but not exclusively, localized within the cell nucleus (Liebermann and Hoffman 1998; Zhan et al. 1994; Zhang et al. 1999; Carrier et al. 1994).

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D.A. Liebermann and B. Hoffman (eds.), *Gadd45 Stress Sensor Genes*, Advances in Experimental Medicine and Biology 793, DOI 10.1007/978-1-4614-8289-5_2,

Gadd45a was cloned as a *g*rowth *a*rrest and *D*NA *d*amage-inducible (GADD) gene that is rapidly induced by UV radiation in Chinese hamster ovary (CHO) cells (Zhan et al. 1994), whereas gadd45b was cloned as a *my*eloid *d*ifferentiation primary response (MyD118) gene, induced in the absence of protein synthesis following induction of differentiation of M1 myeloblastic leukemia cells (Liebermann and Hoffman 1998; Abdollahi et al. 1991). Gadd45g was isolated using a gadd45b cDNA fragment encoding for amino acids 37–92, which is about 80 % homologous to gadd45a, to screen for other potential members of the gadd45 gene family and was found to encode for the murine homologue of human CR6 (Zhang et al. 1999), an immediate early response gene in T cells stimulated by interleukin-2 (Beadling et al. 1993).

Each gadd45 gene has a distinctive pattern and level of expression, consistent with each gadd45 family member playing a different role in response to specific stressors. Gadd45 genes are expressed in multiple murine tissues, including heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testes (Zhang et al; 1999), and are induced in response to multiple environmental and physiological stresses, including methyl methanesulfonate (MMS), γ-irradiation (IR), ultraviolet radiation (UV), VP-16, daunorubicin (DNR), and inflammatory cytokines (Liebermann and Hoffman 1998; Gupta et al. 2005, 2006b). In all cases the level and pattern of expression is gene specific. Furthermore, during myeloid differentiation, using either normal bone marrow (BM) stimulated with different hematopoietic cytokines or various hematopoietic cell lines induced to undergo terminal myeloid differentiation, each gadd45 gene exhibits a distinctive pattern of expression (Zhang et al. 1999; Gupta et al. 2006b).

Consistent with the expression patterns, regulation of expression for each gadd45 gene is unique and cell type dependent. For instance, gadd45a is a p53 target gene, although its induction can also be p53 independent, whereas gadd45b is a primary response gene to both IL-6 and TGF-β, and gadd45g is induced as a primary response to IL-2 and IL-6 (Zhang et al. 1999; Carrier et al. 1994; Beadling et al. 1993; Selvakumaran et al. 1994; Yoo et al. 2003). Interestingly, mice null for each of the gadd45 genes are viable (Hollander et al. 1999; Gupta et al. 2005).

Gadd45 proteins have been shown to have 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 (Hoffman and Liebermann 2009; Liebermann and Hoffman 1998; Liebermann et al. 2011). The proteins encoded by the gadd45 family of genes do not have any enzymatic function nor any recognizable motifs; they function predominantly by interacting with and modulating the structure/function of partner proteins, an ever-expanding group of proteins that participate in cell cycle, stress signaling, DNA repair, genomic stability, and epigenetic regulation (Fig. 2.1) (Hoffman and Liebermann 2009; Liebermann and Hoffman 1998; Liebermann et al. 2011). If protein–protein interactions determine the many functions of gadd45 proteins, an open question is 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 post-translational modifications of both the gadd45 proteins and their interacting

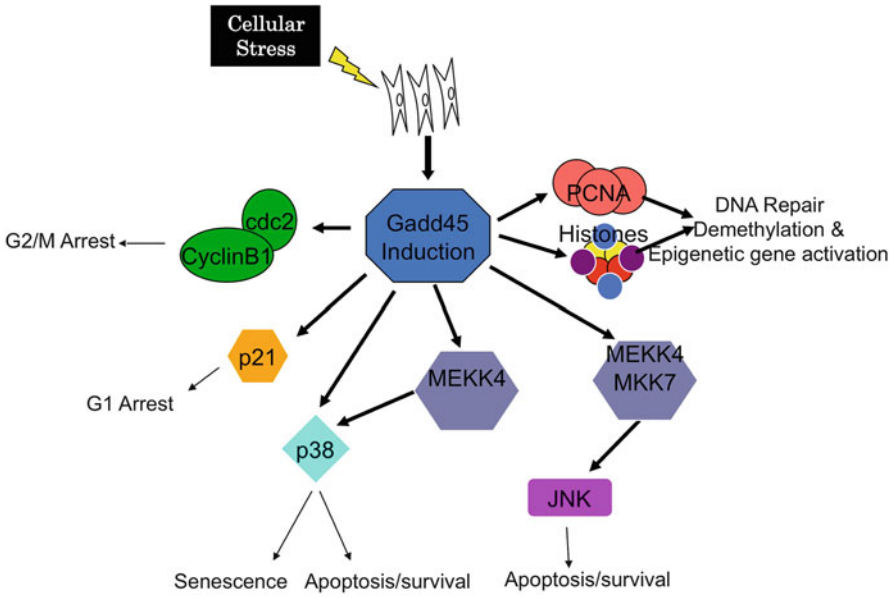

Fig. 2.1 Gadd45 function in stress signaling. Summary of the various protein–protein interactions of gadd45 proteins that affect cellular processes such as cell cycle arrest, DNA repair, survival, apoptosis, senescence, as well as epigenetic gene activation (Liebermann et al. 2011)

partners, which in turn may be determined by the type and magnitude of the stress stimulus and the cell type (Hoffman and Liebermann 2009; Liebermann et al. 2011). Recently, it was reported that gadd45a binds RNA and evidence is provided suggesting that the gadd45a protein is associated with a ribonucleoprotein particle (Sytnikova et al. 2011). Not surprisingly, published reports demonstrate that gadd45 genes participate in development and the function of multiple organ systems, as well as organ dysfunction and carcinogenesis. This chapter will focus on the influence of the gadd45 family of genes on carcinogenesis.

2.2 Gadd45 Is a Sensor of Oncogenic Stress

Gadd45 has a role in DNA repair, survival/apoptosis, cell cycle regulation, senescence, and maintaining genomic stability in response to stress; therefore, it is not surprising that it would play a role in initiation and progression of cancer as well as response to different therapies. There is a large body of evidence showing that gadd45 proteins are sensors of oncogenic stress and can modulate tumor initiation, progression, and response to different therapeutics. Its effect can be as either tumor promoter or tumor suppressor, depending upon the cell/tissue type and transforming events (Hoffman and Liebermann 2009; Liebermann et al. 2011).

Mice null for gadd45a displayed increased susceptibility to radiation-induced carcinogenesis (Hollander et al. 1999), and RAS-driven mammary tumorigenesis is increased in gadd45a null mice (Tront et al. 2006). Consistent with these observations, cells null for gadd45a exhibited genomic instability and escape from senescence (Hollander et al. 1999). Transformation of primary mouse cells requires introduction of two activated oncogenes; however, disruption of certain key growth control genes allows single oncogene transformation (Lundberg et al. 2000; Drayton and Peters 2002). Loss of gadd45a in combination with H-RAS expression was sufficient for transformation of murine embryonic fibroblasts (MEFs) (Hollander et al. 1999). Single oncogene transformation for either gadd45b or gadd45g null MEFs has not been determined.

2.3 Gadd45 Family Members Can Behave as Either Tumor Suppressors or Tumor Promoters

Although mutations in the gadd45 family of genes are infrequent in cancer, reduced expression of the three gadd45 family members has been observed in many tumors and tumor cell lines. Often, reduced expression is correlated with promoter methylation in several types of human cancer. The gadd45a promoter is frequently methylated in breast cancer, resulting in reduced expression when compared with normal breast epithelium (Wang et al. 2005). In pituitary adenomas, reduced expression of the gadd45g gene is seen in 67 % of patients and is often associated with methylation of the gadd45g gene; reversal of this epigenetic change results in reexpression of the protein (Bahar et al. 2004). Gadd45g is also downregulated in anaplastic thyroid cancer and in 65 % of hepatocellular carcinomas due to hypermethylation of its promoter (Sun et al. 2003). In another study, all three gadd45 genes were methylated and silenced in hepatocellular carcinoma as well, indicating a strong linkage between gadd45 gene expression and liver cancer. Furthermore, downregulation of gadd45b expression is associated with hypermethylation of the gadd45b promoter, and this is correlated with hepatitis C virus (HCV) and HCV-associated hepatocellular carcinomas (Higgs et al. 2010). Ying et al. (2005), analyzing the methylation status of two regions in the gadd45g promoter in a total of 75 cell lines as well as primary tissues and tumors, showed frequent promoter hypermethylation, including 85 % of non-Hodgkin's lymphoma, 50 % of Hodgkin's lymphoma, 73 % of nasopharyngeal carcinoma, 50 % of cervical carcinoma, 29 % of esophageal carcinoma, and 40 % of lung carcinoma but not in immortalized normal epithelial cell lines, normal tissues, or peripheral blood mononuclear cells. Primary carcinomas showed less frequent methylation than primary lymphomas. Ectopic express of gadd45g in tumor cells suppressed cell growth and colony formation, supporting a tumor suppressor role for gadd45g (Ying et al. 2005). Methylation-mediated repression of gadd45a was also found in prostate cancer (Ramachandran et al. 2009). The methylation status on all three gadd45 genes was assessed in 139 non-small-cell lung tumor

patient samples; a significant number of tumors (31.6 %) showed promoter methylation of gadd45g and downregulation of its mRNA (Na et al. 2010). Decreased RNA and protein expression of gadd45a and gadd45g, but not gadd45b, was observed in 138 gastric cardia adenocarcinoma (GCA) tumors, and was correlated with increased methylation frequency of the distal promoter of gadd45a and the proximal promoter of gadd45g in GCA tumors, and was inversely correlated with its cognate gene expression (Guo et al. 2013).

In addition to methylation, gadd45 gene expression is suppressed by other mechanisms in different tumors and tumor cell lines. Bioinformatic analysis of gene expression profiles in gonadotrope pituitary tumors, which have high morbidity and limited treatment options, identified repression of gadd45b, with no evidence of hypermethylation of its promoter. The tumor suppressor function of gadd45b was demonstrated by inhibition of growth, increased apoptosis, and reduced colony formation when overexpressed in the LBT2 mouse gonadotrope cell line (Michaelis et al. 2011). It was demonstrated that NFkB-mediated repression of gadd45a and gadd45g is critical for survival and tumor growth of the DU145 prostate cancer cell line and is a possible target for therapy (Zerbini et al. 2004). JunD is another mediator of gadd45a/g repression and cell survival in prostate cancer cells (Zerbini et al. 2011) and is also a potential target for therapy. Another mechanism responsible for reduced gadd45a expression in tumors is by elevated Myc; recently it was shown that Myc inhibits FOXO3a-induced transcription of gadd45a (Amente et al. 2011).

Data from other cancer samples suggests that gadd45 proteins can also have pro-oncogenic effects on different cancers; this appears to be the case in most studies on gadd45a in pancreatic cancer. Pancreatic cancer has the worst prognosis of all major malignancies in humans; therefore, there is a high priority to understand the molecular parameters that contribute to the disease and its response to therapy. Pancreatic ductal adenocarcinoma (PDA), the predominant form of human pancreatic cancer, is usually associated with the activation of K-RAS and inactivation of any or all of the tumor suppressor genes p16INK4a, p53, and SMAD4 (Hingorani et al. 2005; Leach 2004). One study showed that gadd45a expression is elevated in several PDA cell lines, and loss of gadd45a expression limits growth and survival of one cell line in culture (Schneider et al. 2006). We have preliminary data that inhibition of gadd45a expression in the PANC1 cell line also limits cell number, whereas there may be a small increase in cell numbers with elevated gadd45a expression. In contrast to above-cited studies, a recent study reported that overexpressing gadd45a induced apoptosis in a pancreatic cell line (Li et al. 2009).

A report analyzing clinical PDA samples attempted to correlate expression of gadd45a to p53 inactivation and outcome (Yamasawa et al. 2002). This was important since gadd45a is a p53 target gene, although it has been shown by this lab as well as by others to also be expressed independently of p53. Interestingly, elevated gadd45a expression was observed in 54 % of human pancreatic ductal carcinomas and the frequency of point mutations was found to be almost 14 % (Yamasawa et al. 2002). Moreover, overexpression of gadd45a protein, along with possible p53 loss of function, contributed to poor prognosis compared with patients with undetectable

gadd45a (Yamasawa et al. 2002). It should be pointed out that combined high levels of gadd45a and mutant p53 protein have been reported for several non-pancreatic cancer cell lines, such as breast (i.e., HS578T), central nervous system (SF-268), lung (NCI-H226), lymphoid (K562 and WI-L2-NS), prostate (PC3), and renal (TK10) cell lines (Hildesheim and Fornace 2002).

Using a mouse model of breast carcinogenesis, recent work conducted in this laboratory (Tront et al. 2006, 2010) has demonstrated that gadd45a can function as either a tumor suppressor or tumor promoter, depending on the transforming oncogene. MMTV–Myc and MMTV–RAS mouse strains null for gadd45a were generated, and each was compared to its wild-type counterpart. Loss of gadd45a was observed to accelerate the onset of RAS-driven mammary tumor formation; tumors increased more rapidly and had a more aggressive histological phenotype. The accelerated tumor formation was associated with both a decrease in apoptosis, linked to a decrease in JNK activation, and a decrease in senescence, linked with a decrease in p38 phosphorylation (Tront et al. 2006). In contrast to the tumor suppressor function of gadd45a in RAS-mediated breast tumorigenesis, in the MMTV–Myc mouse model of breast tumorigenesis, loss of gadd45a decelerated the onset of breast tumor formation (Tront et al. 2010). The tumor promoter function of gadd45a is mediated through negative regulation of MMP10 expression via the GSK3β/β-catenin signaling cascade, resulting in increased tumor vascularization. These novel results indicate that gadd45a can function to either promote or suppress breast tumor development through engagement of different signaling pathways, which depend on the molecular nature of the activated oncogene (Fig. 2.2).

A study was initiated in this laboratory to assess the level of Gadd45a expression in human breast tumors and to correlate it with clinicopathologic features. Expression was determined by immunohistochemistry. It was concluded that gadd45a levels are associated with hormone receptor status. Low levels of Gadd45a were found in normal breast tissue. High levels of Gadd45a were associated with both luminal A and luminal B subtypes, whereas in the triple-negative subtype, its expression was either low or negative (Tront et al. 2013).

