A Novel Role of DNA Fragmentation Factor As a Tumor Suppressor Through Maintaining Genomic Stability

PhD Thesis

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Abstract

DNA fragmentation is a hallmark of apoptosis (programmed cell death). However, the biological function of apoptotic DNA fragmentation remains unclear. In this study, we show that DNA fragmentation factor plays an important role for maintaining genomic stability. Inhibition or loss of the DNA fragmentation factor (DFF)/caspase dependent Dnase (CAD), whose nuclease activity is responsible for digesting genomic DNA during apoptosis, led to significant increases in spontaneous or induced gene mutations, gene amplifications, and chromosomal instability in primary mouse cells and transformed human cell lines. The role of DFF/CAD in maintaining genomic stability is independent of p53, a classic gatekeeper gene. The mechanism underlying genetic instability in DFF/CAD-deficient cells at least in part involves a small but significant elevation in the survival of cells exposed to ionizing radiation, which suggests that apoptotic DNA fragmentation factor contributes to genomic stability by ensuring the removal of cells that have suffered DNA damage. In consistence with the above observations, we found increased cellular transformation of mouse embryonic cells isolated from the DFF/CAD-null mice and significantly enhanced susceptibility to chemical and radiation carcinogenesis in these mice. These data, in combination with published reports on the existence of tumor-specific gene mutations/deletions in the DFF/CAD genes in human cancer samples, suggest that apoptotic DNA fragmentation factor is required for the maintenance of genetic stability and may play a role in tumor suppression.

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List of Abbreviations

6TG	6-thioguanine
AIF	Apoptosis-inducing factor
CAD	Caspase activated Dnase
cad	Cabamyl-P-synthetase, aspartate transcarbarmylase, dihydro-orotase gene
CIN	Chromosome instability
CIDE domain	Conserved domain of 80 amino acids at their N-terminus
CPAN	Caspase-activated nuclease
CTL	Cytotoxic T lymphocyte
DFF	DNA fragmentation factor
Dhfr	Dihydrofolate reductase
DMBA	7,12-dimethylbenz (a) anthracene
DSBs	Double-strand breaks
ELISA	Enzyme-linked Immunosorbent assay
EndoG	Endonuclease G
FACS	Fluorescence Activated Cell Sorter
FasL	Fas ligand
ICAD	Inhibitor of caspase activated Dnase
ICAD-L	Full-length form of DFF45/ICAD
IFNβ	Interferon β
IR	Irradiation
KO	Knockout
MEF	Mouse embryonic fibroblasts

MICAD	Mutant ICAD
MTX	Methotrexate
NER	Nucleotide excision repair
NK	Natural killer cells
PALA	N-(phosphoacetyl)-L-aspartate
RBC	Red blood cell
RT-PCR	Reverse transcriptase-polymerase chain reaction
SRO	Smallest region of overlap
TFT	Trifluorothymidine
tk	Thymidine kinase
TNF	Tumor necrosis factor
TPA	12-O-tetradecanoylphorbol-13-acetate
TRAIL	TNF-related apoptosis-inducing ligand
WT	Wild type
XPC	Xeroderma pigmentosum group C protein

Chapter 1

Introduction

1.1. DNA fragmentation is a hallmark of apoptosis

What is apoptosis?

Apoptosis, or programmed cell death, is a cell-autonomous form of cell death in an active and inherently controlled manner that eliminates unwanted, incorrect or damaged cells. This process depends on actively controlled degradation of intracellular structures.

Cells undergoing apoptosis exhibit specific morphological changes, which include membrane blebbing, cytoplasmic and chromatin condensation, nuclear breakdown, assembly of membrane-enclosed vesicles termed apoptotic bodies, which are eventually subjected to phagocytosis(Widlak 2000). DNA fragmentation into oligonucleosomal size of fragments is a biochemical hallmark of apoptosis.

The extensive morphological and biochemical changes during apoptosis are likely to ensure that dying cells would leave minimum impact to neighboring tissues *in vivo*. These features of apoptosis contrast those of necrosis, another form of cell death in response to noxious stimuli or injury. Necrosis is accompanied by membrane rupture and leakage of cellular contents, and it thus often causes tissue inflammation(Zhang and Xu 2000).

Apoptosis plays an important role in development and homeostasis. Alterations in apoptosis contribute to the pathogenesis of a number of human diseases, including cancer, viral infections, autoimmune diseases, neurodegenerative disorders, and AIDS (acquired immunodeficiency syndrome). Treatments designed to specifically alter the apoptotic threshold may have the potential to change the natural progression of some of these diseases(Thompson 1995).

Mechanism and pathways of apoptosis

Inducers of apoptosis are relatively diverse and include death factors such as FasL (Fas ligand), TNF (tumor necrosis factor), and TRAIL (TNF-related apoptosis-inducing ligand), growth factor deprivation, genotoxic agents such as anti-cancer drugs, chemical carcinogens and radiation, oxidative stress and granzyme B released from NK or lymphotoxic T cells (Nagata 1997; Ashkenazi and Dixit 1998; Green and Reed 1998; Raff 1998).

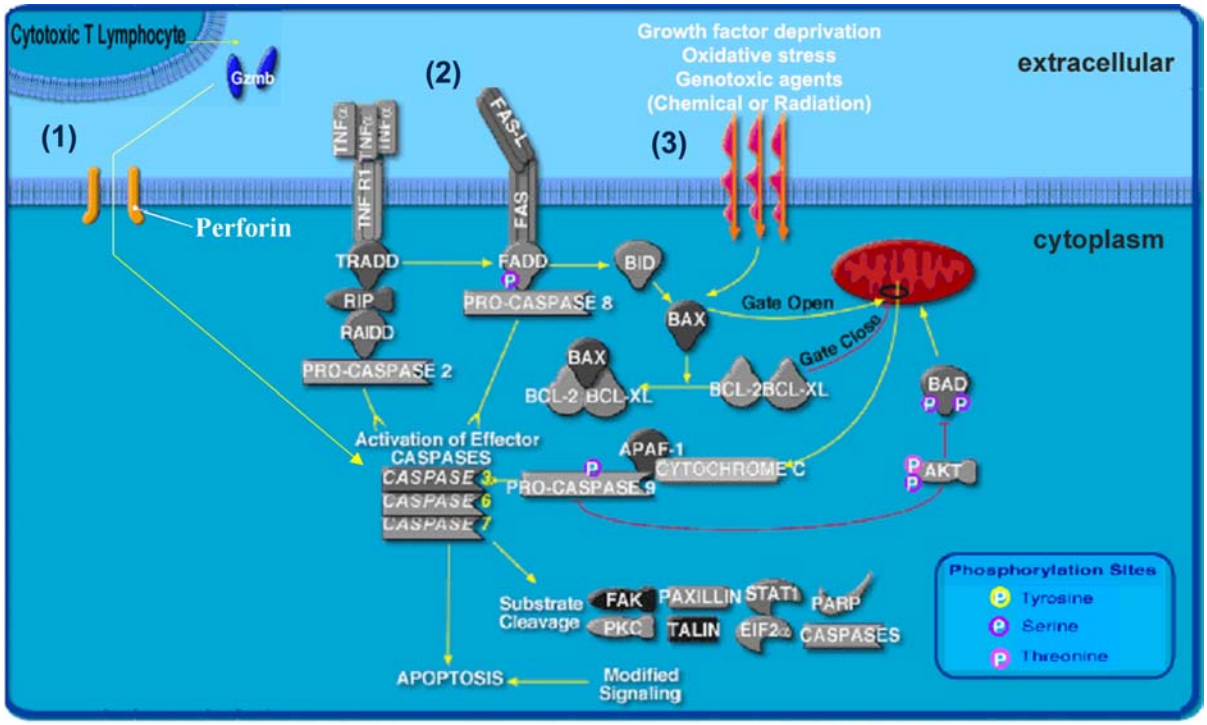


Figure 1.1-1. General apoptosis pathways. (1) Granzyme B pathway in Cytotoxic T lymphocyte (CTL)-mediated cytotoxicity; (2) Death receptor pathway mediates apoptosis induced by TNF, TRAIL or Fas-L; (3) Mitochondria pathway mediates apoptosis induced by growth factor deprivation, genotoxic agents such as radiation or chemotherapeutic drugs, and oxidative stress.

There are 3 general apoptosis pathways (Figure 1.1-1)

1. **Death receptor** (TNF or TRAIL receptor and Fas) **pathway**: Fas and TNF receptor are integral membrane proteins with their receptor domains exposed on cell surface. Binding of the complementary death activator such as Fas ligand (FasL) transmits a signal to the cytoplasm that leads to activation of caspase 8. Activated caspase 8 either directly cleaves and activates procaspase 3 or cleaves Bid, a proapoptotic member of the Bcl-2 family. The cleaved Bid goes to mitochondria, activating the mitochondria pathway and releasing cytochrome C(Nagata 1999).
2. **Mitochondria pathway**: Mitochondrial pathway mediates apoptosis induced by growth factor-deprivation, genotoxic agents such as radiation or chemotherapeutic drugs, and oxidative stress. It comprises two parallel signaling, caspase-dependent and caspase-independent pathways (Figure 1.1- 2).

   Caspase-dependent pathway Mitochondrial protein Bcl-2 is bound to Apaf-1. Cellular damages such as DNA damage can upregulate Bax through P53 activation, which causes Bcl-2 to release Apaf-1. Bax/Bak permeablizes mitochondrial membrane causing release of cytochrome C. Cytochrome C, Apaf-1, ATP and procaspase 9 form apoptosome, in which caspase 9 is activated. Caspase 9 then cleaves its downstream molecules and activates the caspase cascade.

   Caspase-independent pathway In response to apoptotic stimuli, mitochondria can also release factors involved in caspase-independent cell death including apoptosis-inducing factor (AIF) and Endonuclease G (EndoG). AIF translocates

from the mitochondria to the nucleus and causes chromatin condensation and large-scale DNA fragmentation(Susin, Lorenzo et al. 1999). EndoG is able to induce nucleosomal DNA fragmentation in the absence of caspases(Li, Luo et al. 2001). Therefore, release of AIF and EndoG from mitochondria starts an apoptotic program in parallel to caspase activation.

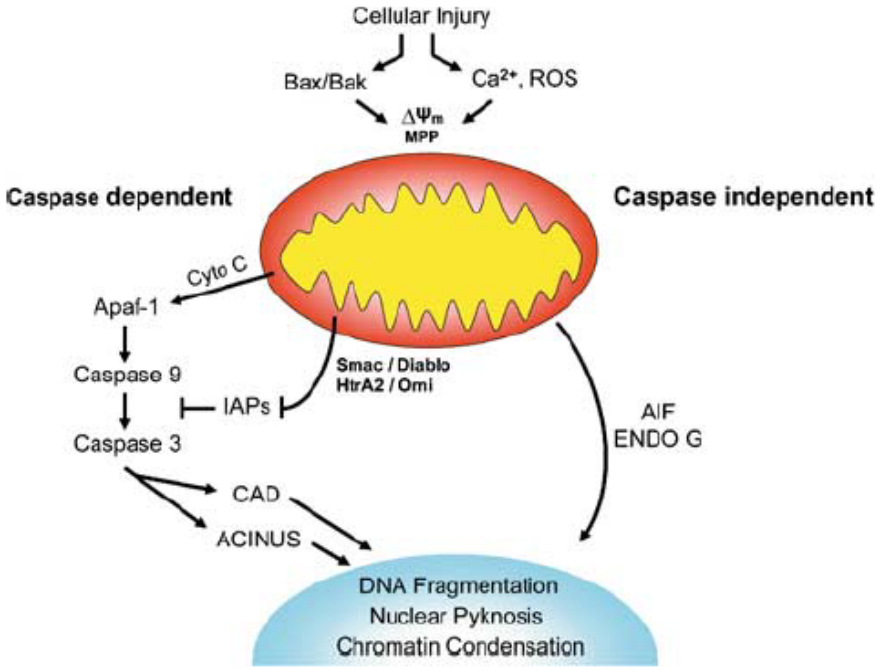


Figure 1.1-2. Release of caspase-dependent and caspase-independent proapoptotic factors from the mitochondria in response to diverse signals.

Apoptotic **ceramide signaling** involves multiple, coordinated pathways including the mitochondrial pathway. Stresses such as radiation, oxidative stress, genotoxic drugs and TNFα can activate sphingomyelinase in the plasma membranes and hydrolyze sphingomyelin to ceramide. Ceramide downregulates PI3K-Akt signaling resulting in decreased phosphorylation of BAD, a pro-apoptotic Bcl-2 family member. Dephosphorylated Bad binds to Bcl-2 and Bcl-xL to replace Bax, which regulate commitment to the apoptotic process via release of mitochondrial cytochrome c.

3. **Granzyme B-mediated pathway**: Cytotoxic T lymphocyte (CTL)-mediated cytotoxicity represents the body's major defense against virus-infected and tumorigenic cells, and contributes to transplant rejection and autoimmune disease. CTL attacks target cells by exocytosing granules, releasing their contents into the intercellular space between the target cell and CTL. Perforin facilitates the entry of cytotoxic serine proteases, the granzymes (A & B), into the target cell, where they induce apoptotic death by cleaving and activating procaspases or directly cleaving DFF45/ICAD to activate DFF40/CAD(Darmon, Nicholson et al. 1995; Thomas, Du et al. 2000).

Various apoptotic stimuli and pathways converge at a common hallmark event of apoptosis, DNA fragmentation. It indicates that DNA fragmentation is a fundamental process in apoptosis. However, the biological significance of DNA fragmentation during apoptosis was yet unclear.

DNA fragmentation in Apoptosis

A complete DNA degradation of apoptotic cells involves three steps.

1. Apoptotic degradation of genomic DNA in mammalian cells starts by excision of large DNA fragments ranging in size from 50 kilobases to more than 300 kilobases, which is via excision of chromosomal DNA loops and their oligomers (Brown, Sun et al. 1993; Oberhammer, Wilson et al. 1993; Lagarkova, Iarovaia et al. 1995). Nucleases involved in this process includes cyclophilin C (Montague, Hughes et al. 1997), Ca^{2+}/Mg^{2+} activated endonuclease(s)(Sun, Jiang et al. 1994) (Cain, Inayat-Hussain et al. 1994), DFF/CAD (Nagata 2000)and topoisomerase II under certain conditions (Li, Chen et al. 1999);
2. Large-scale DNA fragmentation is followed by internucleosomal cleavage that generates mono- and oligonucleosomal DNA fragments, and is termed DNA laddering. DFF/CAD(Liu, Zou et al. 1997; Enari, Sakahira et al. 1998) and endonuclease G(Li, Luo et al. 2001) are the nucleases responsible for this process.
3. Engulfment of apoptotic cells and degradation of DNA by Dnase II in lysosomes of macrophage (McIlroy, Tanaka et al. 2000).

Structure and biochemical properties of DFF/CAD

The nuclease for internucleosomal cleavage is called DFF (DNA fragmentation factor) or CPAN (Caspase-activated nuclease)(Halenbeck, MacDonald et al. 1998) in human, or CAD (caspase activated DNase) in mouse. DFF is a heterodimeric protein composed of DFF40/CAD and DFF45/ICAD (Liu, Zou et al. 1997).

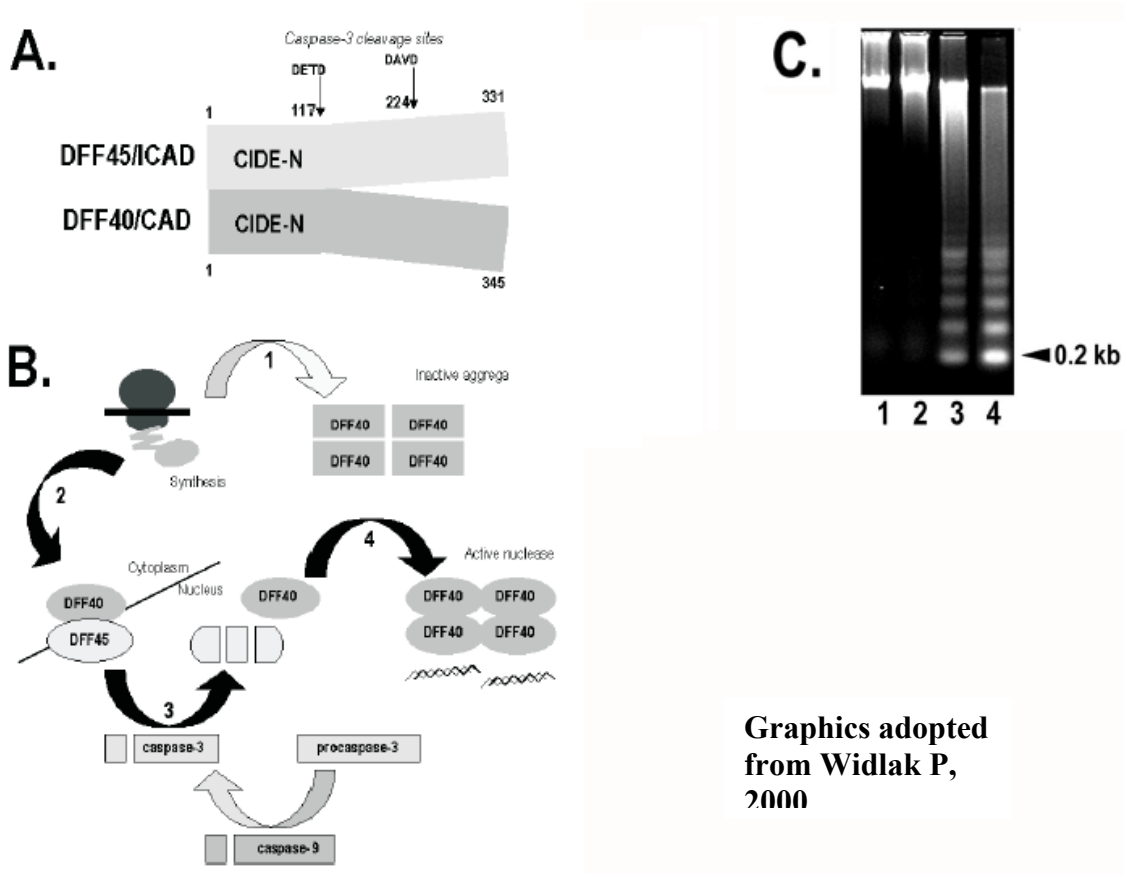


Figure 1.1-3. Structure and mechanisms of activation of DFF. Panel A. Scheme of the DFF40/DFF45 heterodimer. The positions of aspartate residues of DFF45 recognized by caspase-3 are marked with arrows. **Panel B.** Diagram showing mechanisms of DFF activation. DFF40 synthesized in the absence of DFF45 form inactive aggregates (1) or binding of DFF45 promotes proper folding of DFF40 and DFF40/DFF45 heterodimers transfer to nucleus (2), then under apoptotic stimuli DFF45 are cut with caspase-3 (3) and released DFF40 form active homodimers (4). **Panel C.** Nuclei purified from HeLa cells were incubated with caspase-3-activated DFF for 0, 5, 30 and 90 min (lanes **1**, **2**, **3** and **4**, respectively). Nuclear DNA fragmentation was analyzed by standard gel electrophoresis. The position of molecular standards is marked with arrowheads.

Human and Mouse DFF40/CAD are 40-KD basic proteins with pI of 9.3 and 9.7, and composed of 338 and 344 amino acids, respectively. Their amino acid sequences are well conserved, about 75.9% identical. The full-length form of DFF45/ICAD (ICAD-L) is a 45-kDa protein composed of 331 amino acids with a pI of 4.5. . A short form of DFF45, called DFF35 (or ICAD-S), that lacks C-terminus and consists of amino acids 1–265 of ICAD-L, is generated by alternative splicing, and is expressed in a number of different human and mouse cells (Enari, Sakahira et al. 1998; Sabol, Li et al. 1998; Gu, Dong et al. 1999; Kawane, Fukuyama et al. 1999) at a level similar to ICAD-L.

As shown in Figure 1.1-3A, DFF40/CAD and DFF45/ICAD have a conserved domain of 80 amino acids at their N-terminus (CIDE domain), which can be found in other proteins called CIDEs(cell death-inducing DFF45-like effecter) (Inohara, Koseki et al. 1998; Mukae, Enari et al. 1998). CIDE-N domains may be involved in DFF40/DFF45 interactions (Lugovskoy, Zhou et al. 1999; Otomo, Sakahira et al. 2000). The amino acid 3–329 of CAD is sufficient for the CAD DNase activity and its C-terminal residues (amino acids 330–344) are for nuclear transport. DFF40/CAD requires Mg^{2+} for its DNase activity, and functions at a neutral pH(Nagata 2000). The DNase activity of caspase-treated DFF (ICAD/CAD complex) is significantly enhanced by histone or HMG (high mobility group) nuclear proteins (Liu, Li et al. 1998; Liu, Zou et al. 1999). DFF40 is an endonuclease specific for double stranded DNA, but not single stranded DNA or RNA. This introduces double-strand breaks but not single-strand nicks during its cleavage of chromatin (Liu, Zou et al. 1999; Widlak, Li et al. 2000).

DFF45/ICAD is a chaperon as well as inhibitor of DFF40/CAD.

DFF40/CAD, a potentially dangerous protein for the cells, cannot be synthesized on its own. Expression of DFF40/CAD in various systems in the absence of co-expressed DFF45 results in generation of DFF40 inactive aggregates. DFF45/ICAD is required for the proper folding of DFF40/CAD during its synthesis. DFF45/ICAD keeps complexed with DFF40/CAD after production of the functional DFF40/CAD and inhibits its DNase activity (Liu, Zou et al. 1997; Enari, Sakahira et al. 1998; Liu, Li et al. 1998). DFF35/ICAD-S also inhibits DFF40/CAD activity but lacks the chaperon function (Gu, Dong et al. 1999; Sakahira, Enari et al. 1999). During apoptosis, activated caspase 3 cleaves DFF45/ICAD or DFF35/ICAD-S and releases active DFF40/CAD. DFF40/CAD then attacks chromotin at the internucleosomal linker DNA, generating mono- and oligo-nucleosomal fragments which form DNA ladders in electrophoresis (Figure 1.1-3B & 3C). DFF45/ICAD can also be cut by caspase-7 and granzyme B (yet with lower efficiency as compared to caspase-3), but not by caspase-1, -2, -4, -6, or –8 (McIlroy, Sakahira et al. 1999; Wolf, Schuler et al. 1999). There are two conserved caspase cleavage sites in DFF45/ICAD protein, at aspartate residues 117 and 224. Both sites have to be cleaved in order to activate CAD (Sakahira, Enari et al. 1998; McCarty, Toh et al. 1999).

Endonuclease G executes DNA fragmentation in a caspase-independent manner

DFF/CAD is not the only DNase involved in DNA fragmentation during apoptosis. Endonuclease G (EndoG), a 30-kD nuclease in the mitochondria, is encoded by a nuclear gene, translated in the cytosol, and imported subsequently into the

mitochondria. EndoG is released from mitochondria and translocates to nucleus where it degrades chromosomal DNA during apoptosis. Unlike DFF40/CAD, EndoG activity is independent of caspase activation. It has been shown that its activity may be responsible for DNA fragmentation observed in DFF45-deficient MEF cells after induction of apoptosis by UV-irradiation and TNF treatment (Zhang, Liu et al. 1998; Li, Luo et al. 2001). Both DFF/CAD and EndoG are evolutionarily conserved across species, which implicates their significant function in biology.

The biological significance of apoptotic DNA fragmentation was not clearly defined

DNA fragmentation is a hallmark of apoptosis and has been regarded as a critical process in apoptosis. Elucidation of the CAD/ICAD system indicates that cells have developed an elaborate system to regulate this process. DFF40/ CAD is a potentially harmful protein. Degradation of chromosome by DFF40/CAD would be sufficient to kill a cell. However, some reports indicate that cells can undergo apoptotic cell death without DNA degradation (Sakahira, Enari et al. 1998; Zhang, Liu et al. 1998; McIlroy, Sakahira et al. 1999), although DNA fragmentation may accelerate the process (Zhang, Wang et al. 1999). When activated during apoptosis, caspases cleave more than 100 substrates including proteins for DNA replication, transcription, or translation, cytoskeletal proteins, and kinases and phosphatases (Stroh and Schulze-Osthoff 1998). It is likely that caspase activation is sufficient to kill cells.

If cells can die without undergoing DNA fragmentation, why have they developed such an evolutionarily conserved system for cleaving the chromosomal DNA? Mice deficient in CAD or ICAD appear normal, suggesting that this system is dispensable for

mammalian development(Zhang, Liu et al. 1998; Kawane, Fukuyama et al. 2003). Some hypotheses have been proposed for the physiological role of DFF/CAD. An important function of apoptosis is to eliminate harmful cells such as cancer cells and virus-infected cells. DFF/CAD digest the chromosomal DNA before phagocytes engulf the apoptotic bodies so that the recipient cells may avoid transformation by activated oncogenes or viral genes(Holmgren, Szeles et al. 1999). Another possible role for apoptotic DNA fragmentation might be to reduce the autoimmune response. DNA is known to be a strong autoantigen. If massive cell death caused by inflammation or ischemia exceeded the ability of phagocytes to clean up the dead cells, DNA from the dying cells might induce an autoimmune reaction. To reduce such a possibility, DNA may have to be cleaved by the CAD/ICAD system.

