complete adjuvant on day 21 and an intravenous injection of 25 μg of nitrated α-syn in phosphate-buffered saline on day 48. On day 51, the spleen was removed and the lymphocytes were fused to myeloma cells (line Sp2/0-Ag14) by using polyethylene glycol 1500.
24. Y. Z. Ye, M. Strong, Z. Q. Huang, J. S. Beckman, *Methods Enzymol.* **269**, 201 (1996).
25. B. I. Giasson, unpublished observations.
26. C. F. Lippa, M. L. Schmidt, V. M.-Y. Lee, J. Q. Trojanowski, *Ann. Neurol.* **45**, 353 (1999).
27. J. E. Duda, unpublished observations.
28. B. I. Giasson *et al.*, *J. Neurosci. Res.* **59**, 528 (2000).
29. D. W. Dickson *et al.*, *Am. J. Pathol.* **155**, 1241 (1999).
30. B. I. Giasson *et al.*, unpublished observation.
31. J. M. Souza, B. I. Giasson, Q. Chen, V. M.-Y. Lee, H. Ischiropoulos, *J. Biol. Chem.* **275**, 18344 (2000).
32. M. A. Smith, P. L. Rickey Harris, L. M. Sayre, J. S. Beckman, G. Perry, *J. Neurosci.* **17**, 2653 (1997).
33. P. F. Good, P. Werner, A. Hsu, C. W. Olanow, D. P. Perl, *Am. J. Pathol.* **149**, 21 (1998).
34. M. L. Schmidt *et al.*, *Am. J. Pathol.* **139**, 53 (1991)
35. J. Q. Trojanowski, T. Schuck, M. L. Schmidt, V.M-Y. Lee, *J. Histochem. Cytochem.* **37**, 207 (1989).
36. B. I. Giasson, K. Uryu, J. Q. Trojanowski, V. M.-Y. Lee, *J. Biol. Chem.* **274**, 7619 (1999).
37. As described previously (*18*, *34*), blocks of cingulate cortex, hippocampus, and midbrain from postmortem DLB and LBVAD brians, as well as cerebellar white matter from MSA brains and globus pallidus from an NBIA1 brain, were immersion-fixed in 70% ethanol with 150 mM NaCl for 24 to 36 hours. The samples were dehydrated through a series of graded ethanol steps to xylene at room temperature and infiltrated with paraffin at 60°C as described (*35*) and then cut into multiple, near-serial 6-μm sections for immunohistochemistry by incubating the sections with primary antibody at 37°C for 90 min or at 25°C overnight followed by application of avidin-biotin complex (ABC) system (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA) and the chromagen 3,3′-diaminobenzidine (DAB) to visualize immunolabeled structures in sections lightly counterstained with hematoxylin.
38. E. M. Rodriguez, R. Yulis, B. Peruzzo, G. Alvial, R. Andrade, *Histochemistry* **81**, 253 (1984).
39. Gray matter (0.5 g) from the cingulate gyrus or the amygdala of LBVAD and control brains was dissociated with a Dounce homogenizer by using 10 ml of HS buffer [50 mM tris (pH 7.4), 750 mM NaCl, 10 mM NaF, 5 mM EDTA with protease inhibitors] per gram of tissue and sedimented at 100,000*g* for 30 min. The resulting pellets were reextracted with 10 ml of HS buffer–0.5 % Triton X-100 per gram of tissue. After centrifugation, the pellets were resuspended in 1.0 M sucrose–HS buffer and layered on a discontinuous 1.2 M/1.5 M/2.2 M sucrose gradient. After centrifugation at 200,000*g* for 2 hours, each fraction was assayed by Western blot analysis to determine the presence of α-syn. Most of the α-syn was located at the 1.5 M/2.2 M sucrose interphase (250 μl), and this fraction was used to assay for the presence of nitrated protein. Ten microliters of HS-soluble fractions and 1.5 M/2.2 M sucrose-interphase fractions, termed HS/Triton-insoluble, were loaded on separate lanes of 12% polyacrylamide gels for Western blot analysis.
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Role of *BAX* in the Apoptotic Response to Anticancer Agents

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To assess the role of *BAX* in drug-induced apoptosis in human colorectal cancer cells, we generated cells that lack functional *BAX* genes. Such cells were partially resistant to the apoptotic effects of the chemotherapeutic agent 5-fluorouracil, but apoptosis was not abolished. In contrast, the absence of *BAX* completely abolished the apoptotic response to the chemopreventive agent sulindac and other nonsteroidal anti-inflammatory drugs (NSAIDs). NSAIDs inhibited the expression of the antiapoptotic protein Bcl-X_L, resulting in an altered ratio of BAX to Bcl-X_L and subsequent mitochondria-mediated cell death. These results establish an unambiguous role for *BAX* in apoptotic processes in human epithelial cancers and may have implications for cancer chemoprevention strategies.

