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Lack of Obvious 50 Kilobase Pair DNA Fragments in DNA Fragmentation Factor 45-Deficient Thymocytes upon Activation of Apoptosis

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The DNA fragmentation factor 45 (DFF45/ICAD) is a key subunit of a heterodimeric DNase complex critical for the induction of DNA fragmentation during apoptosis *in vivo.* To further assess the importance of DFF45 in chromosomal DNA degradation, we induced apoptosis in wild-type control and DFF45 deficient thymocytes and compared the cleavage of chromosomal DNA to 50 kilobase pair size fragments. We found that there is a lack of obvious large chromosomal DNA fragments upon treatments by various apoptotic agents in DFF45 deficient thymocytes. The major organ systems in the DFF45 mutant mice either two months or fifteen months of age appear normal. These results suggest that functional DFF45 is required for cleavage of DNA into both large size and oligonucleosomal size fragments in thymocytes during apoptosis. However, deficiency in DFF45 apparently does not significantly affect normal mouse development and tissue homeostasis. © 2000 Academic Press

Key Words: **DFF45; apoptosis; thymocytes; 50 kb DNA fragments; development; tissue homeostasis.**

Programmed cell death or apoptosis is a precisely regulated cellular process critical in development and tissue homeostasis (1–4). Alteration of apoptosis is implicated in many human diseases, including cancer and neurodegenerative diseases (5). The main biochemical hallmark of apoptosis is degradation of chromosomal DNA into oligonucleosomal sizes (6, 7). DNA fragmentation and chromatin condensation depend critically on a heterodimeric protein composed of DNA fragmentation factors 45 and 40 (DFF45 or ICAD and DFF40 or CAD, respectively; 8–11) *in vitro.* DFF40 contains an intrinsic DNase activity whereas DFF45 serves both as a molecular chaperone and as an inhibitor of DFF40 (8, 10). It has been suggested that degradation of chromosomal DNA to 50 kilobase pair (kb) size fragments occurs prior to oligonucleosomal DNA fragmentation during apoptosis (12). Candidate endonucleases for such large size chromosomal degradation have also been proposed (13). We previously generated DFF45 mutant mice and showed that DFF45 is essential for DNA fragmentation into oligonucleosomal sizes *in vivo* (14–16). However, whether degrading chromosomal DNA into 50 kb size fragments during apoptosis requires a functional DFF45 remains unclear.

DNA fragmentation *in vivo* may be an important step for disposal of large fragments of DNA from dying cells, which may be critical in maintaining normal tissue homeostasis (17–19). We previously showed that thymocytes from DFF45 mutant mice exhibit slower rate of apoptosis than wild-type control thymocytes in response to multiple apoptotic stimuli (16). Moreover, we found that both the granule cell density and total granule cell number in the hippocampal dentate gyrus region are higher in the DFF45 mutant brains than in the wild-type brains (20). Furthermore, the increased granule cell number correlated with enhanced spatial learning and memory in DFF45 mutant mice compared to wild-type mice (20). Thus, at least in the case of the central nervous system, a lack of DFF45 affected normal neuronal cell number and function. Whether DFF45 plays a role in general development and tissue homeostasis elsewhere remain unknown.

We have used the DFF45 mutant mice to address these two key issues. We found that there is a lack of obvious 50 kb chromosomal DNA fragments upon treatments by various apoptotic agents in DFF45 deficient thymocytes. The major organ systems in the DFF45 mutant mice either two months or fifteen months of age appear normal. These results suggest that a functional DFF45 is required for cleavage of DNA into both large size and oligonucleosomal size fragments in thymocytes during apoptosis. However,

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deficiency in DFF45 apparently does not significantly affect normal mouse development and tissue homeostasis.

MATERIALS AND METHODS

DFF45 mutant mice. All mice were housed in the animal facility on a 12-h light/dark cycle with food and water available *ad libitum*. DFF45 mutant mice and wild-type control mice were generated by crossing heterozygous mutant animals and were identified by genomic Southern blotting as described previously (14). Unless specified, DFF45 mutant and wild-type control litter-mates eight to twelve weeks of age were used for all subsequent analyses.