Dr. Nagata's group has shown that undigested chromosomes in CAD(-/-)DNase II(-/-) mice induced innate immunity and impaired thymus development. However, the thymus of CAD(-/-) mouse was almost normal because it was shown that undigested chromosome in CAD(-/-) apoptotic cells was effectively degraded by DNase II in lysosomes of microphages (Kawane, Fukuyama et al. 2003). It suggested that DNase II instead of CAD is the major player in this role. CAD may play an auxiliary role in preventing chromosome-induced immune responses. Another group reported that DFF45-deficient mice exhibit enhanced spatial learning and memory and longer memory retention in the novel object recognition task(Slane, Lee et al. 2000; Slane McQuade, Vorhees et al. 2002). However, the mechanistic link between DNA fragmentation and enhanced learning and memory has not been established. More studies need to be carried out to clearly define the biological function of DFF/CAD.

1.2. Genetic instability is a hallmark of cancer.

Cancer is a genetic disease

Cancer became the top killer of Americans recently (AACR news 2005). Cancers result from accumulation of mutations in genes that control cell proliferation and cell death. Genetic alterations conferring instability occur early in tumorigenesis. The ensuing genetic instability drives progression of tumor formation by generating mutations in proto-oncogenes and tumor suppressor genes. These mutant genes provide cancer cells growth advantage and therefore lead to clonal outgrowth of a tumor(Cahill, Kinzler et al. 1999).

Molecular basis of multistep carcinogenesis

Several lines of evidence in epidemiologic, pathology morphological and molecular studies of tumors indicate that tumorigenesis in humans is a multi-step process and that these steps reflect genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives(Foulds 1954; Renan 1993; Kinzler and Vogelstein 1996; Hahn, Counter et al. 1999). This is most readily demonstrated in experimental models of chemical carcinogenesis, in which the stages of initiation and progression during cancer development have been described (Tenant R: chemocal carcinogenesis. In Franks LM 1997). Initiation results from exposure of cells to a sufficient dose of a carcinogenic agent (initiator). Initiation causes permanent DNA damage (mutations). It is therefore rapid and irreversible and has “memory”. In order for the genetic alterations in an initiated cell to be heritable, the damaged DNA template

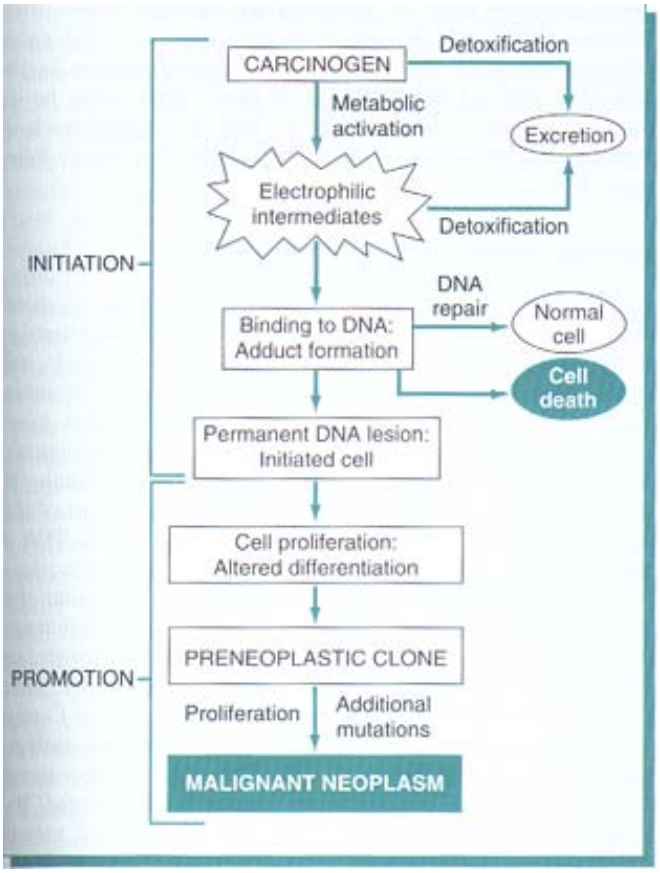


Figure 1.2-1. General schema of events in chemical carcinogenesis. Note that promoters cause clonal expansion of the initiated cells, thus producing a preneoplastic clone. Further proliferation induced by the promoter or other factors causes accumulation of additional mutations and emergence of a malignant tumor (Diagram adopted from Ramzi Cotran, V.K., Tucker Collins, 1999).

must be replicated. Thus, for initiation to occur, carcinogen-altered cells must undergo at least one cycle of proliferation so that the change in DNA becomes fixed or permanent. Initiation by itself is not sufficient for tumor formation (Figure 1.2-1). It must be followed by promotion for carcinogenesis to occur. Promoters can induce tumors in initiated cells, but they are not tumorigenic by themselves. Promoters such as phorbol esters, hormones and phenols leads to proliferation and clonal expansion of initiated (mutated) cells. For example, phorbol ester is a powerful activator of protein kinase C, which phosphorylates several substrates involved in signal transduction pathways, including those activated by growth factors. The promoting effect is linked to stimulation of cell proliferation. The forced sustained cell proliferation increases the risk of mutagenesis and hence accumulation of additional mutations in the initiated cells leading to malignant transformation. Thus, the process of tumor promotion includes multiple steps: proliferation of preneoplastic cells, malignant conversion and eventually tumor progression(Cotran R.S. 1999).

Six traits of cancers

Most human cancers that have been analyzed reveal multiple genetic alterations. There are six essential alterations in cell physiology that collectively dictate malignant growth (Figure 1.2-2). Each of these alterations is conferred by genetic mutations. Transformed cells gain these mutations gradually in a multi-step process. Genetic instability facilitates generation of mutations at a rate higher than normal and thus drives tumor development.

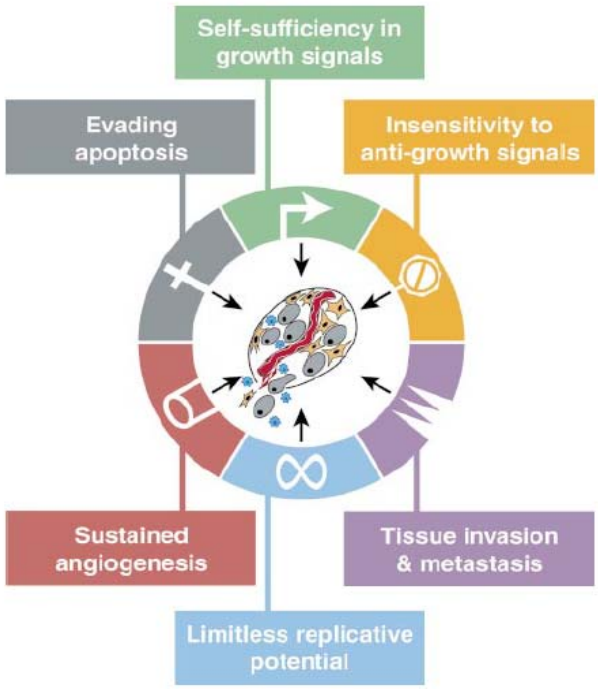


Figure 1.2-2. Acquired Capabilities of Cancer. We suggest that most if not all cancers have acquired the same set of functional capabilities during their development, albeit through various mechanistic strategies. (Graphic adopted from Hanahan, D. & Weinberg, R.A., 2000)

Component	Acquired Capability	Example of Mechanism
	Self-sufficiency in growth signals	Activate H-Ras oncogene
	Insensitivity to anti-growth signals	Lose retinoblastoma suppressor
	Evading apoptosis	Produce IGF survival factors
	Limitless replicative potential	Turn on telomerase
	Sustained angiogenesis	Produce VEGF inducer
	Tissue invasion & metastasis	Inactivate E-cadherin

Figure 1.2-3. Mutational changes leading to acquired abilities. (Graphic adopted from Hanahan, D. & Weinberg, R.A., 2000)

Each of these physiologic changes—novel capabilities acquired during tumor development, represents the successful breaching of an anticancer mechanism hardwired into cells and tissues. These six capabilities are shared in common by most and perhaps all types of human tumors (Hanahan and Weinberg 2000). (1) Self-sufficiency in growth signals. Normal cells require mitogenic growth signals (GS) before they can move from a quiescent state into an active proliferative state while tumor cells invariably show a greatly reduced dependence on exogenous growth stimulation; (2) Insensitivity to growth-inhibitory (antigrowth) signals. Within a normal tissue, multiple antiproliferative signals operate to maintain cellular quiescence and tissue homeostasis. Cancer cells are insensitive to these signals and therefore their growth is out of control; (3) Evasion of programmed cell death (apoptosis). (4) Limitless replicative potential; (5) Sustained angiogenesis; (6) Tissue invasion and metastasis. Examples of genetic alterations conferring these capabilities are shown in (Figure 1.2-3)

What is genetic instability

Genetic instability refers to a dynamic rate of change in genome. Genetic alterations arise at a rate higher than normal in cells showing genetic instability. Therefore, one can not determine the level of genetic instability of a tumor merely by scoring the presence of these alterations. Abnormally elevated levels of genetic instability can be found in many tumors(Lengauer, Kinzler et al. 1997). This instability is reflected in the heterogeneity seen within individual tumors and among tumors of the same type. Although it is still debated whether instability is essential for tumor initiation, it is clear that instability is the driving force for tumor progression(Marx 2002).

Genetic instability exists at two distinct levels. In a small subset of tumors, instability is observed at the nucleotide level and results in base substitutions or deletions or insertions of a few nucleotides. In most other cancers, instability is observed at the chromosome level, resulting in losses and gains of whole chromosomes or large portions thereof. There are four major types of genetic alterations in tumors(Lengauer, Kinzler et al. 1998) as shown in Figure 1.2-4.

Subtle sequence changes. These changes involve base substitutions or deletions or insertions of a few nucleotides (Figure 1.2-4a, b), and, unlike the alterations described below, they cannot be detected through cytogenetic analysis. For example, missense mutations in the *K-ras* gene occur in over 80% of pancreatic cancers(Almoguera, Shibata et al. 1988). Defects in two of the major repair systems, nucleotide-excision repair and mismatch repair, lead to these changes.

Alterations in chromosome number. Alterations in chromosome number involve losses or gains of whole chromosomes (aneuploidy; Figure 1.2-4c). Such changes are found in nearly all major human tumor types(Mitelman 1994). Examples include the loss of chromosome 10 in glioblastomas, often reflecting the inactivation of the tumor-suppressor gene *PTEN*(Wang, Puc et al. 1997), and the gain of chromosome 7 in papillary renal carcinomas, reflecting a duplication of a mutant *MET* oncogene(Zhuang, Park et al. 1998). The molecular basis of chromosomal instability(CIN) remains undefined in most human cancers. However, studies indicated that a large number of gene alterations can lead to CIN. These genes include those involved in chromosome condensation, kinetochore structure and function, sister-chromatid cohesion, and centrosome/microtubule formation and dynamics, as well as checkpoint genes that

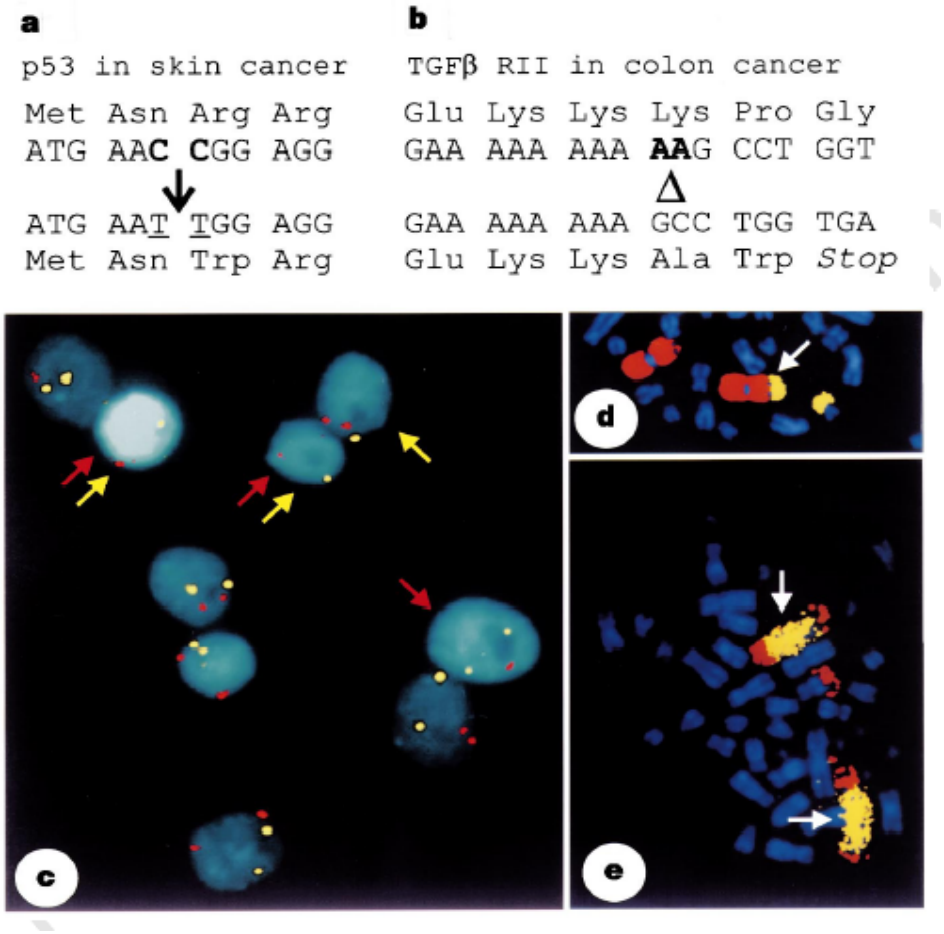


Figure 1.2-4. Examples of genetic alterations in cancer. a, b, Subtle sequence alterations: a, mutation at a dipyrimidine site (bold letters) of the p53 gene (codons 247±248) found in a xeroderma pigmentosum patient with a defect in nucleotide excision repair (NER)94; b, a two-base deletion located within a sequence of ten repeating adenines of the transforming growth factor-b receptor II (TGFb RII) gene (codons 125±128) in a colorectal cancer cell line with mismatch-repair (MMR) deficiency95. **c,** Gross chromosomal change. Loss of chromosomes 3 (red arrows) and 12 (yellow arrows) in colorectal cancer (CRC) cells. A clone of the CRC cell line SW837 was expanded through 25 generations before fluorescence in situ hybridization (FISH). Interphase nuclei were hybridized with labeled centromeric DNA probes specific for chromosome 3 (red spots) and chromosome 12 (yellow spots). The number of signals detected in SW837 cells was diverse, indicating CIN; normal cells, as well as cancer cells exhibiting MIN, had two red and two yellow signals in nearly every nucleus46. **d,** Chromosome translocation. A metaphase plate of the neuroblastoma cell line GIMEN was hybridized by FISH with labelled whole-chromosome-painting probes specific for chromosome 1 (red) and chromosome 17 (yellow), revealing a t(1;17) translocation (arrow). **e,** Gene amplification. FISH with a N-myc probe (yellow) and a whole chromosome-painting probe specific for chromosome 1 (red) revealed an area of N-myc amplification (arrow) within the derivative chromosomes 1 of the neuroblastoma cell line Kelly (Graphic adopted from Lengauer, C., Kinzler, K.W. & Vogelstein, B, 1998)

monitor proper progression of the cell cycle, spindle checkpoint such as hMAD2(Li and Benezra 1996) and DNA-damage checkpoint as described below in "Apoptosis and genetic instability" (Murray 1995; Elledge 1996; Nasmyth 1996; Paulovich, Toczyski et al. 1997)

Chromosome translocations. These alterations can be detected cytogenetically as fusions of different chromosomes or of normally non-contiguous segments of a single chromosome (Figure 1.2-4d). At the molecular level, such translocations can give rise to fusions between two different genes, endowing the fused transcript with tumorigenic properties. An example is provided by the Philadelphia chromosome in chronic myelogenous leukemias; the carboxy terminus of the c-abl gene on chromosome 9 is joined to the amino terminus of the BCR gene on chromosome 22(Nowell 1997). The molecular mechanism underlying translocation is not clear. It is possible that they arise in cells that enter mitosis with unrepaired double-strand breaks (DSBs) (Murray 1995; Elledge 1996; Nasmyth 1996; Paulovich, Toczyski et al. 1997). Therefore defects in genes involved in DSB repair or DNA-damage checkpoint may be responsible for it.

Gene amplifications. Gene amplifications are seen at the cytogenetic level as homogeneously stained regions or double minutes (Figure 1.2-4e). At the molecular level, multiple copies of an "amplicon" containing a growth-promoting gene(s) can be seen.

The amplicons contain 0.5-10 megabases of DNA, and are different from the duplications of much larger chromosome regions that result from aneuploidy and translocations (Brodeur). An example is the amplification of *N-myc* that occurs in 30% of advanced neuroblastomas(Seeger, Brodeur et al. 1985). Gene amplification occurs late in

tumorigenesis. It occurs at a higher rate in cancer cells than in normal cells(Tlsty, Margolin et al. 1989). Gene amplification is associated with tumor progression, prognosis and resistance to chemotherapeutic drugs(Seeger, Brodeur et al. 1985; Brodeur 1998). It is largely unknown how amplification is generated. Some reports indicate that unrepaired DNA breaks and P53 deficiency play a role in this process(Livingstone, White et al. 1992; Yin, Tainsky et al. 1992; Zhu, Mills et al. 2002).

DAN damage, apoptosis and genetic instability

Cells are constantly exposed to exogenous and endogenous DNA-damaging insults. Exogenous agents include chemical mutagens, radiation and oncogenic viruses, which can damage genomic DNA and cause mutations. Cells can also suffer DNA damage spontaneously from endogenous sources including oxidative stress from normal metabolism and errors during DNA replication and mitosis. Left unrepaired, the myriad types of damage that can occur in genomic DNA pose a serious threat to the faithful transmission of the correct complement of genetic material. In order to protect the integrity of the genome, cells have developed an effective system consisting of DNA repair genes and DNA damage checkpoint genes that regulate cell cycle arrest/progression and apoptosis. Cell cycle arrest is induced in response to DNA damage. Such checkpoint mechanisms allow the cell time to repair DNA damage before cell cycle progression is resumed. In cells with unrepairable extensive DNA damage, molecules such as P53 trigger apoptosis or cellular senescence. DFF/CAD is activated during apoptosis to digest the potentially harmful chromosomal DNA with damage and mutations. Genetic instability arises when there is excessive DNA-damaging insults that

overwhelm the organism's protective system or when there are defects in DNA repair or DNA damage checkpoint genes.

The ATM/ATR signaling network is important for the cellular response to DNA damage including detection, signaling, repair of DNA damages and apoptosis. Exactly how cells sense DNA damage is not clearly understood. Pathways of DNA repair are generally equipped with proteins that bind preferentially to certain classes of DNA lesion. For example, the MutS proteins bind to mismatched bases(Jiricny 1998), the Ku heterodimer binds to DSBs(Smith and Jackson 1999), and the *Xeroderma pigmentosum* (XP) group C protein (XPC) involved in NER is one of several proteins that selectively recognize UV-induced DNA photoproducts (de Laat, Jaspers et al. 1999). Over the past few years, evidence has accumulated for the dynamic physical localization of many DNA repair and DNA damage signaling proteins to sites of DNA lesions within the cell. In the case of DNA double strand break, γ-H2AX and Ku heterodimer bind to the site of break. γ-H2AX recruits 53BP1, BRCA1, MDC1, MRE11–RAD50–NBS1 complex to the site of DNA damage. 53BP1, BRCA1 and MDC1 function as adaptor molecules that mediate recruitment of ATM, ATR, and their targets to the sites of DNA damage. ATM, ATR and DNA-PKcs(DNA protein kinase catalytic subunit) complexed with Ku relay the signaling by phosphorylation of their downstream molecules such as Chk1, Chk2, P53 and MDM2(Bassing, Chua et al. 2002; Celeste, Petersen et al. 2002; Fernandez-Capetillo, Chen et al. 2002; Shiloh 2003; Stewart, Wang et al. 2003). MDM2 is an ubiquitin ligase that ubiquitinates P53 and itself, resulting in degradation by the 26S proteosome. Phosphorylation of P53 and MDM2 abrogates P53-MDM2 interaction and interferes with nuclear export of MDM2-P53 complex respectively, reducing degradation of P53. These

events lead to cell cycle arrest or apoptosis. Cells with extensive DNA damage undergo senescence or apoptosis to prevent propagation of carcinogenic genetic alterations. P53 tumor suppressor plays a central role in the decision of a cell to undergo apoptosis.

Apoptosis is one of the crucial mechanisms for maintaining genomic integrity. An important regulator of P53 is Chk2. Chk2 not only activates p53 but also increases its stability in cells exposed to IR(Hirao, Cheung et al. 2002; Takai, Naka et al. 2002). Activation of Chk2 that leads to p53-mediated apoptosis is regulated by both ATM-dependent and ATM-independent pathways, the latter of which might be mediated by the newly identified ATX (Shiloh 2003). Activation of the transcription factor p53 in response to DNA damage results in induction of p53 target genes such as Noxa, Bax, and Puma, leading to apoptosis(Miyashita and Reed 1995; Oda, Ohki et al. 2000; Nakano and Vousden 2001). Chk2 also regulates the activity of the transcription factor E2F1 by phosphorylation of Ser-364 and PML by phosphorylation of Ser-117 in response to DNA damage, again resulting in induction of apoptosis(Yang, Kuo et al. 2002; Stevens, Smith et al. 2003) . PML (promyelocytic leukemia gene) encodes a tumor suppressor that mediates IR-induced apoptosis in a P53-independent manner(Yang, Kuo et al. 2002).

When apoptosis is inhibited in cells exposed to DNA-damaging agents, the surviving cells can show genetic instability. Cells that undergo apoptosis after being treated by genotoxin have extensive DNA damage and genetic alterations. Inhibition of apoptotic cell death helps some of these cells survive with lots of mutations. A small fraction of these cells may be able to proliferate and propagate their genetic alterations, therefore contributing to cells showing genomic instability.

1.3. Abnormality of DFF/CAD genes in human cancers

Abnormality of DFF/CAD gene is frequently found in a variety of tumor types. The DFF40/DFF45 genes map to chromosome 1p36.2-36.3. Alterations of 1p such as 1p deletion characterize a wide range of human malignancies, including both solid tumors and hematological cancers, such as melanoma, colon carcinoma, breast carcinoma, hepatocellular carcinoma, pheochromocytoma and neuroblastoma (Maris, White et al. 1995; Schwab, Praml et al. 1996; Van Gele, Speleman et al. 1998). DFF45 is preferably expressed in low-stage neuroblastoma tumors and to a lesser degree in high-stage neuroblastomas (Abel F 2002). Decreased expression of DFF45/ICAD has been correlated with higher-grade tumors and poor prognosis in patients with esophageal carcinoma(Konishi, Ishiguro et al. 2002). All these evidences suggest a potential role of DFF/CAD in tumor development.

DFF40/DFF45 are implicated to be tumor suppressor genes in neuroblastoma

Neuroblastoma is a malignant childhood tumor of migrating neuroectodermal cells derived from the neural crest and destined for the adrenal medulla and the sympathetic nervous system. Approximately 36% of all neuroblastomas have 1p deletions of variable length (Fong, Dracopoli et al. 1989; Ohtsu, Hiyama et al. 1997; Iolascon, Lo Cunsolo et al. 1998). Allelic loss of chromosome 1p is a predictor of unfavorable outcome in patients with neuroblastoma(Caron H 1996; Iolascon, Lo Cunsolo et al. 1998). Analyses of such chromosome deletion suggest that there is more than one tumor suppressor gene in the region 1p36.1-1p36.3 (Ohtsu, Hiyama et al. 1997; Komuro, Valentine et al. 1998; Caron, Spieker et al. 2001; Hiyama, Hiyama et al. 2001;

White, Thompson et al. 2001). Functional support for a 1p tumor suppressor comes from the observation that the transfer of 1p chromosomal material into the neuroblastoma cell line NGP suppressed tumorigenicity and induced differentiation(Bader, Fasching et al. 1991). Identification and characterization of a 500-Kb homozygously deleted region at 1p36.2-p36.3 in a neuroblastoma cell line showed that the DFF45 gene is located in this 500-Kb region(Ohira, Kageyama et al. 2000).

In addition, it was reported that the DFF45 gene at 1p36.2 is homozygously deleted and encodes variant transcripts in another neuroblastoma cell line(Yang, Chen et al. 2001). Rare allele variants of DFF45 have also been reported in human neuroblastoma tumors(Yang, Chen et al. 2001; Abel F 2002). Moreover, tumor-specific DFF45 gene mutations or deletions were identified in human germ cell tumor and neuroblastoma tumors from patients (Abel F 2002; Abel, Sjoberg et al. 2004), indicating the involvement of this gene in tumor development.

DFF deficiency and genetic instability in human cancers

N-myc amplification and 1p deletion are two most important factors in predicting the outcomes of neuroblastomas. There is a strong correlation between 1p deletion and *N-myc* amplification(Fong, Dracopoli et al. 1989). Nearly all 1p-deleted tumors showed *N-myc* amplification whereas majority of myc-amplified tumors had 1p deletion (Iolascon, Lo Cunsolo et al. 1998; Komuro, Valentine et al. 1998; Caron, Spieker et al. 2001; Spitz, Hero et al. 2002). 1p deletions of *N-myc*-amplified tumors are very large, always at least including a region from 1p35-1p36.1 to telomere. In contrast, in *N-myc* single copy cases, 1p deletions were described to be consistently smaller. Analysis of SRO (smallest region

of overlap) of the *N-myc* single copy tumors and SRO of *N-myc*-amplified tumors implied that a suppressor locus in 1p36.2-3 must be deleted in *N-myc* -amplified tumors, a region that DFF40 and DFF45 genes are located(Westermann and Schwab 2002). Chromosome 1p deletion is also associated with higher telomerase activity(Hiyama, Hiyama et al. 2001). Other aberrations such as gain or loss of portions of chromosomes and chromosomal rearrangement are commonly seen in 1p-deleted tumors. In addition to the above evidences, the role of apoptosis in preventing genetic instability and the fact that DFF/CAD is a DNase activated during apoptosis lead us to speculate that DFF/CAD may protect the integrity of genome through degrading chromosomal DNA of cells suffering extensive DNA damage and mutations. Therefore loss of DFF/CAD may result in increased genetic instability and consequently tumorigenesis.