The induction of apoptosis, or programmed cell death, in cancer cells is thought to be fundamental to the success of treatments for cancer. The *Bcl-2* family members are intimately involved in the apoptosis (*1*, *2*), but the role of these proteins in drug-induced death has been confusing. *BAX*, the prototypic death-promoting member of the *Bcl-2* family, provides a good example of the complications that have arisen. Many studies have relied on overexpression of BAX protein, conditions that may not faithfully reproduce its normal activity, and have yielded conflicting results (*3*). Drugs induce endogenous *BAX* expression through p53-dependent transcription in some cancer cell lines, but not others (*4*). In mice, *BAX* plays no role in the most well-studied examples of drug- or radiation-induced and p53-dependent apoptosis, involving thymocytes and intestinal epithelium (*5*–*7*). Unlike the human gene, the murine *BAX* gene has no p53-binding site in its promoter (*8*). Nevertheless, *BAX* deficiency promotes drug resistance in murine fibroblasts by partially attenuating p53-dependent apoptosis, but only when such cells are transformed with the adenoviral *E1A* oncogene (*3*, *9*). This picture is further confounded by the finding that *BAX* deficiency can promote rather than inhibit apoptosis in some murine cell types (*10*).

The most important targets of chemotherapeutic agents are human epithelial cells, which give rise to the vast majority of naturally occurring cancers. To clarify the role of *BAX* in drug-induced apoptosis in such cells, we created and studied isogenic derivatives that differ only in the presence or absence of the *BAX* gene. HCT116 colorectal cancer cells were chosen as the parental cells because they contain normal *p53* and *BAX* genes and undergo apoptosis in response to 5-fluorouracil (5-FU) and sulindac. 5-FU is the mainstay of treatment for colorectal cancer and is an antimetabolite that induces cell death in a p53-dependent manner (*11*). Sulindac is the prototypic chemopreventive agent for patients with colorectal cancer predisposition and is a nonsteroidal anti-inflammatory drug (NSAID) that binds to and inhibits cyclooxygenases and other cellular proteins (*12*–*14*).

To obtain isogenic cells differing in *BAX* status, we first exploited the innate propensity of mismatch repair (MMR)–deficient cells to mutate mononucleotide tracts (*15*). *BAX* contains an unstable G_8 tract at nucleotides 114 to 121 (codons 38 to 41) that is often mutated in MMR-deficient tumors (*16*). Through analysis of unselected subclones (*17*), we found that 2% of HCT116 cells had two intact *BAX* alleles, 94% had one intact allele (+/−, with one allele containing a deletion or insertion of a G within the G_8 tract), and 4% had two mutant alleles (−/− cells). Western blots confirmed the absence of BAX protein in the $BAX^{-/-}$ cells (Fig. 1A). In $BAX^{+/+}$ and $BAX^{+/-}$ cells, there was a slight induction of BAX protein by agents that activate p53 (Fig. 1A). Induction of p53 by 5-FU caused equivalent amounts of apoptosis in $BAX^{+/+}$ and heterozygous $BAX^{+/-}$ cells. The extent of apoptosis was somewhat reduced in the $BAX^{-/-}$ cells, but *BAX* deficiency did not recapitulate the effects of *p53* deficiency, which provided nearly complete protection against 5-FU–induced apoptosis (Fig. 1B) (*11*). In marked contrast, *BAX* deficiency completely eliminated the apoptosis induced by sulindac (Fig. 1B). In this case, *p53* deficiency had no effect, indicating that the apoptosis induced by sulindac was *p53*-independent. A similar effect of BAX deficiency was observed on the apoptosis in-

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duced by indomethacin, another NSAID, but little protection was observed against ceramide, a non-NSAID inducer of apoptosis [Fig. 1B and Web fig. 1 (*18*)].