Endocrine disruptors (ED), chemicals that alter or mimic physiological hormones (Casals-Casas and Desvergne 2011), have been found to originate from a wide variety of sources frequently encountered by humans. Given the role of estradiol in breast cancer causation, EDs that interfere with estrogen signaling have the potential to contribute to breast carcinogenesis (Jenkins et al. 2012). In support of this prediction, experiments in rodent breast cancer models show that the plastic component, bisphenol A (BPA) (Betancourt et al. 2010), the synthetic estrogen diethylstilbestrol (DES) (Rothschild et al. 1987; Kawaguchi et al. 2009), and the soy component genistein (Murrill et al. 1996; Lamartiniere et al. 1995) modulate breast carcinogenesis. Many of the EDs, including DES, BPA, and genistein, have been shown to increase gadd45 expression (Knight et al. 2009a, b; Regenbrecht et al. 2008; Salleh et al. 2003, 2004; Sowa et al. 2004). Thus, it is possible that some ED functions that modulate breast cancer are mediated through gadd45 stress signaling.

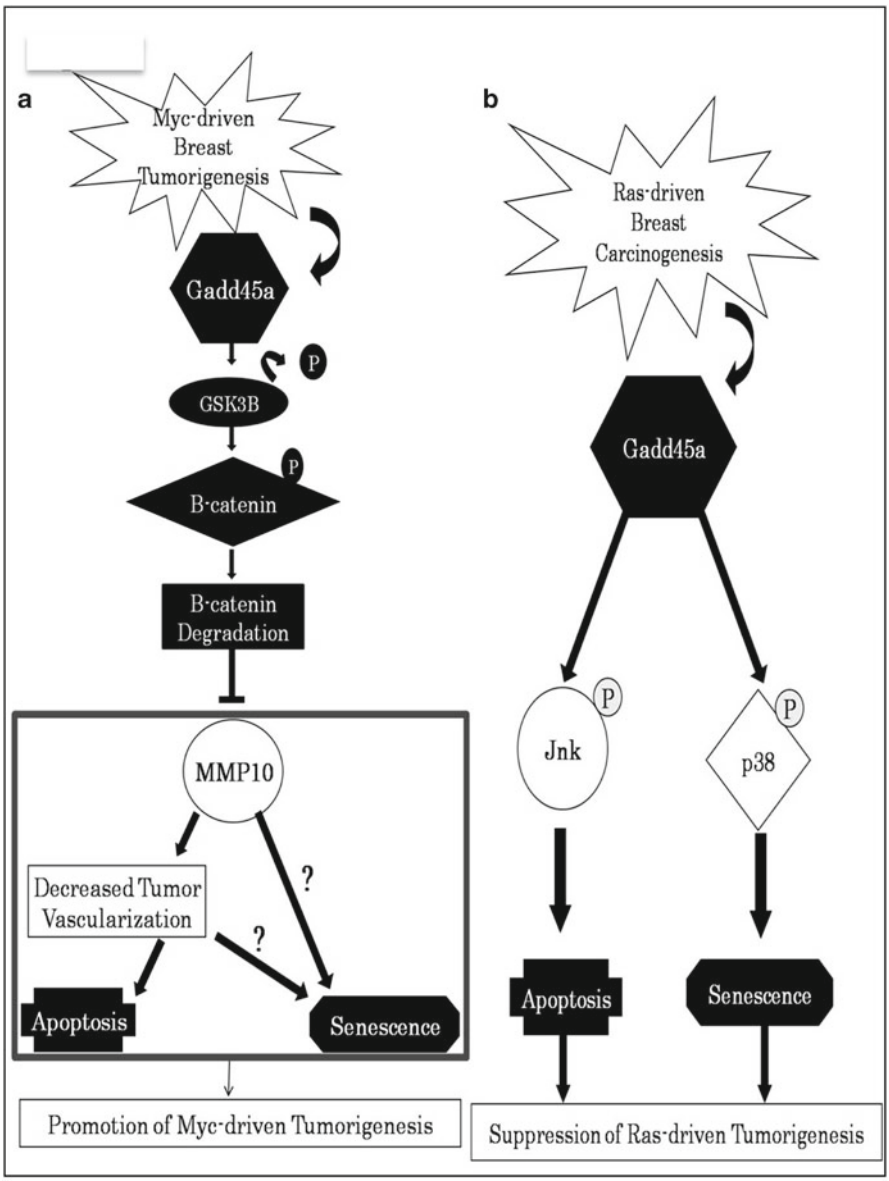

Fig. 2.2 Schematic diagram demonstrating how gadd45a modulates mammary tumor development in RAS-driven compared to Myc-driven tumors. Gadd45a suppresses RAS-driven tumorigenesis via Jnk-mediated apoptosis and p38-mediated senescence. In contrast gadd45a promotes Myc-driven tumorigenesis via GSK3β/β-catenin signaling which suppresses MMP10 expression, resulting in increased tumor vascularization and decreased apoptosis and senescence, ultimately accelerating tumor growth (Tront et al. 2010)

2.4 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 hematological stress, including acute stimulation with cytokines, myelo-ablation, 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), suggests 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 in 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 recent report showed that gadd45a methylation in AML is predictive of poor survival (Perugini et al. 2012).

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; Reimann et al. 2006; Miyauchi et al. 1994). 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 preliminary 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), further supporting the hypothesis that the gadd45 oncogenic stress function depends upon cell type, developmental stage, and specific 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.

BCR–ABL is the most common translocation in the myeloproliferative (MPD) disorder chronic myelogenous leukemia (CML); it is associated with a consistent cytogenetic abnormality, termed the Philadelphia chromosome (Ph1) (Nowell and Hungerford 1960), an acquired somatic mutation in the hematopoietic stem cell (Fialkow et al. 1967). A chimeric mRNA (8.5 kb) is translated to an activated BCR–ABL oncoprotein, most commonly 210 kDa in size. BCR–ABL displays constitutively active tyrosine kinase activity that leads to the recruitment of downstream effectors of cell proliferation and survival by several adapter proteins (e.g., GRB2, GAB2, CRKL) and signaling pathways (e.g., RAS, PI3K, JAK-STAT, PDk2-NFkB), each contributing to the pathogenesis of CML (Tefferi and Gilliland 2007). Data obtained in this laboratory indicates that loss of either gadd45a or gadd4b accelerates the development of BCR–ABL-driven leukemia using the mouse BMT model. In addition, gadd45a expression was observed to be altered in human CML samples, correlating with disease progression, thereby identifying gadd45 as a tumor suppressor in the context of BCR–ABL-driven leukemia (Liebermann et al. 2011, unpublished). How gadd45 functions as a tumor suppressor of BCR–ABL-driven leukemia, including which signaling pathways and downstream effectors are modulated by gadd45 to suppress leukemogenesis, is important to better understand the molecular pathology of CML.

2.5 Concluding Remarks

There is a large body of evidence showing that Gadd45 proteins are sensors of oncogenic stress and can modulate tumor initiation, progression, and response to different therapeutics. Its effect can be as either tumor promoter or tumor suppressor, depending upon the cell/tissue type and transforming events (Hoffman and Liebermann 2009; Liebermann et al. 2011). Altered expression of different gadd45 genes has been observed in many types of solid tumors as well as in hematopoietic malignancies. There are several instances when restoring gadd45 expression in low-expressing tumors/cells inhibits growth and/or increases apoptosis, suggesting reexpressing gadd45 as a goal for cancer therapy, separately or in combination with

other treatments. The role of gadd45 as a tumor suppressor or promoter for a specific tumor must be unequivocally established prior to elevating its expression.

The ability of gadd45 genes to respond to stressors used in chemotherapy can be switched off by methylation or overexpression of negative regulators such as NFkB or JunD, thereby limiting the effectiveness of the therapy. Drugs can be targeted to reexpress gadd45 in tumors, thereby rendering chemotherapy more effective.

Using human samples, cell lines, and mouse models, evidence is accumulating that gadd45 genes are attractive target(s) for therapy. Since gadd45 expression can impact on initiation and progression of many malignancies, and its role is very specific for each type of malignancy, it is important to precisely establish its role and to decipher the signaling pathways with which it interfaces.

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Chapter 3
Gadd45 Proteins: Key Players of Repair-Mediated DNA Demethylation

Andrea Schäfer

Abstract The three growth arrest and DNA damage 45 (Gadd45) family genes encode for stress-response proteins that are rapidly induced upon cellular stress or differentiation cues. They are well-characterized regulators of cell cycle, senescence, survival, and apoptosis. More recently, it has become clear that Gadd45 proteins promote active DNA demethylation thereby mediating gene activation. This epigenetic function of Gadd45 is important for differentiation and transcriptional regulation during development. Mechanistically, Gadd45 acts as an adapter for DNA repair factors at gene-specific loci to promote removal of 5-methylcytosine from DNA. Hence, Gadd45 is a nexus between DNA repair and epigenetic gene regulation.

3.1 Introduction

Gadd45a, -b, and -g, the three proteins of the growth arrest and DNA damage 45 (Gadd45) family, were originally identified as growth suppressing genes rapidly induced by stress or differentiation cues (Abdollahi et al. 1991; Beadling et al. 1993; Zhan et al. 1994; Zhang et al. 1999). Gadd45 functions are diverse including regulation of cell cycle, senescence, survival, apoptosis, and DNA repair (Fornace et al. 1988, 1992; Smith et al. 1996, 2000; Hollander and Fornace 2002; Mak and Kultz 2004; Barreto et al. 2007). Gadd45 exerts these various functions mainly via interaction with effector proteins of cell cycle, apoptosis, or DNA repair (Fig. 3.1). Since the protein does not harbor any obvious enzymatic activity, Gadd45a was rather surprisingly found as the top hit of a screen for an active DNA demethylase activity (Barreto et al. 2007). This expressing screen aimed to identify the "amazing demethylase" (Cedar and Verdine 1999), the elusive enzyme that actively removes

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D.A. Liebermann and B. Hoffman (eds.), *Gadd45 Stress Sensor Genes*, Advances in Experimental Medicine and Biology 793, DOI 10.1007/978-1-4614-8289-5_3,

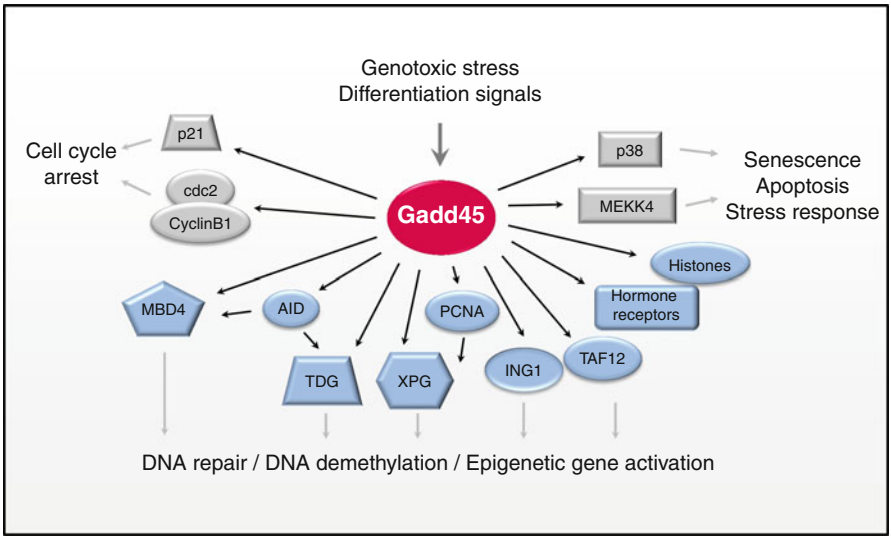

Fig. 3.1 Gadd45 interaction partners. The diagram shows the various interaction partners of Gadd45 and their effect on cell cycle regulation, senescence, apoptosis, DNA repair, and DNA demethylation. The following Gadd45 interacting proteins are depicted: Cdc2/cyclin B1 (Zhan et al. 1999; Vairapandi et al. 2002), proliferating cell nuclear antigen (PCNA) (Smith et al. 1994; Azam et al. 2001), p21 (Kearsey et al. 1995), p38 (Bulavin et al. 2003), MAPK kinase kinase MEKK4 (Takekawa and Saito 1998), nuclear hormone receptors (Yi et al. 2000), histones (Carrier et al. 1999), activation-induced (cytidine) deaminase (AID), methyl-CpG-binding domain protein 4 (MBD4) (Rai et al. 2008), thymine DNA glycosylase (TDG) (Cortellino et al. 2011), xeroderma pigmentosum group G protein (XPG) (Barreto et al. 2007), transcription initiation factor TFIID subunit 12 (TAF12) (Schmitz et al. 2009), and inhibitor of growth 1 (ING1) (Cheung et al. 2001; Schäfer et al. 2013). The figure is modified and updated from Niehrs and Schäfer (2012)

the epigenetic mark 5-methylcytosine from DNA (Gjerset and Martin 1982; Wilks et al. 1982; reviewed in Niehrs 2009). With the identification of Gadd45 as promoter of active DNA demethylation, many open questions arose: Is Gadd45-mediated DNA demethylation physiologically relevant? Is it important for development and disease? What is the mechanism of Gadd45-mediated DNA demethylation? Meanwhile, the crucial role of Gadd45-mediated DNA demethylation has been recognized in many different contexts. Mechanistically, Gadd45 engages cellular DNA repair enzymes to remove 5mC from the DNA. Hence, not only Gadd45 but also DNA repair proteins have an unexpected epigenetic function, which is the focus of the following chapter.

3.2 DNA Methylation and Demethylation

In eukaryotes, methylation of cytosine is the most prevalent covalent modification in DNA. In animals, cytosine methylation is predominantly confined to CpG dinucleotides yet also found in non-CpG residues in embryonic stem cells (Lister et al.

2009; Stadler et al. 2011). DNA methylation serves as an epigenetic mark whose presence in regulatory or promoter regions typically confers transcriptional silencing. DNA methylation functions in X chromosome inactivation, imprinting, tissue-specific gene expression, embryonic development, and cancer (reviewed in Jones and Takai 2001; Bird 2002; Deaton and Bird 2011; Hackett and Surani 2013).

