

Life and death in paradise



Or Gozani, Michael Boyce, Lina Yoo, Philip Karuman and Junying Yuan

Over 500 researchers participated in a recent American Association for Cancer Research special conference, entitled "Apoptosis and Cancer: Basic Mechanisms and Therapeutic Opportunities in the Post-Genomic Era" (February 13–17, 2002) in sunny Hawaii (Hilton Waikoloa village, Kona, Hawaii). The meeting participants presented the most recent findings on the mechanisms regulating cell death in cancer. In the past decade, apoptosis research has undergone a quantum leap, metamorphosing from a descriptive, phenomenological discipline into a molecularly defined, highly complex signalling field. This transformation was highlighted in the conference's opening talk by meeting co-organizer, John Reed (The Burnham Institute, La Jolla, CA). Reed and colleagues used published protein functional information and bio-informatic mining of the available human genome databases to tabulate the number of human proteins predicted to be involved in regulating apoptosis. The list includes 11 catalytically active caspases, 26 CARD (caspase associated recruitment domain)-, 32 DD (death domain)-, 12 DED (death effector domain)-, 8 BIR (baculovirus inhibitor of apoptosis protein region)-, 24 BH (Bcl-2 homology)-, and 34 PAAD/PYD (pyrin/PAAD)-containing sequences.

The founding paradigms of the apoptosis field are based largely on original genetic analysis of the nematode *C. elegans*¹. This elegant work established that the basic genetic program responsible for the programmed deaths of 131 somatic cells during *C. elegans* development involves only four genes: *egl-1*, *ced-3*, *ced-4* and *ced-9*. Surprisingly, recent analyses of germline apoptosis in *C. elegans* revealed that additional mechanisms of cell death regulation exist. Indeed, although germline apoptosis requires *ced-3*, *ced-4* and *ced-9* (ref. 2), *egl-1* is not essential. Instead, in germline apoptosis, the Ras/mitogen-activated protein kinase (MAPK) pathway and DNA damage response genes can carry out an *egl-1*-like function². In this meeting, Michael Hengartner (University of Zurich, Zurich, Switzerland) reported that the checkpoint genes *rad-1* and *hus-1* are critical for the DNA damage-induced cell death of germ cells, through upregulation of *egl-1* in a p53-independent fashion. These data indicate that *egl-1* can participate in environmentally-induced apoptosis. Overall, these results are consistent with mammalian studies demonstrating that diverse upstream signalling pathways converge to activate the core components of cell death machinery.

The genetic analysis of *Drosophila* has also provided considerable insight into the mechanisms of apoptosis. Interestingly, in addition to the canonical apoptotic machinery, *Drosophila* has three pro-apoptotic proteins, named Reaper, Hid and Grim, which all reside on the well-known H99 chromosomal locus. The death-inducing activity of these three proteins lies within a four amino acid stretch (consensus: A (V/I/T) (P/A) (F/Y/V/I)^{3,4}) at the amino terminus, known as the IBM (IAP Binding Motif) or RHG (Reaper, Hid and Grim) motif^{5,6}. In this meeting, John Abrams (UT Southwestern Medical Center, Dallas, TX)