Chapter 2

Materials and Methods

Cell Culture

HCT116 colon cancer cells, TK6 and WTK1 lymphoblastoid cells were obtained from ATCC (Manassas, VA). TK6 and WTK1 cells were cultured in RPMI1640 supplemented with 10% equine serum. HCT116 cells were cultured with DMEM medium supplemented with 10% fetal bovine serum. L929 cells were maintained in DMEM medium supplemented with 10% equine serum.

Irradiator

The high-energy heavy ion beam of 1GeV/amu 56Fe ions was generated from the Alternating Gradient Synchrotron (AGS) accelerator in NASA Space Radiation Laboratory (NSRL) at Brookhaven National Laboratory (BNL), Upton, NY 11973, USA.

X-ray was generated by the XRAD 320 irradiator (Precision X-ray Inc. East Haven, CT), which is a facility of the Comprehensive Cancer Center of Duke University.

Transgenic animal and mouse embryonic fibroblast cells

The CAD (-/-) transgenic mice and immortalized CAD (-/-) MEF cells were obtained from Dr. Shigekazu Nagata's group at Osaka University(Kawane, Fukuyama et al. 2003). The original CAD(-/-) mice were in the 129/Sv background (Kawane, Fukuyama et al. 2003). When they were shipped from Dr. Nagata's lab, they had been crossed into C57BL/6 three generations. In our laboratory, they underwent additional backcrosses into the C57/BL6 background. All the mice used for experiments were maintained on C57BL/6 background after at lease 6 backcrosses from the original 129Sv/C57BL/6 founder mice. To further reduce the effect of the difference in genetic

background, all the experiments involving CAD-/- and wild type mice were carried out using littermates.

Early passage CAD(-) cells were obtained from pregnant female mice at day 14-15. They were cultured in DMEM medium supplemented with 10% fetal bovine serum.

Plasmid construction and gene transduction

The human DFF45/ICAD gene was amplified by PCR from mRNA of normal lymphocytes and cloned into the vector pTOPO-1(Clontech, Palo Alto, CA). They were then sequence-verified. Two point mutations were introduced into the human DFF45 gene using a PCR-based approach similar to a described method(Sakahira, Enari et al. 1998). In addition, a commonly used hematoaglutinin(HA) tag was engineered into the 3'end of the protein, The modified gene, mICAD, was then cloned into a retroviral vector pLPCX (Clontech. Palo Alto, CA), which has a constitutively activated cytomegalovirus(CMV) promoter controlling the expression of a transgene, to derive pLPCX-mICAD. Recombinant retroviruses were generated by transfecting pLPCX-mICAD into φX-ampho packaging cells(Kinsella and Nolan 1996). The φX-ampho cells were kindly provided by Dr. Gary P. Nolan of Stanford University. The derived retroviral vector, LPCX-mICAD, was used to infect HCT116, TK6, WTK1, and L929 cells. Puromycin selection was used to identify cell clones that have stably integrated the transgene. Western blot analysis by use of an anti-HA antibody (Roche Molecular Biology) was carried out to identify clones that were stably expressing the mICAD protein.

Western Blotting

Cells were collected, washed in PBS and lysed in 1% Triton lysis buffer (20mM Tris PH 7.5, 100mM NaCl, 5mM EDTA, 1% Triton X-100, 10mM NaF, 1mM Na_3VaO_4, 20μg/ml Leupeptin, 10μg/ml Pepstatin, 10μg/ml aproptinin). Samples were denatured at 100°C for 5 min. Equal amount of total protein were loaded to each well for electrophoresis in 10% SDS polyacrylamide gels and then transferred to polyvinylidene fluoride (PVDF) microporous membranes (Millipore Corporation). Membranes were then incubated with primary antibody followed by incubation with horseradish peroxidase-linked secondary antibodies. Antibody-antigen complexes were detected using chemiluminescence (Pierce). The primary antibody is anti-HA tag (Santa Cruz).

Apoptosis evaluation by annexin V staining

To quantify the amount of cellular apoptosis in mICAD transfected cells, a method based on the staining of externalized phosphotidyl serine by annexin V staining was adopted. A commercially available annexin V staining kit(Clontech, Palo Alto, CA) was used. Control and radiation treated cells were stained according to manufacturer's instruction. Mouse bone marrow cells were isolated and the red blood cells were lysed with a RBC lysis buffer (QIAGEN 79217) before the cells were stained with annexin V and propidium iodide or subjected to DNA fragmentation analysis. Double annexin V/propidium iodide staining was used to distinguish cells that were undergoing early vs late apoptosis. The fraction of cells with positive annexin V staining was defined as cells undergoing apoptosis. Quantification of annexin V staining was carried out by the Duke University Cancer Center Flow Cytometry Facility.

Quantification of apoptotic DNA fragmentation

To quantify the effect of mICAD expression and CAD knockout on the incidence of cells with apoptotic DNA fragmentation, a commercially available sandwich ELISA kit was purchased from Roche Diagnostics(Mannheim, Germany, Cat#1 774 425). The kit quantifies cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) in cells undergoing programmed cell death. It was chosen because of its high specificity and sensitivity for apoptotic DNA fragments.

DNA fragmentation was also evaluated by quantification of SubG1 fraction through cell cycle analysis and by showing the DNA ladder in electrophoresis. For SubG1 fraction quantification, cells were fixed in 70% ethanol and stained with propidium iodide and then subG1 fraction was determined by cell cycle analysis. DNA fragments were extracted from irradiated cells using an apoptotic DNA ladder isolation kit (BioVision K170-50, Mountain View, CA 94043). This kit selectively extracts the cleaved small DNA fragments from apoptotic cells leaving out most undigested genomic DNA.

Clonogenic assay of cellular survival after irradiation

To evaluate the effect of apoptotic DNA fragmentation on cellular survival, clonogenic assays were used. For HCT116 cells, different number of cells were plated in 10-cm Petri dishes and irradiated with different doses of ionizing radiation. The number of cells plated at different doses was empirically determined so that in each dish the surviving cells numbered around 30-150. Two weeks after irradiation, the colonies that

emerged in the Petri dish were stained and counted and used to calculate the surviving fraction of cells under each irradiation dose.

For TK6 and WTK1 cells, 96-well plates were used because the cells grew in suspension. Different numbers cells were plated in each well for each radiation dose. Two weeks after irradiation, the number of wells with cellular growth was counted and the surviving fractions for each irradiation dose were calculated according to Poisson distribution by use of a method established by Furth et al(Furth, Thilly et al. 1981).

Gene mutation assays

To evaluate radiation-induced mutation frequency at the *tk* and *hprt* loci, established methods were adopted. Briefly, cells were treated with CHAT medium for 2 days to eliminate pre-existing mutants at these two gene loci. The CHAT medium consists of 10 mM deoxycytidine, 200 mM hypoxanthine, 0.2 mM aminopterin, and 17.5mM thymidine. Cells with mutated *tk* or *hprt* genes will be killed by this medium(Liber and Thilly 1982). Subsequently, they were placed in THC (CHAT without aminopterin) medium to recover before cells were treated with gamma-irradiation. Three days (for TK) or 6 days (for HPRT) after irradiation, TK6 (40000cells/well) and WTK1 (2000-4000/well) were plated into 96-well plates in 2μg/ml trifluorothymidine (TFT) to isolate TK mutants. For *hprt* mutants, TK6 (40000 cells/well) and WTK1 (5000/well) were plated in 0.5μg/ml 6-thioguanine (6TG). Cells were also plated at 1.6 cell/well in the absence of the selective agent to determine plating efficiency. Cells with mutations at the *hprt* locus were scored after 11 days. For cells with *tk* mutations, the cells were re-fed with TFT at 11days and mutant cell growth in each plates was scored at 18 days post

plating. Mutation frequencies were calculated from the Poisson distribution, as described previously(Furth, Thilly et al. 1981; Liber and Thilly 1982).

Gene amplification assays

Clonogenic assays were used to measure the frequency of gene amplification in L929 and HCT116 cells. The selective agent for *cad* gene amplification, PALA, was obtained from the Drug Synthesis Branch, Division of Cancer treatment, National Cancer Institute. The selective agent for *dhfr* gene amplification, MTX, was purchased from Sigma (St Lois, MO). To estimate the frequency of gene amplification, we determined the LD_{50}(the dose that inhibited 50% of treated cell growth) for each drug in each cell line. For estimation of resistant clones, about 1-2 $\times$ 10^6-cells/10cm dish were employed. Cells were then selected in 3.5-5 $\times$ LD_{50} of PALA or MTX. Resistant colonies usually appeared in 2-3 weeks and were counted subsequently. Frequency of amplification is expressed as the number of resistant colonies relative to the number of colonies formed without PALA and MTX.

Fluctuation Analysis

About 1000 cells were inoculated into each well of 24-well plates. After growth without selection to about 10^6 cells per well, cells were trypsinized. Cells from each well were plated into 10cm dishes, and grew in the presence of 3.5$\times$$LD_{50}$ (for HCT116 cells) or 5$\times$$LD_{50}$ (for L929 cells) PALA. Resistant colonies appeared in 2-3 weeks and were fixed with methanol, stained with crystal violet for counting.

Analysis of chromosomal aberrations

To analyze in vivo chromosomal aberrations, mice were given 3 Gy whole-body γ-irradiation. Three or seven days later, they were sacrificed. One hour before sacrifice, 0.1ml of 0.5% colchicine was injected intraperitoneally. After the sacrifice, the bone marrow cells were flushed out by use of hypotonic solution (0.075M KCl) and incubated at 37^{o}C for 10 minutes. Cells were then fixed by Carnoy's fixative (methanol: acetate 3:1) and dropped onto slides for analysis. To analyze chromosomal aberrations in MEF cells, colcemid was added to the cells for 1 hour, the cells were then incubated in hypotonic solution and fixed by Carnoy's fixative and dropped onto slides for analysis. At least 100 metaphase spreads were analyzed for chromosomal aberrations for each sample.

To examine translocations, chromosome painting was done with whole chromosome probes for mouse chromosome 1,2 and 8 according to manufacturer's protocol (Cambio Ltd, Cambridge, UK). Translocations were examined in 100 cells for each sample.

Detection of Aneuploid cells by FACS analysis

Cells were collected, washed with PBS + 1% FBS, fixed with cold 70% ethanol, stained with propidium iodide (10 ug/mL) and ribonuclease A (100ug/mL), and subjected to cell cycle analysis using FACS (Flow Cytometry Facility, Duke University Comprehensive Cancer Center). Percentage of aneuploid cells was calculated with ModFit LT cell-cycle analysis software (Verity Software House, Topsham, ME).

Micronucleus assay

Cells were plated in the 6-well plates 24 hours before the irradiation by high-energy heavy ion beam of 1GeV/amu 56Fe ions. One day after irradiation, Cytochalasin B (5 ug/ml) (Sigma, St. Louise, U.S.A.) was added to the culture medium and the cells were cultured for 24-48 hours. Then the culture medium was removed and the cells were rinsed with phosphate-buffered saline (PBS) and fixed by Carnoy fixative (Methanol:Acetic acid 3:1) for 5 minutes at room temperature. The air-dried cells were then immersed in the 2x sodium chloride-sodium citrate (SSC) buffer with 0.1% Nonidet® P 40 (NP 40) for 1 minute and then were stained in 5ug/ml acridine orange (Sigma-Aldrich) in PBS for 2-5 minute with gentle shaking. Then cells were washed with PBS and the micronuclei were scored under a fluorescent microscope.

Soft agar assay for tumorigenic transfomation

The soft agar assays were conducted according to established protocols(Cox and Der 1994) For each cell line, single cell suspensions were prepared in duplicate, ranging in 5-fold increments from10^4 to 2.5×10^5 cells per 60-mm dish, in a 0.33% top agar suspension, overlaid onto a 0.5% agar bottom layer, and incubated at 37°C for colony formation. Both the top and bottom agar layer were made from bacto-agar(DIFCO, Detroit, MI). Cells were fed twice a week by dropwise addition of growth medium. The presence of colony growth was scored after 3 weeks under a microscope.

To confirm the tumorigenicity of the colonies that emerged in the soft agar assay, the colonies were picked, transferred into DMEM medium and expanded. Cells from each colony (about $3\text{-}5x10^6$) were then injected subcutaneously into the right flanks of

6–8-week-old athymic nude mice (Charles River Laboratories, Raleigh, NC). After inoculation, the incidence and growth of tumors were evaluated at least once a week for at least 16 weeks. Mice were sacrificed when tumor size reaches 15mm in diameter.

Tumor growth assay

For tumor growth assays, about 10^6 L929 cells were injected s.c. into nude mice in 50 μL of PBS solution. Tumor growth was then followed and measured by a caliper every 2 to 3 days. Tumor volume was calculated using the following formula: volume =length × $width^2$ × p/6. For histological evaluation, tumors were removed at 4 weeks, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4-Am thickness, and subjected for immunohistochemistry using Ki67 antibody. Wilcoxon Ranksum test was used for the statistical analysis.

Immunohistochemistry

Paraffin embedded tissue sections were dewaxed in xylene, rehydrated and washed in TBS, pH7.4. For antigen retrieval, paraffin sections were heated in a microwave oven (900W) in 10 mM citrate buffer followed by treatment with 3% H2O2 and blocking with 20% normal goat serum. Sections were then incubated with antibody against Ki67 (Novacastra Laboratories, NCL-Ki67P) followed by incubation with a biotinylated rabbit secondary. An avidin-biotin complex was formed and developed using diaminobenzidine substrate. Slides were counterstained with hematoxylin.

Two-stage skin carcinogenesis assay

In this assay, 7-10 weeks old mice were used. The dorsal skin of the mice was shaved 3 days before the start of topical treatments. The mice were initiated with 100 µg dimethylbenz[a]anthracene (DMBA) in 200µl acetone applied topically under yellow light, and control mice were treated with 200 µl acetone. All the mice remained in the dark for 12-24 hours following DMBA or acetone vehicle treatment. One week after DMBA treatment, twice-weekly topical treatment with 2.5µg TPA (12-O-tetradecanoylphorbol-13-acetate) in 100µl acetone or 100µl acetone alone was initiated and continued for 20 weeks. Mice were observed twice a week for skin papillomas greater than 1-2mm in height. One week following the end of experiment mice were euthanized, dorsal skin with or without papillomas was removed, fixed and stained for pathological examination.

Ionizing radiation carcinogenesis.

3-4-week-old mice received 3 Gy whole-body IR at a dose rate of 96cGy/min from an X-ray irradiator on day 1 and day 3, for a total dose of 6 Gy. Following treatment, mice were observed three times per week for morbidity. Mice exhibiting signs of distress or obvious tumors were necropsied. Tissues with lesions were fixed in formalin or frozen by liquid nitrogen for histological examinations.

Chapter 3

A novel role of DNA fragmentation factor as a tumor suppressor through maintaining genomic stability

3.1 Introduction

Although DNA fragmentation has long been associated with apoptosis (Williams, Little et al. 1974; Wyllie 1980), its physiological roles are not clearly defined. Cell death, the eventual outcome of the apoptotic process, was not significantly affected by DNA fragmentation, neither were many other apoptosis-associated biochemical processes (Sakahira, Enari et al. 1998; Zhang, Liu et al. 1998). The only suggested physiological role for DNA fragmentation is that it may play some roles in spatial learning and memory (Slane, Lee et al. 2000).

It has been reported that the genes encoding the nuclease that are responsible for the fragmentation of DNA, DFF40/CAD and DFF45/ICAD(Liu, Zou et al. 1997; Enari, Sakahira et al. 1998), are aberrantly expressed in many tumor types. In addition, the abnormalities in this gene are associated with poor prognosis in cancer patients (Ohira, Kageyama et al. 2000; Yang, Chen et al. 2001; Konishi, Ishiguro et al. 2002; Hsieh, Liaw et al. 2003). Most significantly, tumor-specific DFF45 gene mutations or deletions have been identified in human germ cell tumor and neuroblastomas from patients (Abel F 2002; Abel, Sjoberg et al. 2004), indicating the involvement of this gene in tumor development.

The present study was initiated to explore the potential relationship between apoptotic DNA fragmentation and tumor development. We reasoned that the process that is involved in the destruction of genomic DNA might have a direct effect on genomic integrity, especially when cells are under assault from DNA-damaging agents. This effect on genomic instability may be responsible for the observed association between DFF abnormality and cancer. Such a relationship is consistent with the view that genomic

instability is an important factor during carcinogenesis/malignant tumor development(Loeb 1991; Lengauer, Kinzler et al. 1997; Stoler, Chen et al. 1999; Nowak, Komarova et al. 2002). Our results indicate that impairment of DNA fragmentation leads to increased genetic instability, cellular transformation and susceptibility to chemical carcinogenesis. They suggest that DNA fragmentation factor may be an important gene for maintaining genetic stability and preventing tumorigenesis.

3.2 Results

(1) Efficient inhibition of apoptotic DNA fragmentation by engineered mutant ICAD gene expression

In order to examine the potential role of DNA fragmentation in maintaining genetic instability, we created cell lines in which the nuclease activities of DFF/CAD(Liu, Zou et al. 1997; Enari, Sakahira et al. 1998) were genetically blocked. This was achieved by stable transduction of a modified copy of the gene DFF45/ICAD(Sakahira, Enari et al. 1998), which is an inhibitor as well as a chaperone of DFF40/CAD(Enari, Sakahira et al. 1998; Liu, Li et al. 1998; Sakahira, Enari et al. 1998), the nuclease that is responsible for fragmentation of genomic DNA during cellular apoptosis. Under normal circumstances, DFF45/ICAD is physically associated with DFF40/CAD, preventing its activation. However, when cellular apoptosis is initiated, DFF45/ICAD is digested by activated caspases(e.g. caspase 3) and dissociates from DFF40/CAD, which allows for the activation of the nuclease activities of the latter and subsequent fragmentation of genomic DNA. We followed a published approach to modify the ICAD protein(Sakahira, Enari et al. 1998). The two caspase-sensitive sites of

DFF45/ICAD were mutagenized (D117E and D224E) to derive the mutated ICAD (mICAD) protein (Figure 3.2-1A). The modification rendered the protein resistant to caspases, thereby inhibiting its dissociation from the CAD protein and preventing the activation of the latter. Over-expression of the gene has been shown to block apoptotic DNA fragmentation in cells of human and murine origin(Sakahira, Enari et al. 1998).

The mICAD gene was then transduced into three human cell lines HCT116, TK6, WTK1, and a mouse cell line L929 by a retrovirus-mediated approach. HCT116 is a colon cancer cell line while TK6 and WTK1 are lymphoblastoid cell lines derived from the same individual with differing p53 status(Little, Nagasawa et al. 1995). L929 is a transformed mouse fibroblast cell line. Stable expression of the mICAD gene was readily detectable in transduced cells (Figure 3.2-1B). The expression of the mICAD gene effectively inhibited the activation of the CAD/DFF40 nuclease, consistent with previous reports(Sakahira, Enari et al. 1998). This was evidenced by the fact that ionizing radiation-induced apoptotic DNA fragmentation in mICAD-transduced cells was effectively suppressed by the expression of mICAD ($p \leq 0.001$) (Figure 3.2-2A). Similar to previous studies(Sakahira, Enari et al. 1998), the inhibition of DNA fragmentation had little effect on the rate of apoptosis. When externalized cell membrane phosphotidyl serine, an indicator of early apoptosis, was stained (by fluorescence labeled annexin V), no difference was observed between cells transfected with an empty vector and cells transfected with the modified ICAD gene ($p > 0.1$, Figure 3.2-2B).

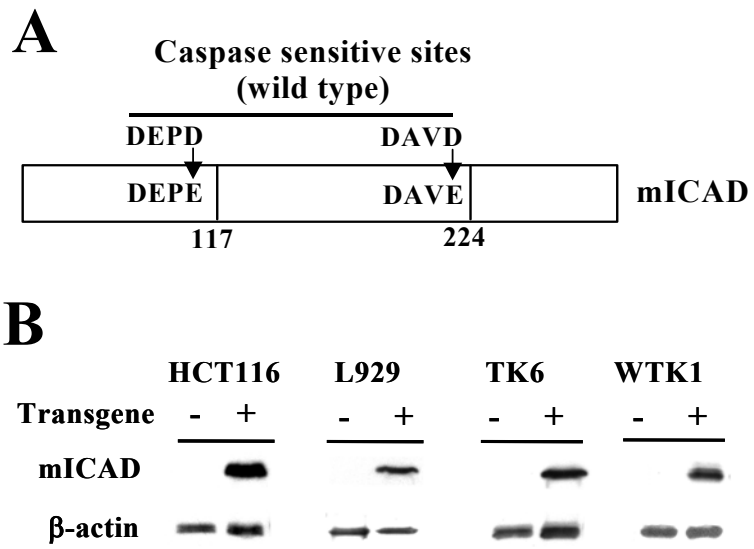


Figure 3.2-1. Generation of genetically modified cell lines expressing mutant ICAD. (A) The structure of the modified ICAD/DFF45 protein. The mutations lead to amino acids changes that render the mutant ICAD protein resistant to caspase cleavage. **(B)** Western blot analysis of mICAD protein expression in cells that have been stably transduced with a mICAD gene. An antibody (from Roche Molecular Biology) against the HA tag that was engineered into the 3' end of the mICAD gene was used so that only the modified ICAD gene is detected. It is clear that only those cells that were transduced with the mICAD gene express the mutant form of the ICAD protein.

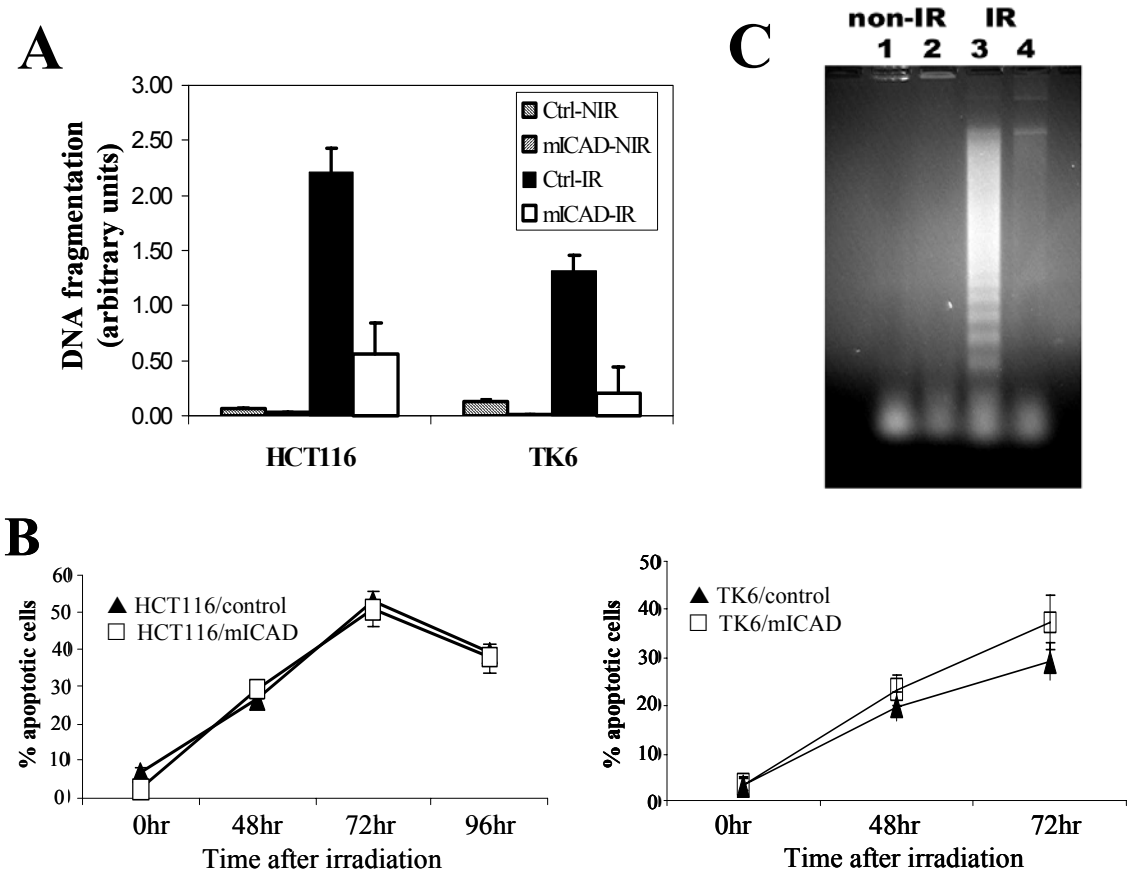


Figure 3.2-2 DNA fragmentation is inhibited in cells transduced with mutant ICAD (A) Evaluation of the extent of radiation-induced apoptotic DNA fragmentation in cells that were transduced with the mICAD gene. Control and mICAD-transduced cells were irradiated with γ-rays(3 Gy for HCT116 and 1.5 Gy for TK6) and the DNA fragmentation was quantified by an ELISA kit at 72 hours after radiation. NIR: non-irradiated; IR: irradiated. **(B)** Quantification of apoptosis induced by γ-irradiation in mICAD-transduced HCT116 and TK6 cells. Apoptosis were evaluated by an annexin V staining kit. Error bars represent standard deviation. **(C)** DNA fragments were extracted from HCT116 cells 72 hours after 4 Gy γ-ray irradiation, using a kit that selectively isolates DNA fragments from apoptotic cells. Lane 1: non-irradiated control cells; Lane 2: non-irradiated mICAD cells; Lane 3: irradiated control cells; Lane 4: irradiated mICAD cells.