During replication, the maintenance DNA methyltransferase 1 (Dnmt1) copies the methylation pattern to the newly synthesized DNA strand with high accuracy. Thereby, Dnmt1 passes the existing methylation pattern on to the next cell generation. Hence, DNA methylation was considered as a very stable epigenetic mark. However, it is meanwhile widely accepted that DNA methylation is a dynamic modification which can be erased via DNA demethylation.

One distinguishes passive versus active DNA demethylation. Passive DNA demethylation refers to the loss of 5mC during replication in the absence of Dnmt1 (Fig. 3.2). If Dnmt1 or its essential cofactor Uhrf1 (Np95) is inhibited, degraded, or excluded from the nucleus, every round of replication leads to 50 % reduction of 5mC and thereby to a progressive loss of DNA methylation. Since passive DNA demethylation requires replication, it can only operate in dividing cells and is therefore ineffective in terminally differentiated cells. A prominent example of passive DNA demethylation is the replication-dependent removal of 5mC from the maternal pronucleus in the mouse zygote (Mayer et al. 2000; Oswald et al. 2000; Santos et al. 2002). DNA replication likely also contributes to global hypomethylation in primordial germ cells (Seisenberger et al. 2012).

In contrast, active DNA demethylation is accomplished by an enzymatic activity (Fig. 3.2). By definition, DNA demethylation is active when replication-dependent demethylation can be excluded. Hence, reports of DNA demethylation in non-dividing cells, of non-replicating plasmids, or in vitro using cell extract indicate an active, enzymatic process (Gjerset and Martin 1982; Jost 1993; Weiss et al. 1996; Agius et al. 2006). Examples of active DNA demethylation are frequently associated with epigenetic reprogramming, cellular differentiation, or stress response in animals and plants. For example, reprogramming of the paternal pronucleus in the mouse zygote is intimately connected with its rapid DNA demethylation (Mayer et al. 2000; Oswald et al. 2000; Iqbal et al. 2011; Wossidlo et al. 2011). Similarly, reprogramming in developing germ cells requires active DNA demethylation of parental imprints (Hajkova et al. 2010; Hackett et al. 2013), although a contribution of passive demethylation cannot be ruled out (Kagiwada et al. 2013). Furthermore, induced reprogramming in cell culture systems involves active DNA demethylation and activation of key pluripotency genes (Zhang et al. 2007; Mikkelsen et al. 2008; Bhutani et al. 2010).

DNA demethylation is also induced by differentiation stimuli. For example, T-cell activation leads to demethylation of the *interleukin*-2 promoter (Bruniquel and Schwartz 2003). Similarly, glucocorticoid treatment induces demethylation of the *tyrosine aminotransferase* (*tat*) gene (Kress et al. 2006). Likewise, estradiol treatment has been shown to induce periodical demethylation of the *pS2* promoter (Kangaspeska et al. 2008; Metivier et al. 2008). Moreover, DNA demethylation and transcriptional activation of memory promoting genes are essential in the hippocampus in rats during fear conditioning (Miller and Sweatt 2007).

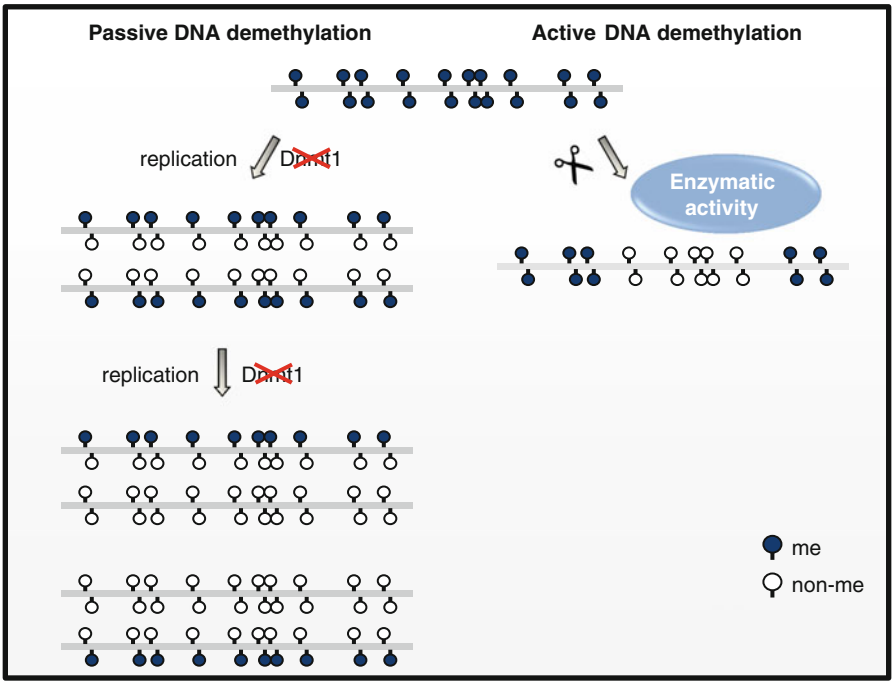

Fig. 3.2 Passive versus active DNA demethylation. During passive DNA demethylation, a first round of replication leads to two daughter strands that are only hemimethylated. If Dnmt1 activity is lacking, those daughter strands are not subjected to re-methylation. The methylation level is reduced with every round of replication and results only after the second replication in 50 % completely unmethylated DNA molecules. In contrast to the passive mechanism, active DNA demethylation is DNA replication independent, is mediated by enzymatic activities, and is targeted

The multitude of examples illustrates that DNA demethylation crucially affects the cellular methylation pattern in many different contexts and thereby contributes to the epigenetic signature of a cell (reviewed in Niehrs 2009; Wu and Zhang 2010).

3.3 Mechanisms of Active DNA Demethylation

The process of active 5mC removal appears complex, and it emerges that multiple mechanisms exist. It is still unclear what engages a specific mode of DNA demethylation in a specific context. However, tissue-specific expression of DNA demethylating factors and regulators is one possible determinant. The three *Gadd45* isoforms, for instance, are differentially expressed (Kaufmann et al. 2011) and upregulated upon various differentiation cues to induce demethylation of key differentiation genes (Ma et al. 2009; Le May et al. 2010b; Sen et al. 2010; Guo et al. 2011a; Zhang et al. 2011).

In general, one discriminates oxidative- from repair-based DNA demethylation. 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). For example, recent studies propose that Tet1/Tet2 or Tet3 convert 5mC to 5hmC in primordial germ cells (Hackett et al. 2013) and in the paternal pronucleus in the mouse zygote, respectively (Inoue and Zhang 2011; Iqbal et al. 2011; Wossidlo et al. 2011). In both systems, the gradual loss of 5hmC agrees with kinetics of replication-coupled dilution and indicates therefore a passive replacement of 5hmC. However, also active 5hmC removal by further oxidation to 5-formyl- and 5-carboxylcytosine (Ito et al. 2011) followed by excision from the DNA has been reported (He et al. 2011; Ito et al. 2011; Maiti and Drohat 2011).

In contrast to oxidative DNA demethylation, repair-based 5mC removal involves canonical cellular DNA repair machineries. This indicates that DNA repair proteins not only maintain genomic stability but also crucially influence epigenetic gene regulation. Three major repair modes can be distinguished: (1) excision of the methylated cytosine base or its oxidized derivatives by base-excision repair (BER), (2) BER after deamination of 5mC to thymine, and (3) excision of nucleotides encompassing the 5mC by nucleotide-excision repair (NER). In a common final step, DNA demethylation is completed by incorporation of unmethylated cytosine. As Gadd45 proteins play a key role in the latter two processes, these will be the focus of the following sections.

3.3.1 Gadd45-Mediated 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 not only conserved in frog, zebrafish, mouse, and man but also 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 and unpublished observation). Gadd45-mediated demethylation seems restricted to single-copy genes (Table 3.1), whereas global methylation appears unaffected (Jin et al. 2008; Engel et al. 2009; Schäfer et al. 2010). Therefore, it might not be surprising that Gadd45a mice display no major changes in global methylation (Engel et al. 2009). In addition, 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 only combined knockdown of all Gadd45 isoforms impairs DNA demethylation (Rai et al. 2008).

Gadd45-mediated DNA demethylation is active as it operates on non-replicating plasmid DNA. Furthermore, Gadd45a mediates DNA demethylation in nondividing *Xenopus* oocytes (Simonsson and Gurdon 2004; Barreto et al. 2007). Upon microinjection, *Xenopus* oocytes rapidly demethylate the methylation-silenced mouse *oct4* promoter (Simonsson and Gurdon 2004). This demethylation is fully dependent on Gadd45a which is physically recruited to the *oct4* promoter during DNA demethylation (Barreto et al. 2007; Schäfer et al. 2013).

Examples of Gadd45 demethylation targets are numerous and found in various biological systems (Table 3.1). A recurrent feature of Gadd45-mediated DNA demethylation is that it is often initiated by differentiation stimuli or cellular stress

Table 3.1 Examples of DNA demethylation implicating Gadd45

Gadd45 gene	System	Target	Cofactor	Reference
Gadd45a	*Xenopus* oocytes	*oct4* promoter plasmid	XPG	Barreto et al. (2007)
Gadd45a	HEK293T NIH 3T3	*rDNA*	XPG, XPA, XPF, TAF12	Schmitz et al. (2009)
Gadd45a	Hela cells, RA stimulated	*RARβ2*	XPC, XPA, XPG, XPF/ERCC1	Le May et al. (2010b)
Gadd45a	CD4+ T cells (autoimmune)	*CD11a*, *CD70*		Li et al. (2010)
Gadd45a	Osteogenic differentiation	*Dlx5*, *Runx2*, *Bgp*, *Osterix*		Zhang et al. (2011)
Gadd45a	MEFs	*(Crabp2*, *Efs*, *Hoxa5*, *Rbp1)*	AID, TDG	Cortellino et al. (2011)
Gadd45a	HEK293T MEFs	*MAGEB2*, *DHRS2*, *TAF7L* *Mageb2*, *Hoxd8*, *Cxcl1*	Ing1	Schäfer et al. (2013)
Gadd45b	Mouse adult neurogenesis	*Bdnf IX*, *Fgf-1b* *Per2*, *Crebbp*, *Grip1*		Ma et al. (2009) Guo et al. (2011a)
Gadd45b	Parietal cortex in psychosis	*BDNF IX*		Gavin et al. (2012)
Gadd45b	Treatment of schizophrenia	*Reelin*, *BDNF*, *GAD67*		Matrisciano et al. (2011)
Gadd45a, *-b*	Epidermal differentiation	*S100*		Sen et al. (2010)
Gadd45a, *-b*, *-g*	Zebrafish embryos	*Plasmid DNA* *Bulk genome (Neurod2*, *Sox2*, *Sox1a)*	Aid/Apobec, Mbd4	Rai et al. (2008)

Parentheses mark genes where the evidence for a direct demethylation by Gadd45 is circumstantial
Table modified from Niehrs and Schäfer (2012)

that both lead to Gadd45 gene induction. For example, *Gadd45a* is upregulated during osteogenic differentiation of mesenchymal stem cells and demethylates key differentiation genes like *Osterix* and *Runx2* (Zhang et al. 2011). Similarly, *Gadd45a* and *Gadd45b* are induced upon calcium-induced differentiation of epidermal progenitor cells and demethylate the differentiation marker *S100P* (Sen et al. 2010). Furthermore, *Gadd45* promotes neurogenesis by preventing epigenetic silencing of proneural markers like *Neurod2* during zebrafish development (Rai et al. 2008). Gadd45b is essential for adult neurogenesis by demethylating and activating key neurogenic genes like *Bdnf IX* and *Fgf1* (Ma et al. 2009; Guo et al. 2011a). Conversely, *Bdnf IX* promoter hypermethylation due to reduced Gadd45b recruitment is a hallmark of psychotic disorders (Gavin et al. 2012). Notably, antipsychotic medication restores *Gadd45b* recruitment to *Reelin* and *Bdnf* promoters and their physiological hypomethylation (Matrisciano et al. 2011).

3.3.2 Repair-Mediated DNA Demethylation by Gadd45

The various examples point out a central role of Gadd45 proteins in active DNA demethylation. However, the lack of any obvious enzymatic activity of Gadd45 raises the question about the mechanism. The implication of Gadd45 in base- and nucleotide-excision repair (BER, NER, Box 3.1) suggested an attractive mechanism of repair-mediated DNA demethylation by Gadd45.

Box 3.1 Base- and Nucleotide-Excision Repair

BER is the main DNA repair pathway to correct small base lesions arising from metabolic by-products and exogenous stresses (reviewed in Fromme and Verdine 2004; Robertson et al. 2009). Oxidation, alkylation, deamination, and depurinatiation/depyrimidination cause lesions like 8-oxoguanine, 7-methylguanine, and uracil. BER repairs these lesions in a two-step process. First, damage-specific DNA glycosylases, the key enzymes of BER, recognize the lesion. To date, 11 different glycosylases have been identified that allow repair of a broad range of alterations. These enzymes hydrolyze the N-glycosidic bond creating an apyrimidinic/apurinic (AP) site intermediate. Typically an AP endonuclease generates a nick, 5′ of the AP site. The resulting gap contains a 3′-hydroxyl and a 5′-phosphate that prime DNA repair synthesis by specified DNA polymerases. DNA ligases seal the nick and restore thereby the integrity of the double helix. Efficient BER is important to avoid accumulation of mutations which contribute to cancerogenesis, aging, and neurodegeneration.

NER is the main pathway to repair UV-induced damage in human cells. More generally, it removes bulky helix-distorting lesions including those induced by mutagens like cisplatin. NER inactivation causes severe autosomal inherited disorders like xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD) (reviewed in de Boer and Hoeijmakers 2000; Cleaver 2005). The affected proteins are named accordingly, for example, xeroderma pigmentosum complementation group proteins (XPA to XPG). NER is a multistep process involving the concerted action of a multiprotein complex. Specific DNA damage recognizing protein complexes recruit the NER machinery. Stalling of the RNA polymerase II by bulky DNA lesions engages transcription-coupled repair (TC-NER) by recruitment of the Cockayne syndrome proteins, CSA and CSB. In contrast, global genomic repair (GG-NER) is initiated by the XPE-XPC recognition complex. Both recognition events activate a common downstream pathway. The helicases XPB and XPD unwind the DNA and recruit XPA which serves as platform for the binding of the 3′- and 5′ endonucleases XPG and XPF-ERCC1, respectively.