To demonstrate that *BAX* deficiency was responsible for the observed effects, we prospectively inactivated the wild-type (WT) *BAX* allele in a *BAX* heterozygote. A targeting vector was constructed (Fig. 2A) and transfected into HCT116 cells for this purpose (*19*). We recovered two clones in which the remaining WT *BAX* allele had been targeted (KO1 and KO2) (*19*), as verified by Southern and Western blotting (Fig. 2B) and DNA sequencing [Web fig. 2 (*18*)]. Treatment of these cells with 5-FU and NSAIDs (Fig. 2C) revealed that they behaved identically to the $BAX^{-/-}$ clones shown in Fig. 1; deletion of *BAX* provided slight protection against ceramide-induced apoptosis [Web fig. 3 (*18*)], partial protection against 5-FU–induced apoptosis (Fig. 2C), and profound protection against the apoptosis induced by the NSAIDs sulindac and indomethacin (Fig. 2C). Controls for these experiments were provided by heterozygous sister clones that arose after transfection with the targeting vectors; the behavior of these $BAX^{+/-}$ clones was identical to that of the HCT116 parental cells, as expected (Fig. 2C).

If *BAX* deficiency so profoundly affects the sensitivity to NSAIDs, one might expect that parental cell populations treated with NSAIDs would be enriched in cells with mutations of *BAX*. This hypothesis was tested by recovery of the clones growing after indomethacin treatment (Fig. 3A). Of 60 clones analyzed, 70% (42 out of 60) had insertions or deletions in the G_8 tracts of both *BAX* alleles, as compared with only 4% (4 out of 96) in the parental population ($P < 10^{-12}$, χ^2 test). The clones with *BAX* mutations were resistant to both indomethacin and sulindac (Fig. 3B).

What are the mechanisms by which NSAIDs cause *BAX*-dependent apoptosis? Previous studies have shown that alterations in the ratio between proapoptotic and antiapoptotic members of the *Bcl-2* family, rather than the absolute expression level of any single *Bcl-2* family member, can determine apoptotic sensitivity (*20*). Bcl-2 was not detectably expressed before or after treatment with NSAIDs, but the expression of the antiapoptotic Bcl-X_L protein was substantially reduced by NSAIDs, both in parental HCT116 cells and in their $p53^{-/-}$ derivatives (Fig. 4A). The inhibition of *Bcl-X_L* expression was also observed at the RNA level [Web fig. 4 (*18*)], suggesting a transcriptional basis for this decrease.

The hypothesis that the ratio between BAX and Bcl-X_L proteins plays a major role in sensitivity to NSAIDs led to several testable predictions. First, this ratio should change in other colorectal cancer cell lines