(2) Increased frequency of radiation-induced gene mutations in mutant ICAD-expressing cells.

To evaluate the effects of apoptotic DNA fragmentation on DNA-level genetic instability, we examined radiation-induced mutation frequencies at the autosomal thymidine kinase (*tk*) locus and the X-linked hypoxanthine guanine phosphoribosyl transferase (*hprt)* locus in the TK6 and WTK1 lymphoblastoid cells. These two gene loci were chosen because the availability of quantitative mutation assays and the abundance of previously published data using these lines. The hemizygous status of *tk* and *hprt* genes in the TK6 and WTK1 cells makes it possible to observe the mutation frequencies at these two gene loci with a reasonable number ($<10^7$) of cells. Mutations in the *tk* gene allow the host cells to be resistant to the cytotoxic drug trifluorothymidine (TFT) while mutations in the *hprt* gene allow host cells to be resistant to the cytotoxic drug 6-thioguanine (6TG). The addition of these two drugs will therefore allow for the selective outgrowth of cells that have suffered mutations in these two genes, which in turn will allow for an evaluation of the mutation frequencies of the two genes in the general cell population.

In TK6 cells, mutant ICAD/DFF45 expression significantly enhanced radiation-induced mutation frequencies at both the *tk* and *hprt* loci ($p \leq 0.001$) (Figure 3.2-3, upper panels). In WTK1 cells, where the background frequencies of mutation were much higher, the difference was smaller but still significant ($p<0.02$) (Figure 3.2-3, lower panels). One plausible explanation for the smaller difference in WTK1 is the very high background mutation rates in the WTK1 cells, which may overshadow the contribution of DNA fragmentation on induced mutations at these gene loci. These results suggest that

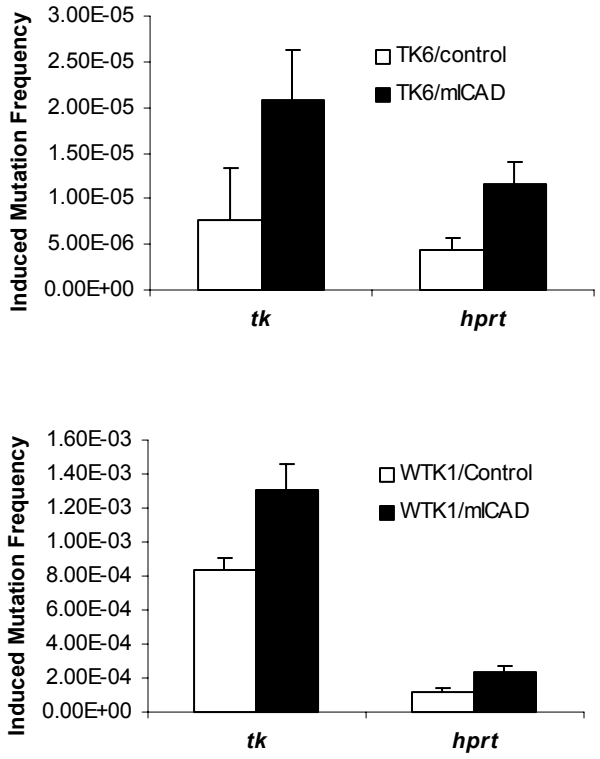


Figure 3.2-3 The frequency of radiation-induced gene mutations at the *tk* and *hprt* in TK6 (top panel) and WTK1 (lower panel) cells. Both control and mICAD-transduced cells were used in all experiments. In each experimental series, results from three independent experiments were shown. For each experiment the radiation-induced gene mutation frequencies of control as well as in mICAD-transduced cells were shown. In both panels, the results for *tk* and *hprt* loci were statistically significant ($p<0.05$, paired *t*-test). Background mutation frequencies (from non-irradiated cell populations) were subtracted in each of the experiments shown. The background mutation frequency is $4.11\pm0.62\times10^{-6}$ at *tk* and $2.47\pm0.73\times10^{-6}$ at *hprt* loci in TK6/control cells, $4.05\pm1.03\times10^{-6}$ at *tk* and $1.35\pm0.82\times10^{-6}$ at *hprt* in TK6/mICAD cells, $2.23\pm0.91\times10^{-4}$ at *tk* and $1.43\pm0.62\times10^{-5}$ at *hprt* in WTK1/control cells, $1.44\pm0.43\times10^{-4}$ at *tk* and $1.98\pm0.57\times10^{-5}$ at *hprt* in WTK1/mICAD cells.

apoptotic DNA fragmentation may function as a checkpoint to guard against DNA-damage-induced gene mutations.

(3) Significantly elevated frequency of gene amplification in cells expressing mutant ICAD

We next examined the effects of apoptotic DNA fragmentation on spontaneous and DNA-damage-induced gene amplification(Schimke, Kaufman et al. 1978; de Saint Vincent, Delbruck et al. 1981), which is a commonly used measure of genomic instability in transformed cells(Tlsty, Margolin et al. 1989; Livingstone, White et al. 1992; Yin, Tainsky et al. 1992; Smith, Agarwal et al. 1995). It has been shown previously that ionizing radiation can increase the frequency of gene amplification significantly(Sharma and Schimke 1989; Hahn, Nevaldine et al. 1990). The frequencies of gene amplification were estimated at two gene loci: the cabamyl-P-synthetase, aspartate transcarbarmylase, dihydro-orotase (*cad*) gene and the dihydrofolate reductase (*dhfr*) gene. Amplifications of these two genes will allow the cells to be resistant to the drugs N-(phosphoacetyl)-L-aspartate(PALA) and methotrexate (MTX), respectively. Therefore, it is possible to estimate the frequency of the cells that have undergone *cad* or *dhfr* gene amplifications by quantifying the number of cell colonies that are resistant to PALA or MTX, respectively.

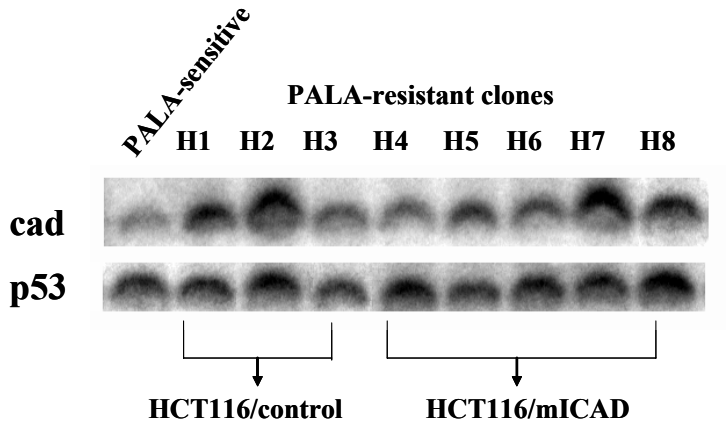
As shown in Table 1, stable mICAD expression in HCT116 caused a significant increase in the level of spontaneously arising PALA-resistant clones compared with parental cells. Furthermore, the frequencies of induced gene amplification in irradiated cells were also much higher(≈ 5 fold) in mICAD-expressing cells than that in the control

Table 1. Frequency of gene amplification in control or mICAD-expressing HCT116 and L929 cells

Drug selection / Cell types		PALA selection (for *cad* gene amplification)				MTX selection (for *dhfr* gene amplification)			
	Irradiation Dose	PE (%)	LD_{50} (μM)	Total# selected cells ($x10^6$)	$PALA^r$ Frequency ($x10^{-5}$)	PE (%)	LD_{50} (μM)	Total# selected cells ($x10^6$)	MTX^r Frequency ($x10^{-5}$)
HCT116/control	None	45.3	31.4	4.5	5.54	42.0	17.5	15.6	0.046
HCT116/mICAD	None	64.7	15.0	1.5	27.4	41.0	13.3	15.6	0.25
HCT116/control	3Gy	59.3	31.4	2.4	8.50	13.4	17.5	14.3	0.21
HCT116/mICAD	3Gy	66.0	15.0	0.6	41.7	18.0	13.3	11.5	1.01
L929/control	None	47.3	21.3	20	0.032	47.3	31.0	10	0.15
L929/mICAD	None	55.5	9.9	3	21.3	55.5	16.7	12	0.81
L929/control	4.5Gy	22.8	21.3	4.8	3.66	22.8	31.0	4.8	10.5
L929/mICAD	4.5Gy	11.8	9.9	2.4	81.6	11.8	16.7	3	37.4

PE = plating efficiency; LD_{50}=dose at which 50% cell growth were inhibited; $PALA^r$ = PALA-resistant clone, MTX^r=MTX resistant clone.

A.



B.

Clone#	control	H1	H2	H3	H4	H5	H6	H7	H8
Relative *cad* gene copy#	1.0	1.98	2.64	1.96	1.72	2.39	1.92	3.05	1.85

Figure 3.2-4 Southern blot analysis of the relative gene dosage for PALA-resistant HCT116-clones. (A) Photograph of southern blot results for selected PALA-resistant clones. **(B)** Densitometric analysis of the relative copy number of *cad* genes in the PALA-resistant clones by ImageQuant TL software (Molecular Dynamics).

cells. An even more impressive increase in PALA-resistant clones was seen in L929 cells. The spontaneously arising frequency of PALA resistant colonies in mICAD-expressing cells is 670 fold higher than that in control cells (Table 1). In addition, radiation-induced frequency of PALA-resistant clones was about 16 fold higher in mICAD-expressing cells than in control cells. Amplification of the *cad* gene in PALA-resistant clones was confirmed by southern blotting shown in supplemental data. In most of the clones examined, *cad* gene dosage was increased 2-3 folds in these cells (Figure 3.2-4).

At the *dhfr* site, a similar pattern was observed. In both HCT116 and L929 cells, mICAD expression caused significant increases in the frequencies of spontaneous and radiation-induced gene amplification (Table 1).

In addition, fluctuation analysis (Table 2) was used to estimate the rate of spontaneous gene amplification in HCT116 and L929 cells, which is a measure of the incidence of new gene amplification per cell per generation. Our data indicate that inhibition of apoptotic DNA fragmentation caused a ≈5 fold increase in the rate of gene amplification in HCT16 cells (from 9.21 $x10^{-6}$ to $44.5x10^{-6}$/cell/generation) and ≈6 fold increase (from $<1.45x10^{-7}$ to $9.34x10^{-7}$/cell/generation) in L929 cells. These data indicate that apoptotic DNA fragmentation is not only important in regulating gene amplification induced by DNA-damaging agents, it is also important in regulating gene amplification arising under normal growth conditions.

Table 2. Fluctuation analysis of spontaneous gene amplification rate in HCT116 and L929

HCT116/control		HCT116/mICAD	
Replicate cultures (C)	24	Replicate cultures (C)	24
Initial cells per culture (N_0)	1000	Initial cells per culture (N_0)	1000
Final cells per culture (N_t)	3.07×10^6	Final cells per culture (N_t)	2.86×10^6
$PALA^r$ Colonies per culture (N)	Replicates with N $PALA^r$ Colonies	$PALA^r$ Colonies per culture (N)	Replicates with N $PALA^r$ Colonies
0-20	0	0-50	0
21-40	6	51-100	2
41-60	5	101-150	7
61-80	4	151-200	10
81-100	2	201-250	3
101-120	2	251-300	0
121-520	5	301-350	2
Average per sample (r')	101.54	Average per sample (r')	168.32
Variance	11323.1	Variance	4323.43
Plating efficiency (PE)	55%	Plating efficiency (PE)	66%
Average per culture (r) *(Corrected by PE)*	184.62	Average per culture (r) *(Corrected by PE)*	1020.12
Mutation Rate ($3.5\times LD_{50}$)	**9.21×10^{-6}**	**Mutation Rate($3.5\times LD_{50}$)**	**44.5×10^{-6}**
SD/Average(Exp.)	1.05	SD/Average(Exp.)	0.39
SD/Average(Calc.)	0.75	SD/Average(Calc.)	0.61
L929/control		**L929/mICAD**	
Replicate cultures (C)	24	Replicate cultures (C)	24
Initial cells per culture (N_0)	600	Initial cells per culture (N_0)	600
Final cells per culture (N_t)	8.28×10^5	Final cells per culture (N_t)	9.76×10^5
$PALA^r$ Colonies per culture (N)	Replicates with N $PALA^r$ Colonies	$PALA^r$ Colonies per culture (N)	Replicates with N $PALA^r$ Colonies
0	24	0	15
1-2	0	1-2	5
3-4	0	3-4	2
5-6	0	5-6	1
7-8	0	7-8	0
9-10	0	9-10	1
Average per culture (r')	0	Average per culture (r')	1.167
Variance	0	Variance	4.75
Plating efficiency (PE)	31.75%	Plating efficiency (PE)	41.5%
Average per culture (r)	0	Average per culture (r)	2.811
Mutation Rate ($5xLD_{50}$)	**$<1.45\times10^{-7}$**	**Mutation Rate ($5\times LD_{50}$)**	**9.34×10^{-7}**
SD/Average(Exp.)	N/A	SD/Average(Exp.)	1.87
SD/Average(Calc.)	N/A	SD/Average(Calc.)	1.59

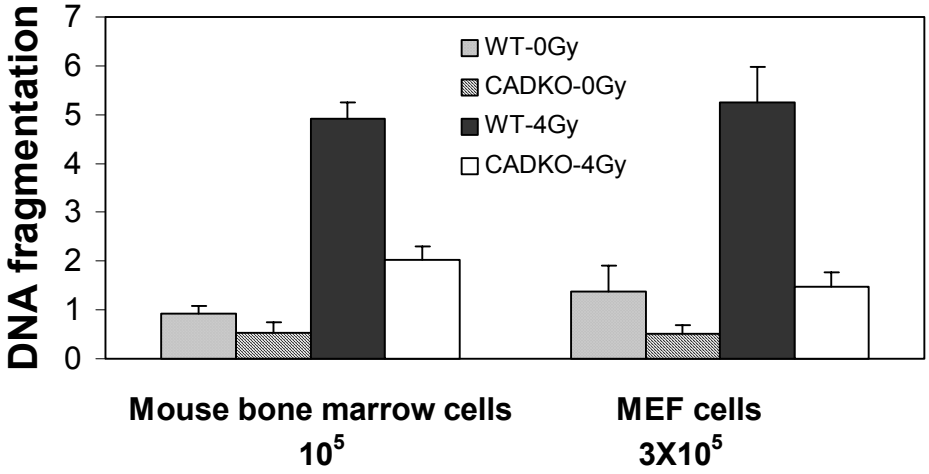
Mutation rates and the calculated value for the ratio of the SD to the average were determined by using equations 8 and 12, respectively, of Luria & Delbruck (1). The value for the experimental SD/average for these fluctuation experiments is similar to that predicted by the Luria & Delbruck (equation 12 in the same reference). These values are consistent that PALA selects for preexisting CAD amplification events that occur spontaneously in HCT116 and L929 cells.

(4) Elevated chromosome instability in *CAD*-deficient mice.

In order to examine the effects of *CAD* gene on genetic instability *in vivo*, we conducted studies in transgenic mice with targeted disruption of mouse CAD/DFF40 gene (Kawane, Fukuyama et al. 2003). Bone marrow cells in irradiated CAD-/- mice and irradiated MEFs isolated from CAD-/- mice underwent apoptosis at rates slightly lower than those of the CAD+/+ mice (Figure 3.2-5B). DNA fragmentation was significantly decreased in these apoptotic CAD-/- cells($p<0.05$, paired *t*-test)(Figure 3.2-5A).

Mutant (CAD-/-) and wild-type (CAD+/+) mice from the same litter were exposed to sublethal doses of whole body γ-rays. Metaphase chromosomal spreads from bone marrow cells were then prepared and examined for chromosomal aberrations. Aberrations scored included chromatid break, double minute, chromosome terminal deletion, ring chromosome and dicentric chromosome. Most of these were acute, short-term aberrations that would be lost with cellular proliferation. A significant increase of chromosomal aberrations was seen in the CAD(-/-) mice when compared with wild type control cells at both 3($p<0.006$, paired *t*-test) and 7($p<0.04$, paired *t*-test) days after irradiation of the mice(Figure 3.2-6). Quantitatively, the fraction of CAD(-/-) cells with induced aberrations was on the average twice as high as that of the wild type cells. In addition, in non-irradiated CAD(-/-) animals about 1-1.5% metaphase chromosomal spreads prepared from CAD(-/-) bone marrow cells showed chromosome aberrations in the form of chromatid breaks. No chromosomal aberrations were observed in wild type control cells. A tabulated breakdown of aberrations is shown in Table 3.

A.



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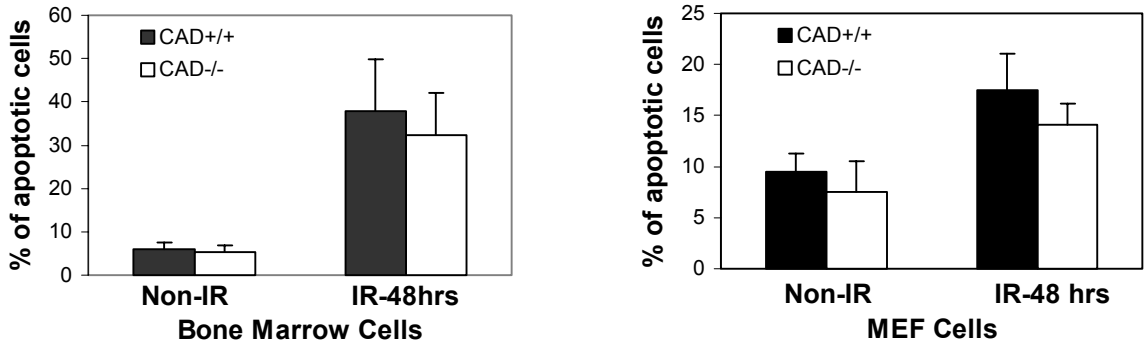


Figure 3.2-5 Radiation-induced apoptosis and DNA fragmentation in mice bone marrow cells and MEFs. (A). IR-induced DNA fragmention: Mice received 4Gy whole body X-ray and bone marrow cells were isolated for quantification of DNA fragmentation by a ELISA kit at 48 hours after irradiation. 3×10^5 MEF cells/sample were used for quantification of DNA fragmentation at 48 hours after 4 Gy X-ray irradiation. **(B)** Apoptosis rate of mice bone marrow cells and MEFs were determined by annexin V-propidium iodide staining 48hours after 4Gy X-ray irradiation.

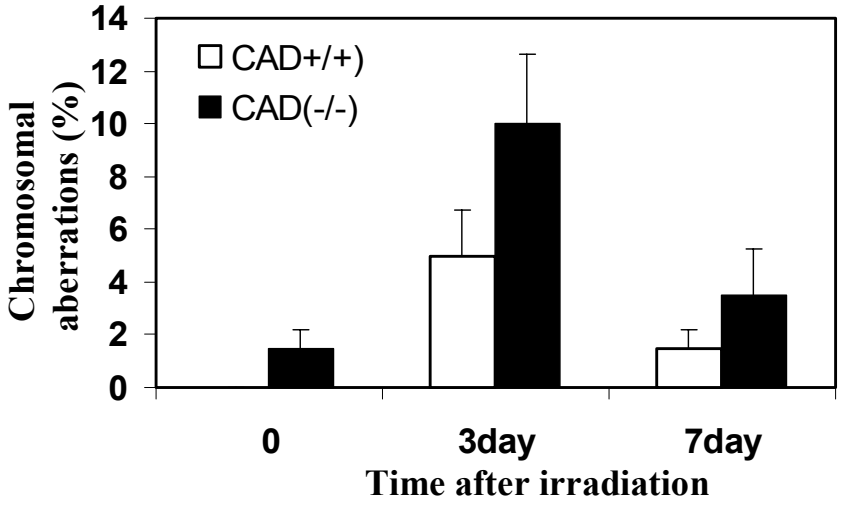


Figure 3.2-6 Elevation of radiation induced chromosomal aberrations in cells with targeted disruption of the CAD gene. Chromosomal aberrations in bone marrow cells derived from irradiated mice. CAD(-/-) and CAD(+/+) mice from the same litter was exposed to sublethal dose of γ-radiation (3 Gy). They were then scored for chromosome aberrations at different time points.

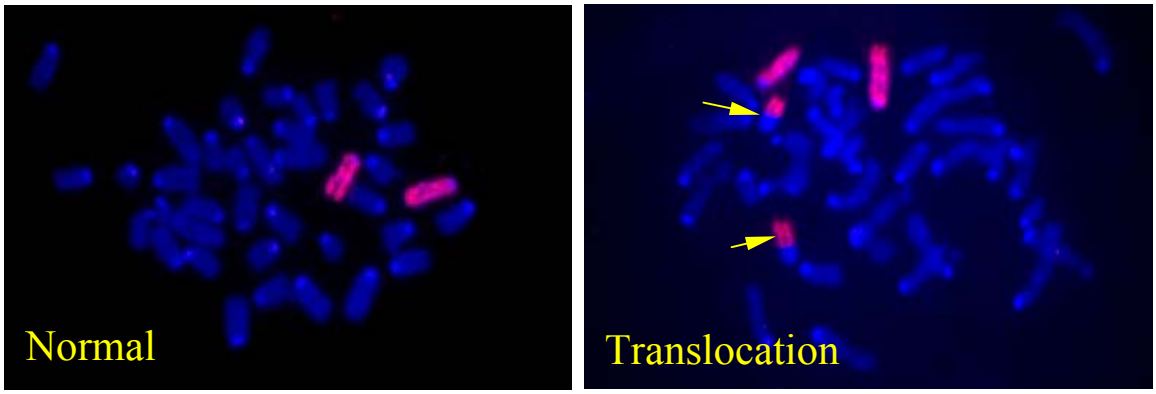
Table 3. Radiation-induced chromosome aberrations in CAD-/- mice

Aberrations \ Mice & treatment	Non-irradiated		3days after 3Gy		7days after 3Gy	
	WT	KO	WT	KO	WT	KO
Chromatid break	0	1.5%	2.65%	3.67%	1.32%	2.98%
Terminal deletion	0	0	1.0%	1.32%	0	0.5%
Double minute	0	0	2.5%	9.0%	0.25%	1.49%
Dicentric chromosome	0	0	0	0.33%	0	0.25%
Aberrant cells	0	1.5%	4.5%	9.5%	1.25%	3.47%

Subsequently, whole chromosome painting was conducted to evaluate the frequencies of chromosomal translocations, which were indicative of more stable chromosomal abnormalities that will be transmitted to progeny cells. The results revealed abnormally high levels of radiation-induced translocations and rearrangements involving chromosome 2 in mouse embryonic fibroblasts (MEF) isolated from CAD (-/-) mice when compared with those from wild type mice (Figure 3.2-7). In three pairs of CAD(-/-) and CAD(+/+) cells examined, the fractions of cells with chromosome 2-associated translocations were consistently higher in CAD(-/-) cells after exposure to ionizing radiation ($p<0.03$, paired *t*-test). These data indicate that disruption of the CAD gene leads to elevated frequency of DNA-damage-induced chromosomal instability *in vivo* as well as *in vitro*.

(5) Mechanisms underlying genetic instability in cells deficient in DNA fragmentation.

What are the mechanisms responsible for the observed increase in genetic instability in cells with disrupted DFF/CAD genes? A series of experiments were conducted to characterize potential anomalies associated with the disruption of the DFF/CAD genes. Cell cycle analysis indicated that DFF/CAD disruption had no effect on cell cycle progression of the non-irradiated or irradiated cells (data not shown). Subsequently clonogenic survival assays were carried out to evaluate long-term survival of cells that have suffered DNA damage. In contrast to the early apoptosis assays (Figure 3.2-2), a small but statistically significant ($p\leq0.02$, *t*-test) increase in clonogenic survival in mICAD-expressing HCT116 (Figure 3.2-8A) and L929 cells (data not shown) was observed. The same difference was also seen in immortalized mouse embryonic



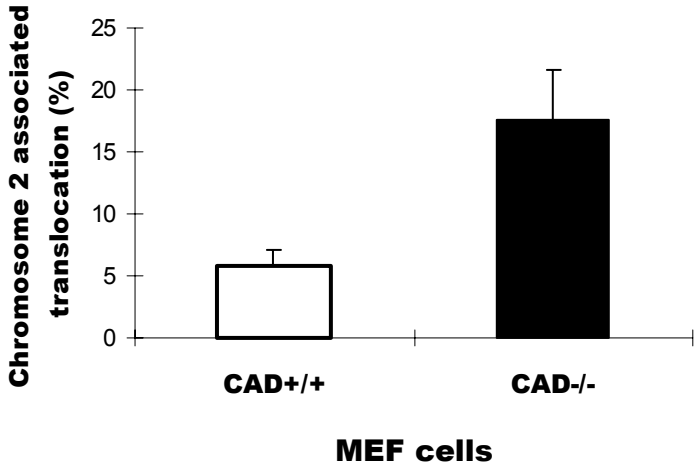


Figure 3.2-7 Elevated frequency of radiation-induced chromosome 2 translocations in embryonic fibroblast cells derived from CAD(-/-)mice. The top two panels shows the fluorescent photomicrographs of typical spreads with normal (left) and translocated (right) chromosome 2. The yellow arrows show the chromosomes that are involved in translocations. The lower panel shows the average of chromosome 2-associated translocation frequency from three independent pairs of CAD(+/+) and CAD(-/-) mouse embryonic fibroblasts.