Primary cell culture and genomic DNA isolation. Primary thymocytes from both DFF45 mutant and wild-type control mice were isolated and resuspended at 2×10^6/ml in DMEM and were cultured in the absence or presence of dexamethasone (1 μM), etoposide (50 μM) or staurosporine (2 μM) respectively for different lengths of time at 37°C (14). DNA samples were then extracted by overnight incubation at 56°C in 0.1 ml of lysis buffer (50 mM Tris–HCl at pH 7.5, 10 mM EDTA, 150 mM NaCl, 50 μg/ml of freshly made proteinase K). These DNA samples were recovered by isopropanol precipitation, resuspended in TE-RNase as described (14).

Pulsed-field gel and conventional gel analysis of DNA. Detection of large size DNA fragments was carried out by using a Bio-Rad CHEF Mapper XA Pulsed Field Electrophoresis system. DNA samples were loaded onto 1% agarose gels and run in 0.5 × TBE buffer (89 mM Tris–borate, 89 mM boric acid and 2 mM EDTA at pH 8.0) at 14°C for 20 h at a voltage gradient of 6V/cm with a linear switch interval ramp from 0.5 to 45.0 s. Parameters were chosen to optimally separate 10 kb to 500 kb DNA fragments (13). Detection of oligonucleosomal size DNA fragments was carried by using 1.0% agarose gels (14). All DNA samples were visualized by ethidium bromide staining.

Histological analysis. Histological examination was performed on representative tissues from all major organ systems as described previously (21). Tissues were fixed in 10% neutral buffered formalin, dehydrated through a gradient of alcohols and embedded in paraffin. Sections were cut at a thickness of 4–5 μm and stained with haematoxylin and eosin. Age-matched DFF45 mutant and wild-type control litter-mates were used in the study. Sections were examined and photographed under light microscopy using different magnifications.

RESULTS AND DISCUSSION

Lack of obvious 50 kilobase pair DNA fragments in DFF45 deficient thymocytes upon apoptotic exposure. It has been reported that prior to oligonucleosomal size DNA fragmentation, chromosomal DNA degrades into 50 kb size fragments upon activation of apoptosis (12). More recently, Cidlowski and colleagues demonstrated that cyclophilin C can produce 50 kb DNA fragments but not oligonucleosomal DNA fragments (13). While the physiological importance of the initial DNA degradation into 50 kb sizes remains unknown, we investigated whether such DNA degradation depends on a functional DFF45. We treated DFF45 deficient and wild-type control thymocytes for different lengths of time with etoposide and separated the resulting DNA samples together with those from untreated thymocytes using pulsed-field gel electrophoresis as described (13). As shown in Fig. 1A, etoposide induced

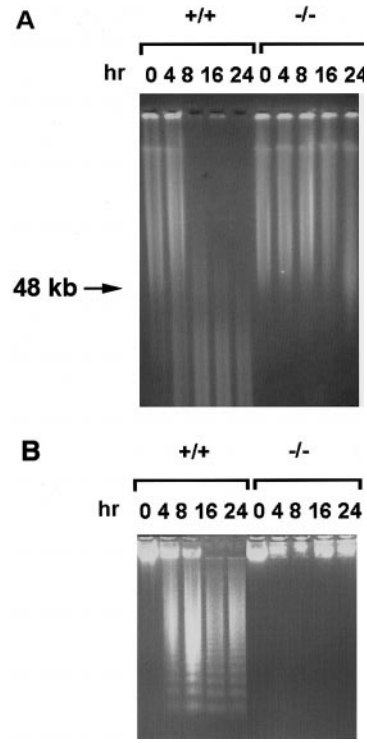

FIG. 1. Lack of obvious 50 kilobase pair size DNA fragments in DFF45 deficient thymocytes upon activation of apoptosis. Wild-type (+/+) and DFF45 deficient (−/−) thymocytes (n = 8 mice each) were isolated and treated with etoposide (50 μM) for different lengths of time. DNA samples were extracted. Pulsed-field gel electrophoresis of the DNA was performed using the Bio-Rad CHEF Mapper XA system in 0.5 × TBE buffer at 14°C for 20 h at a voltage gradient of 6 V/cm (A). Conventional gel electrophoresis was performed using 1.0% agarose gels (B). All DNA samples were visualized by ethidium bromide staining. The lambda phage DNA was used as a molecular weight marker. h, hour.