(continued)

Box 3.1 (continued)

These endonucleases nick the DNA for removal of an ~24–32 nucleotide-long oligonucleotide. The resulting gap is filled by DNA polymerase activity, a process which is positively influenced by PCNA. Finally, DNA ligases seal the nick. Inactivation of NER leads to aging, neurological defects, and increased cancer predisposition. For example, XP patients have a 1,000-fold higher risk of developing skin cancers, including malignant melanoma.

Gadd45a has previously been implicated in BER. Engagement of the BER machinery by the base-damaging agent methanesulfonate (MMS) induces *Gadd45a* which is required for efficient repair of the base lesions (Zhan et al. 1996; Jung et al. 2007). The central enzymes of BER are DNA glycosylases that recognize the damage and excise the base by hydrolyzing the N-glycosidic bond. In zebrafish, the thymine glycosylase Mbd4, which specifically recognizes G:T mismatches, interacts with Gadd45 and the 5mC deaminases Aid and Apobec2a/2b (Rai et al. 2008). This complex is vital for demethylation of methylated DNA fragments and proneural genes like *neurod2* during development. The data point to a model in which the deaminases convert 5mC in a first step to thymine. The resulting G:T mismatch is subsequently recognized and excised by the Mbd4 glycosylase. The gap is filled with unmethylated cytosine to accomplish DNA demethylation (Fig. 3.3). In this model, Gaddd45 targets Aid/Mbd4 to the DNA demethylation site as a scaffold protein. Yet, a further Gadd45/Aid demethylation complex containing another thymine DNA glycosylase Tdg has been identified in mammalian cells (Cortellino et al. 2011). Tdg knockout cells fail to demethylate retinoic acid- and glucocorticoid-responsive target genes (Cortellino et al. 2011). In these contexts, Gadd45a is proposed to recruit the Tdg/Aid complex to the target genes. Subsequently Tdg excises as glycosylase the product of 5mC deamination, namely, thymine. However, Tdg can also excise oxidative DNA demethylation intermediates like 5-carboxylcytosine or 5-formylcytosine generated by the Tet proteins (Maiti and Drohat 2011). Hence, Tdg might create a functional nexus between oxidative- and base-excision-repair-mediated DNA demethylation, interconnecting oxidized 5mC derivatives (Maiti and Drohat 2011), and Gadd45a (Cortellino et al. 2011). Whether Gadd45 is involved in the removal of oxidized bases remains to be shown. However, this is a tempting hypothesis given the intriguing overlap between Tet1 and Gadd45b demethylation targets in the brain (Ma et al. 2009; Guo et al. 2011b).

In addition to BER, Gadd45 has been implicated in NER. Gadd45a knockdown in colon cancer cells induces NER defects and thereby hypersensitivity to UV irradiation and DNA damaging agents like cisplatin (Smith et al. 1996). Similarly, genetic loss of Gadd45a leads to reduced global genomic NER as the cells are hypersensitive to DNA damage stress (Smith et al. 2000; Hollander et al. 2001; Gupta et al. 2005). This likely contributes to chromosomal abnormalities in Gadd45a knockout cells and increased radiation- and carcinogen-induced tumorigenesis in Gadd45a knockout mice (Hollander et al. 1999, 2001; Hildesheim et al. 2002). Furthermore, Gadd45a and the NER gene

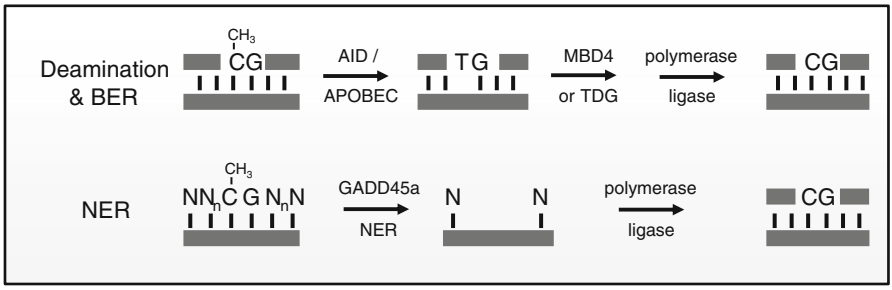

Fig. 3.3 Gadd45-mediated DNA demethylation by BER and NER. DNA demethylation by deamination of 5mC followed by base-excision repair (BER) or nucleotide-excision repair (NER); N_n in the NER model indicates up to 32 nucleotides, which may be excised. Key: *AID* activation-induced (cytidine) deaminase, *MBD4* methyl-CpG-binding domain protein 4, *TDG* thymine DNA glycosylase. The figure is modified from Niehrs and Schäfer (2012)

XPC genetically interact in lung tumorigenesis in double-mutant mice (Hollander et al. 2005). 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). Furthermore, Gadd45 proteins influence 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).

Typical NER characteristics are (1) the excision and replacement of a stretch of nucleotides by endonucleases and (2) the requirement of xeroderma pigmentosum (XP) group proteins. Notably, Gadd45-dependent *oct4* demethylation in *Xenopus* oocytes is accompanied by nucleotide incorporation, indicative for DNA synthesis and DNA repair (Barreto et al. 2007). In addition, the 3′ NER endonuclease XPG binds Gadd45a and is required for demethylation in mammalian cells as well as in *Xenopus* oocytes (Barreto et al. 2007; Schmitz et al. 2009). The data argue for 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 filled with unmethylated nucleotides by unscheduled DNA synthesis, completing DNA demethylation (Barreto et al. 2007) (Fig. 3.3). A paradigm of this model is the rDNA locus in mammalian cells. Upon Gadd45a-mediated DNA demethylation, the rDNA locus incorporates BrdU, a nucleotide analogue, in arrested NIH3T3 cells indicating unscheduled DNA repair synthesis (Schmitz et al. 2009). Furthermore, the NER components XPA, XPG, or XPF are essential for this demethylation, as their knockdown induces *rDNA* promoter hypermethylation associated with heterochromatic histone marks and transcriptional silencing (Schmitz et al. 2009). Similarly, Gadd45a and NER components co-associate at the *RARb2* promoter to demethylate the locus upon retinoic acid stimulation (Le May et al. 2010b). Many NER factors not only function in DNA repair but are also linked to transcriptional activation as part of the transcription factor complex TFIIH (Ito et al. 2007; Le May et al. 2010b; reviewed in Le May et al. 2010a). This raises the question whether they are required for Gadd45a-mediated demethylation as true repair

enzymes or rather as transcription factors. The observation that rDNA demethylation requires the NER endonuclease activity of XPG suggests that indeed the enzymatic repair role of XPG is required for Gadd45a-mediated demethylation (Schmitz et al. 2009).

In a nutshell, the data support two distinct models for BER- and NER-mediated DNA demethylation by Gadd45 which may either be interconnected or independent. The first scenario suggests that the two repair models are employed sequentially creating a hemimethylated intermediate: Gadd45-mediated DNA demethylation of the first strand by NER might be followed by BER-mediated DNA demethylation of the second strand to achieve full demethylation. However, at least Gadd45-mediated demethylation of rDNA is not affected by knockdown of the BER enzyme TDG (Schmitz et al. 2009) or by BER inhibitors but only by the NER inhibitor gemcitabine (Schmitz et al. 2009; Schäfer et al. 2010). This rather suggests that NER- and BER-mediated demethylation are employed independently. However, the factors influencing the pathway choice in a specific cell type and context are currently unknown.

3.4 Targeting Gadd45-Mediated DNA Demethylation

A common observation is that Gadd45-mediated demethylation is a highly selective process. It is not only gene-specific, but within a given gene, it typically affects only distinct CpGs (Barreto et al. 2007; Schmitz et al. 2009; Schäfer et al. 2013). Therefore, one of the primary questions is how targeting specificity of the Gadd45 demethylation complex is achieved.

An important determinant for target specificity is a distinct chromatin pattern at a given target gene promoter. Gadd45-mediated DNA demethylation typically affects promoter proximal CpGs, whereas distal regions remain unaffected (Barreto et al. 2007; Schmitz et al. 2009; Le May et al. 2010b; Cortellino et al. 2011; Schäfer et al. 2013). Proximal regions are often associated with specific histone modifications like histone H3 lysine 4 trimethylation (H3K4me) (Santos-Rosa et al. 2002; Schneider et al. 2004). Interestingly, the inhibitor of growth 1 (Ing1), a reader of H3K4me3, interacts with Gadd45a to promote gene-specific DNA demethylation (Schäfer et al. 2013). Ing1 recognizes sites marked by H3K4me3 via its PHD domain which is required to recruit Gadd45a. Reduction of H3K4 methylation impairs Gadd45a/Ing1 recruitment and target gene demethylation. Genome-wide profiling identified novel Gadd45a/Ing1 target genes depending on the Ing1-PHD domain and H3K4me3. Hence, Ing1 acts as an adaptor between chromatin and Gadd45a-mediated DNA demethylation (Schäfer et al. 2013). However, though H3K4me3 is essential for Gadd45a recruitment, it is not sufficient as it is found at many more loci than are regulated by DNA methylation. Hence, further yet unknown factors are required to specify Gadd45a recruitment.

Additional candidates mediating target specificity might be the nuclear hormone receptors (NR). The NRs could recruit the DNA demethylation complex to their cognate hormone-responsive elements. Gadd45 proteins are known to bind several

NRs including RXRa, RARa, ERa, PPARa, -b, and -g and to co-activate nuclear hormone-responsive reporters (Yi et al. 2000). Indeed, retinoic acid- (Le May et al. 2010b), dexamethasone- (Zhang et al. 2011), or constitutive androstane receptor ligand treatment (Tian et al. 2011) induce Gadd45 binding to the hormone-responsive target genes followed by DNA demethylation and activation. A further prerequisite for Gadd45 targeting seems to be low-level transcription (Le May et al. 2010b). Gadd45 interacts with TAF12, a component of the PolI transcription complex. TAF12 and hence transcription are required for Gadd45-mediated rDNA demethylation (Schmitz et al. 2009). Notably, transcription is also required for histone acetylation-induced DNA demethylation (D'Alessio et al. 2007). Eventually, Gadd45a might also be involved in this process as it preferentially interacts with acetylated chromatin (Carrier et al. 1999).

The presence of 5mC is another determinant for Gadd45 targeting. For example, Gadd45 enhances Mbd4/Aid recruitment to methylated but not to unmethylated DNA substrates (Rai et al. 2008). Interestingly, Gadd45a binds hemimethylated DNA with high avidity (Lee et al. 2011). Furthermore, Gadd45a binds Dnmt1 and impairs re-methylation of the hemimethylated substrate (Lee et al. 2011). Thus, Gadd45a function in DNA demethylation might not be limited to recruit the repair machinery, but might extend to protect a (hemimethylated) DNA demethylation intermediate from re-methylation.

Finally, Gadd45 targeting might involve noncoding RNAs (ncRNAs). ncRNAs fulfill a variety of epigenetic functions, and there is an intimate cross talk between ncRNAs and DNA methylation. In addition to their function in targeting DNA methylation (Matzke et al. 2007), there is evidence for ncRNA-targeted DNA demethylation (Imamura et al. 2004; Zheng et al. 2008). Interestingly, Gadd45, as member of the RNA binding L7Ae ribosomal protein family, can bind RNA and is part of a ribonucleoprotein complex (Sytnikova et al. 2011). However, the physiological relevance, the identity of cognate RNAs, and a role of this complex in DNA demethylation remain unknown.

3.5 Concluding Remarks

Gadd45 emerged as key player in active DNA demethylation in recent years. This was as surprising as the implication of the canonical DNA repair proteins in epigenetic gene activation. Far from merely protecting the genome from inflicting lesions, the repair proteins present the central enzymes of the majority of different DNA demethylation models. Despite major improvements in the understanding of this complex process, several different issues remain unresolved. The direct implication of nuclear hormone receptors needs to be established. The relation of NER- and BER-mediated DNA demethylation requires more investigation. The possible role of Gadd45 in oxidative DNA demethylation requires proof of principle. In sum, although many puzzle pieces seem uncovered, some connections still have to be identified to recognize the big picture of active DNA demethylation.

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Chapter 4
Gadd45 Proteins in Immunity

Ingo Schmitz

Abstract The vertebrate immune system protects the host against invading pathogens such as viruses, bacteria and parasites. It consists of an innate branch and an adaptive branch that provide immediate and long-lasting protection, respectively. As the immune system is composed of different cell types and distributed throughout the whole body, immune cells need to communicate with each other. Intercellular communication in the immune system is mediated by cytokines, which bind to specific receptors on the cell surface and activate intracellular signalling networks. Growth arrest and DNA damage-inducible 45 (Gadd45) proteins are important components of these intracellular signalling networks. They are induced by a number of cytokines and by bacterial lipopolysaccharide. 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. Moreover, Gadd45β regulates autophagy, a catabolic pathway that also degrades intracellular pathogens. Regarding adaptive immunity, Gadd45 proteins are especially well characterized in T cells. For instance, Gadd45β and Gadd45γ regulate cytokine expression and Th1 differentiation, while Gadd45α inhibits p38 kinase activation downstream of the T cell receptor. Due to their many functions in the immune system, deficiency in Gadd45 proteins causes autoimmune diseases and less efficient tumour immunosurveillance.