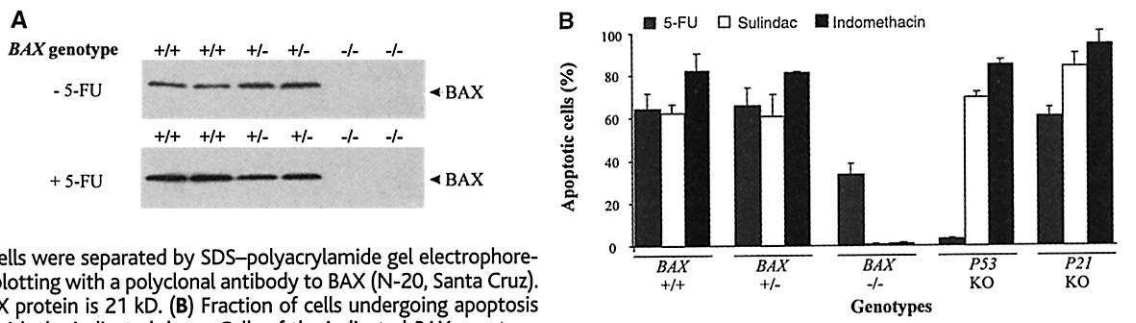

Fig. 1. Drug effects on cells of varying *BAX* genotypes. (**A**) BAX protein expression in cells treated with 5-FU. HCT116 cells with the indicated *BAX* genotypes were treated for 48 hours with 5-FU (375 μM, Roche). Equal amounts of total cellular proteins from these cells were separated by SDS–polyacrylamide gel electrophoresis and subjected to immunoblotting with a polyclonal antibody to BAX (N-20, Santa Cruz). The molecular size of the BAX protein is 21 kD. (**B**) Fraction of cells undergoing apoptosis after 48 hours of treatment with the indicated drugs. Cells of the indicated *BAX* genotype were tested, along with cells in which *p53* or *p21* genes were disrupted through gene targeting (*p53* KO and *p21* KO, respectively) (*24*). 5-FU (Roche), sulindac sulfide (Merck), and indomethacin (Sigma) were used at final concentrations of 375, 120, and 500 μM, respectively. Cells (attached plus those floating in the medium) were harvested at varying time points after drug treatment and fixed in a solution containing final concentrations of 3.7% formaldehyde, 0.5% Nonidet P-40, and Hoescht 33258 (10 μg/ml) in phosphate-buffered saline. Apoptosis was assessed through microscopic visualization of condensed chromatin and micronucleation after staining of cells with DAPI (*25*). At least two independent experiments were carried out for each condition, and a minimum of 300 cells were counted for each measurement. The sulindac and indomethacin responses of the *BAX*-deficient cells were significantly different from the other cells tested ($P < 10^{-8}$, Student's *t* test).

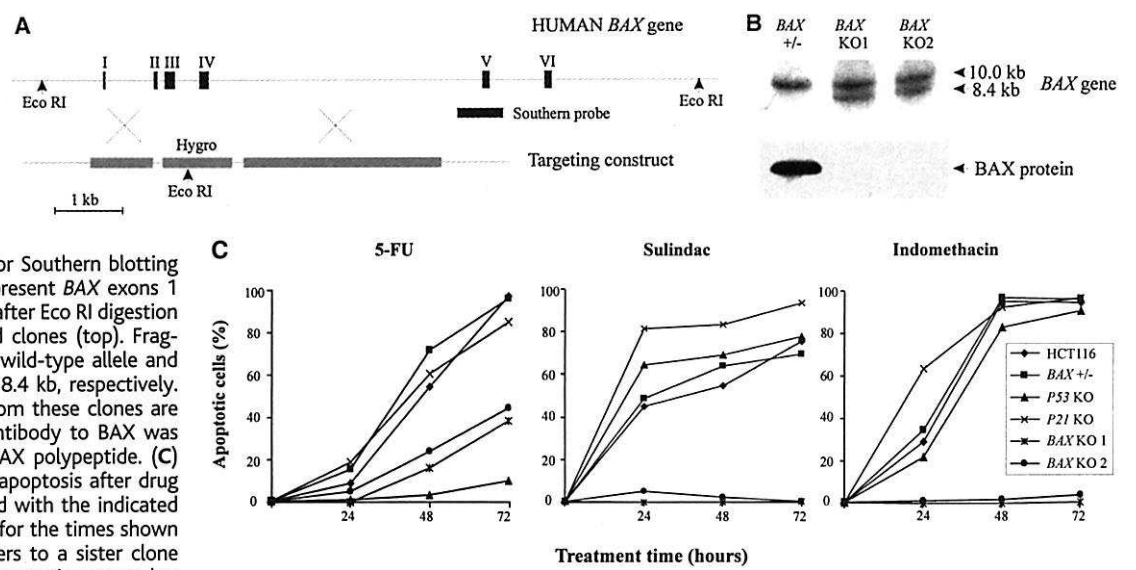

Fig. 2. Targeted deletion of *BAX*. (**A**) *BAX* genomic locus and the targeting construct. The targeting construct consists of two homologous arms and a hygromycin-resistant gene (Hygro). The Eco RI sites within the *BAX* gene and the targeting construct and position of the probe used for Southern blotting are shown. Boxes I to VI represent *BAX* exons 1 through 6. (**B**) Southern blot after Eco RI digestion of genomic DNA of selected clones (top). Fragments corresponding to the wild-type allele and targeted allele are 10.0 and 8.4 kb, respectively. Western blots of proteins from these clones are shown at the bottom; an antibody to BAX was used to detect the 21-kD BAX polypeptide. (**C**) Fraction of cells undergoing apoptosis after drug treatment. Cells were treated with the indicated drugs as described in Fig. 1B for the times shown on the *x* axis. "$BAX^{+/-}$" refers to a sister clone isolated from the transfection experiment used to generate the knockout clones. The fraction of apoptotic cells was assessed by fluorescence microscopy of DAPI-stained cells.