abundant DNA fragmentation after 8 h of exposure in wild-type control thymocytes. In sharp contrast, etoposide treatment produced very little 50 kb DNA fragments in DFF45 deficient thymocytes at this time point. To be certain that etoposide was effective in inducing apoptosis, we also checked the DNA samples from the above treated thymocytes for oligonucleosomal size DNA fragmentation using conventional gel electrophoresis. Etoposide treatment produced an expected time-dependent increase in DNA fragmentation in the wild-type thymocytes but not in the DFF45 deficient thymocytes (Fig. 1B). Thus, our results suggest that a functional DFF45 is required for cleavage of DNA into 50 kb size fragments during apoptosis.

To strengthen this conclusion, we performed similar pulsed-field gel electrophoresis experiments using either dexamethasone or staurosporine as additional apoptotic stimuli. Whereas both dexamethasone and staurosporine induced abundant DNA degradation after 8 h of exposure in wild-type control thymocytes, they failed to induce any DNA degradation in DFF45

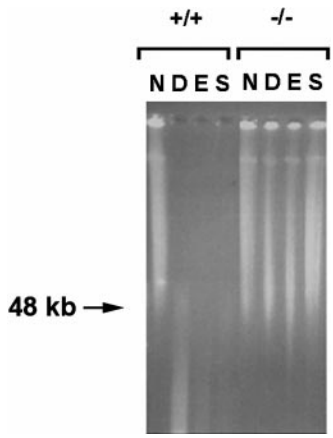

FIG. 2. Lack of obvious 50 kilobase pair size DNA fragments in DFF45 deficient thymocytes upon activation of apoptosis. Wild-type and DFF45 deficient thymocytes (n = 8 mice each) were treated with etoposide (50 μM), dexamethasone (1 μM) or staurosporine (2 μM) for 24 h. Pulsed-field gel electrophoresis of DNA was performed and DNA samples were visualized by ethidium bromide staining as in Fig. 1. N, no treatment; D, dexamethasone; E, etoposide; S, staurosporine.

deficient thymocytes after the same apoptotic treatments (data not shown). Indeed, unlike in the wild-type thymocytes, etoposide, dexamethasone or staurosporine treatment all failed to induce the appearance of 50 kb DNA fragments in DFF45 deficient thymocytes even after 24 h of apoptotic exposure (Fig. 2). Additional experiments showed that both dexamethasone and staurosporine produced a time-dependent increase in oligonucleosomal size DNA fragmentation in wild-type thymocytes but not in DFF45 deficient thymocytes, indicating the effectiveness of the apoptotic stimuli (data not shown).

Our findings suggest that a functional DFF45 is required for cleavage of DNA into both 50 kb size and oligonucleosomal size fragments during apoptosis in thymocytes. Nagata and colleagues reported that a caspase-resistant DFF45/ICAD can inhibit both large-scale (50–200 kb) and oligonucleosomal DNA fragmentation in staurosporine-treated Jurkat cells (22). More recently, the same group reported in primary thymocytes isolated from a DFF45/ICAD dominant-negative transgenic mouse, 50 kb DNA fragments could be barely detected after 6 h of glucocorticoid treatment (23). Thus, inhibition of the endogenous DFF45 activity at least attenuated the appearance of 50 kb DNA fragments upon activation of apoptosis. These results from the dominant-negative studies are consistent with our findings.

How might DFF45 function in influencing the production of 50 kb size DNA fragments during apoptosis is currently unknown. One possibility is that DFF45 may somehow mediate signaling events to the endonuclease(s) responsible for the large size DNA degradation upon activation of apoptosis in the thymus. Alternatively, DFF45 may be an integral part of a large nuclease complex responsible for cleavage of DNA into both 50 kb size and oligonucleosomal size fragments during apoptosis. Due to the complexity of the mammalian system, different cell types may also use a slightly different machinery during apoptosis. Whether DFF45 is required for cleavage of DNA into 50 kb size fragments during apoptosis in other tissues and in other species remain to be investigated.