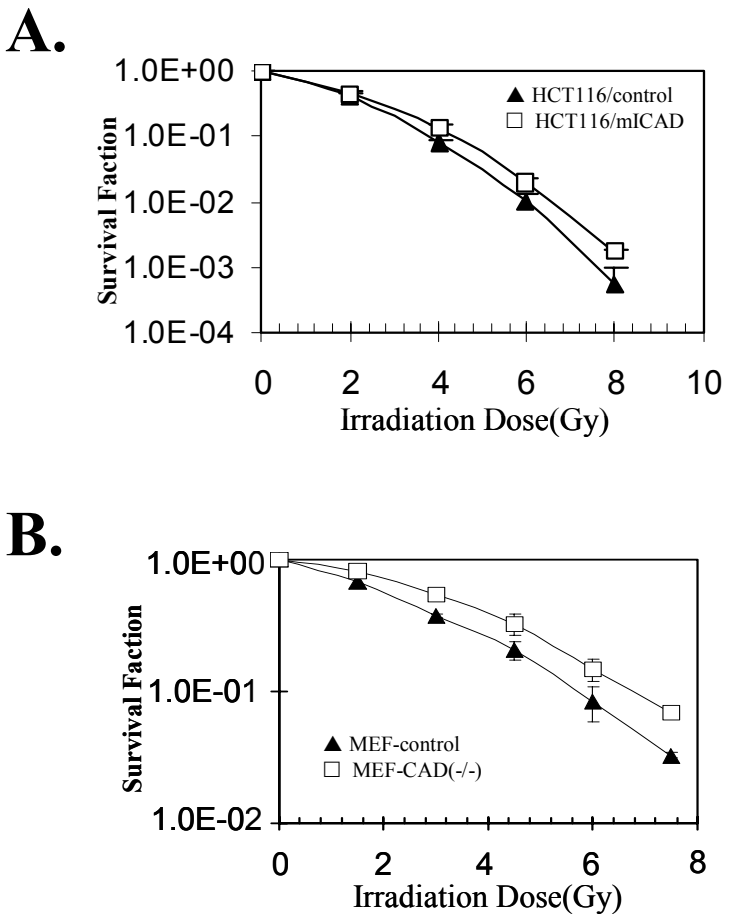


Figure 3.2-8. Increased clonogenic survival in CAD/DFF impaired cells. Clonogenic survival of mICAD-expressing HCT116 **(A)** and CAD(-/-) MEF cells **(B)** after γ-irradiation.

fibroblast (MEF) cells derived from mice with targeted disruption in the CAD gene when they were compared with wild type control cells (Figure 3.2-8B).

The difference in clonogenic survival of the cells after irradiation suggested a potential mechanism for the role of DFF/CAD in maintenance of cellular genomic stability: increased survival of genetically damaged cells that were otherwise destined to die and therefore removed from the cell population. This mechanism was supported by the observations made in HCT116-mICAD cells that were subjected to ionizing radiation exposure. At an earlier time point (6 hrs) post-irradiation, the incidence of radiation-induced chromosomal aberrations were similar between wild type and CAD/DFF-inhibited cells ($p=0.42$, *t*-test Figure 3.2-9). However, at a later time point (96 hrs), the CAD-inhibited cells showed significantly more aberrations than the wild type controls ($p<0.005$, *t*-test) (Figure 3.2-9). A tabulated breakdown of aberrations is shown in Table 3. Similar observations were made in control and mICAD- transduced TK6 and WTK1 cells (Figure 4.2-5). These data suggest that CAD/DFF plays a role that is similar to the archetypical checkpoint gene p53: the removal of cells that have suffered severe DNA damage. However, we can not rule out the possibility that other yet unidentified functions of CAD/DFF may also be involved.

(6) Cellular transformation in CAD-null MEF cells.

The increased susceptibility to radiation-induced genetic instability in CAD-deficient cells was reminiscent of similar characteristics observed in mouse cells with disrupted p53 or gadd45 gene(Lowe, Jacks et al. 1994; Hollander, Sheikh et al. 1999). To further examine if the CAD/DFF40 gene possesses functions that are similar to

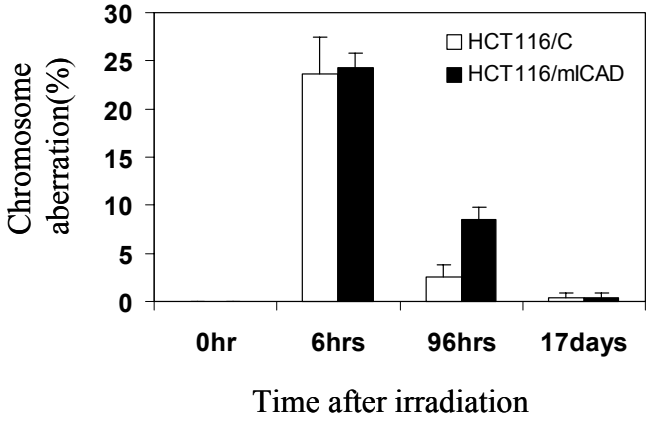


Figure 3.2-9. **Delayed removal of cells with chromosomal aberrations in mICAD-expressing HCT116 cells.** Control and mICAD-transduced HCT116 cells were exposed to ionizing radiation (3 Gy). They are then examined for chromosomal aberrations at different time points. For each time point, at least 100 metaphases were counted. The error bars represent standard deviation.

Table 4. Radiation-induced chromosome aberrations in HCT116 cells

Treatment of HCT116 / Aberrations	Non-irradiated		6 hrs after 3Gy		4 days after 3Gy	
	Ctrl	mICAD	Ctrl	mICAD	Ctrl	mICAD
Chromatid break	0	0	98%	84%	1.33%	4.67%
Ring chromosome	0	0	2%	2%	0.67%	1.33%
Double minute	0	0	4%	6%	12%	19.3%
Dicentric chromosome	0	0	0	0	0	0.67%
Aberrant cells	0	0	56%	46%	6.67%	16.7%

checkpoint genes such as p53 or gadd45, the soft agar assay, which is a very stringent *in vitro* assay for malignant cellular transformation, was carried out to examine the ability of MEF cells derived from CAD(-/-) and wild type mice (littermates of the CAD-/- mice) to undergo anchorage-independent growth. The observed frequency for the growth of soft agar colonies in CAD(-/-) MEF cells was much higher than those observed in CAD(+/+) MEF cells($p<0.01$, Figure 3.2-10). When these soft agar colonies were expanded and inoculated subcutaneously into athymic nude mice, 100%(20 out of 20) of the colonies formed tumors in 3-12 weeks time. These results indicated that loss of the CAD gene increased the susceptibility of host MEF cells to oncogenic transformation.

(7) Increased susceptibility to chemical carcinogenesis in CAD-null mice.

To further examine the potential roles of CAD/DFF in carcinogenesis, a skin carcinogenesis model was adopted to examine the susceptibility of CAD(-/-) mice to chemically induced skin tumors. Wild type control (CAD(+/+)) and CAD(-/-) mice, which were littermates in C57BL/6 background (see Material and Methods section for a description of the breeding program), were subjected to the chemical mutagen DMBA (7,12-dimethylbenz (a) anthracene) and tumor promoter TPA(12-O-tetradecanoylphorbol-13-acetate) treatment according to a standard protocol that induces skin tumors in treated mice. Significant difference in tumor latency, incidence and number of tumors per mouse was observed between the two groups of mice(Figure 3.2-11). At 20 weeks post-treatment, 39% of the wild-type mice remained tumor-free compared with only 8% CAD(-/-) mice($p<0.002$)(Figure 3.2-11A). Furthermore, the average number of tumors per mouse in the CAD(-/-) group was 4.3 compared with 1.0

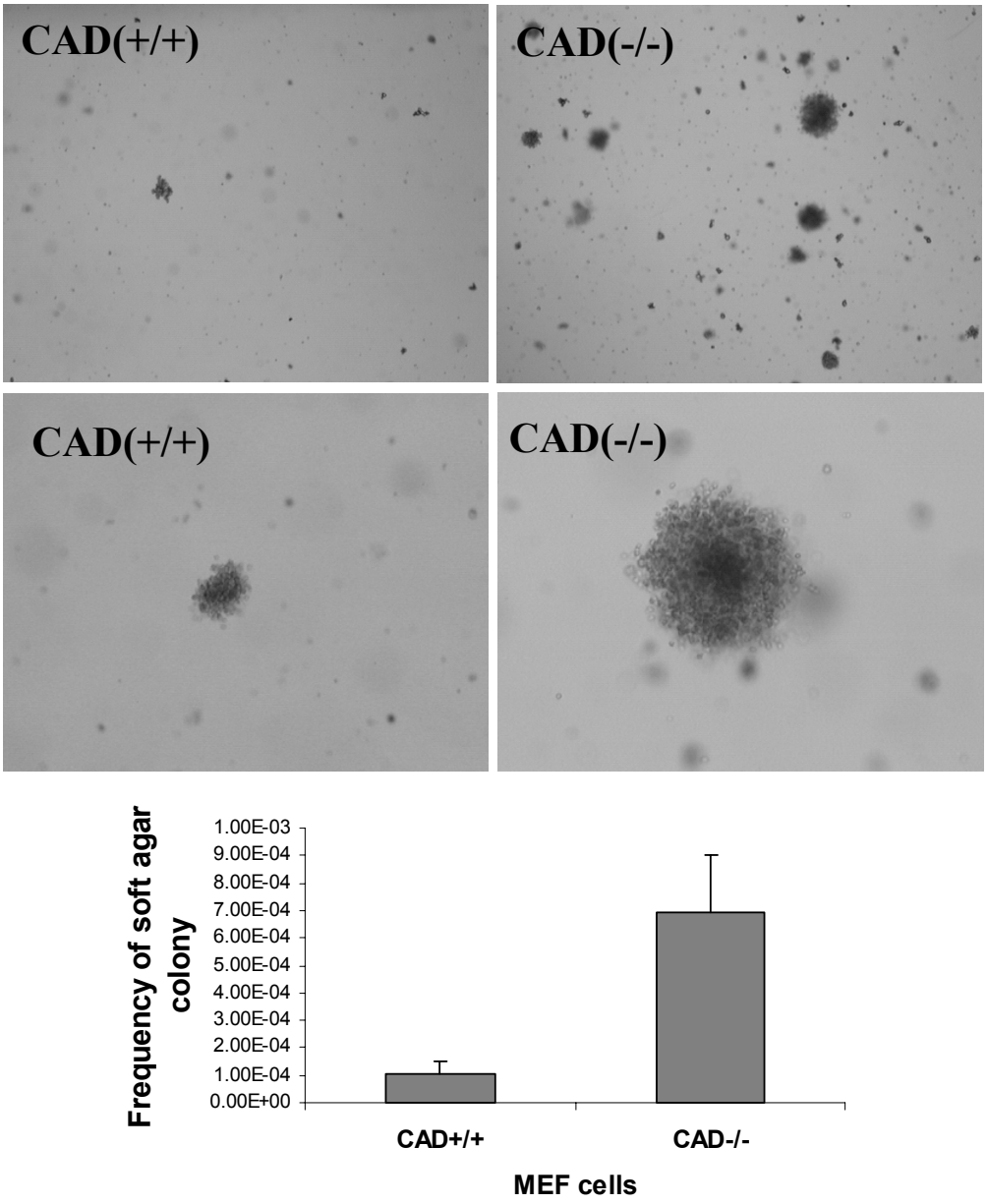


Figure 3.2-10. **Elevated frequency of cellular transformation in embryonic fibroblast cells derived from CAD(-/-) mice.** Shown in top panels for photomicrographs of typical colony growth in CAD(+/+) and CAD(-/-) plates. The black spots in the photos are the transformed colonies. The lower panel shows the average frequency of soft agar colonies from three independent pairs of embryonic fibroblasts isolated from CAD(+/+) and CAD(-/-) littermates.

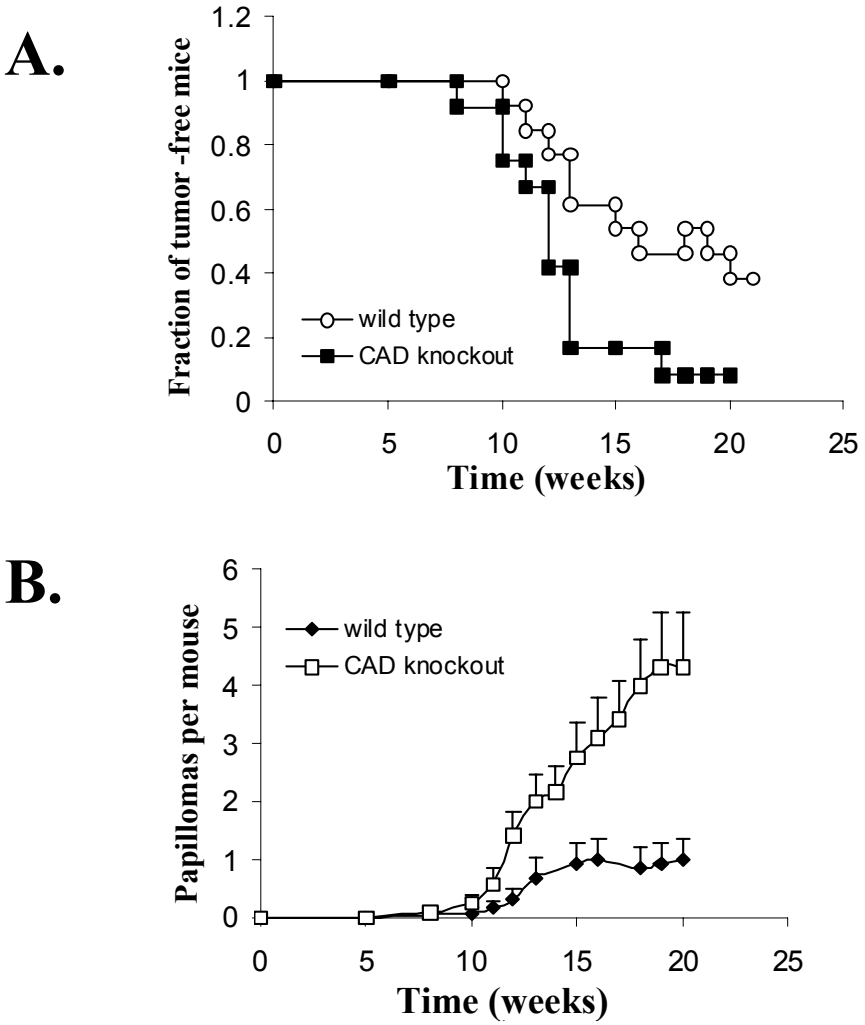
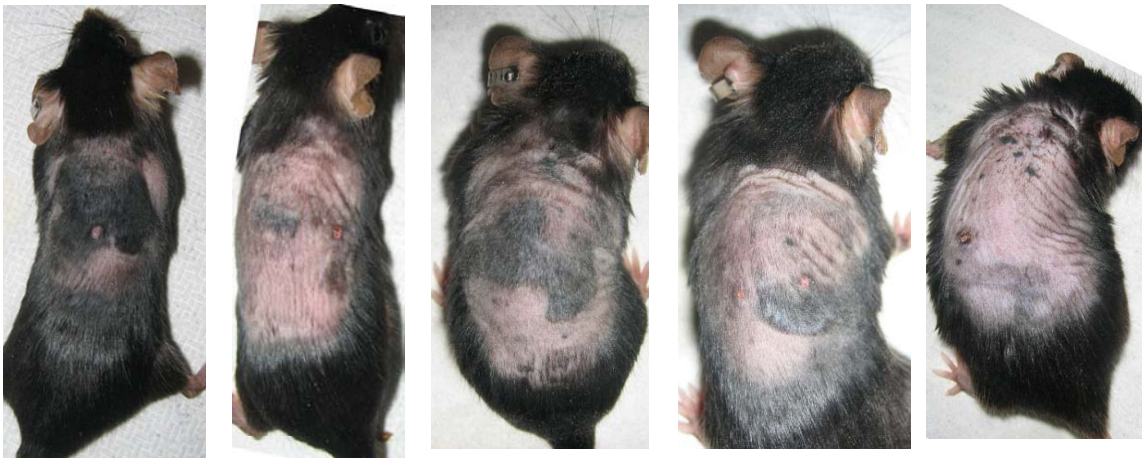


Figure 3.2-11. Increased susceptibility to chemical carcinogenesis in CAD(-/-) mice. Control CAD(+/+) and CAD(-/-) mice were treated with DMBA (at 100 μg /mouse once to initiate tumorigenesis) and TPA (2.5 μg/mouse twice weekly for 20 weeks). The mice were closely followed for skin tumor formation. **(A).** Upper panel shows the fraction of mice that remain tumor-free at different time points. **(B).** Lower panel shows the average number of tumors per mouse at different time points. In either case, the difference between the two groups is significant ($p<0.002$ and 0.001 respectively). Each group had 24 mice. The error bars represent standard error of the mean.



Top panels : CAD(+/+) mice. Lower panels: CAD(-/-) mice

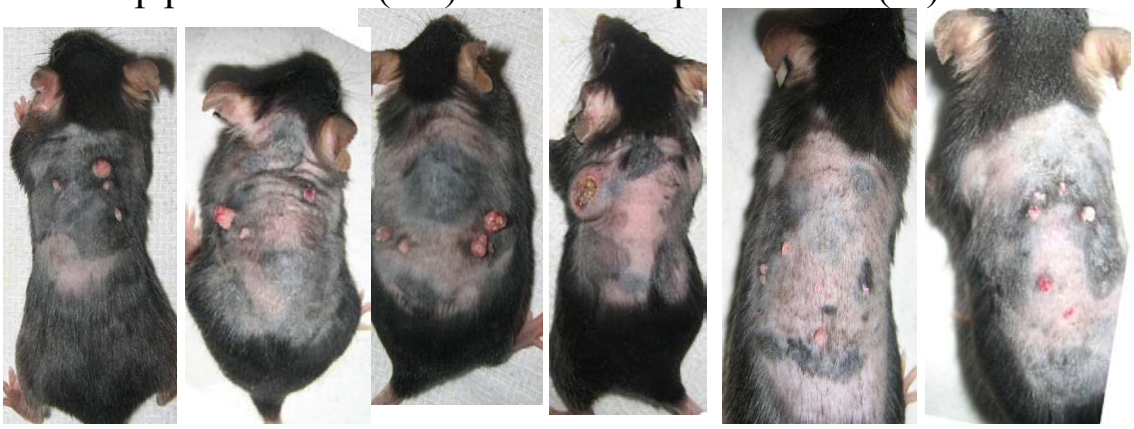


Figure 3.2-12. DMBA and TPA induced skin papillomas in CAD(-/-) and CAD(+/+) mice. The mice were treated with DMBA(100 ug/per mouse) once and TPA (2.5 ug) twice weekly for 20 weeks. Skin papilloma formation was followed closely. Shown were pictures taken for representative mice in each group (24 mice per group were used to carry out the experiments.

in the CAD(+/+) group (Figure 3.2-11B). Therefore, CAD(-/-) mice was significantly more susceptible to DMBA-TPA-induced skin tumors than the wild-type mice ($p<0.001$, SAS survival analysis).

In addition to increased number of tumors per mouse, it was also observed that papillomas in the CAD(-/-) mice grew significantly faster and reached bigger sizes than those of the wild type mice (Figure 3.2-12). Papillomas in the wild type mice were smaller and slower growing. Some of them showed spontaneous regression. Interestingly, two of the CAD(-/-) mice developed malignant ulcerative squamous carcinomas compared to none in wild type mice. These observations suggest a more aggressive progression of skin tumors in CAD knockout mice. Taken together, our observations indicate a significant role for the DFF/CAD genes in the suppression of chemically induced skin carcinogenesis.

(8) Increased radiation carcinogenesis in CAD knockout mice.

To investigate radiation-induced carcinogenesis in *cad-/-* mice, 80 littermates from heterozygote crosses were irradiated with a sublethal schedule as described in "Materials and Methods". One year after treatment, there was a 2.4 fold increase in frequency of *cad–/–* mice succumbing to tumours compared with wild type (Figure 3.2-13). Tumour incidence for *cad*+/– mice was intermediate between that of wild type and null mice. 21% of wild-type mice developed tumors, compared with 40% and 50%, respectively, of *cad*+/– and *cad–/–* mice. Histologic examination demonstrated that the majority of tumors were thymus lymphomas. A few mice also developed leukemias. Necropsy often revealed large yellow whitish thymic masses, enlarged lymph nodes and

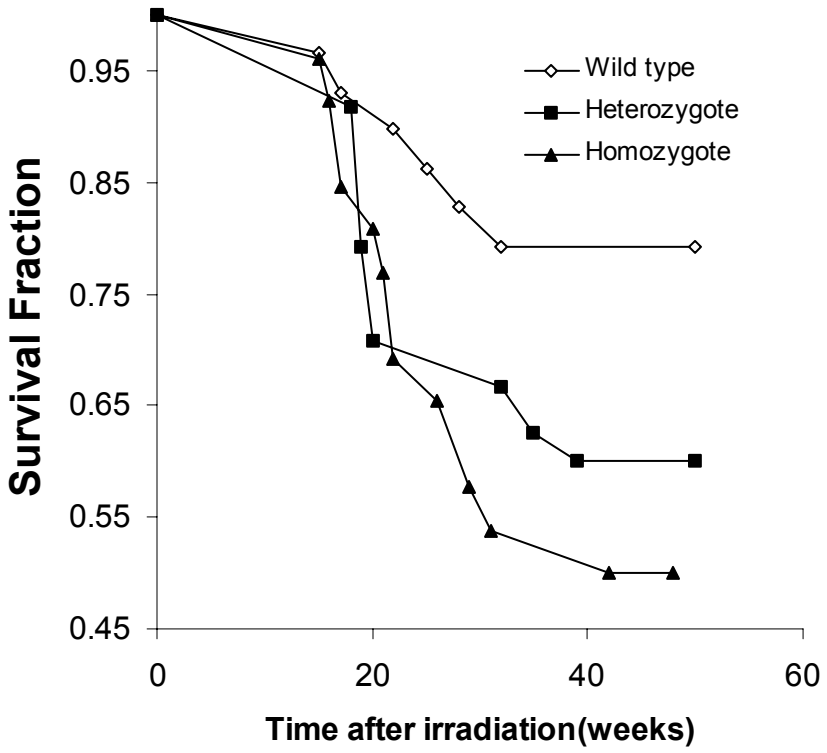


Figure 3.2-13. Enhanced susceptibility to radiation carcinogenesis in CAD(-/-) mice. 29 wild type, 24 heterozygous and 26 homozygous littermates received a total of 6 Gy X-ray whole body irradiation. The mice were then observed for one year for tumor development. The tumor-free survivals of these mice are shown. The difference between CAD+/+ and CAD-/- group is significant (P=0.048, SAS survival analysis)

hepatosplenomegaly. Spontaneous tumor formation was not observed in non-irradiated mice at age of 12-18 months. Our results are comparable to those for IR carcinogenesis in Gadd45a-/- mice(Hollander, Sheikh et al. 1999).

3.3 Discussion

Apoptosis plays an important role in development and homeostasis. Alterations in apoptosis contribute to the pathogenesis of a number of human diseases, including cancer, viral infections, autoimmune diseases, neurodegenerative disorders, and AIDS (acquired immunodeficiency syndrome). However, the biological function for apoptotic DNA fragmentation, one of the most important hallmarks of the apoptotic process, has not been defined clearly. Our results indicate that apoptotic DNA fragmentation is an important step to maintain genetic stability and suppress cellular transformation/carcinogen-induced tumorigenesis. They suggest the novel role of apoptotic DNA fragmentation as a checkpoint for genomic stability and involvement in tumor suppression.

Clinical relevance of DFF/CAD-deficiency to human cancers

Our results are consistent with previous studies on the potential roles of the DFF/CAD genes in human cancers. In human neuroblastomas, a high percentage (approximately 28-47%) contains deletions of chromosome 1p(Fong, Dracopoli et al. 1989; Ohtsu, Hiyama et al. 1997; Iolascon, Lo Cunsolo et al. 1998; Hiyama, Hiyama et al. 2001). Analyses of such chromosome deletions suggest that there may be tumor suppressor gene(s) in the region 1p36.1-1p36.3, which contains both DFF40/CAD and

DFF45/ICAD (Ohtsu, Hiyama et al. 1997; Komuro, Valentine et al. 1998; Caron, Spieker et al. 2001; Hiyama, Hiyama et al. 2001; White, Thompson et al. 2001). In agreement with this are two reports showing homozygous deletions of DFF45 in neuroblastoma cell lines(Ohira, Kageyama et al. 2000; Yang, Chen et al. 2001). Deletions of the DFF45 gene have been shown to disrupt the function of the DFF/CAD complex completely(Zhang, Liu et al. 1998). In addition to these studies, tumor-specific mutations from human patients provide additional evidence for potential role of the loss of DFF/CAD functions in human carcinogenesis(Abel, Sjoberg et al. 2004). RT-PCR gene expression study showed that DFF45 is preferably expressed in low-stage neuroblastoma tumors and to a lesser degree in high-stage neuroblastomas (Abel F 2002). Decreased expression of DFF45/ICAD is correlated with poor prognosis in patients with esophageal carcinoma(Konishi, Ishiguro et al. 2002). Taken together, the evidence for the involvement of DFF/CAD involvement in human cancer development is indeed strong. The chemical and radiation carcinogenesis data provided in this study (Figure 3.2-11, 3.2-12 and 3.2-13) suggested a role of CAD/DFF in tumor suppression, albeit in a murine model.