treated with sulindac. This prediction was tested in a total of eight cell lines. Despite the heterogeneity in response to sulindac previously noted in such lines (*21*), we found that five of eight lines responded similarly, with no increase in BAX but substantial increases in the ratio of BAX:Bcl-X_L due to decreases in Bcl-X_L expression (Fig. 4B). Second, NSAIDs with little structural similarity to sulindac should induce similar changes in the ratio between BAX and Bcl-X_L; this prediction was confirmed by analysis of cells treated with indomethacin (Fig. 4B). Third, the change in BAX/Bcl-X_L ratio should cause apoptosis through a mitochondrial pathway (*1*, *2*). This pathway was indeed affected in sulindac-treated cells, as the mitochondrial membrane potential was disrupted [Web fig. 5 (*18*)], caspase 9 was activated (Fig. 4C and Web fig. 6), and DNA was subsequently degraded in cells with intact *BAX* genes but not in *BAX*-deficient cells [Web figs. 7 and 8 (*18*)]. Fourth, a biologically significant difference in NSAID sensitivity should be reflected in standard colony-formation assays, in which not just apoptosis but all parameters related to cellular growth and death can be simultaneously assessed. We found that *BAX* disruption led to a profound difference in sensitivity to NSAIDs, whereas disruptions of *p53* or *p21* had no effect in standard colony-formation assays (Fig. 4D and Web figs. 9 and 10 (*18*)]. Finally, the forced expression of Bcl-X_L should rescue cells with

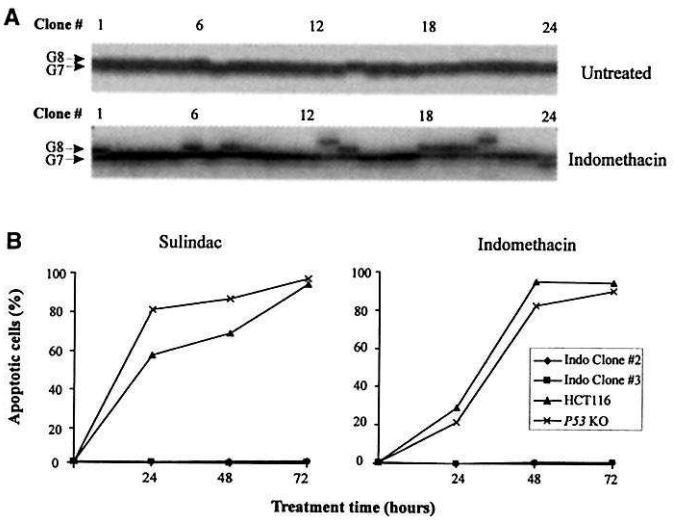

Fig. 3. Cells selected for resistance to indomethacin contain *BAX* mutations. **(A)** HCT116 cells were treated with indomethacin (500 μM) for 72 hours. Cells were harvested and plated into 96-well plates without drug. Single clones were recovered and expanded. PCR analysis of the *BAX* gene shows that 17 out of 24 clones that arose after indomethacin treatment contained mutations of both *BAX* alleles (lower panel), whereas such mutations were rare in clones that arose in the absence of indomethacin treatment (upper panel). Most mutants had a deletion of a G within the normal G_8 tract, whereas clones 13 and 21 had an insertion (creating G_9) and clone 24 had a 2–base pair deletion (creating G_6). **(B)** Clones 2 and 3 (labeled Indo Clone #2 and #3) were expanded and tested for apoptosis after indomethacin treatment, along with control clones of the indicated genotypes.