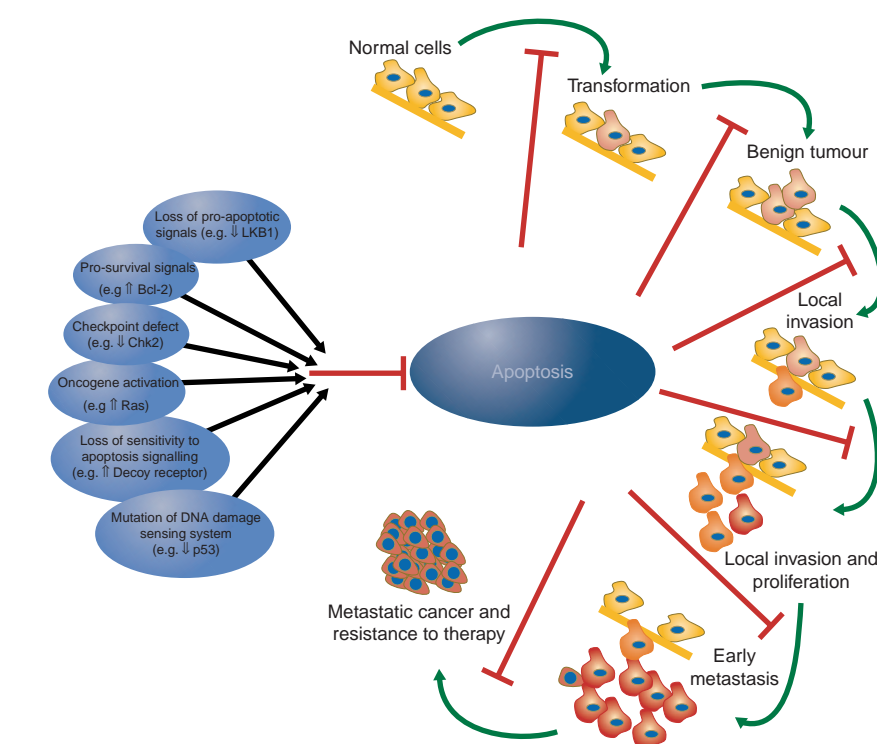


Figure 1 A schematic representation demonstrating the role of apoptosis in the prevention of cancer. Normal cells can become cancerous as a consequence of suffering multiple oncogenic 'hits'. Signalling pathways that sense homeostatic imbalances induce apoptosis, and are critical checkpoints that prevent the formation and progression of cancers. Mutations that damage the apoptosis machinery eliminate these checkpoints and severely compromise the ability of the body to battle tumorigenesis. The number of clinical scenarios where mutations in genes that render cells deficient in apoptosis, and thus causative in disease pathology, has rapidly grown over the last few years. Shown here are a few examples where alterations in the apoptosis machinery (for example, LKB1 mutations) result in a defect in apoptosis and the inability to prevent multiple cellular transformation events. Red lines denote inhibitory pathways.

and Emad Alnemri (Thomas Jefferson University, Philadelphia, PA) reported the identification of a fourth pro-apoptotic

Reaper family member, Sickie, that is found near the H99 locus and also has an N-terminal IBM/RHG motif^{6,7}. Sickie does not

seem to be generally involved in programmed cell death in *Drosophila*, as it is expressed normally in H99 deletion mutants and does not compensate for the global apoptotic defects found in these flies. However, expression of *sickle* is induced in response to genotoxic stimuli, suggesting a more specific role in apoptosis, perhaps functioning as a death enhancer that coordinates appropriate cell death in conjunction with other members of the Reaper family.

In mammals, Smac/Diablo is a putative orthologue of *Drosophila* Reaper, Grim, and Hid, and has an N-terminal IBM/RHG motif⁵. After its release from mitochondria during apoptosis, Smac is thought to disrupt the interaction between the inhibitor of apoptosis protein XIAP and caspase-9, through its IBM/RHG domain⁵. By searching the human genome databank for proteins with potential IBM/RHG motifs, several groups concurrently identified Omi/HtrA2 as another pro-death molecule released from mitochondria during cell death⁸. Omi/HtrA2 is a serine protease that inhibits IAP activity. Emad Alnemri and Julian Downward (Imperial Cancer Research Fund, London, England) both proposed models for how Omi/HtrA2 may induce cell-death. One model is that Omi/HtrA2 may cleave Smac to expose the IBM/RHG domain of Smac. On the other hand, Omi/HtrA2, but not Smac, seems to be required for UV-induced cell death, suggesting that Omi/HtrA2 may cleave other targets and thus function in Smac-independent pathways. The short sequence of the IBM/RHG domain raises the intriguing possibility that many proteins may contain this motif, though the number of functionally relevant candidates may be limited if the IBM/RHG motif functions only when located at the extreme N-terminus of the protein. However, proteolytic cleavage events may expose internal IBM/RHG motifs and thereby unmask the pro-apoptotic activity of certain proteins.

It is believed that the pro-apoptotic activity of Reaper-Hid-Grim is caused by their ability to displace IAP proteins from caspases, thereby relieving the inhibitory state imposed on caspases by IAPs. Work presented by Hermann Steller (Rockefeller University, New York, NY) provided further mechanistic insight into the antagonism between DIAP1 and Reaper-Hid, and how this interplay regulates caspase activation. Consistent with findings reported for mammalian IAPs, Steller's group has found that DIAP1 seems to function as an E3 ubiquitin ligase of *Drosophila* caspases. In conjunction with the E2 ubiquitin-conjugating enzyme UbcD1, DIAP1 mediates the proteasome-dependent degradation of caspases. Steller also reported that mutations in *Drosophila* which decrease UbcD1 dosage result in increased levels of DIAP1,

suggesting that DIAP1 is also degraded by the ubiquitin-proteasome pathway. As a consequence of the increased DIAP1 dosage, Reaper-, Grim- and Hid-induced apoptosis is suppressed in these mutants. However, although both Reaper and Hid activate caspases by binding to DIAP, Reaper, but not Hid, can induce the auto-ubiquitination and destruction of DIAP1. This result distinguishes the mechanisms by which Reaper and Hid antagonize DIAP and induce apoptosis: Hid may function primarily by blocking DIAP1 from binding caspases, whereas Reaper has an additional function that facilitates DIAP1 auto-ubiquitination and proteasome-dependent destruction.