Normal development and homeostasis of major organ systems in DFF45 mutant mice. We previously showed that DFF45 mRNA is ubiquitously expressed throughout mouse development (16). Moreover, thymocytes from DFF45 mutant mice exhibit slower rate of apoptosis than wild-type control thymocytes in response to multiple apoptotic stimuli. To investigate the possible *in vivo* consequences of decreased DNA fragmentation and cell death on major organ systems in mouse development and tissue homeostasis, we performed a systematic histological survey of all the major internal organs using DFF45 mutant mice either two months or fifteen months of age. In two months old DFF45 mutant mice, there were no significant alterations including hypertrophy in brain, muscle, eye, thymus, spleen, liver, heart, lung, kidney, and colon organs when compared to those from the litter-mate control mice (Figs. 3A–3J). No major differences in uteri and testes were observed although subtle changes in spermatogenesis may exist in the testes from the DFF45 mutant mice (data not shown). A more detailed analysis of spermatogenesis is currently underway to determine the significance of these changes in DFF45 mutant mice. Thus, the major organ systems developed normally despite defects in proper DNA fragmentation and cell death in response to apoptotic stimuli.

In fifteen months old DFF45 mutant mice (n = 4), there were again no significant alterations including hypertrophy in any of the above organs when compared to those from the litter-mate control mice. Two DFF45 mutant females had successfully given births to about 10 litters each and they were pregnant at the time of histological analysis, indicating they were still capable of reproducing at this age. The only notable changes we observed in the DFF45 mutant mice were severe perivascular lymphocyte cuffing in the salivary gland and lung. There were also mild to moderate lymphocyte cuffing in kidney and liver of the mutant mice. Finally, there were multifocal areas of mineralization in the uterine myometrium in the DFF45 mutant females. These are common aging changes of mice. Therefore, the DFF45 mutation apparently does not significantly affect tissue homeostasis in the mutant mice.

Our histological analyses indicate that DFF45 is not indispensable for the development and tissue homeostasis of virtually all major organ systems *in vivo*. There are two possible reasons for our findings. One