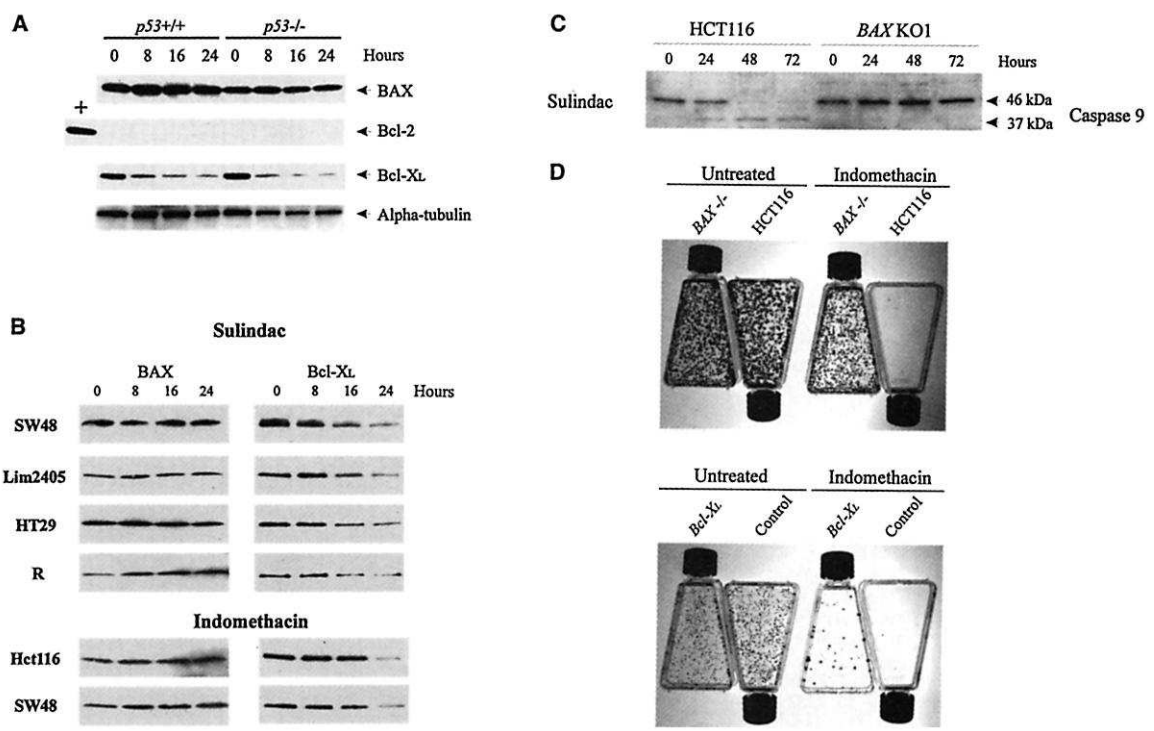

Fig. 4. Mechanisms underlying *BAX*-dependent, NSAID-induced apoptosis. **(A)** Expression of *Bcl-2* family proteins after sulindac treatment. Parental HCT116 cells and *p53* knockout cells were treated with sulindac sulfide (120 μM). Equal amounts of total cellular proteins collected at the indicated time points were analyzed by immunoblotting with antibodies against BAX, Bcl-2 (N-19, Santa Cruz), Bcl-X_L (Transduction Laboratories), and α-tubulin (TU-02, Santa Cruz) as loading control. The lane labeled "+" represents protein from HL-60 cells, which normally express Bcl-2. **(B)** Bax and Bcl-X_L proteins from the indicated colorectal cancer cell lines were analyzed by immunoblotting after treatment with either sulindac or indomethacin. **(C)** Caspase 9 Western blot of parental and *BAX* knockout cells after treatment with sulindac for the indicated times (in hours). Equal amounts of total cellular proteins were separated and immunoblotted with a caspase 9–specific antibody (H-83, Santa Cruz), which recognizes the COOH-terminus of the protein. The molecular size of the intact caspase 9 polypeptide is 46 kD. A degraded fragment of caspase 9 (37 kD) is detected in parental cells, but not in *BAX* knockout cells. **(D)** Colony-formation assays. (Top) About 5000 cells were treated with indomethacin as described in Fig. 1B. Cells (attached plus those floating in the medium) were harvested after 72 hours of treatment and plated into T25 flasks without drug. (Bottom) Cells were transfected with a Bcl-X_L expression vector or a control (empty) vector and treated with zeocin (to select for transfectants) or zeocin plus indomethacin for 48 hours (*26*). In all cases, clones were visualized by crystal violet staining 7 to 10 days later, and the results shown are representative of at least three independent experiments. *BAX* gene disruption resulted in a greater than 100-fold increase in colony number compared with parental cells, whereas exogenous Bcl-X_L expression resulted in a greater than 10-fold increase in colony number compared with cells transfected with the empty control vector.

intact *BAX* genes from NSAID-mediated apoptosis. This prediction was confirmed by introducing a Bcl-X_L expression vector into HCT116 cells and assessing colony formation after treatment with NSAIDs (Fig. 4D).