Discrepancy between apoptosis assay and clonogenic survival assay

How does one reconcile the fact that disruption of apoptotic DNA fragmentation has minimal effect on apoptosis (Figure 3.2-2) but significant influence on clonogenic survival(Figure 3.2-8)? The discrepancies between the two may reflect different endpoints these assays were measuring. Apoptosis assays measure the fraction of cells undergoing apoptosis at the time of observation while clonogenic assays take into the

account of the cumulative effects of cell death over the entire period of experimentation (2-3 weeks), which is more likely to detect smaller survival differences between two cell populations.

The increase in clonogenic survival after radiation is surprising given the fact that DNA fragmentation is almost at the end stage of the "execution" phase of the whole apoptotic process. One would predict that alterations at this stage should cause no change in the overall survival of the cells. There may be two potential explanations of our observation to the contrary. The first is that each step in the multi-step apoptotic process, including those in the execution phase, may influence eventual cell death. This is evidenced by two published reports that indicated that processes at the end stage of apoptotic death, such as the engulfment (phagocytosis) of apoptotic bodies could affect the overall death rate (Hoeppner, Hengartner et al. 2001; Reddien, Cameron et al. 2001). Therefore, it is possible that disruption of DNA fragmentation, which occurs prior to phagocytosis, may also affect the whole apoptotic process. A second possibility is that apoptotic fragmentation may be part of a feedback loop that can affect the outcome of eventual cell death. This has been suggested by a published report (Boulares, Zoltoski et al. 2001), which indicated that disruption of the apoptotic DNA fragmentation gene makes murine fibroblast cells resistant to the apoptosis-inducing effects of TNF-α, thereby supporting our conclusion that CAD gene disruption can lead to increased clonogenic survival.

Mechanism underlying genetic instability in DFF/CAD-impaired cells

A key question about the role of DFF/CAD in carcinogenesis is the molecular mechanism through which it regulates genetic stability. From the evidence we obtained in various cell lines constructed in this study, we conclude that DFF/CAD maintains genetic stability in a manner that is very similar to another tumor suppressor gene, p53, by removing cells with severely damaged DNA. Evidence for this comes from the fact that CAD(-/-) cells and mICAD transduced cells showed a small but clear increase in clonogenic survival (Figure 3.2-8) and that mICAD transduced cells showed an attenuated rate of removing cells with severe chromosome aberrations (Figure 3.2-9).

DNA fragmentation is at the late stage of apoptosis after caspase activation. It is generally understood that once caspase activation is initiated, survival of the cell would be unlikely whether or not DNA fragmentation is initiated. In order to address this issue, we provided further evidence to show that DNA fragmentation is essential to cell death during apoptosis. We irradiated cells with or without CAD deficiencies to initiate apoptosis. Then, we used flow cytometry to sort out those cells that show signs of early apopotosis, i.e., those cells that show positive staining for annexin V but negative for propidium iodide. These cells were then plated and observed for potential recovery. As shown in Figure 3.3-1**,** CAD deficiency allowed significantly more cells to survive and form colonies on plates in both HCT116-mICAD cells and in MEF(CAD-/-) cells.

How does a relatively modest difference in clonogenic survival cause a significant increase in mutation frequency dramatically? This is possible because the small percentage of cells that are kept alive (which should die normally) may have suffered

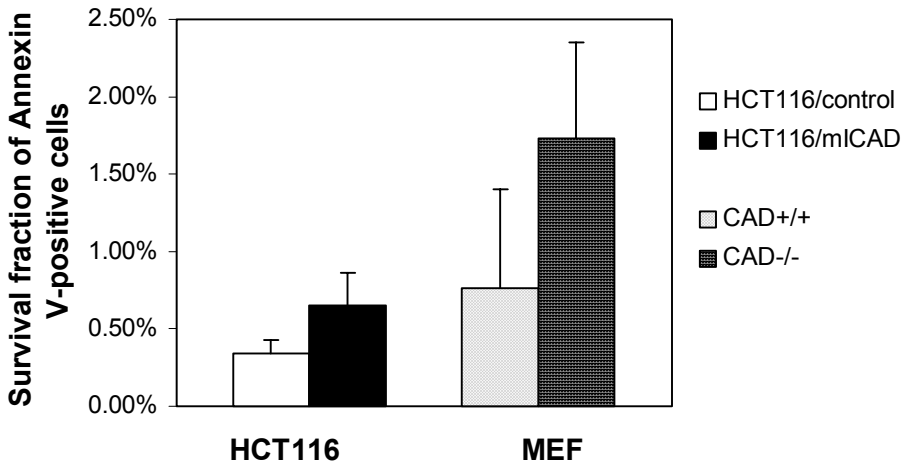


Figure 3.3-1. Clonogenic survival of Annexin V-positive cells. Apoptosis was induced by radiation in cells. Early apoptotic cells which bind to FITC-conjugated Annexin V were sorted out by flow cytometry. Certain number of annexin V-binding cells were seeded into plates and allowed to recover and grow for 2 weeks. The survival fraction was corrected by plating efficiency of each cell line. P=0.0459 and 0.0286 for HCT116 and MEF cells respectively by t test.

quite severe DNA damage that should otherwise be destined to die. Therefore the mutation rate among these cells may be much higher than the normal cell population, which may influence the overall mutation rate significantly. As an example, if we assume the following scenario where the mutation frequency of normal cells is 10^{-6}, the increased survival is 1%, and we further assume that among this 1% of additional survivor cells, 1 out of 1000 cells has mutations, which is quite possible(Chang and Little 1992), then the increased frequency of mutation in the population will be $1\% \times 10^{-3} = 10^{-5}$, 10 times of the normal frequency (10^{-6}). So a small increase in survival of DNA-damaged cells can have a big impact on the mutation rate.

Spontaneous and induced tumorigenesis

In our studies, spontaneous tumor development has not been observed in CAD(-/-)mice, in contrast to p53-deficient mice. However, CAD-null mice showed significantly increased susceptibility to tumors when exposed to genotoxic stress. In this respect, the CAD or ICAD genes are similar to quite a few other tumor suppressor genes such as gadd45, $p16^{INK4a}$, chk2 and ku80. Mice deficient in these genes do not develop tumors spontaneously. However, they do develop tumors at an elevated frequency when mice were exposed to carcinogens or when they are juxtaposed together with mutations in other tumor suppressor genes(Hollander, Sheikh et al. 1999; Difilippantonio, Zhu et al. 2000; Krimpenfort, Quon et al. 2001; Hirao, Cheung et al. 2002) .

In summary, the results presented here clearly established the roles of apoptotic DNA fragmentation in regulating mammalian genomic instability and cancer

development. To our knowledge, this is the first demonstration of a direct functional role for DFF/CAD in maintaining genetic stability and prevention of tumorigenesis.

Chapter 4

Apoptotic DNA fragmentation factor maintains chromosome stability in a P53-independent manner

4.1 Introduction

DNA fragmentation factor (DFF) or caspase-activated deoxyribonuclease(CAD) is a heterodimeric protein complex that functions downstream of Caspases to execucte DNA fragmentation during apoptosis. The smaller subunit DFF40/CAD is a DNase that is normally complexed with its inhibitor and chaperon, DFF45/ICAD. During apoptosis, activated caspases cleave DFF45/ICAD, allowing DFF40/CAD to enter the nucleus and degrade chromosomal DNA(Liu, Zou et al. 1997; Enari, Sakahira et al. 1998). Defects in either DFF40/CAD or DFF45/ICAD lead to deficiency in DNA fragmentation during apoptosis.

Deficiency of DFF40/DFF45 genes was revealed in a number of neoplasms (Maris, White et al. 1995; Caron H 1996; Schwab, Praml et al. 1996; Van Gele M 1998; Van Gele M 1998; Judson H 2000). Decreased DFF45 expression is shown to associate with higher-stage neuroblastoma(Abel F 2002) and a poorer prognosis in some cancer patients (Konishi, Ishiguro et al. 2002). DFF40/45 is also candidate tumor suppressor for neuroblastomas. Deletion or tumor-specific mutations of DFF45 gene were found in neuroblastoma and other tumor specimen and neuroblastoma cell lines(Ohira, Kageyama et al. 2000; Abel, Sjoberg et al. 2004). However, the molecular mechanism underlying the association between DFF deficiency and human malignancies has not been clarified.

Chromosome instability (CIN), involving gains and losses of whole or sections of chromosomes, occurs frequently in most human malignancies. The molecular basis of chromosome instability is not well understood. Functional alterations in many genes have been implicated in the generation chromosome instability. Some of these genes are directly involved in maintaining chromosome structures. Examples of these include genes

involved in chromosome condensation, sister-chromatid cohesion, kinetochore structure and function and centrosome/microtubule formation and dynamics(Hoyt, Stearns et al. 1990; Spencer, Gerring et al. 1990). An example is tubulin-encoding TUB genes. TUB gene mutants displayed increased chromosome instability(Hoyt, Stearns et al. 1990). Other genes are indirectly involved. An example is checkpoint genes that monitor the proper progression through the cell cycle(Murray 1995; Elledge 1996; Nasmyth 1996; Paulovich, Toczyski et al. 1997). One of the most important checkpoint genes is the DNA-damage checkpoint gene P53. P53 mutations are seen in over 50% of all human tumors and P53 deficiency is an important contributing factor for CIN.

In this study, we show that apoptotic DNA fragmentation is an important step to maintain chromosome stability in mammalian cells and suppress tumor development. It achieves this through elimination of cells that have already suffered extensive damage. In addition, it appears to function independently of p53.

4.2 Results and Discussion

(1). Effective inhibition of apoptotic DNA fragmentation in cells expressing mutant ICAD

DFF45/ICAD is a chaperon as well as an inhibitor of DFF40/CAD. ICAD is normally complexed with CAD and inhibits its DNase activity. When apoptosis is induced, ICAD is cleaved by caspase and CAD is released to carry out DNA fragmentation in the nucleus. There are two caspase cleavage sites in ICAD, D117 and D224. We engineered a mutant ICAD according to a published method by introducing point mutations at the two cleavage sites, D117E and D224E. Mutant ICAD (mICAD) is

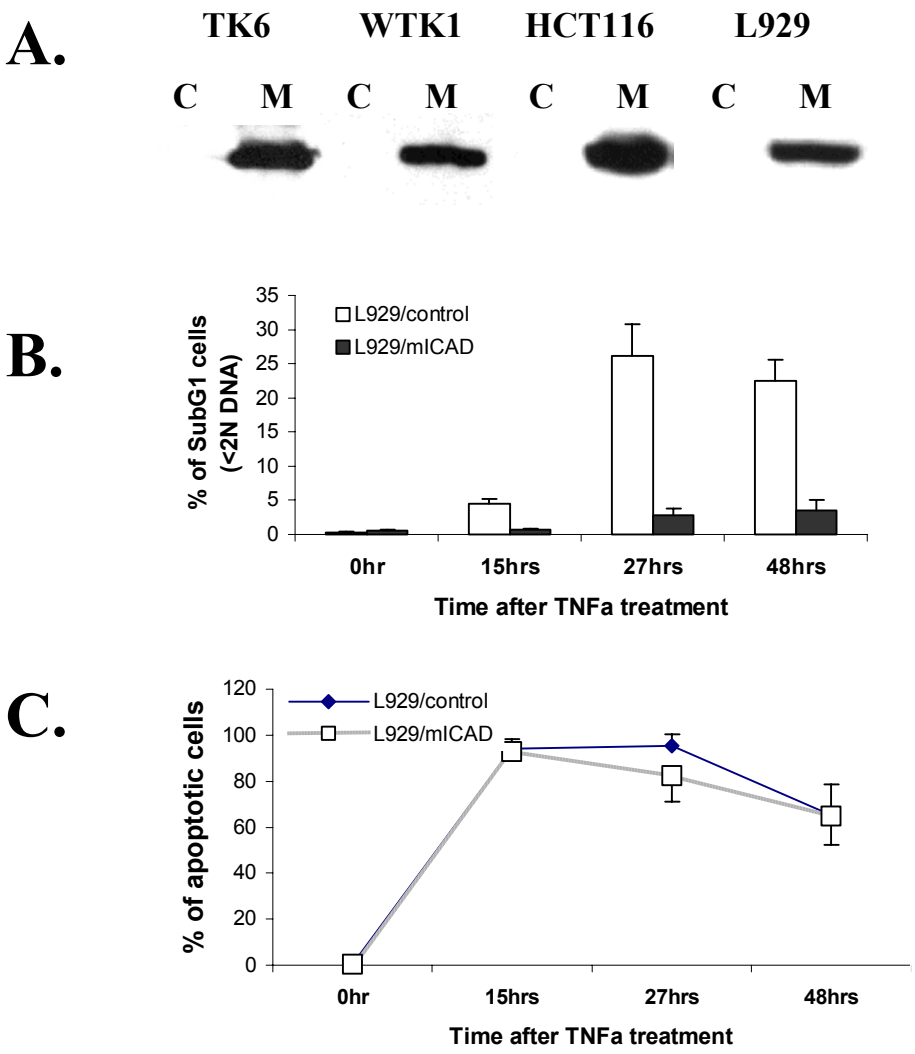


Figure 4.2-1 Inhibited DNA fragmentation in mutant ICAD-expressing cells. (A). Expression of mutant ICAD protein was shown by western blotting in fours cell lines. C: control cells; M: mutant ICAD cells. **(B).** DNA fragmentation was determined by fraction of cells with <2N DNA content by cell cycle analysis in L929 cells treated with TNFα. **(C).** Apoptosis rate was determined by annexin V-propidium iodide staining at different point after TNFα treatment.

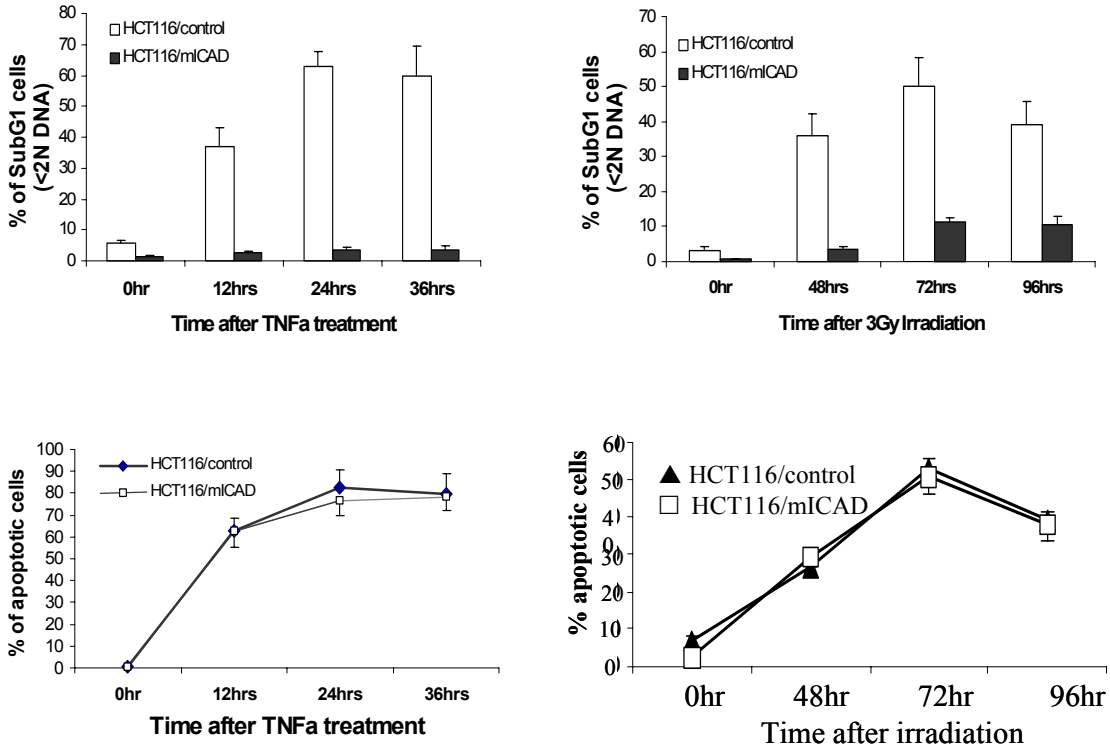


Figure 4.2-2 Inhibited DNA fragmentation in mutant ICAD expressing HCT116 cells. Apoptosis was induced by TNFα(left panels) or radiation(right panels) and DNA fragmentation was reflected by fraction of SubG1 cells by cell cycle analysis(upper panels). Apoptosis rate was measured by annexin V-propidium iodide staining(lower panels).

resistant to caspase cleavage and inhibits DNA fragmentation during apoptosis in both human and mouse cells(Sakahira, Enari et al. 1998). We established four cell lines expressing mICAD, HCT116, TK6, WTK1 and L929, as shown in Figure 4.2-1A.

DNA fragmentation was analyzed by DNA histogram in these stable cells. Small DNA fragments produced by CAD digestion of the cellular chromosomes can leak out of the ethanol-fixed cells. Therefore cells that undergo DNA fragmentation have <2N DNA content. As shown in Figure 4.2-1B and Figure 4.2-2 (upper panels), DNA fragmentation is significantly inhibited in mICAD transduced HCT116 and L929 cells when apoptosis is induced by γ-irradiation or TNFα. Reduced DNA fragmentation in mICAD cells is not due to decreased apoptosis because the control and mICAD-expressing cells underwent apoptosis at about the same rate (Figure 4.2-1B and Figure 4.2-2 lower panels). These data are consistent with a previous report(Sakahira, Enari et al. 1998).

(2). Inhibition of DNA fragmentation by mICAD led to increased chromosome aberrations in a P53-independent manner

Genetic instability is a hallmark of human cancers. It is the driving force for tumor development. In order to understand pontential roles of DFF deficiency in cancer development, we examined the effect of mICAD expression on chromosome instability. Radiation induces chromosome aberrations such as chromatid break, double minute, ring chromosome, dicentric chromosome and polyploidy. We examined metaphase spreads of cells exposed to γ-irradiation for these aberrations. MICAD-expressing cells had a markedly higher level of chromosomal aberrations before and after radiation($p<0.05$, t test) (Figure 4.2-3). TK6 and WTK1 are lymphoblastoid cell lines derived from the same

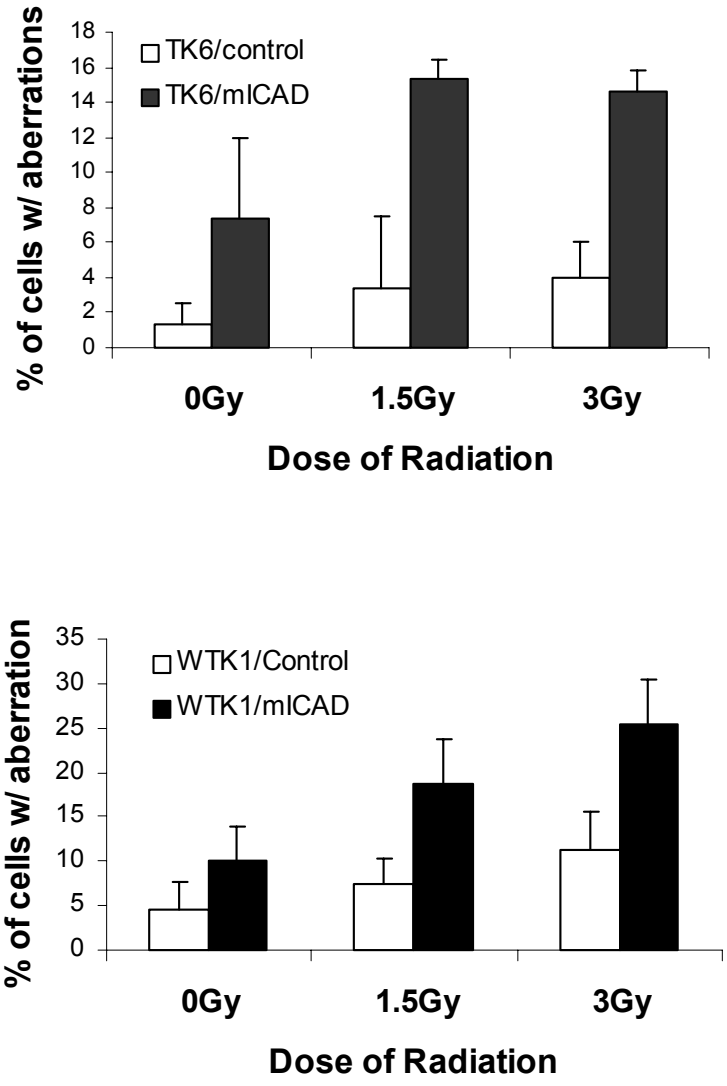


Figure 4.2-3 Increased radiation-induced chromosome aberrations in mutant ICAD cells. Cells were irradiated with different doses of γ-irradiation. 48 hours after irradiation, metaphase spreads were prepared for examination of chromosome aberrations. Cells with chromosome aberrations were scored in TK6 (upper panel) and WTK1 cells (lower panel).

individual with differing p53 status(Little, Nagasawa et al. 1995). The fact that higher levels of chromosome aberration in mICAD-expressing cells were observed in both p53-normal TK6 cells and p53-deficient WTK1 cells indicated that the effect of mICAD is independent of P53, a master regulator of genomic stability.

(3). Increased heavy ion radiation-induced micronucleus in CAD-/- cells

Micronucleus (MN) assay is a preferred method for assessing chromosome damage because they enable both chromosome loss and chromosome breakage to be measure reliably. High energy heavy ion (HZE) radiation is an important component of radiation environment in the space, which is a health hazard for astronauts. Like ionizing radiation, HZE can cause DNA damages. We showed that HZE radiation led to significantly more chromosome damages in CAD-deficient cells. As shown in Figure 4.2-4, mouse embryo fibroblasts derived from CAD-null mice had much more MNs than the wild type cells after HZE radiation ($p<0.02$).

(4). Increased aneuploidy in cells with impaired DNA fragmentation

The majority of cancer cells show an aneuploid karyotype. Aneuploidy is an important manifestation of chromosome instability in cancers. In order to study the effect of DNA fragmentation on aneuploidy, we analyzed the chromosomal ploidy of TK6 and WTK1 cells stably transfected with mICAD. Parental TK6 and WTK1 show basic diploid cell karyotypes(Skopek, Liber et al. 1978; Liber and Thilly 1982; Yandell and Little 1986). Consistent with previous reports, FACS analysis showed that by far the majority (98%) of TK6/control cells were diploid. TK6/mICAD cells had significantly increased

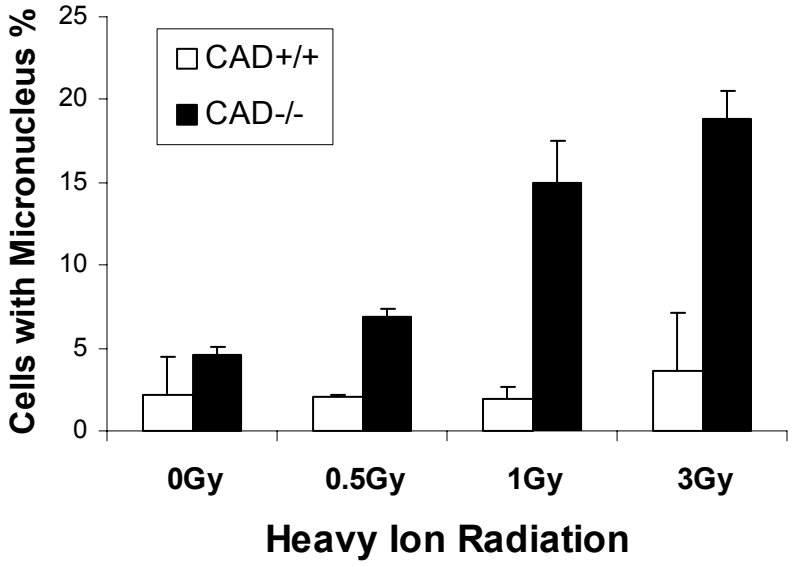


Figure 4.2-4. HZE radiation-induced micronucleus in CAD-null MEF cells. Wild type and CAD-/- MEF cells were irradiated with different doses of heavy ion beams. 12 hours after radiation, cytochalasin B were added to block cytokinesis for 48 hours. Then micronucleus was scored in at least 500 dividing cells (doublet cells) for each sample.