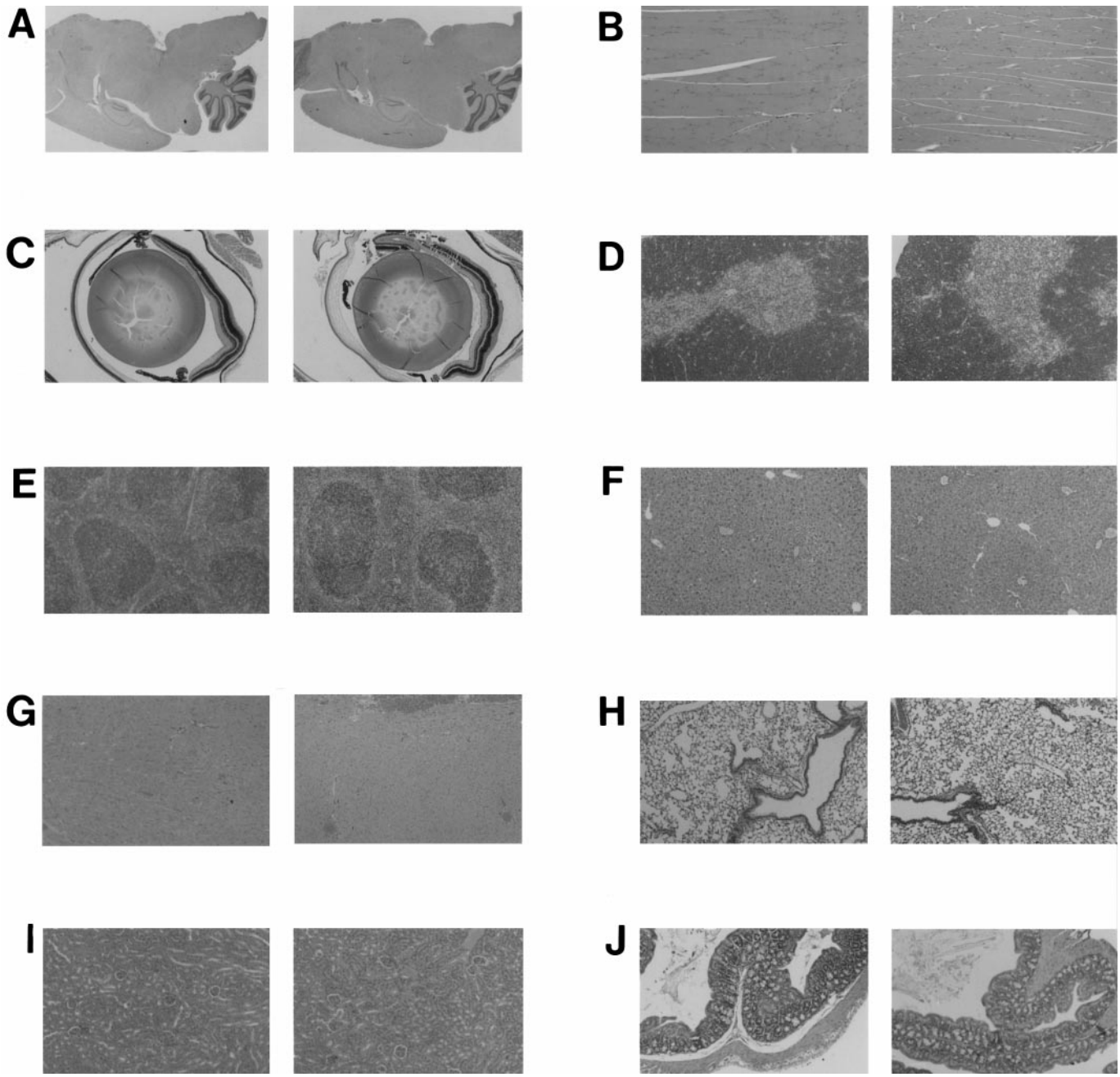

FIG. 3. Normal development of major organs in DFF45 mutant mice. Hematoxylin and eosin (H&E)-stained sections from different organs from wild-type control ($n = 2$) and DFF45 mutant mice ($n = 4$) two months of age. A, brain; B, muscle; C, eye; D, thymus; E, spleen; F, liver; G, heart; H, lung; I, kidney; J, colon. Wild-type control sections are on the left and mutant sections are on the right. Magnifications: A, 2.5×; B, 25×; C, 10×; D–J, 25×.

possibility is that altered DNA fragmentation and cell death are not detrimental to development and tissue homeostasis of these systems. Studies of apoptotic regulating or executioner molecules, such as p53, caspases-1, 2 and 11, have shown that a lack of any of these molecules individually does not significantly affect mouse development despite their key roles in apoptosis (24).

A more likely possibility is that there may be additional ways to degrade chromosomal DNA from dying cells in DFF45 mutant mice. Nagata and colleagues recently reported that, in a dominant-negative DFF45 transgenic mouse in which the DFF45 activity is inhibited, DNA fragmentation in phagocytes could occur and DNase II is the likely endonuclease for degrading DNA after the apoptotic cells have been engulfed (23). Hor-

vitz and colleagues also showed that the completion of DNA degradation requires activity from engulfing cells and NUC-1 (25). NUC-1 is a homolog of DNase II and it plays an important role in an intermediate step of DNA degradation upon activation of apoptosis in C. elegans (25). Moreover, candidate apoptotic endonucleases, such as DNase I (26), inducible-lymphocyte Ca^{2+}/Mg^{2+}-dependent endonuclease (27), cyclophilins (13), as well as yet to be identified nucleases could all contribute to DNA fragmentation during apoptosis *in vivo*. The DFF45 mutant mice provide a very useful model system to address the significance of these systems in DNA degradation during apoptosis in the future.