It was not expected that deletion of a single gene could so profoundly affect cell death in a human cancer cell. Previous experiments have shown that NSAIDs induce heterogeneous changes in human tumor cells, including growth arrest, apoptosis, and necrosis (*21*). Despite this heterogeneity, we found that most colorectal cancer cell lines exhibited markedly similar changes in BAX:Bcl-X_L ratios, whereas the others died through mechanisms that were independent of BAX:Bcl-X_L. Furthermore, the importance of *BAX* was rigorously demonstrated in HCT116 cells through three different approaches for generating cells with disrupted *BAX* genes. In addition to their implications for understanding basic determinants of drug responsiveness in human cancer cells, these results may have important clinical implications. It is currently believed that chemoprevention offers the best hope for nonsurgical management of patients with hereditary predispositions to colorectal cancer. The most common form of such predisposition is hereditary nonpolyposis colorectal cancer, which is caused by defects in mismatch repair (*22*). Our results suggest that such tumors may easily develop resistance to NSAIDs through an inherent instability in the mononucleotide tract in *BAX*. By analogy with the successful strategy used to combat the highly mutable retroviruses that cause acquired immunodeficiency syndrome (*23*), it may therefore be important to consider combinations of chemopreventive drugs, rather than single agents, in such patients.

Note added in proof: It has recently been shown that tumor cells without BAX undergo less apoptosis when xenografted in mice (*27*).

References and Notes

1. A. Gross, J. M. McDonnell, S. J. Korsmeyer, *Genes Dev.* **13**, 1899 (1999).
2. D. R. Green, J. C. Reed, *Science* **281**, 1309 (1998).
3. Reviewed in M. E. McCurrach, T. M. Connor, C. M. Knudson, S. J. Korsmeyer, S. W. Lowe, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 2345 (1997).
4. Reviewed in T. G. Huang, S. M. Ip, W. S. Yeung, H. Y. Ngan, *Eur. J. Cancer* **36**, 249 (2000).
5. C. M. Knudson, K. S. K. Tung, W. G. Tourtellotte, G. A. J. Brown, S. J. Korsmeyer, *Science* **270**, 96 (1995).
6. D. M. Pritchard, C. S. Potten, S. J. Korsmeyer, S. Roberts, J. A. Hickman, *Oncogene* **18**, 7287 (1999).
7. C. M. Coopersmith, J. I. Gordon, *Oncogene* **15**, 131 (1997).
8. T. Schmidt *et al.*, *Cell Death Differ.* **6**, 873 (1999).
9. A. Gross, J. Jockel, M. C. Wei, S. J. Korsmeyer, *EMBO J.* **17**, 3878 (1998).
10. J. Lewis *et al.*, *Nature Med.* **5**, 832 (1999).
11. F. Bunz *et al.*, *J. Clin. Invest.* **104**, 263 (1999).
12. C. Williams, R. L. Shattuck-Brandt, R. N. DuBois, *Ann. N.Y. Acad. Sci.* **889**, 72 (1999).
13. T. C. He, T. A. Chan, B. Vogelstein, K. W. Kinzler, *Cell* **99**, 335 (1999).
14. Y. Yamamoto, M. J. Yin, K. M. Lin, R. B. Gaynor, *J. Biol. Chem.* **274**, 27307 (1999).
15. Y. Ionov, M. A. Peinado, S. Malkhosyan, D. Shibata, M. Perucho, *Nature* **363**, 558 (1993).
16. N. Rampino *et al.*, *Science* **275**, 967 (1997).
17. HCT116 subclones were obtained by single-cell dilution. Genomic DNA from individual clones was isolated in 96-well plates. To measure the length of the *BAX* G-tract, we labeled polymerase chain reaction (PCR) primers with [γ^{32}P]adenosine 5′-triphosphate using T4 kinase and used them to amplify *BAX* genomic DNA fragments. The primers used for amplification were 5′- CAGTTCGTCCCCGATGCGC-3′ and 5′-AGGAGTGACACCCCGTTCTG-3′.
18. Supplemental Web material is available at *Science* Online at www.sciencemag.org/feature/data/1055633.shl.
19. An HCT116 clone heterozygous for *BAX* was transfected with the targeting construct (Fig. 2A) using Lipofectamine (Life Technologies, Rockville, MD). Twenty-four hours after transfection, cells were plated at clonal density in 96-well plates in media containing hygromycin (0.1 mg/ml). Hygromycin-resistant clones were expanded and screened for targeting events with PCR. Two PCR-positive clones were recovered from ~5000 hygromycin-resistant clones. Homologous recombination at the *BAX* locus was verified by genomic Southern blotting with the probe indicated in Fig. 2A, sequencing of the G_8-tract and surrounding nucleotides of the remaining *BAX* allele, and analysis of BAX protein expression. Details of the targeting construct and the primers used for PCR are available from the authors upon request.
20. M. G. Vander Heiden, C. B. Thompson, *Nature Cell Biol.* **1**, E209 (1999).
21. M. L. Smith, G. Hawcroft, M. A. Hull, *Eur. J. Cancer* **36**, 664 (2000).
22. H. T. Lynch, A. de La Chapelle, *J. Med. Genet.* **36**, 801 (1999).
23. S. C. Johnson, J. G. Gerber, *Adv. Intern. Med.* **45**, 1 (2000).
24. F. Bunz *et al.*, *Science* **282**, 1497 (1998).
25. T. Waldman, C. Lengauer, K. W. Kinzler, B. Vogelstein, *Nature* **381**, 713 (1996).
26. HCT116 cells were transfected with a Bcl-X_L expression plasmid (Invitrogen) or a control plasmid with no insert (pcDNA3.1/GS, Invitrogen). Twenty-four hours after transfection, cells were treated with indomethacin (500 μM) for 48 hours. About 10^7 indomethacin-treated cells and 10^5 untreated cells were inoculated in separate T25 flasks and treated with Zeocin (0.5 mg/ml, Invitrogen) for 3 days to select for transfected cells. Colonies were visualized by crystal violet staining 7 to 10 days later.
27. Y. Ionov *et al. Proc. Natl. Acad. Sci. U.S.A.* **97**, 10872 (2000).