Abbreviations

ATG	Autophagy-related
Bcl-x_L	B cell lymphoma x large
c-FLIP	Cellular FLICE inhibitory protein

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D.A. Liebermann and B. Hoffman (eds.), *Gadd45 Stress Sensor Genes*, Advances in Experimental Medicine and Biology 793, DOI 10.1007/978-1-4614-8289-5_4,

Cbl-b	Casitas B-lineage lymphoma proto-oncogene b
CD	Cluster of differentiation
$CD4^+$	Cluster of differentiation 4-positive
$CD8^+$	Cluster of differentiation 8-positive
CLR	C-type lectin receptor
CR6	Cytokine response gene 6
DISC	Death-inducing signalling complex
EAE	Experimental autoimmune encephalomyelitis
Egr	Early growth response
G-CSF	Granulocyte colony-stimulating factor
Gadd45	Growth arrest and DNA damage 45
GM-CSF	Granulocyte–macrophage colony-stimulating factor
GRAIL	Gene related to anergy in lymphocytes protein
IFN	Interferon
IL	Interleukin
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
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
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
PRR	Pattern recognition receptor
RLR	Retinoic acid-inducible gene (RIG)-I-like receptor
ROS	Reactive oxygen species
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TGF-β	Transforming growth factor beta
Th	T helper
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
TNFR1	Tumour necrosis factor receptor 1
vMIA	Viral mitochondrial-localized inhibitor of apoptosis
ZAP-70	Zeta-chain associated protein of 70 kDa

4.1 Introduction

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 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 triggering, these receptors activate the NF-κB signalling 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 then mount an adaptive immune response. T cells are lymphocytes that develop in the thymus (hence, named T cell) and mediate cellular immunity. There are different types of T cells, namely, cytotoxic T cells and helper T cells. Cytotoxic T cells express the co-receptor CD8 ($CD8^+$ CTLs) and kill infected target cells via the induction of apoptosis. Helper T cells express the co-receptor CD4 ($CD4^+$ Th cells) 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 CD40. 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 reinfection 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 and Th17 cells, 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). They develop upon stimulation of naïve T cells with both the immunosuppressive cytokine transforming growth factor beta (TGF-β) and with the pro-inflammatory cytokine IL-6 and 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 signalling networks in immune cells. Here, I will first discuss signals that induce expression of Gadd45 proteins in cells of the immune system. The next two sections will be on the function of Gadd45 proteins in the innate and adaptive immune system, respectively. Finally, I will discuss the role of Gadd45 proteins in pathological settings, such as autoimmune diseases (Fig. 4.1).

4.2 Expression of Gadd45 Proteins in Immune Cells

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 (Sanjuan et al. 2007; Selvakumaran et al. 1994; Yang et al. 2001); and Gadd45γ transcription is stimulated by interleukin-2 (Beadling et al. 1993).

With respect to innate immune signals, it has been shown that LPS, a cell wall component of gram-negative bacteria, induced Gadd45β in vivo (Zhang et al. 2005). Of note, Gadd45β induction could be inhibited by co-administration of the

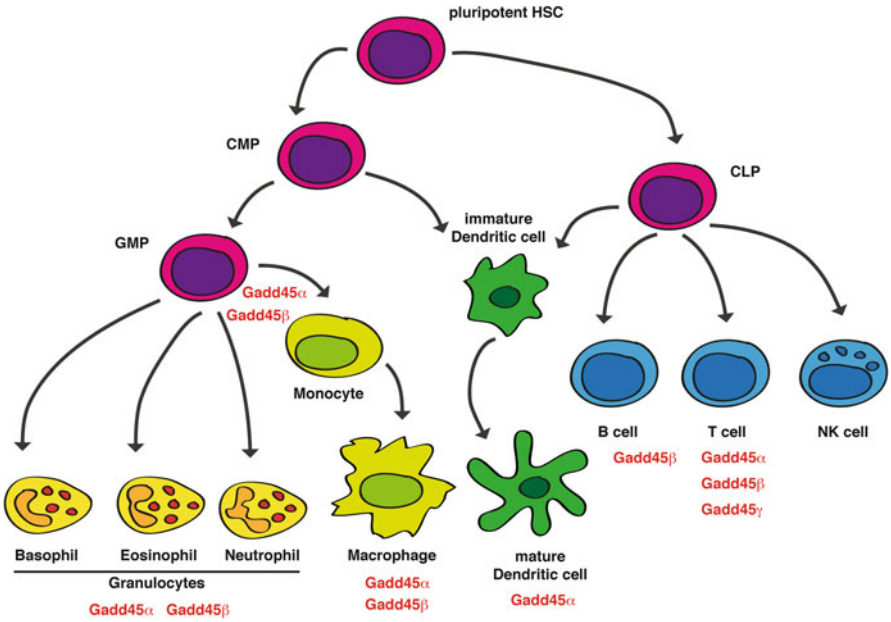

Fig. 4.1 Gadd45 proteins in hematopoiesis and function of immune cells. Immune cells develop from pluripotent hematopoietic stem cells (HSC) that give rise to myeloid (granulocytes (*yellow*) and monocytes/macrophages (*light green*)) and lymphoid (B cells, NK cells and T cells (*blue*)) lineages. Dendritic cells (*dark green*) can develop from both the myeloid and lymphoid lineages. Progenitor cells are shown in *purple*. Gadd45 proteins are shown in *red* next to the cell type, in which they play a crucial role in development or function. *CMP* common myeloid progenitor, *GMP* granulocyte monocyte progenitor, *CLP* common lymphoid progenitor. Certain progenitor cell types and red blood cells are not shown for clarity reasons

glucocorticoid analogue dexamethasone or by thalidomide. Since both drugs are inhibitors of the pro-inflammatory transcription factor NF-κB (Keifer et al. 2001; Majumdar et al. 2002; Neumann and Naumann 2007), these data suggested that *Gadd45b* is an NF-κB target gene. Indeed, bortezomib, a proteasome inhibitor that impairs degradation of IκB proteins and thereby NF-κB activation, but not pharmacological inhibitors of mitogen-activated protein kinases (MAPKs), prevented Gadd45β induction by LPS in mice (Zhang et al. 2005). In line with this observation, analysis of the *Gadd45b* gene indicated several functional NF-κB binding sites (Balliet et al. 2001; Jin et al. 2002). It was shown that NF-κB-induced Gadd45β inhibits activation of the MAPK JNK via inhibition of the upstream kinase MKK7 in TNFα-treated T cell hybridomas (De Smaele et al. 2001; Papa et al. 2004). However, it is currently unknown whether this pathway operates in immune cells in vivo. Furthermore, so far it has not been addressed so far whether other stimuli of the innate immune systems (i.e. PAMPs) that activate the NF-κB pathway also induce Gadd45β protein expression.

A number of cytokines have been reported to induce the expression of Gadd45 proteins in hematopoietic cells and cells of the immune system. First,

hematopoietins such as granulocyte–macrophage colony-stimulating factor (GM-CSF), M-CSF, G-CSF and interleukin-3 (IL-3) were shown to induce expression of all *Gadd45* genes at the mRNA level in bone marrow cells (Gupta et al. 2006). Hematopoietic cells from the bone marrow, which are either deficient for Gadd45α or Gadd45β and are more sensitive to apoptosis upon several kinds of cellular stresses, suggesting that these two proteins act in an anti-apoptotic fashion in this cell type (Gupta et al. 2005, 2006). IL-6, a cytokine that supports differentiation of hematopoietic cells and that has pro-inflammatory activity, induces Gadd45β in the murine myelomonocytic cell line M1 (Zhan et al. 1994). Another pro-inflammatory cytokine that induces Gadd45 expression and belongs to the IL-1 family is IL-18 (Arend et al. 2008). IL-18 induced the expression of *Gadd45b* and *Gadd45g* genes in $CD4^+$ Th cells, which was dramatically enhanced by co-treatment with IL-12 (Yang et al. 2001). Of note, Gadd45α was not induced by IL-12 and IL-18. Similarly to IL-18 in $CD4^+$ Th cells, IL-33, another IL-1 family cytokine, synergized with IL-12 to induce Gadd45β expression in $CD8^+$ cytotoxic T cells (Yang et al. 2011). In sum, pro-inflammatory cytokines appear to be crucial inducers of Gadd45 gene expression in hematopoietic cells.

Since the action of these pro-inflammatory cytokines on T cells often depends on the activation status of T cells, it is not surprising that the stimulation of the T cell receptor (TCR) also induced *Gadd45* gene expression. Thus, stimulation of naïve $CD4^+$ T cells with anti-CD3 antibodies (triggering the TCR complex) led to Gadd45β expression at early time points (within 4 h), an effect that was augmented by co-stimulation via CD28 (Lu et al. 2004). This early induction of Gadd45β was also observed in vivo in thymocytes using TCR transgenic mice and injection of cognate peptide antigens (Schmitz et al. 2003). In contrast, induction of Gadd45γ required prolonged stimulation of naïve $CD4^+$ T cells for 48–96 h (Lu et al. 2001). This may be related to the fact that IL-2 signalling rather than TCR or CD28 signals induces Gadd45γ expression (Hoffmeyer et al. 2001).

Surprisingly, Gadd45β expression is not only induced by the above-mentioned immunostimulatory signals but also by transforming growth factor beta (TGF-β), which is a strong immunosuppressive cytokine (Li and Flavell 2008). Thus, Gadd45β can be induced by TGF-β in the murine myelomonocytic cell line M1 and in the T cell line EL-4 (Selvakumaran et al. 1994). Accordingly, *Gadd45b* gene expression is regulated by Smad proteins, which are transcription factors that are activated at the TGF-β receptor complex (Takekawa et al. 2002; Ungefroren et al. 2005; Yoo et al. 2003). However, it is currently unknown whether Gadd45β is actually required for the immunosuppressive function of TGF-β on immune cells in vivo.

4.3 The Function of Gadd45 Proteins in Innate Immunity

Within the innate immune system, the function of Gadd45 proteins is best studied in myeloid cells such as granulocytes and macrophages. For instance, in the absence of either Gadd45α or Gadd45β, in vitro differentiation of bone marrow cells into

macrophage or granulocyte lineages resulted in reduced frequencies of these cell types (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 signalling by Gadd45 proteins. In summary, Gadd45 proteins play a crucial role in the differentiation, proliferation and function of myeloid cells.

As stated previously, LPS induces Gadd45β via the NF-κB pathway in vivo (Zhang et al. 2005). However, what could be the function of Gadd45β downstream of LPS? A recent study provides evidence that Gadd45β regulates macroautophagy (Keil et al. 2013), a process that 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 called ATG5, without which 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). This essential macroautophagy protein is targeted by a Gadd45β-MEKK4-p38 signalling pathway since Gadd45β and MEKK4 direct p38

to the autophagosome (Keil et al. 2013). MEKK4 is a mitogen-activated protein kinase kinase kinase (MAP3K) that activates p38 and JNK mitogen-activated protein kinases and is present in a closed conformation and, therefore, inactive (Gerwins et al. 1997; Takekawa et al. 1997). Upon binding of Gadd45 proteins, MEKK4 adopts an open conformation that allows dimerization, autophosphorylation and activation of downstream kinases (Miyake et al. 2007; Takekawa and Saito 1998). 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. Accordingly, Gadd45β-deficient fibroblasts and macrophages exhibited enhanced macroautophagy upon LPS treatment (Keil et al. 2013) (Fig. 4.2).

4.4 The Function of Gadd45 Proteins in Adaptive Immunity

With respect to adaptive immunity, most of the work on Gadd45 proteins has concentrated on T cells. However, there are also a few reports on other cell types of the adaptive immune system. For instance, it was shown that B cells strongly induce Gadd45β along with known anti-apoptotic proteins such as Bcl-x_L 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 signalling cascade such as the formation of the death-inducing signalling 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-x_L 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 signalling 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.

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).

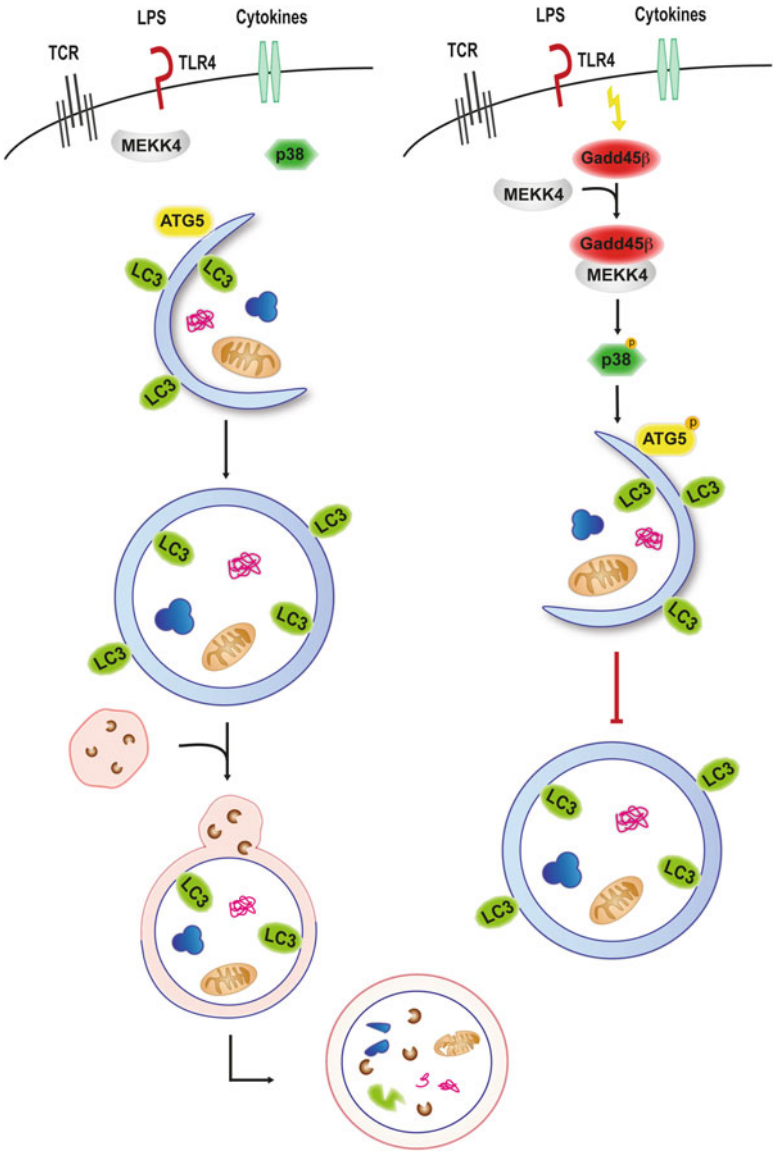

Fig. 4.2 Gadd45β and autophagy. Upon starvation or infection with intracellular pathogens, the cell mounts an autophagic response. The autophagosomal membrane (*light blue*) forms de novo and elongates in an LC3-II- (*light green*) and ATG5-dependent (*yellow*) manner to engulf protein aggregates, organelles or intracellular pathogens. Subsequently, the autophagosome (*light blue*) fuses with a lysosome (*light red*) leading to vesicle acidification and subsequent cargo degradation. Gadd45β (*red*) and MEKK4 (*grey*) together direct the p38 mitogen-activated protein kinase (*dark green*) to the autophagosomal membrane, where it phosphorylates ATG5. This event inhibits maturation of the autophagosome and, thus, blocks autophagy. Gadd45β expression is induced by TCR triggering, by binding of lipopolysaccharide (LPS) to toll-like receptor 4 (TLR4) or by certain cytokines

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 *Gadd45*β (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.