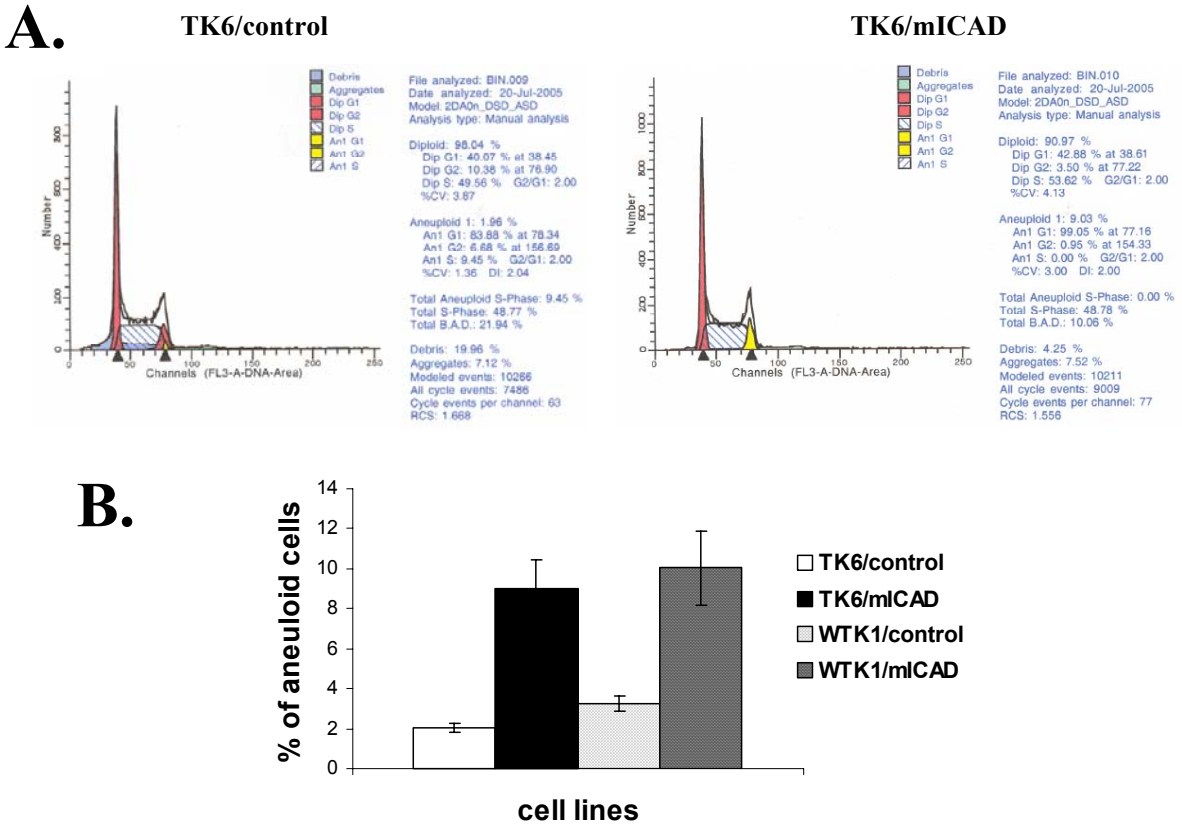


Figure 4.2-5. FACS analysis of aneuploidy in TK6 and WTK1 cells expressing mutant ICAD. (A) Representative FACS profiles from control (left panel) and mutant ICAD-expressing TK6 cells (right panel), showing the increase in the proportion of aneuploid cells. **(B)** Diagram showing the percentage of aneuploid cells in control and mutant ICAD-expressing TK6 and WTK1 cells.

aneuploid cells(9%, $p<0.01$, t test). Similar finding was made in WTK1 cells. Cells that overexpress mICAD had significantly more aneuploid cells than the control ($p<0.03$, t test) (Figure 4.2-5). It indicates that inhibition of apoptotic DNA fragmentation leads to chromosome number instability regardless of P53 gene status.

(5). Inhibition of DNA fragmentation during apoptosis delayed removal of cells with DNA damages induced by radiation

In order to understand the mechanism underlying increased chromosome instability in DNA fragmentation-inhibited cells, we examined the time course of chromosome aberrations in irradiated cells at different time points after radiation. Immediately after radiation, a similarly high level of aberrations was induced in both control and mICAD cells. It suggests that mICAD did not protect the chromosomes from being damaged by radiation. At 40 and 87 hours after radiation, the majority of aberrant cells were eliminated from the control population, however; significantly more aberrant cells remained in the mICAD population (Figure 4.2-6). These data indicate that mICAD expression interfered with the removal of the cells that have suffered chromosome damage through apoptosis. This effect is very similar to that of P53. As shown in Figure 3B, there were much more aberrant cells in WTK1 cells(p53-/-) than in TK6 cells(p53+/+) at 40 and 87 hours after radiation. In WTK1 cells, p53 and DFF-deficiencies seemed to have additive effect to delay the removal of DNA-damaged cells. The removal of aberrant cells was further delayed by p53 deficiency in mICAD-expressing WTK1 cells. The amounts of aberrant cells in control and mICAD-expressing

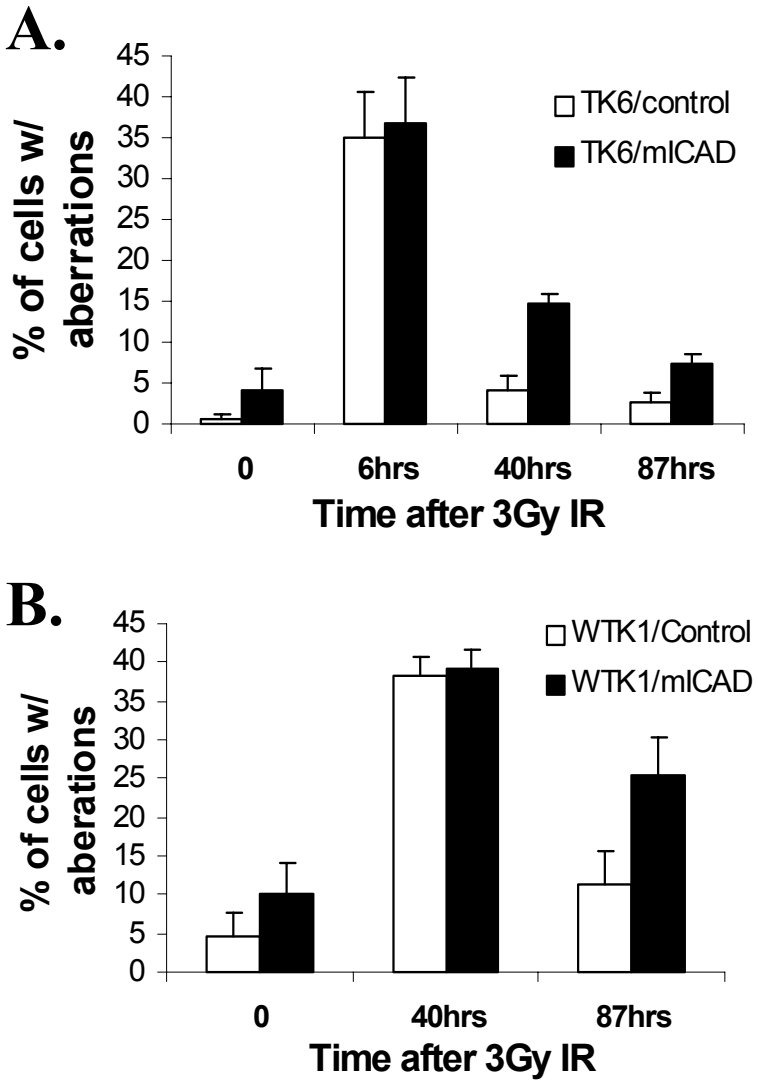


Figure 4.2-6. Delayed removal of cells with chromosomal aberrations in mICAD-expressing cells. Control and mICAD-transduced TK6 **(A)** and WTK1 **(B)** cells were exposed to ionizing radiation (3 Gy). They are then examined for chromosomal aberrations at different time points. For each time point, at least 100 metaphases were counted. The error bars represent standard deviation.

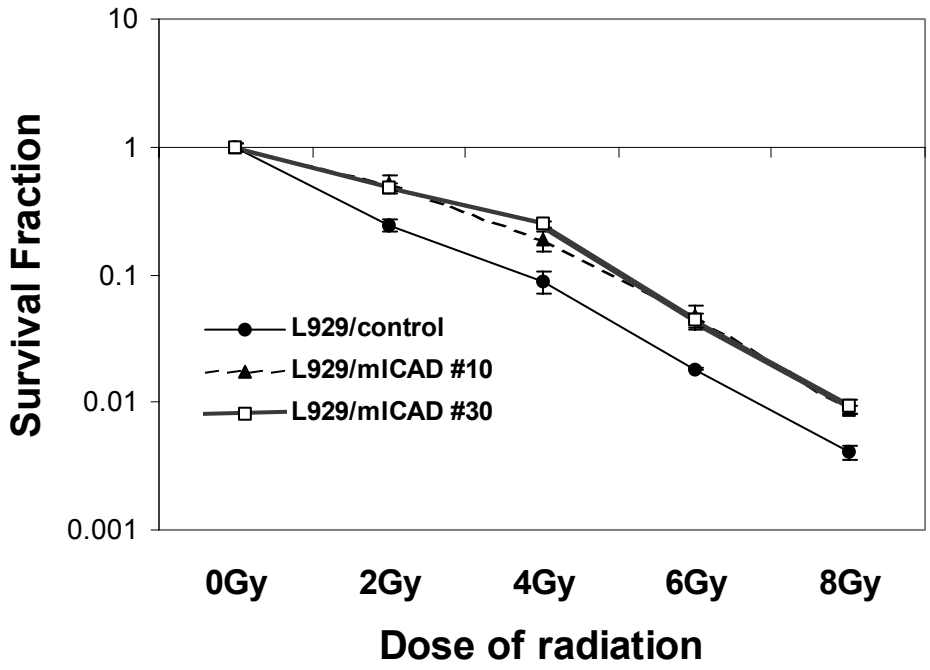


Figure 4.2-7. Clonogenic survival after radiation in mutant ICAD expressing cells. Control and two clones of mutant ICAD-expressing L929 cells were irradiated with different doses of γ-irradiation. Certain number of cells were seeded into 10cm plates and allowed to grow for 2 weeks after γ-irradiation. Colonies were stained with crystal violet and counted to calculate the survival fraction.

WTK1 cells are about the same even 40 hours after radiation. The difference did not appear until 87 hours after radiation exposure(Figure 4.2-6B).

The delayed removal of DNA-damaged cells is caused by a small but significantly increased survival of irradiated mICAD cells. This is revealed in L929 cells by clonogenic survival assay which measures the long-term survival of irradiated cells(Figure 4.2-7). A potential explanation for the observation of increased chromosome instability in CAD-inhibited cells is that CAD-inhibition resulted in survival of a very small fraction of genetically-damaged cells that should otherwise be removed though apoptosis. This fraction, though small in number, can contribute significantly to the genomic instability in exposed cells because survival cells with genetic instability is a low frequency event in general. This hypothesis would require that inhibition of ICAD gene to attenuate the overall amount of apoptosis in cells exposed to radiation. This is supported both by our clonogenic data (Figure 4.2-7).

(6). Enhanced growth of tumors expressing mutant ICAD`

Next we examined the ability of mICAD-tranduced L929 cells to form tumors in vivo. L929-mICAD cells were inoculated subcutaneously into nude mice and tumor growth was observed twice a week. All three clones of mICAD-expressing L929 tumors showed significantly faster growth rate than the control L929 tumor (Figure 4.2-8).

The increased growth of tumors could result from either enhanced survival or accelerated proliferation. However, there is no consistent difference in proliferation between control tumors and different clones of mICAD tumors. Ki67 antigen is a marker for proliferation. Staining of tumor tissues by Ki67 antibody showed that proliferation

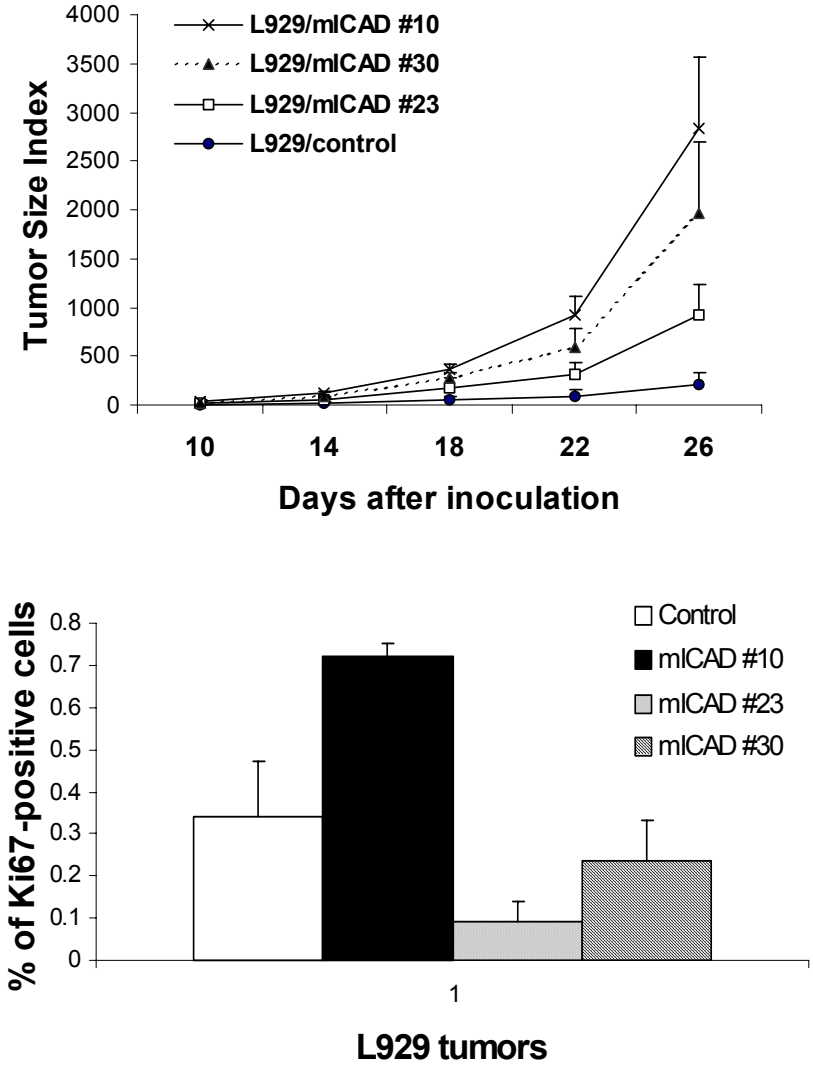


Figure 4.2-8. Enhanced tumor growth of mutant ICAD expressing L929 tumors. (A). Growth curve of L929 tumors. 106 cells were inoculated subcutaneously into each nude mice. There were at least 6 mice in each group ($p<0.05$, Wilcoxon Ranksum test). **(B).** Fraction of Ki67-positive cells in L929 tumors. Proliferating cells were identified by immunohistochemical staining with Ki67 antibody and the percentage of Ki67-positive cells were scored in five random areas on each slides. Error bar represents standard error.

rate is correlated with different growth rate among tumors of three mICAD clones. However, it does not explain the difference between control and mICAD groups. The proliferation rate of control tumor is higher than one mICAD clone, but similar to the second and lower than the third. Despite the conflicting rate of proliferation, all 3 clones of mIACD tumors grew faster than the control tumors. It is very likely that the better survival of tumor cells as shown by the clonogenic assay is the reason for the faster growth of mICAD tumors. The mICAD-expressing cells may survive the stressful tumor microenvironment better than control cells.

Although apoptotic DNA fragmentation is a process evolutionarily conserved across species, its physiological role remains elusive. It was reported that digestion of chromosomes in apoptotic cells prevents innate immunity (to produce IFNβ which is toxic to thymocytes) and therefore ensure the normal development of thymocytes. However, thymus of mouse with disprupted DNA fragmentation (DFF40/CAD(-/-) mouse) is only slightly different from that of wild type mouse. Loss of DNA fragmentation by DFF40/CAD is mostly compensated by DNase II in the lysosome of macrophage, which degrade chromosomal DNA in engulfed apoptotic bodies(Kawane, Fukuyama et al. 2003). Others suggested that DFF45 might play a role in learning and memory(Slane, Lee et al. 2000). In this study, we clearly identified a novel role of DFF/CAD as a checkpoint to prevent chromosome instability.

The precise mechanism for genetic instability as a result of CAD deficiency is unknown. However, our data suggest that removal of genetically damaged cells is probably the main mechanism. This is based on two pieces of evidence: 1) the fact that CAD deficiency leads to increased clonogenic survival after irradiation; 2) the fact that

cells with harmful chromosomal aberrations are removed less rapidly in cells with CAD deficiency than in normal cells. This mode of action of CAD is reminiscent of the checkpoint gene p53, which keeps genomic stability by eliminating cells with DNA damage. It is of interest to note that CAD appears to function independently of p53 in maintaining chromosome instability. A potential rationale may be that it sits at one of the last stages of apoptotic pathway while p53 is quite upstream.

In summary, our results showed that inhibition of DNA fragmentation led to chromosome instability independent of p53 status and tumors with deficient DNA fragmentation had enhanced growth *in vivo*. Therefore, we identified a novel role of apoptotic DNA fragmentation as a p53-independent checkpoint to maintain chromosome stability.

Chapter 5

Perspective

DNA fragmentation is a hallmark of apoptosis. It is defined by the internucleosomal cleavage of genomic DNA in apoptotic cells. Cellular endonucleases including DFF40/CAD and endonuclease G are responsible for this process. DFF40/CAD is activated by caspase cleavage of its chaperon and inhibitor, DFF45/ICAD while endonuclease G (EndoG) is released from mitochondria together with apoptosis inducing factor(AIF), a process independent of caspases.

Although apoptotic DNA fragmentation is an evolutionarily conserved process and has been regarded as critical in apoptosis, its biological function remains elusive. Why does it exist at all throughout evolution? Mice deficient in CAD or ICAD appear normal, suggesting that this system is dispensable for mammalian development. Various studies have been conducted to explore the potential functions of DNA fragmentation. Studies from one group showed that DFF45-deficient mice exhibited enhanced spatial learning and memory and longer memory retention in the novel object recognition task. However, the mechanistic link between DFF45 and its role in learning and memory is not established. It was also proposed that undigested chromosome DNA in apoptotic cells is a strong autoantigen which can induce innate immunity and therefore may result in autoimmune disease or impaired thymus development. However, the CAD-/- mice have normal thymus development and show no increased incidence of autoimmune diseases. In our study, we clearly showed that DFF/CAD is an important gene to maintain genomic stability and suppress tumor development. Our finding agrees well with the clinical correlation between DFF/CAD deficiency and human cancers, which was reported in a number of publications. DFF deficiency was found in a variety of human cancers and associated with higher stage of tumor and poorer prognosis. Therefore, it has long been

postulated to be a candidate tumor suppressor gene of neuroblastoma. Our discovery provides a strong evidence for this hypothesis.

DNA fragmentation is a late stage event downstream of caspase activation in apoptosis. It was thought that activated caspases, which cleave more than 100 cellular substrates, are sufficient to kill apoptotic cells. Nagata's group reported that DNA fragmentation is not essential to apoptotic cell death based solely on apoptosis assays of CAD-/- cells or cells expressing mutant ICAD. However, other groups showed that DFF45-deficient cells are more resistant to apoptosis and suggested that DNA fragmentation together with other apoptotic events may contribute to an amplification loop in the death process. Our data support the latter by showing a significantly increased long-term survival of irradiated CAD-/- cells with clonogenic assay. Apoptosis assays only measure the apoptosis rate at a single time point while clonogenic assay takes into account of the accumulative effect of cell death during the whole time of experiment. Therefore, it is a more sensitive assay than apoptosis assays in detecting small difference in cell death. Our data clearly suggest that DNA fragmentation is an important component of effective killing machinery and contribute to ensure the complete destruction of apoptotic cells.

Whether apoptosis is a reversible process is controversial and debated by major apoptosis groups. Apoptotic cells have membrane phosphotydyl serine externalized which can bind annexin V. We isolated annexin V-binding apoptotic cells by cell sorting to study whether some of these cells can survive apoptosis. Our data showed that majority of these cells eventually died with only a small fraction survived and form colonies. Annexin V-positive CAD-/- cells formed much more colonies than the normal

wild type Annexin V-positive cells. The clonogenic survival of a small fraction of normal wild type Annexin V positive cells does not necessarily indicate that apoptosis is reversible because they may well be contaminated non-apoptotic cells due to inaccuracy of cell sorting. However, the presence of more clonogenic survival of Annexin V-binding CAD-/- cells does suggest that apoptosis may indeed be reversed in some apoptotic cells deficient in DNA fragmentation. And this, again, indicates that DNA fragmentation is essential to apoptotic cell death at least in some apoptotic cells.

Genetic instability is a hallmark of cancers and is fundamental to tumor development. We identified DFF/CAD as a novel checkpoint gene, which suppresses tumor development through maintaining genomic stability especially in DNA damage-induced carcinogenesis. It highlights once more the close interplays among apoptosis, genomic instability, and tumorigenesis and provides further insights into these biological processes. It also provides a potential therapeutic strategy for cancers with DFF/CAD deficiency. Delivery of normal DFF genes by gene therapy or recombinant DFF proteins may be applied as an alternative in treatment of these cancer patients.

There are also a number of interesting questions that remain to be answered.

Cancer susceptibility genes have been classified into two groups: gatekeepers and caretakers. Gatekeepers are genes that control cell proliferation, differentiation and death, whereas caretakers are DNA repair genes whose inactivation leads to genetic instability. DFF/CAD belongs to the "gatekeeper" category because it is involved in cell death, the removal of DNA-damaged cells. It will be interesting to study the cooperation between DFF/CAD and other gatekeepers and/or caretakers in maintaining genetic stability and prevention of tumorigenesis. Ku80 and DNAPK are involved in DNA repair and the

knockout mice for Ku80 and DNAPK are available. It would be interesting to study genetic inbstatbility and carcinogenesis in mice with double knockout of CAD/DFF and Ku80 or DNAPKcs. We can cross the CAD-/- mice with Ku80-/- or DNAPKcs-/- mice to produce CAD-/- Ku80-/- or CAD-/- DNAPKcs-/- double knockout mice and isolate MEF cells from these mice. Then we can compare the level of chromosome instability (by studying chromosome aberrations and translocations) in wild type, CAD-/-, Ku80-/- or DNAPKcs-/-, and CAD-/- Ku80-/- or CAD-/- DNAPKcs-/- double knockout mice. We will also observe the spontaneous and radiation-induced tumor development in these mice. We expect that the double knockout mice will develop tumors spontaneously.

Endonuclease G is another gene responsible for DNA fragmentation during apoptosis. It will be interesting to explore the role of EndoG in genetic instability and tumorigenesis. CAD-/- mice do not develop tumor spontaneously. A potential reason may be compensation of the remaining EndoG under normal condition. When treated by radiation or chemical carcinogens, it may decompensate and develop tumors. Therefore, it will be particularly interesting to study spontaneous carcinogenesis in EndoG-/- CAD-/- mice. Similar to the above, EndoG-/- CAD-/- mice will be produced. Chromosome instability, cellular transformation and spontaneous as well as induced tumorigenesis will be studied in these mice and MEFs derived from them. We expect to see a more significant phenotype in these double knockout mice.

References

Abel, F., R. M. Sjoberg, et al. (2004). "Mutations in the N-terminal domain of DFF45 in a primary germ cell tumor and in neuroblastoma tumors." Int J Oncol **25**(5): 1297-302.

Abel F, S. R., Ejeskar K, Krona C, Martinsson T. (2002). "Analyses of apoptotic regulators CASP9 and DFFA at 1P36.2, reveal rare allele variants in human neuroblastoma tumours." Br J Cancer **86**(4): 596-604.

Almoguera, C., D. Shibata, et al. (1988). "Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes." Cell **53**(4): 549-54.

Ashkenazi, A. and V. M. Dixit (1998). "Death receptors: signaling and modulation." Science **281**(5381): 1305-8.

Bader, S. A., C. Fasching, et al. (1991). "Dissociation of suppression of tumorigenicity and differentiation in vitro effected by transfer of single human chromosomes into human neuroblastoma cells." Cell Growth Differ **2**(5): 245-55.

Bassing, C. H., K. F. Chua, et al. (2002). "Increased ionizing radiation sensitivity and genomic instability in the absence of histone H2AX." Proc Natl Acad Sci U S A **99**(12): 8173-8.

Boulares, A. H., A. J. Zoltoski, et al. (2001). "Roles of DNA fragmentation factor and poly(ADP-ribose) polymerase in an amplification phase of tumor necrosis factor-induced apoptosis." J Biol Chem **276**(41): 38185-92.

Brodeur, G. M. H., M. D. (1998). The Genetic Basis of Human Cancer 1st edn (eds Kinzler, K. W. & Vogelstein, B.). New York, McGraw-Hill.

Brown, D. G., X. M. Sun, et al. (1993). "Dexamethasone-induced apoptosis involves cleavage of DNA to large fragments prior to internucleosomal fragmentation." J Biol Chem **268**(5): 3037-9.

Cahill, D. P., K. W. Kinzler, et al. (1999). "Genetic instability and darwinian selection in tumours." Trends Cell Biol **9**(12): M57-60.

Cain, K., S. H. Inayat-Hussain, et al. (1994). "DNA fragmentation into 200-250 and/or 30-50 kilobase pair fragments in rat liver nuclei is stimulated by Mg2+ alone and Ca2+/Mg2+ but not by Ca2+ alone." FEBS Lett **349**(3): 385-91.

Caron, H., N. Spieker, et al. (2001). "Chromosome bands 1p35-36 contain two distinct neuroblastoma tumor suppressor loci, one of which is imprinted." Genes Chromosomes Cancer **30**(2): 168-74.

Caron H, v. S. P., de Kraker J, Bokkerink J, Egeler M, Laureys G, Slater R, Westerveld A, Voute PA, Versteeg R. (1996). "Allelic loss of chromosome 1p as a predictor of unfavorable outcome in patients with neuroblastoma." N Engl J Med. **334**(4): 225-30.

Celeste, A., S. Petersen, et al. (2002). "Genomic instability in mice lacking histone H2AX." Science **296**(5569): 922-7.

Chang, W. P. and J. B. Little (1992). "Persistently elevated frequency of spontaneous mutations in progeny of CHO clones surviving X-irradiation: association with delayed reproductive death phenotype." Mutat Res **270**(2): 191-9.

Cotran R.S., K. V., Collins T (1999). Robbins pathologic basis of disease. Philadelphia, W.B. Sauders Company.

Cox, A. D. and C. J. D. Der (1994). "Biological Assays for Cellular transformation." Methods in Enzymology **238**: 277-294.

Darmon, A. J., D. W. Nicholson, et al. (1995). "Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme B." Nature **377**(6548): 446-8.

de Laat, W. L., N. G. Jaspers, et al. (1999). "Molecular mechanism of nucleotide excision repair." Genes Dev **13**(7): 768-85.

de Saint Vincent, B. R., S. Delbruck, et al. (1981). "The cloning and reintroduction into animal cells of a functional CAD gene, a dominant amplifiable genetic marker." Cell **27**(2 Pt 1): 267-77.