28. We thank L. Zawel, I. Rhee, E. Carson-Walter, S. Zhou, T. Chan, and F. Bunz for experimental advice; C. Lengauer and L. Meszler for help with cell imaging; and J. Flook for assistance with fluorescence-activated cell sorting. Supported by the Clayton Fund and by NIH grants CA 43460, CA 57345, CA 62924, GM 41690, and GM07309. B.H.P. is a recipient of the Howard Hughes postdoctoral fellowship for physicians. K.W.K. received research funding from Genzyme Molecular Oncology (Genzyme). B.V. and K.W.K. are consultants to Genzyme. The university and researchers (L.Z., B.V., and K.W.K.) own Genzyme stock, which is subject to certain restrictions under university policy. The terms of these arrangements are being managed by the university in accordance with its conflict of interest policies.

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A PEST-Like Sequence in Listeriolysin O Essential for *Listeria monocytogenes* Pathogenicity

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Establishment and maintenance of an intracellular niche are critical to the success of an intracellular pathogen. Here, the pore-forming protein listeriolysin O (LLO), secreted by *Listeria monocytogenes*, was shown to contain a PEST-like sequence (P, Pro; E, Glu; S, Ser; T, Thr) that is essential for the virulence and intracellular compartmentalization of this pathogen. Mutants lacking the PEST-like sequence entered the host cytosol but subsequently permeabilized and killed the host cell. LLO lacking the PEST-like sequence accumulated in the host-cell cytosol, suggesting that this sequence targets LLO for degradation. Transfer of the sequence to perfringolysin O transformed this toxic cytolysin into a nontoxic derivative that facilitated intracellular growth.

Intracellular pathogens reside in specific cellular compartments, e.g., a modified phagosome (*Mycobacterium tuberculosis*) (*1*), the Golgi apparatus (*Chlamydia trachomatis*) (*2*), or the cytosol (*L. monocytogenes*) (*3*). How pathogens establish and maintain these intracellular niches is the essence of pathogenesis. Although we know little of the molecular mechanisms by which intracellular pathogens achieve compartmentalization, one emerging theme is that pathogens exploit the existing cellular machinery of the host (*4*).

The secreted pore-forming protein LLO of the facultative intracellular bacterial pathogen *L. monocytogenes* is an essential virulence determinant that allows the bacterium to escape from the host vacuole and reach the host cytosol (*5*). Although LLO is produced by bacteria in both the cytosol and the vacuole, LLO activity is restricted to the vacuolar

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