Dendritic cells are the most potent professional antigen-presenting cells (Shortman and Liu 2002). They 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 signalling by Gadd45 proteins is crucial for mounting a Th1 response via activation of dendritic cells. Currently, 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 (Ronkina et al. 2010).

Regarding Th1-mediated immunity, Gadd45 proteins were also described to affect Th1 cells in an intrinsic manner. 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 wild-type 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). However, Gadd45γ was not required for hematopoiesis, T cell proliferation or T cell responsiveness to IL-2 (Hoffmeyer et al. 2001; 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 also supports 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 a mitogen-activated protein kinase kinase kinase (MAP3K) that activates p38 and JNK (Gerwins et al. 1997; Takekawa et al. 1997). 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 wild-type 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.

Next to IFN-γ production, Gadd45β was shown to be important for IL-2 secretion in differentiated Th1 cells as well as in naïve T cells (Lu et al. 2004). As in differentiated Th1 cells, Gadd45β-deficient naïve T cells showed reduced p38 MAPK activity suggesting reduced T cell activation (Lu et al. 2004). Surprisingly, Gadd45β was also identified by DNA array technology as a gene induced during induction of T cell anergy, a state of unresponsiveness that is induced in T cells when they receive TCR stimulation in the absence of a co-stimulatory signal (Safford et al. 2005). Accordingly, anergy is a mechanism to ensure immunological tolerance along with clonal deletion by apoptosis or suppression of immune responses by regulatory T cells (Schwartz 2003). Besides other mechanisms, anergy is mediated on the molecular level by 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). Recently, a connection between T cell anergy and Gadd45β was reported. When analysing 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). Next to induction of the E3 ubiquitin ligase Cbl-b, Deltex1 induced transcriptional activation of the *Gadd45b* gene (Hsiao et al. 2009). 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 functional role for Gadd45β in T cells anergy has not been directly tested (Fig. 4.3).

As described above, Gadd45β and Gadd45γ are involved in activation of the p38 mitogen-activated protein kinase via a classical kinase cascade. Thus, these two Gadd45 proteins activate MEKK4 (a mitogen-activated protein kinase kinase kinase) in T cells, which activates MKK3 or MKK6 (mitogen-activated protein kinase kinases) leading to the activation of p38. In contrast to Gadd45β and Gadd45γ, 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-tier 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). The importance of this alternative pathway was demonstrated by the generation of knock-in mice harbouring a Tyr323Phe

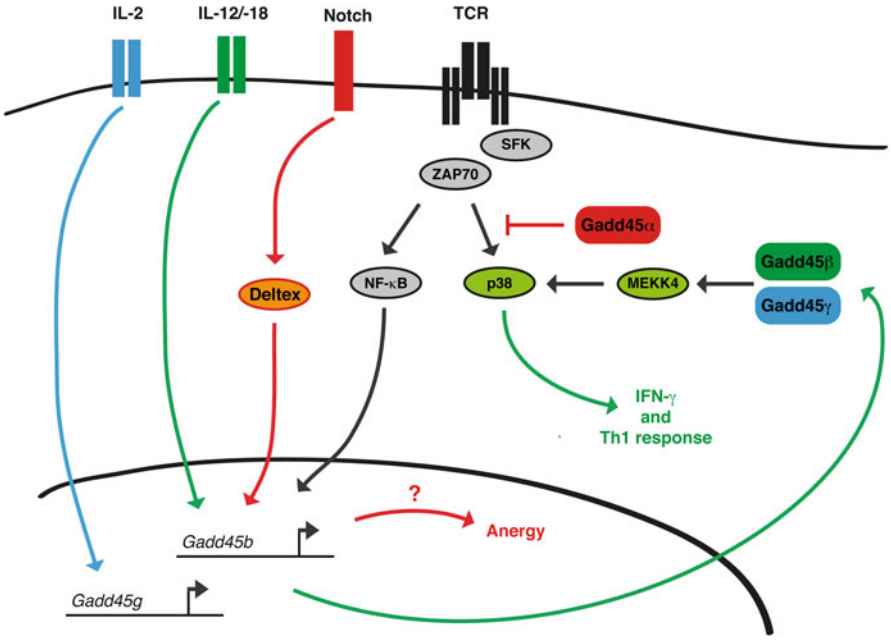

Fig. 4.3 Gadd45 proteins in T cells. Triggering of the T cell receptor (TCR) activates Src family kinases (SFK) and the zeta-chain-associated protein of 70 kDa (ZAP-70). These tyrosine kinases lead to the activation of the transcription factor NF-κB and the p38 mitogen-activated protein kinase. NF-κB 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. Gadd45α is constitutively expressed in T cells and can inhibit the alternative activation of p38 by ZAP70-mediated tyrosine phosphorylation. Activation of *Gadd45b* by Notch and Deltex may lead to T cell anergy by a yet unknown mechanism

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). 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.

4.5 Gadd45 Proteins in Autoimmunity and Tumour Immunosurveillance

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 harbouring a Tyr323Phe mutation in both p38α and p38β, the two isoforms of p38MAPK 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 wild-type T cells became even more obvious in a transfer EAE model, in which naïve T cells of either wild-type 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 wild-type 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). In addition, 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 scores in this murine model

of serum-induced arthritis (Svensson et al. 2009). 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.

Next to the suppression of autoimmunity, Gadd45 proteins seem to promote anti-tumour 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 tumour T cell line was inhibited in immunized mice compared to non-immunized mice. Supporting the idea of a Gadd45 protein-aided anti-tumour immune response, tumour 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 tumour 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, tumour vaccination failed in mice double deficient for Gadd45β and Gadd45γ (Ju et al. 2009). Taken together, Gadd45 proteins have important functions in tumour immunosurveillance.

Acknowledgements I am grateful to Dr. Yvonne Rauter and Alisha Walker for critically reading the manuscript and for helping with the figures. This work was supported by grants of the Deutsche Forschungsgemeinschaft (SCHM1586/3-1) and the Helmholtz Association cross-programme activity “Metabolic Dysfunction and Human Disease”.

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Chapter 5
Gadd45 in the Liver: Signal Transduction and Transcriptional Mechanisms

Jianmin Tian and Joseph Locker

Abstract Injury and growth stimulation both remarkably increase the hepatic expression of Gadd45β. In liver cancer, promoter methylation frequently silences Gadd45β, demonstrating due to a suppressive function that is often proapoptotic. This contrasts with normal hepatocytes, where 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 *Gadd45b−/−* genotype increases cell injury and decreases cell proliferation during liver regeneration (i.e., compensatory growth and proliferation). Liver hyperplasia (i.e., de novo growth and proliferation) is an alternate form of growth, caused by drugs that activate the nuclear receptor, CAR. As in regeneration, the *Gadd45b−/−* 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.

Abbreviations

CAR	Constitutive androstane receptor
Gadd45	Growth arrest and DNA damage-inducible 45 proteins
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus

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D.A. Liebermann and B. Hoffman (eds.), *Gadd45 Stress Sensor Genes*, Advances in Experimental Medicine and Biology 793, DOI 10.1007/978-1-4614-8289-5_5,

IL6	Interleukin 6
JNK	c-Jun N-terminal kinase
MKK4/JNKK1	MAPK kinase 4/JNK kinase 1
MKK7/JNKK2	MAPK kinase 7/JNK kinase 2
NFκB	Nuclear factor kappa B
PH	Partial hepatectomy
TCPOBOP	1,4-bis[2-(3,5-dichloropyridyloxy)]benzene
TNFα	Tumor necrosis factor alpha
TNFR1, TNFR2	Tumor necrosis factor a receptor 1, 2

5.1 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 kDa), each protein has a surprising number of functions and binding partners. As part of its adaptations to different kinds of stress, the liver stimulates expression of individual Gadd45 protein in various responses to injury, inflammation, chemicals, and drugs. Liver function is diverse and includes essential processes like 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 effect of each Gadd45 protein probably reflects 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, and a variable number of inflammatory cells. It is often unclear which of these cells expresses a particular Gadd45 protein, so this review will highlight the main liver cell, i.e., the hepatocyte. 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 the liver or any other tissue, the last section reviews these mechanisms in detail.

5.2 Gadd45α and Gadd45γ

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 (Su et al. 2002; Locker et al. 2003; Fallsehr et al. 2005; 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 three times more mutations in the *Gadd45a*-null mouse. (Hollander et al. 2001) Thus, as in other tissues, Gadd45α responds to DNA damage and facilitates DNA repair.

Transcriptional regulation of *Gadd45a* links to DNA damage through factors expressed in the liver and most other tissues, including p53 (Zhan et al. 1998; Kastan and Bartek 2004), 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 factors in the liver, CREBH activated by unfolded proteins and ER stress (Luebke-Wheeler et al. 2008), ATF5 by fasting, ER stress, and oxidative stress (Zhou et al. 2008; Shimizu et al. 2009).

Foxo3a, a transcriptional mediator of oxidative stress, also regulates *Gadd45a*, 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 Foxo3a (Amente et al. 2011; Notas et al. 2012). These findings also suggest feedback inhibition among Gadd45 proteins. Their prior expression might block activation of Foxo3a, since they are strong inhibitors of JNK2 (Papa et al. 2008) (see below).

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 *Gadd45g*, 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).

5.3 Induction of Gadd45β in the Liver

Two types of hepatocyte proliferative responses induce striking immediate-early expression of Gadd45β: regeneration, the compensatory proliferation after the loss of hepatocytes, and hyperplasia, the de novo proliferation caused by drugs and

xenobiotic activators of the constitutive androstane receptor (CAR). The induction is exceptionally high—150-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). Gadd45β is also induced by agents that activate another nuclear receptor of xenobiotics, the pregnane X receptor (PXR) (Kodama and Negishi 2011), that is not associated with proliferation.

Regeneration and hyperplasia stimulate Gadd45β through separate transcriptional mechanisms. PH, or liver damage from toxic agents like CCl_4, activates two known signaling pathways that stimulate Gadd45β transcription, TNFα-NFκB, and TGFβ-SMAD. This 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). The impairment of Gadd45β induction by PH in the *Tnfr1−/−* mouse confirms this relationship (Papa et al. 2008). NFκB also accounts for the induction of Gadd45β by *S*-adenosylmethionine (Seewoo et al. 2012).

TGFβ, another inducer of Gadd45β 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 and Smad4, which stimulate *Gadd45b* transcription through a downstream enhancer (Major and Jones 2004). Three other agents induce hepatic transcription of *Gadd45b* but through undefined transcriptional regulators and binding sites. The multikinase inhibitor sorafenib strongly induces Gadd45β in sensitive, but not resistant, HCC cell lines, acting through a 72-bp upstream regulatory region (Ou et al. 2010). Oxaliplatin, a DNA-damaging drug, and insulin also induce Gadd45β in HCC cell lines, by unknown mechanisms (Bortoff et al. 2010; Seewoo et al. 2012).

The induction of Gadd45β by CAR is independent of the TNFα and TGFβ pathways, since TCPOBOP treatment activates neither (Columbano et al. 2005). The full induction of by TCPOBOP in *Tnfr1−/−* and *Tnfr1−/− Tnfr2−/−* knockout mice confirms this independence. In contrast, the *Car−/−* genotype prevents induction by TCPOBOP. CAR binds to a specific site near the Gadd45β promoter and activates reporter genes through this site (unpublished results). PXR also directly stimulates *Gadd45b*, in this case through a specific upstream binding site (Kodama and Negishi 2011).

Chronic hepatitis C virus (HCV) infection causes inflammation, hepatocyte loss, compensatory proliferation, and strong TNFα signaling. Surprisingly, these processes do not induce Gadd45β expression in HCV-infected liver, HCV transgenic mice, or HCV-induced liver cancer (Higgs et al. 2010). This suppression of Gadd45β occurs via promoter methylation. Thus, hepatocytes from HCV transgenic mice fail to arrest their cell cycles after UV-C treatment. However, treatment with the DNA demethylating agent, 5-azacytidine, restores Gadd45β expression and UV-induced cell cycle arrest. Although the mechanism that induces the methylation is unclear, these studies do confirm a relationship between hepatocyte DNA repair and Gadd45β.

5.4 Gadd45 Proteins in HCC

The Gadd45β promoter is hypermethylated in many HCC (Qiu et al. 2003, 2004; Higgs et al. 2010), a change that correlates with low or absent expression. Treatment with 5-azacytidine induces reexpression of Gadd45β and growth inhibition, suggesting a suppressive impact on cancer cells, the opposite of its growth effects in nonneoplastic liver. However, the mechanism of tumor suppression is unresolved. In contrast to Gadd45β, studies of Gadd45γ in HCC do not indicate suppressive effects. One paper reported that increased Gadd45γ expression is 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 indicates DNA repair functions similar to Gadd45α (Hollander et al. 2001).

5.5 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 a moderate 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 the compensatory proliferation. Nevertheless, an opposite effect on the proliferation of hyperplasia, however, indicates that Gadd45β does not have in intrinsic role in direct cell cycle or replication processes. In addition, PXR stimulation activates expression of Gadd45β, but not proliferation (Kodama and Negishi 2011). The effects of Gadd45β on hepatocyte proliferation are therefore variable and facultative. They depend on the specific inducing process and the context of concurrent changes.

5.6 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 stimulation of Gadd45α or Gadd45γ. This confirms their separate transcriptional regulation and demonstrates that the 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 *Gadd45b*−/− 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 *Gadd45b*−/− 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 *Gadd45b*−/− so the effect is JNK specific. A further experiment confirmed this relationship, because JNK2 knockout introduced into the *Gadd45b*−/− background fully restored liver regeneration (Papa et al. 2008). The experiments also confirm an antiproliferative effect of JNK2 observed in isolated hepatocytes (Sabapathy et al. 2004). The effects also contrast the functions of JNK1 of 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.

5.7 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 (Columbano and Shinozuka 1996; Ledda-Columbano et al. 2000; Tian et al. 2011). Nevertheless, CAR activation produces extremely rapid liver growth (Tian et al. 2011).