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REFERENCES

1. Ellis, R. E., Yuan, J. Y., and Horvitz, H. R. (1991) Mechanisms and functions of cell death. *Ann. Rev. Cell Biol.* **7,** 663–698.
2. Raff, M. C. (1992) Social controls on cell survival and cell death. *Nature* **356,** 397–400.
3. White, E. (1996) Life, death and pursuit of apoptosis. *Genes Dev.* **10,** 1–15.
4. Jacobson, M. D., Weil, M., and Raff, M. C. (1997) Programmed cell death in animal development. *Cell* **88,** 347–354.
5. Thompson, C. B. (1995) Apoptosis in the pathogenesis and treatment of disease. *Science* **267,** 1456–1462.
6. Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. (1972) Apoptosis: A basic biological phenomenon with wide-ranging implication in tissue kinetics. *Br. J. Cancer* **26,** 239–257.
7. Wyllie, A. H. (1980) Glucocorticoid induced thymocyte apoptosis is associated with endogeneous endonuclease activation. *Nature* **284,** 555–556.
8. Liu, X., Zou, H., Slaughter, C., and Wang, X. (1997) DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell* **89,** 175–184.
9. Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A., and Nagata, S. (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature* **391,** 43–50.
10. Liu, X., Li, P., Widlak, P., Zou, H., Luo, X., Garrard, W. T., and Wang, X. (1998) DFF40 induces DNA fragmentation and chromatin condensation during apoptosis. *Proc. Natl. Acad. Sci. USA* **95,** 8461–8466.
11. Sakahira, H., Enari, M., and Nagata, S. (1998) Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* **391,** 96–99.
12. Oberhammer, F., Wilson, J. W., Dive, C., Morris, I. D., Hickman, J. A., Wakeling, A. E., Walker, P. R., and Sikorska, M. (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,** 3679–3684.
13. Montague, J. W., Hughes, F. M., Jr., and Cidlowski, J. A. (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,** 6677–6684.
14. Zhang, J., Liu, X., Scherer, D. C., Van Kaer, L., Wang, X., and Xu, M. (1998) Resistance to DNA fragmentation and chromatin condensation in mice lacking the DNA fragmentation factor 45. *Proc. Natl. Acad. Sci. USA* **95,** 12480–12485.
15. Cory, S. (1998) Cell death throes. *Proc. Natl. Acad. Sci. USA* **95,** 12077–12079.
16. Zhang, J., Wang, X., Bove, K. E., and Xu, M. (2000) 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,** 37450–37454.
17. Peitsch, M. C., Mannherz, H. G., and Tschopp, J. (1994) The apoptosis endonucleases: Cleaning up after cell death? *Trends Cell Biol.* **4,** 37–41.
18. Bortner, C. D., Oldenburg, N. B., and Cidlowski, J. A. (1995) The role of DNA fragmentation in apoptosis. *Trends Cell Biol.* **5,** 21–27.
19. Nagata, S. (2000) Apoptotic DNA fragmentation. *Exp. Cell Res.* **256,** 12–18.
20. Slane, J., Lee, H., Vorhees, C., Zhang, J., and Xu, M. (2000) DNA fragmentation factor 45 deficient mice exhibit enhanced spatial learning and memory compared to wild-type control mice. *Brain Res.* **867,** 70–79.
21. Boivin, G. P., Ormsby, H., Jones-Carson, J., O'Tool, B. A., and Doetschman, T. (1997) Germ-free and barrier-raised TGFβ1-deficient mice have similar inflammatory lesions. *Transgenic Res.* **6,** 197–202.
22. Sakahira, H., Enari, M., Ohsawa, Y., Uchiyama, Y., and Nagata, S. (1999) Apoptotic nuclear morphological change without DNA fragmentation. *Current Biology* **9,** 543–546.
23. McIlroy, D., Tanaka, M., Sakahira, H., Fukuyama, H., Suzuki, M., Yamamura, K.-i., Ohsawa, Y., Uchiyama, Y., and Nagata, S. (2000) An auxiliary mode of apoptotic DNA fragmentation provided by phagocytes. *Genes Dev.* **14,** 549–558.
24. Vaux, D. L., and Korsmeyer, S. J. (1999) Cell death in development. *Cell* **96,** 245–254.
25. Wu, Y.-C., Stanfield, G. M., and Horvitz, H. R. (2000) NUC-1, a Caenorhabditis elegans DNase II homolog, functions in an intermediate step of DNA degradation during apoptosis. *Genes Dev.* **14,** 536–548.
26. Peitsch, M. C., Polzar, B., Stephan, H., Crompton, T., MacDonald, H. R., Mannherz, H. G., and Tschopp, J. (1993) Characterization of the endogenous deoxyribonuclease involved in nuclear DNA degradation during apoptosis (programmed cell death). *EMBO J.* **12,** 371–377.
27. Khodarev, N. N., and Ashwell, J. D. (1996) An inducible lymphocyte nuclear Ca2+/Mg(2+)-dependent endonuclease associated with apoptosis. *J. Immunol.* **156,** 922–931.