Difilippantonio, M. J., J. Zhu, et al. (2000). "DNA repair protein Ku80 suppresses chromosomal aberrations and malignant transformation." Nature **404**(6777): 510-4.

Elledge, S. J. (1996). "Cell cycle checkpoints: preventing an identity crisis." Science **274**(5293): 1664-72.

Enari, M., H. Sakahira, et al. (1998). "A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD." Nature **391**(6662): 43-50.

Fernandez-Capetillo, O., H. T. Chen, et al. (2002). "DNA damage-induced G2-M checkpoint activation by histone H2AX and 53BP1." Nat Cell Biol **4**(12): 993-7.

Fong, C. T., N. C. Dracopoli, et al. (1989). "Loss of heterozygosity for the short arm of chromosome 1 in human neuroblastomas: correlation with N-myc amplification." Proc Natl Acad Sci U S A **86**(10): 3753-7.

Foulds, L. (1954). The Experimental Study of Tumor Progression., London: Academic Press.

Furth, E. E., W. G. Thilly, et al. (1981). "Quantitative assay for mutation in diploid human lymphoblasts using microtiter plates." Anal Biochem **110**(1): 1-8.

Green, D. R. and J. C. Reed (1998). "Mitochondria and apoptosis." Science **281**(5381): 1309-12.

Gu, J., R. P. Dong, et al. (1999). "Functional interaction of DFF35 and DFF45 with caspase-activated DNA fragmentation nuclease DFF40." J Biol Chem **274**(30): 20759-62.

Hahn, P., B. Nevaldine, et al. (1990). "X-ray induction of methotrexate resistance due to dhfr gene amplification." Somat Cell Mol Genet **16**(5): 413-23.

Hahn, W. C., C. M. Counter, et al. (1999). "Creation of human tumour cells with defined genetic elements." Nature **400**(6743): 464-8.

Halenbeck, R., H. MacDonald, et al. (1998). "CPAN, a human nuclease regulated by the caspase-sensitive inhibitor DFF45." Curr Biol **8**(9): 537-40.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.

Hirao, A., A. Cheung, et al. (2002). "Chk2 is a tumor suppressor that regulates apoptosis in both an ataxia telangiectasia mutated (ATM)-dependent and an ATM-independent manner." Mol Cell Biol **22**(18): 6521-32.

Hiyama, E., K. Hiyama, et al. (2001). "Biological characteristics of neuroblastoma with partial deletion in the short arm of chromosome 1." Med Pediatr Oncol **36**(1): 67-74.

Hoeppner, D. J., M. O. Hengartner, et al. (2001). "Engulfment genes cooperate with ced-3 to promote cell death in Caenorhabditis elegans." Nature **412**(6843): 202-6.

Hollander, M. C., M. S. Sheikh, et al. (1999). "Genomic instability in Gadd45a-deficient mice." Nat Genet **23**(2): 176-84.

Holmgren, L., A. Szeles, et al. (1999). "Horizontal transfer of DNA by the uptake of apoptotic bodies." Blood **93**(11): 3956-63.

Hoyt, M. A., T. Stearns, et al. (1990). "Chromosome instability mutants of Saccharomyces cerevisiae that are defective in microtubule-mediated processes." Mol Cell Biol **10**(1): 223-34.

Hsieh, S. Y., S. F. Liaw, et al. (2003). "Aberrant caspase-activated DNase (CAD) transcripts in human hepatoma cells." Br J Cancer **88**(2): 210-6.

Inohara, N., T. Koseki, et al. (1998). "CIDE, a novel family of cell death activators with homology to the 45 kDa subunit of the DNA fragmentation factor." Embo J **17**(9): 2526-33.

Iolascon, A., C. Lo Cunsolo, et al. (1998). "Interstitial and large chromosome 1p deletion occurs in localized and disseminated neuroblastomas and predicts an unfavourable outcome." Cancer Lett **130**(1-2): 83-92.

Jiricny, J. (1998). "Eukaryotic mismatch repair: an update." Mutat Res **409**(3): 107-21.

Judson H, v. R. N., Strain L, Vandesompele J, Van Gele M, Speleman F, Bonthron DT. (2000). "Structure and mutation analysis of the gene encoding DNA fragmentation factor 40 (caspase-activated nuclease), a candidate neuroblastoma tumour suppressor gene." Hum Genet. **106**(4): 406-13.

Kawane, K., H. Fukuyama, et al. (1999). "Structure and promoter analysis of murine CAD and ICAD genes." Cell Death Differ **6**(8): 745-52.

Kawane, K., H. Fukuyama, et al. (2003). "Impaired thymic development in mouse embryos deficient in apoptotic DNA degradation." Nat Immunol **4**(2): 138-44.

Kinsella, T. M. and G. P. Nolan (1996). "Episomal vectors rapidly and stably produce high-titer recombinant retrovirus." Hum Gene Ther **7**(12): 1405-13.

Kinzler, K. W. and B. Vogelstein (1996). "Lessons from hereditary colorectal cancer." Cell **87**(2): 159-70.

Komuro, H., M. B. Valentine, et al. (1998). "Fluorescence in situ hybridization analysis of chromosome 1p36 deletions in human MYCN amplified neuroblastoma." J Pediatr Surg **33**(11): 1695-8.

Konishi, S., H. Ishiguro, et al. (2002). "Decreased expression of DFF45/ICAD is correlated with a poor prognosis in patients with esophageal carcinoma." Cancer **95**(12): 2473-8.

Krimpenfort, P., K. C. Quon, et al. (2001). "Loss of p16Ink4a confers susceptibility to metastatic melanoma in mice." Nature **413**(6851): 83-6.

Lagarkova, M. A., O. V. Iarovaia, et al. (1995). "Large-scale fragmentation of mammalian DNA in the course of apoptosis proceeds via excision of chromosomal DNA loops and their oligomers." J Biol Chem **270**(35): 20239-41.

Lengauer, C., K. W. Kinzler, et al. (1997). "Genetic instability in colorectal cancers." Nature **386**(6625): 623-7.

Lengauer, C., K. W. Kinzler, et al. (1998). "Genetic instabilities in human cancers." Nature **396**(6712): 643-9.

Li, L. Y., X. Luo, et al. (2001). "Endonuclease G is an apoptotic DNase when released from mitochondria." Nature **412**(6842): 95-9.

Li, T. K., A. Y. Chen, et al. (1999). "Activation of topoisomerase II-mediated excision of chromosomal DNA loops during oxidative stress." Genes Dev **13**(12): 1553-60.

Li, Y. and R. Benezra (1996). "Identification of a human mitotic checkpoint gene: hsMAD2." Science **274**(5285): 246-8.

Liber, H. L. and W. G. Thilly (1982). "Mutation assay at the thymidine kinase locus in diploid human lymphoblasts." Mutat Res **94**(2): 467-85.

Little, J. B., H. Nagasawa, et al. (1995). "Absence of radiation-induced G1 arrest in two closely related human lymphoblast cell lines that differ in p53 status." J Biol Chem **270**(19): 11033-6.

Liu, X., P. Li, et al. (1998). "The 40-kDa subunit of DNA fragmentation factor induces DNA fragmentation and chromatin condensation during apoptosis." Proc Natl Acad Sci U S A **95**(15): 8461-6.

Liu, X., H. Zou, et al. (1997). "DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis." Cell **89**(2): 175-84.

Liu, X., H. Zou, et al. (1999). "Activation of the apoptotic endonuclease DFF40 (caspase-activated DNase or nuclease). Oligomerization and direct interaction with histone H1." J Biol Chem **274**(20): 13836-40.

Livingstone, L. R., A. White, et al. (1992). "Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53." Cell **70**(6): 923-35.

Loeb, L. A. (1991). "Mutator phenotype may be required for multistage carcinogenesis." Cancer Res **51**(12): 3075-9.

Lowe, S. W., T. Jacks, et al. (1994). "Abrogation of oncogene-associated apoptosis allows transformation of p53-deficient cells." Proc Natl Acad Sci U S A **91**(6): 2026-30.

Lugovskoy, A. A., P. Zhou, et al. (1999). "Solution structure of the CIDE-N domain of CIDE-B and a model for CIDE- N/CIDE-N interactions in the DNA fragmentation pathway of apoptosis." Cell **99**(7): 747-55.

Maris, J. M., P. S. White, et al. (1995). "Significance of chromosome 1p loss of heterozygosity in neuroblastoma." Cancer Res **55**(20): 4664-9.

Marx, J. (2002). "Debate surges over the origins of genomic defects in cancer." Science **297**(5581): 544-6.

McCarty, J. S., S. Y. Toh, et al. (1999). "Multiple domains of DFF45 bind synergistically to DFF40: roles of caspase cleavage and sequestration of activator domain of DFF40." Biochem Biophys Res Commun **264**(1): 181-5.

McIlroy, D., H. Sakahira, et al. (1999). "Involvement of caspase 3-activated DNase in internucleosomal DNA cleavage induced by diverse apoptotic stimuli." Oncogene **18**(31): 4401-8.

McIlroy, D., M. Tanaka, et al. (2000). "An auxiliary mode of apoptotic DNA fragmentation provided by phagocytes." Genes Dev **14**(5): 549-58.

Mitelman, F., Johansson, B. &Mertens, F. (1994). "Catalog of Chromosome Aberrations in Cancer." **2**.

Miyashita, T. and J. C. Reed (1995). "Tumor suppressor p53 is a direct transcriptional activator of the human bax gene." Cell **80**(2): 293-9.

Montague, J. W., F. M. Hughes, Jr., et al. (1997). "Native recombinant cyclophilins A, B, and C degrade DNA independently of peptidylprolyl cis-trans-isomerase activity. Potential roles of cyclophilins in apoptosis." J Biol Chem **272**(10): 6677-84.

Mukae, N., M. Enari, et al. (1998). "Molecular cloning and characterization of human caspase-activated DNase." Proc Natl Acad Sci U S A **95**(16): 9123-8.

Murray, A. W. (1995). "The genetics of cell cycle checkpoints." Curr Opin Genet Dev **5**(1): 5-11.

Nagata, S. (1997). "Apoptosis by death factor." Cell **88**(3): 355-65.

Nagata, S. (1999). "Biddable death." Nat Cell Biol **1**(6): E143-5.

Nagata, S. (2000). "Apoptotic DNA fragmentation." Exp Cell Res **256**(1): 12-8.

Nakano, K. and K. H. Vousden (2001). "PUMA, a novel proapoptotic gene, is induced by p53." Mol Cell **7**(3): 683-94.

Nasmyth, K. (1996). "At the heart of the budding yeast cell cycle." Trends Genet **12**(10): 405-12.

Nowak, M. A., N. L. Komarova, et al. (2002). "The role of chromosomal instability in tumor initiation." Proc Natl Acad Sci U S A **99**(25): 16226-31.

Nowell, P. C. (1997). "Genetic alterations in leukemias and lymphomas: impressive progress and continuing complexity." Cancer Genet Cytogenet **94**(1): 13-9.

Oberhammer, F., J. W. Wilson, et al. (1993). "Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation." Embo J **12**(9): 3679-84.

Oda, E., R. Ohki, et al. (2000). "Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis." Science **288**(5468): 1053-8.

Ohira, M., H. Kageyama, et al. (2000). "Identification and characterization of a 500-kb homozygously deleted region at 1p36.2-p36.3 in a neuroblastoma cell line." Oncogene **19**(37): 4302-7.

Ohtsu, K., E. Hiyama, et al. (1997). "Clinical investigation of neuroblastoma with partial deletion in the short arm of chromosome 1." Clin Cancer Res **3**(7): 1221-8.

Otomo, T., H. Sakahira, et al. (2000). "Structure of the heterodimeric complex between CAD domains of CAD and ICAD." Nat Struct Biol **7**(8): 658-62.

Paulovich, A. G., D. P. Toczyski, et al. (1997). "When checkpoints fail." Cell **88**(3): 315-21.

Raff, M. (1998). "Cell suicide for beginners." Nature **396**(6707): 119-22.

Reddien, P. W., S. Cameron, et al. (2001). "Phagocytosis promotes programmed cell death in C. elegans." Nature **412**(6843): 198-202.

Renan, M. J. (1993). "How many mutations are required for tumorigenesis? Implications from human cancer data." Mol Carcinog **7**(3): 139-46.

Sabol, S. L., R. Li, et al. (1998). "Inhibition of apoptosis-associated DNA fragmentation activity in nonapoptotic cells: the role of DNA fragmentation factor-45 (DFF45/ICAD)." Biochem Biophys Res Commun **253**(1): 151-8.

Sakahira, H., M. Enari, et al. (1998). "Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis." Nature **391**(6662): 96-9.

Sakahira, H., M. Enari, et al. (1999). "Functional differences of two forms of the inhibitor of caspase- activated DNase, ICAD-L, and ICAD-S." J Biol Chem **274**(22): 15740-4.

Schimke, R. T., R. J. Kaufman, et al. (1978). "Gene amplification and drug resistance in cultured murine cells." Science **202**(4372): 1051-5.

Schwab, M., C. Praml, et al. (1996). "Genomic instability in 1p and human malignancies." Genes Chromosomes Cancer **16**(4): 211-29.

Seeger, R. C., G. M. Brodeur, et al. (1985). "Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas." N Engl J Med **313**(18): 1111-6.

Sharma, R. C. and R. T. Schimke (1989). "Enhancement of the frequency of methotrexate resistance by gamma-radiation in Chinese hamster ovary and mouse 3T6 cells." Cancer Res **49**(14): 3861-6.

Shiloh, Y. (2003). "ATM and related protein kinases: safeguarding genome integrity." Nat Rev Cancer **3**(3): 155-68.

Skopek, T. R., H. L. Liber, et al. (1978). "Isolation of a human lymphoblastoid line heterozygous at the thymidine kinase locus: possibility for a rapid human cell mutation assay." Biochem Biophys Res Commun **84**(2): 411-6.

Slane, J. M., H. S. Lee, et al. (2000). "DNA fragmentation factor 45 deficient mice exhibit enhanced spatial learning and memory compared to wild-type control mice." Brain Res **867**(1-2): 70-9.

Slane McQuade, J. M., C. V. Vorhees, et al. (2002). "DNA fragmentation factor 45 knockout mice exhibit longer memory retention in the novel object recognition task compared to wild-type mice." Physiol Behav **76**(2): 315-20.

Smith, G. C. and S. P. Jackson (1999). "The DNA-dependent protein kinase." Genes Dev **13**(8): 916-34.

Smith, K. A., M. L. Agarwal, et al. (1995). "Regulation and mechanisms of gene amplification." Philos Trans R Soc Lond B Biol Sci **347**(1319): 49-56.

Spencer, F., S. L. Gerring, et al. (1990). "Mitotic chromosome transmission fidelity mutants in Saccharomyces cerevisiae." Genetics **124**(2): 237-49.

Spitz, R., B. Hero, et al. (2002). "Fluorescence in situ hybridization analyses of chromosome band 1p36 in neuroblastoma detect two classes of alterations." Genes Chromosomes Cancer **34**(3): 299-305.

Stevens, C., L. Smith, et al. (2003). "Chk2 activates E2F-1 in response to DNA damage." Nat Cell Biol **5**(5): 401-9.

Stewart, G. S., B. Wang, et al. (2003). "MDC1 is a mediator of the mammalian DNA damage checkpoint." Nature **421**(6926): 961-6.

Stoler, D. L., N. Chen, et al. (1999). "The onset and extent of genomic instability in sporadic colorectal tumor progression [see comments]." Proc Natl Acad Sci U S A **96**(26): 15121-6.

Stroh, C. and K. Schulze-Osthoff (1998). "Death by a thousand cuts: an ever increasing list of caspase substrates." Cell Death Differ **5**(12): 997-1000.

Sun, D. Y., S. Jiang, et al. (1994). "Separate metabolic pathways leading to DNA fragmentation and apoptotic chromatin condensation." J Exp Med **179**(2): 559-68.

Susin, S. A., H. K. Lorenzo, et al. (1999). "Molecular characterization of mitochondrial apoptosis-inducing factor." Nature **397**(6718): 441-6.

Takai, H., K. Naka, et al. (2002). "Chk2-deficient mice exhibit radioresistance and defective p53-mediated transcription." Embo J **21**(19): 5195-205.

Tenant R: chemocal carcinogenesis. In Franks LM, T. N. e. (1997). "An introduction to the cellular and molecular Biology of Cancer 3rd ed." 106-125.

Thomas, D. A., C. Du, et al. (2000). "DFF45/ICAD can be directly processed by granzyme B during the induction of apoptosis." Immunity **12**(6): 621-32.

Thompson, C. B. (1995). "Apoptosis in the pathogenesis and treatment of disease." Science **267**(5203): 1456-62.

Tlsty, T. D., B. H. Margolin, et al. (1989). "Differences in the rates of gene amplification in nontumorigenic and tumorigenic cell lines as measured by Luria-Delbruck fluctuation analysis." Proc Natl Acad Sci U S A **86**(23): 9441-5.

Van Gele M, S. F., Vandesompele J, Van Roy N, Leonard JH (1998). "Characteristic pattern of chromosomal gains and losses in Merkel cell carcinoma detected by comparative genomic hybridization." cancer research **58**(7): 1503-8.

Van Gele, M., F. Speleman, et al. (1998). "Characteristic pattern of chromosomal gains and losses in Merkel cell carcinoma detected by comparative genomic hybridization." Cancer Res **58**(7): 1503-8.

Van Gele M, V. R. N., Ronan SG, Messiaen L, Vandesompele J, Geerts ML, Naeyaert JM, Blennow E, Bar-Am I, Das Gupta TK, van der Drift P, Versteeg R, Leonard JH, Speleman F. (1998). "Molecular analysis of 1p36 breakpoints in two Merkel cell carcinomas." Genes Chromosomes Cancer **23**(1): 67-71.

Wang, S. I., J. Puc, et al. (1997). "Somatic mutations of PTEN in glioblastoma multiforme." Cancer Res **57**(19): 4183-6.

Westermann, F. and M. Schwab (2002). "Genetic parameters of neuroblastomas." Cancer Lett **184**(2): 127-47.

White, P. S., P. M. Thompson, et al. (2001). "Detailed molecular analysis of 1p36 in neuroblastoma." Med Pediatr Oncol **36**(1): 37-41.

Widlak, P. (2000). "The DFF40/CAD endonuclease and its role in apoptosis." Acta Biochim Pol **47**(4): 1037-44.

Widlak, P., P. Li, et al. (2000). "Cleavage preferences of the apoptotic endonuclease DFF40 (caspase- activated DNase or nuclease) on naked DNA and chromatin substrates." J Biol Chem **275**(11): 8226-32.

Williams, J. R., J. B. Little, et al. (1974). "Association of mammalian cell death with a specific endonucleolytic degradation of DNA." Nature **252**(5485): 754-5.

Wolf, B. B., M. Schuler, et al. (1999). "Caspase-3 is the primary activator of apoptotic DNA fragmentation via DNA fragmentation factor-45/inhibitor of caspase-activated DNase inactivation." J Biol Chem **274**(43): 30651-6.

Wyllie, A. H. (1980). "Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation." Nature **284**(5756): 555-6.

Yandell, D. W. and J. B. Little (1986). "Chromosome 14 marker appearance in a human B lymphoblastoid cell line of nonmalignant origin." Cancer Genet Cytogenet **20**(3-4): 231-9.

Yang, H. W., Y. Z. Chen, et al. (2001). "DNA fragmentation factor 45 (DFF45) gene at 1p36.2 is homozygously deleted and encodes variant transcripts in neuroblastoma cell line." Neoplasia **3**(2): 165-9.

Yang, S., C. Kuo, et al. (2002). "PML-dependent apoptosis after DNA damage is regulated by the checkpoint kinase hCds1/Chk2." Nat Cell Biol **4**(11): 865-70.

Yin, Y., M. A. Tainsky, et al. (1992). "Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles." Cell **70**(6): 937-48.

Zhang, J., X. Liu, et al. (1998). "Resistance to DNA fragmentation and chromatin condensation in mice lacking the DNA fragmentation factor 45." Proc Natl Acad Sci U S A **95**(21): 12480-5.

Zhang, J., X. Wang, et al. (1999). "DNA fragmentation factor 45-deficient cells are more resistant to apoptosis and exhibit different dying morphology than wild-type control cells." J Biol Chem **274**(52): 37450-4.

Zhang, J. H. and M. Xu (2000). "DNA fragmentation in apoptosis." Cell Res **10**(3): 205-11.

Zhu, C., K. D. Mills, et al. (2002). "Unrepaired DNA breaks in p53-deficient cells lead to oncogenic gene amplification subsequent to translocations." Cell **109**(7): 811-21.

Zhuang, Z., W. S. Park, et al. (1998). "Trisomy 7-harbouring non-random duplication of the mutant MET allele in hereditary papillary renal carcinomas." Nat Genet **20**(1): 66-9.

Biography

Bin Yan was born in Changsha, the capital of Hunan Province, China on November 12, 1972. He received his M.D. degree from Peking Union Medical College in Beijing, China in 1998. He entered the PhD program in the Department of Pathology at Duke University in Durham, North Carolina, USA in 2001. His dissertation research was supervised by Dr. Chuan-Yuan Li in the Department of Radiation Oncology and his co-advisor, Dr. Mark W. Dewhirst in the Department of Pathology, Radiation Oncology and Biomedical Engineering. His publications are listed as following.

Publications

Articles

1. Zhang X, **Yan B(5th)**, Li CY et al. Increased resistance of tumor cells to hyperthermia mediated by integrin-linked kinase, *Clinical Cancer Research.* 2003 Mar;9(3):1155-60.

2. Duan ZJ, **Yan B(3rd),** Hou YD et al. Isolation and characterization of anti-TNF peptides from phage-displayed CDR-based peptide library. *Chinese Journal of Biochemistry and Molecular Biology*. 2003 Oct;19(5):547-551

3. **Yan B**, Lilly M et al. The Pim-2 kinase phosphorylates BAD on serine-112 and reverses BAD-induced cell death. *Journal of Biological Chemistry* 2003 Nov 14;278(46):45358-67

4. Huang Q, **Yan B(4th)**, Li CY et al. A novel conditionally replicative adenovirus vector targeting telomerase-positive tumor cells. *Clinical Cancer Research*. 2004 Feb 15;10(4):1439-45

5. **Yan B**, Li CY et al. Pim-1 kinase inhibits activation of the reporter gene expression in Elk-1 and c-Fos reporting systems but not the endogenous gene expression: an artifact of the reporter gene assay by transient co-transfection. *Braz J Med Biol Res.* 2006 Feb;39(2):169-76.

6. **Yan B**, Dewhirst MW, Li CY et al. A novel role of DNA fragmentation factor gene in maintaining genomic stability. *Proc Natl Acad Sci U S A*. 2006 Jan 31;103(5):1504-9.

7. **Yan B**, Dewhirst MW, Li CY et al. Inhibition of apoptotic DNA fragmentation results in increased chromosomal instability in a P53-independent manner. *Oncogene* (in press)

8. **Yan B**, Dewhirst MW, Li CY et al. Caspase-activated DNAse regulates horizontal gene transfer by uptake of apoptotic bodies. Submitted to *Cancer Res.*

9. Li F, **Yan B(5th),** Dewhist MW, Li CY et al. Regulation of HIF-1 through S-nitrosylation during radiation therapy. Under review by *Science*

10. Wang H, **Yan B (3rd)**, Goldschmidt-Clermont PJ et al. Thrombosis-prone polymorphism of platelet integrin αIIbβ3 differentially regulates intracellular signaling pathways. Under review by *Blood*

11. Kon T, **Yan B (6th)**, Li CY et al. Down regulation of DNA-PKcs by SiRNA increases radiosensitivity of cancer cells. Submitted to *Cancer Res.*

12. **Yan B**, Dewhirst MW, Li CY et al. TNFα increases genetic instability and cellular transformation. (In preparation)

Abstracts

1. **Yan B**, Dewhirst MW, Li CY et al. A novel role of DNA fragmentation factor as a tumor suppressor through maintaining genetic stability. *Cancer Res.* (*97th Annual Meeting of American Association of Cancer Research)*

2. **Yan B**, Lilly MB et al. The pim-2 Serine kinase phosphorylates the proapoptotic protein BAD on Serine-112 and opposes BAD-induced cell death, *Blood.* 2002 Nov. 16; 100(11) part 1:738a, (*44th Annual Meeting of American Society of Hematology Program and Abstracts)*,

3. **Yan B**, Wang H, Goldschmidt-Clermont PJ Role of alphaIIbbeta3 in the megakaryocyte apoptosis through inhibition of MAPK pathway, *Molecular Biology of the Cell*. 2002 Dec. 14; abstract L66 (*42nd American Society for Cell Biology Annual Meeting Abstracts*)

4. Wang H, **Yan B(2nd)**, Goldschmidt-Clermont PJ et al. Changes in expression profiling resulting from alphaIIbbeta3 and its polymorphism suggests a role in apoptosis and differentiation of hematopoietic cells, *Blood.* 2002 Nov. 16; 100(11) part 2:168b, (*44th Annual Meeting of American Society of Hematology Program and Abstracts)*

5. **Yan B,** Lilly M, Li CY et al. Pim-2 kinase mediates the survival signal of IL-3 and inhibits apoptosis of hematopoietic cells via phosphorylation of BAD. *11th*

Annual Biological Sciences Graduate Student Symposium. 2002 Nov. 1; 44: abstract #81

6. **Yan B**, Lilly MB, Li CY et al. The antiapoptotic effect of Pim-1 kinase is not through regulation of MAP kinase pathway, *Molecular Targets for Cancer Therapy: Second Biennial Meeting Conference Syllabus*, 2002 October 11-15; *abstract #71*