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

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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

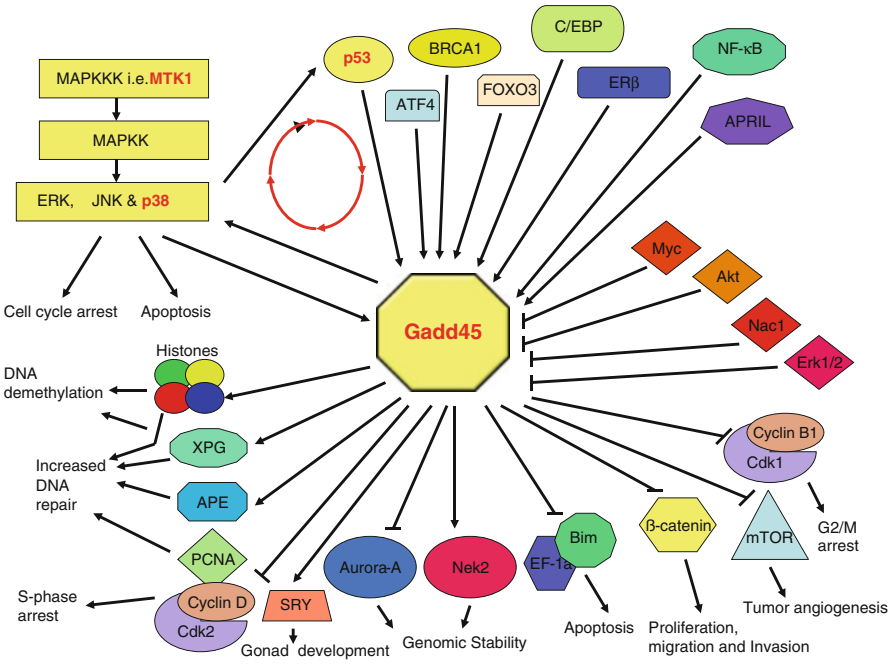

Fig. 1.1 Proteins with well-known roles in regulation of the Gadd45 genes and proteins are shown, as well as effector proteins. *Arrows* indicate positive regulation, while other *lines* indicate negative regulation. In some cases, regulation is complex such as for the p38 and JNK stress MAPK, which 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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