Following treatment with TCPOBOP, liver mass increases 30 % in 3 h and doubles by 18 h. This anabolic response occurs the G1 cell cycle phase. Growth then pauses during S phase—beginning at 24 h—but resumes at 40 h after cell division. Hyperplasia is part of an adaptive response to xenobiotic and toxic exposure, and rapid growth results from synthesis of inactivating and conjugating enzymes, transport molecules, and membrane scaffolds for these proteins. Cell division presumably makes hepatocyte more efficient in detoxification by increasing the surface to volume ratio. In the *Gadd45b*−/− mouse, the proliferation was increased but rapid liver growth was impaired. This inhibition reflected blunting of early mRNA

synthesis induced by TCPOBOP—less upregulation of induced transcripts, more downregulation of inhibited transcripts, and de novo inhibition of other transcripts. By 48 h, however, the growth of the mutant liver was equivalent to wild type. The effect is most apparent at early time points because the essential function of Gadd45β is to enable rapid adaptation through transcription.

5.8 Gadd45β Is a Transcriptional Coactivator

The general 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 by Yi et al. showed that Gadd45 proteins act as direct transcriptional coactivators of nuclear receptors (Yi et al. 2000). These authors noted the characteristic coactivator sequence 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 PPARγ2 in pull-down assays. (3) Gadd45α and -γ coactivated nuclear receptors RXRα, PPARα, and PPARγ2 in assays of transfected reporter plasmids. Similarly, a recent study characterized direct binding of Gadd45β to CAR (Yamamoto et al. 2010).

Essential coactivator functions are intrinsic to different domains of Gadd45β (Fig. 5.1) (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 C-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. 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 regulatory target, the cytochrome P450 2b10 gene (*Cyp2b10*).

Gadd45β has strong coactivator function comparable to 160-kDa Src1, and there is significant homology between their α-helical LXXLL-containing segments (Fig. 5.1). In Src1, mutation of these motifs blocks transcriptional activation (Heery et al. 1997). In Gadd45β, similar mutations convert the protein to a dominant-negative inhibitor, although they do not block binding by a more proximal domain. LXXLL mutations have a similar effect on another coactivator, nuclear receptor binding factor 2 (Nrbf2) (Yasumo et al. 2000; Flores et al. 2004). When bound to nuclear receptors, an LXXLL domain of Src1 simultaneously aligns with Helix 3 and Helix 12 of the ligand-binding domain (Shiau et al. 1998; Pike 2006).

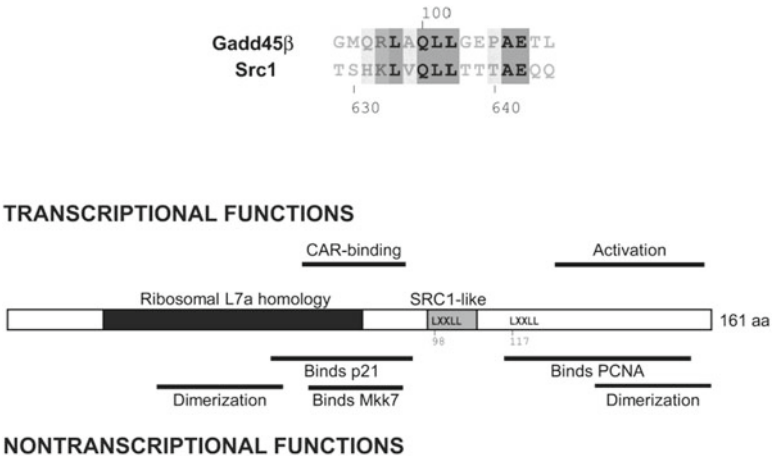

Fig. 5.1 Gadd45β domain structure. Above, alignment of LXXLL regions of Gadd45β and Src1/Ncoa1. There is strong sequence homology between the LXXLL motifs of Gadd45β at aa 98 and Src1 at aa 633, and each is within a short amphipathic α-helical segment, an essential property for binding and activation of nuclear receptors (Torchia et al. 1997; Tornatore et al. 2008). *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). 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, 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

Ketoconazole—an agent that binds this region of CAR and blocks binding of Src1—has the same effect on 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.

The baseline levels of Gadd45β are comparable to many other LXXLL-containing coactivators in liver cells, but 150-fold induction more than doubles the total of all LXXLL-containing coactivators, an extraordinary change in the transcriptional resources of the hepatocyte. CAR activation stimulates a detoxification response, and the rapid growth of the liver is a vigorous adaptation to potentially toxic agents. The early induction of Gadd45β by CAR is a feed-forward mechanism that enables the high-level transcription necessary for adaptation. Although CAR is exclusively an activating transcription factor, treatment with TCPOBOP also causes CAR-dependent downregulation of many genes without a clear relationship to CAR, growth, or xenobiotic metabolism (Columbano et al. 2005). The increased

downregulation in the *Gadd45b−/−* mouse may explain the puzzling transcriptional mechanism. The findings suggest an indirect effect, downregulation by unsuccessful competition for limiting levels of coactivators.

5.9 Conclusions

The three Gadd45 proteins are so similar that they are likely to share almost all functions. The critical features of their different biological roles in the liver are the processes that induce them, which strongly favor Gadd45β in the liver. 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. All of these roles clearly depend 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. 5.1). This functional dichotomy seems to reflect two biological circumstances in the liver. During injury, especially with inflammation, the signal transduction mechanisms are dominant. In the absence of injury, the transcriptional mechanisms dominate. Perhaps the striking high-level induction of Gadd45β reflects 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, an indirect 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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Chapter 6
The Role of the Gadd45 Family in the Nervous System: A Focus on Neurodevelopment, Neuronal Injury, and Cognitive Neuroepigenetics

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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.

6.1 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 kDa), acidic homologs with both nuclear and cytoplasmic expression (Tamura et al. 2012). Expression of the *gadd45* genes was confirmed in a variety of tissues including the skeletal muscle, heart, kidney, lungs, brain, and testis (Zhang et al. 1999). Consistent with their discovery, the *gadd45*

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D.A. Liebermann and B. Hoffman (eds.), *Gadd45 Stress Sensor Genes*, Advances in Experimental Medicine and Biology 793, DOI 10.1007/978-1-4614-8289-5_6,

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.

6.2 Nervous System Development

6.2.1 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 CNS. 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. 6.1.

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

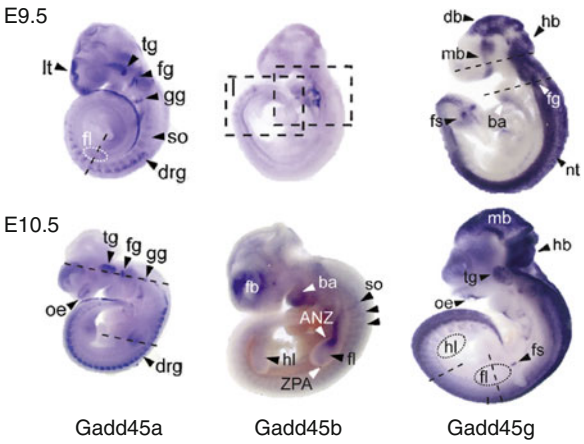

Fig. 6.1 Expression of the *gadd45* genes in mouse embryonic development. Whole mount in situ hybridization on E9.5 (*top row*) and E10.5 (*bottom row*) murine embryos was used to visualize expression of *gadd45a* (*left*), *gadd45b* (*middle*), and *gadd45g* (*right*). Lateral views are shown. Key: *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)

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, 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.

6.2.2 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 its 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 promote 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.

6.2.3 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. 6.7a, 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 (BER) 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, and 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 c-Jun N-terminal kinase (JNK), 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.

Gadd45a has also been identified as 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 proapoptotic 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 upregulated 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 promotes 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).

6.3 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.

6.3.1 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 result 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. 6.2). 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

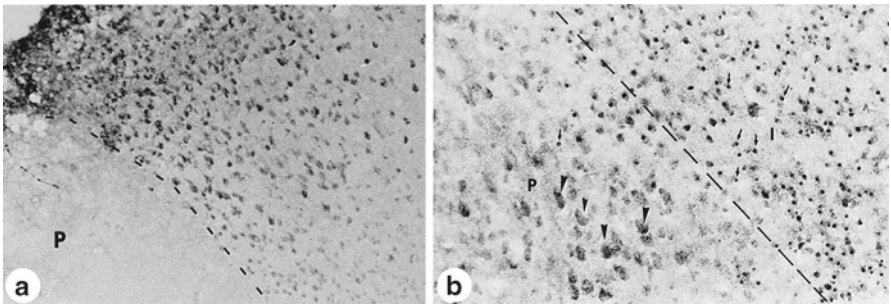

Fig. 6.2 Expression of Gadd45a protein in the cortex after ischemia induced by unilateral MCA occlusion in the neonatal rat. (**a**) After 24 h, Gadd45a was detected in layers II and III and largely absent from the penumbra (P). (**b**) Double staining for Gadd45a immunoreactivity and TUNEL assay performed 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)

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 the infarct; additionally, this elevation was found in 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 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).

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. 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.

6.3.2 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.

6.3.2.1 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 upregulated 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. 6.3). This is a surprising finding because these motor neurons and DRG cells have cell bodies located in

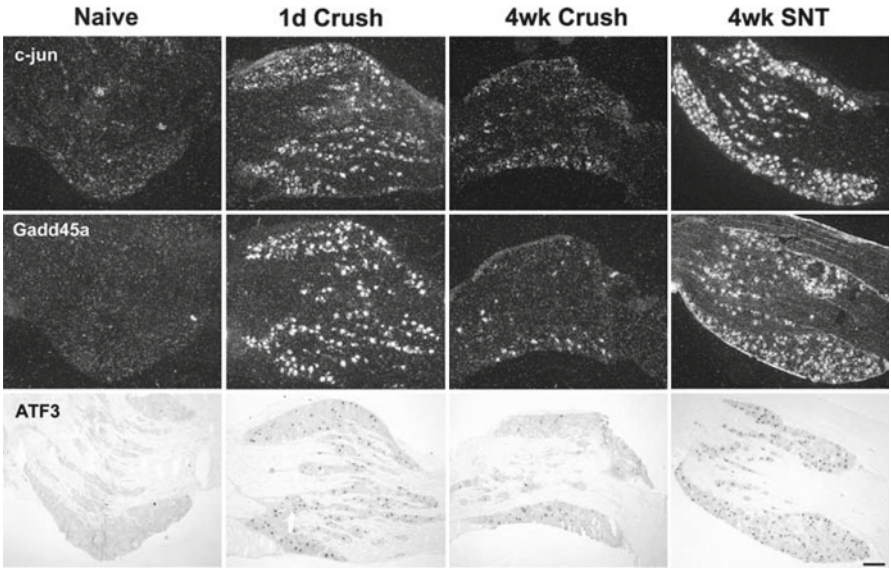

Fig. 6.3 Expression of *gadd45a* mRNA in the dorsal root ganglion (DRG) after sciatic nerve injury. Shown are sections of L4 DRGs from naïve, 1 day or 4 weeks after crush injury sections. Additionally, 4-week post-sciatic nerve transection (SNT) sections are displayed. Transcripts of c-jun (*top row*), Gadd45a (*middle row*), and ATF3 (*bottom row*) are presented. One day after crush injury, all markers are upregulated, and Gadd45a expression remains elevated when regrowth is prevented (4 weeks transection with ligation). Gadd45a expression is downregulated in the DRG when peripheral nerve regrowth after crush injury has completed (4-week crush). *Scale bar*, 200 **μ**m. Reproduced with permission from Befort et al. (2003)

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 upregulated 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 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 upregulate *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.

Recently, 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.

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 wild-type 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.

6.3.2.2 Neuronal Injury by Nonphysical 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 proapoptotic 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 upregulated within the lesion core, composed mostly of reactive astrocytes. These results suggest Gadd45a is associated with neuroprotection and preventing the core lesion site from expanding and that its protective effects may not be limited to neurons.

6.3.3 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 antitumor 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 upregulated 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.

6.3.4 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 upregulate *gadd45a* in response to insults in an effort 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. 6.4) (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

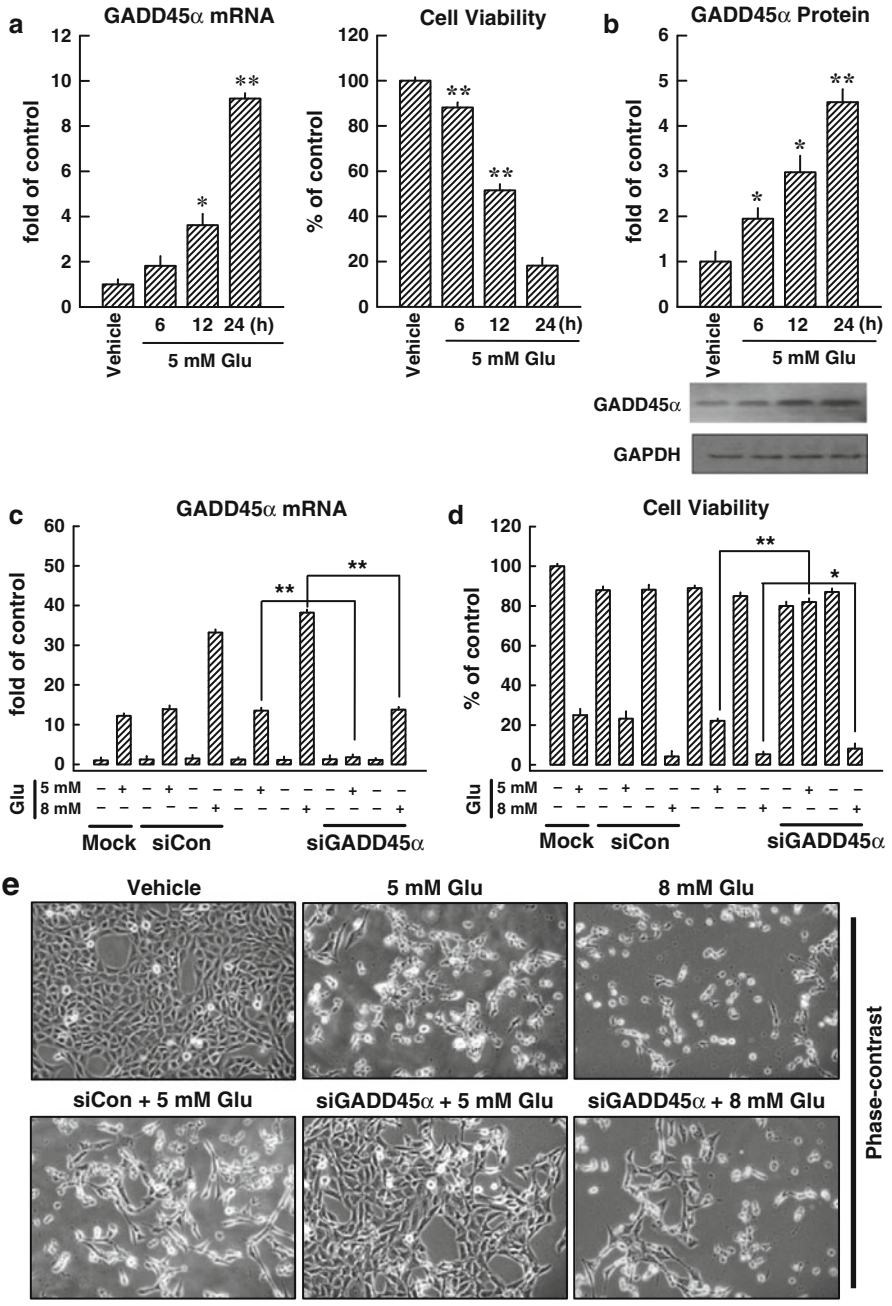

Fig. 6.4 Gadd45a mediates glutamate-induced oxidative cytotoxicity in HT22 hippocampal neuronal cell line. (**a**) *Gadd45a* transcripts and (**b**) Gadd45a protein levels were quantified after 5 mM glutamate treatment. Enhanced expression coincided with attenuated cell viability, assessed through MTT assay. (**c**–**e**) Cells were treated with 5 or 8 mM glutamate for 24 h after 24 h of pre-treatment 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)

* - $p < 0.05$, ** - $p < 0.01$

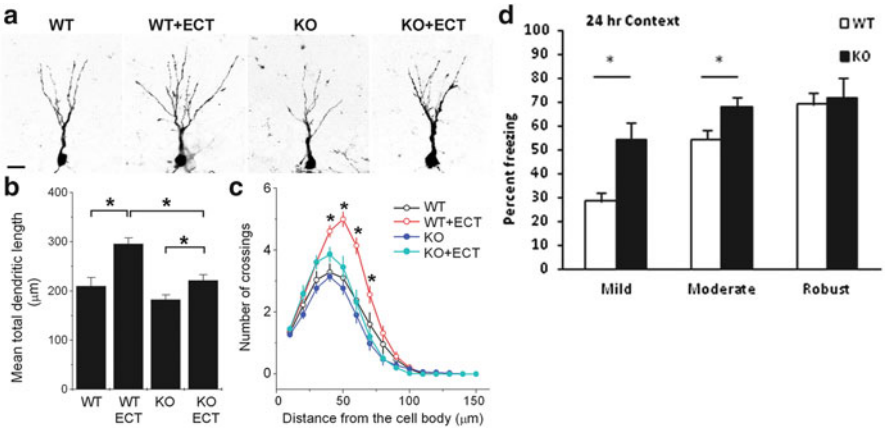

Fig. 6.5 Essential role of Gadd45b in activity-associated phenotypes in the adult CNS. (**a**–**c**) Wild-type and *gadd45b*-null mutant mice were injected with retroviruses expressing GFP to label adult-generated neural progenitors and their progeny. A single episode of electroconvulsive treatment (ECT) 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**) Wild-type 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**)
In sections b and c, * means $p < 0.01$; In d, * means $p < 0.05$

epileptogenesis. Seizures were found to upregulate *gadd45g* and, more so, *gadd45b* in the dentate gyrus granule cell layer (Ma et al. 2009). Furthermore, Gadd45b knockdown or knockout impaired activity-driven proliferation of neural progenitors and dendritic development of newborn neurons (Fig. 6.5a–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.

6.4 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 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. Methylcytosine 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).

6.4.1 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. 6.6a) (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 wild-type 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

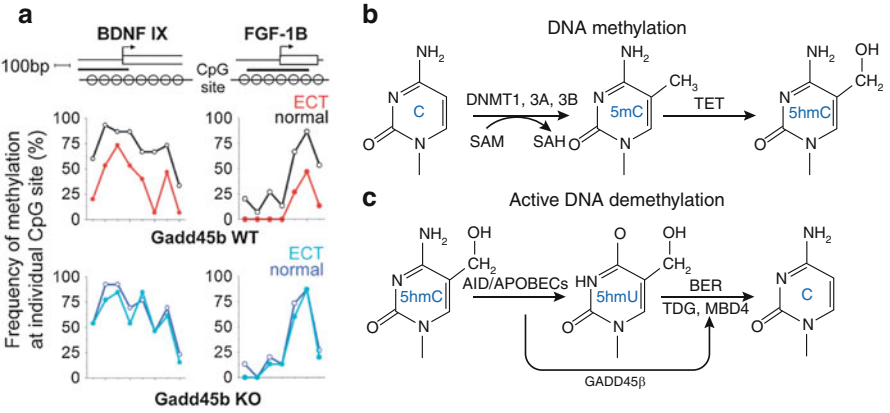

Fig. 6.6 Gadd45b regulates active DNA demethylation in an activity-associated manner. (**a**) Wild-type and *gadd45b*-null mutant mice were subjected to ECT or sham treatment. After 4 h, demethylation was found at the transcriptional start sites of *BDNF exon IX* and *FGF-1B* genes by sodium bisulfite 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**)

be upregulated 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-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. 6.5d). 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). 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. 6.7c). 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 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 recent 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 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. 6.6b, 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.

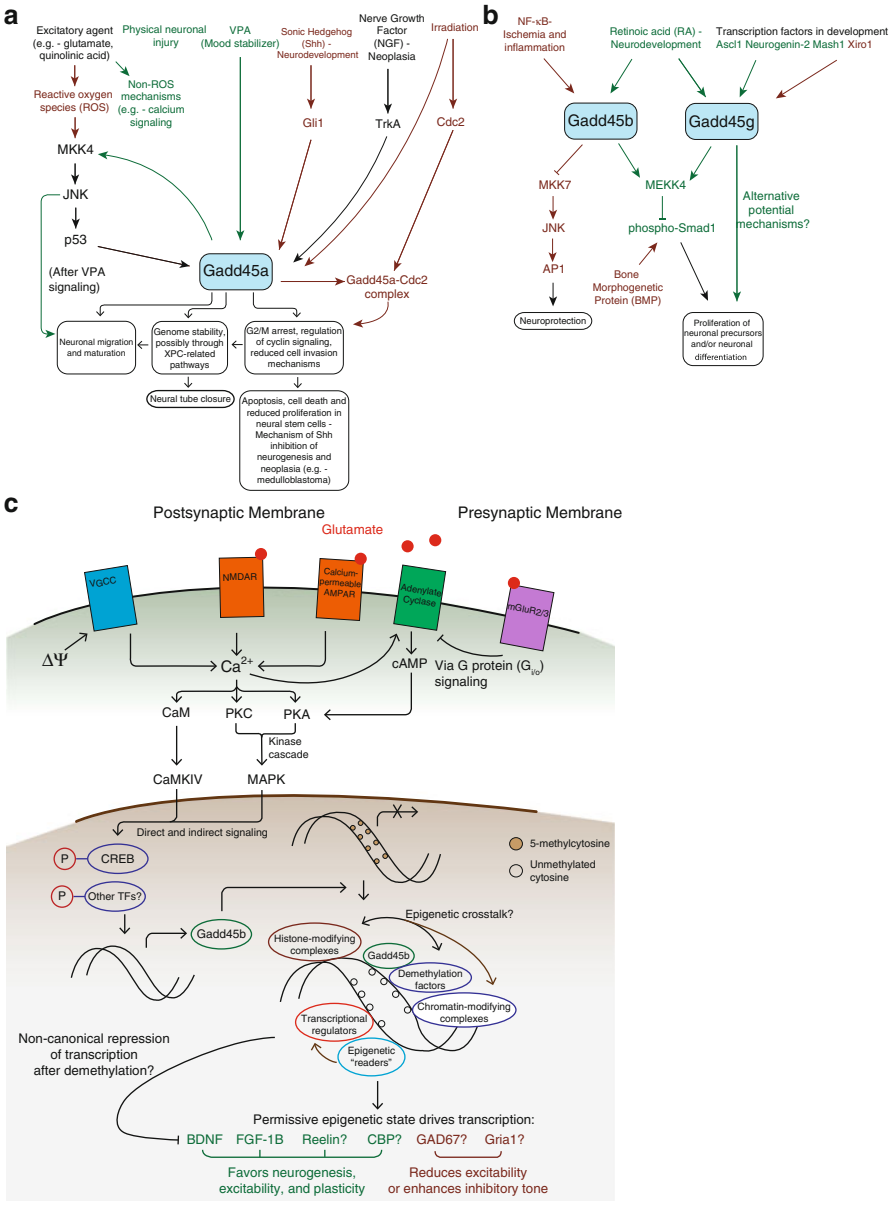

Fig. 6.7 Neuronal molecular signaling by the Gadd45 proteins. (**a**) Numerous upstream cascades impinge on *gadd45a* expression in neuronal development, injury, and tumor formation. Still, the function of Gadd45a in the balance between cell survival and death is largely dependent on the cell type and 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

6.4.2 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 wild-type 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).

6.4.3 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 procognitive 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 upregulated 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.

6.4.4 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 withdrawal, 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.

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 co-localizes 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 receptors (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 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 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).

6.5 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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Chapter 7
Gadd45 Stress Sensors in Preeclampsia

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, 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.

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D.A. Liebermann and B. Hoffman (eds.), *Gadd45 Stress Sensor Genes*, Advances in Experimental Medicine and Biology 793, DOI 10.1007/978-1-4614-8289-5_7,

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 2002). Multiple stresses were found contributing to the preeclamptic 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 6th 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 2002; Dekker 1999). Recent updates, on the other hand, showed that reducing uteroplacental blood flow in pregnant rats can reproduce characteristic preeclamptic manifestations (Li et al. 2012; Makris et al. 2007).

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).

7.1.2 Immune Activation

7.1.2.1 Multiple Factors Triggers 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 a new risk for preeclampsia. The maternal immune system, therefore, has to reestablish 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 (AT1-AAs) that share the same AT1 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 (Zhou et al. 2008). Therefore, triggering AT1 receptor signaling by circulating autoimmune antibodies (AT1-AAs) is notable evidence of how immune activation is involved in preeclamptic pathology (Zhou et al. 2008). In addition, angiotensin II by itself was elevated in preeclamptic placentas and increases systemic sensitivity to angiotensin II in preeclampsia (Shah 2005).

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 (Christopher and Sargent 2004).

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, and 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 et al. 1996). Chronic infusion of TNF-α into normal pregnant rats results in significant increases in arterial pressure and a decrease in renal hemodynamics (Babbette et al. 2007). TNF-α infusion in pregnant rats also triggered AT1-AAs production (LaMarca et al. 2008), suggesting that TNF-α can cause both inflammatory and immune activation in preeclampsia.

IL-1, including IL-1α and 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). Intracisternal or intravenous infusion of IL-1 beta increases blood pressure in a prostaglandin-dependent manner in rats (Takahashi et al. 1992).

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). 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 placental, renal, and vascular tissues, whereas IL-6 stimulates the renin–angiotensin system. 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 neurohumoral 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 oxidoreductases (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, preeclampsia, intrauterine growth restriction (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 and

Sargent 2007). 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). Moreover, early-onset preeclampsia, which is frequently associated with intrauterine growth retardation (IUGR), was reported with high levels of ER stress in the placenta (Burton et al. 2009).

Autoantibodies AT1-AAs also trigger oxidative stress in preeclampsia. They stimulate NADPH oxidase, resulting in an increase in ROS production (Dechend et al. 2003).

Cellular response to oxidative stress is via the mitogen-activated protein kinases (MAPK) pathway. For examples, 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; Lu et al. 2004), 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 placental examination. Placental tissues from both preeclamptic and normotensive (control) patients were examined for the mRNA levels of the Gadd45 family genes (a, b, and g). Although the expression of all three genes were 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 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 p38 protein. With dual 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 p38 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 p38 activation (Xiong et al. 2009, 2011).

7.2.2 AT1-AAs and Gadd45a in Preeclampsia

Angiotensin II is a vessel constrictor which causes increasing blood pressure and shares the same AT1 receptor with AT1-AAs. In order to study the interaction of Gadd45a and AT1-AAs in preeclampsia, angiotensin II was introduced to cultured placental explants. Treatment of placental explants with angiotensin II resulted in Gadd45a induction, p38 phosphorylation (i.e., activation), and elevation of sFlt-1 in the supernatant (Xiong et al. 2011). 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. 2011).

7.2.3 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 attenuated sFlt-1 secretion in the culture medium, whereas blocking JNK activation had no effect on sFlt-1 levels (Xiong et al. 2011).

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

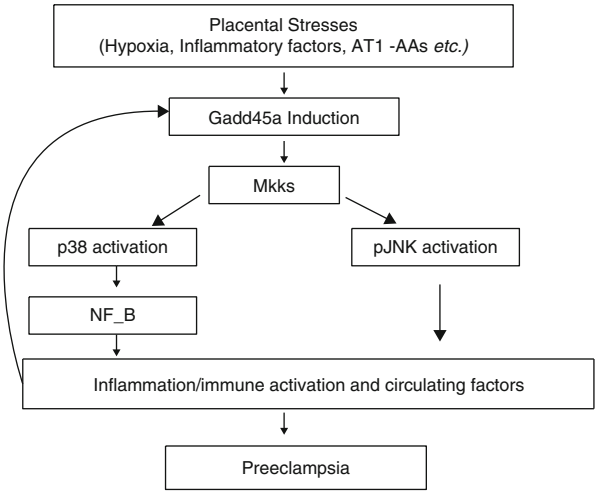

Fig. 7.1

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. 2011).

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/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 (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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D.A. Liebermann and B. Hoffman (eds.), *Gadd45 Stress Sensor Genes*, Advances in Experimental Medicine and Biology 793, DOI 10.1007/978-1-4614-8289-5,