Apoptosis and tumorigenesis

Apoptotic dysfunction is intimately associated with cancer aetiology (Fig. 1). To understand tumorigenesis, it is critical to understand the mechanisms by which apoptotic checkpoints are nullified or bypassed in cancers, and how tumour suppressor genes trigger apoptosis to eradicate potentially dangerous cells. One example discussed at the meeting was the chimaeric fusion protein E2A-HLF, which is produced by the leukaemogenic translocation t(17;19). The translocation has both transforming and anti-apoptotic effects through an impairment of normal E2A function and activation of the survival pathway triggered by the HLF bZip DNA binding and dimerization domains. The E2A-HLF fusion protein has been shown to activate Slug, a zinc-finger transcription factor which is a mammalian homologue of the *C. elegans* cell death specification protein, CES-1 (ref. 9). Thomas Look (Dana-Farber Cancer Institute, Boston, MA) presented the surprising observation that *Slug*^{-/-} mice have supernumerary bone marrow and spleen leukocyte progenitor cells, but normal peripheral blood cell counts. This discrepancy may suggest that leukocyte progenitors are overproduced in *Slug*^{-/-} animals to compensate for increased apoptosis in the periphery. Although the progenitors of *Slug*^{-/-} mice seem to cycle normally, they are more sensitive to apoptosis, and the animals are hypersensitive to gamma irradiation, probably as a result of subsequent bone marrow failure. Interestingly, ionizing radiation causes a stronger induction of the pro-apoptotic proteins, Bax and Noxa, in *Slug*^{-/-} animals than in control animals. Thus, Slug may normally inhibit the expression of pro-apoptotic members of Bcl-2 family, and may therefore function analogously to the *C. elegans* protein Tra-1, which suppresses *egl-1* expression in HSN cells¹⁰. It is an intriguing possibility that other Slug/Ces-1-like proteins may regulate cell-death by suppressing the transcription of apoptotic factors.

DNA damage is a fundamental cause of cellular transformation (see Fig. 1). Therefore, it is imperative for higher metazoans to detect damaged DNA and efficiently eliminate cells in which the damage is too severe to repair. These checkpoints must ultimately result in the activation of caspases and the subsequent enzymatic suicide of the damaged cell. The human genome encodes 11 catalytically active caspases, but which upstream caspases respond specifically to DNA damage is not known. Yuri Lazebnik (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) described a new role for caspase-2 as an apical responder to genotoxic stress. Lazebnik reported that RNAi-mediated reduction in caspase-2 rendered a prostate cancer cell line resistant to DNA damage-induced apoptosis. These results, in conjunction with data demonstrating that caspases-2 is present in the nucleus¹¹, suggest that caspase-2 may function as a sentinel for DNA damage. If this model is correct, it will be of great interest to determine the mechanism of DNA-damage-induced nuclear caspase-2 activation.

Tumour suppressor genes regulate cellular homeostasis by coordinating the induction of apoptosis and cell cycle arrest. Thus, understanding how cells choose between death and growth arrest fates is a crucial issue in the tumour suppressor field. Yoichi Taya (National Cancer Center Research Institute, Tokyo, Japan) discussed a model for how p53-dependent fates are regulated. In this model, different p53 target-genes are transactivated, depending on which specific residues of p53 are phosphorylated. Taya presented evidence that the p53-dependent damage-induced nuclear protein (p53DINP1), which is induced by p53 during the growth arrest response, helps recruit casein kinase 2 to p53 and phosphorylate p53 on Ser 46. This phosphorylation causes p53 to preferentially transactivate pro-apoptotic genes, but not DNA repair or cell cycle arrest genes, thereby switching the p53 response from arrest to death. To test this intriguing model, it will be important to determine whether *p53DINP1*-null cells fail to switch from arrest to apoptosis in response to increasing levels of DNA damage.

In a similar vein, Tak Mak (University of Toronto, Ontario Cancer Institute, Toronto, Canada) discussed how Chk2, a G2 checkpoint kinase activated by DNA damage, might regulate p53. In previous work, the Mak group showed that *Chk2*^{-/-} cells are defective for the p53 response after gamma irradiation. Now, Mak presents evidence that the *Chk2*^{-/-} animals are viable and not prone to spontaneous tumours. However, when *Chk2*^{-/-} animals are treated with mutagens, they develop larger and more numerous tumours than wild-type animals, providing evidence for a proposed role of Chk2 as a tumour suppressor. Based on

these data, Mak proposed that Chk2 phosphorylates distinct target-sites on p53 to control the apoptotic functions of p53. Verifying the elements of this model may provide information on G2 checkpoint pathways that could serve as therapeutic targets in humans.

Jean Wang (University of California, San Diego, La Jolla, CA) described an *in vivo* study examining the function of caspase-mediated cleavage of the Retinoblastoma tumour suppressor protein (Rb) in apoptosis by generating a caspase-uncleavable knock-in Rb mutant. The Rb knock-in mice are born apparently normal, but embryonic fibroblasts (MEFs) from these mice are resistant to apoptosis induced by tumour necrosis factor (TNF), but not by DNA damage. This work may therefore reveal an unexpected function for Rb in regulating TNF signalling, though it remains to be seen whether the phenotype of the knock-in MEFs is a result of Rb stabilization *per se* or an indirect gain-of-function effect of uncleavable Rb on apoptotic signalling. Regardless, this is the first instance of an uncleavable form of a caspase substrate being knocked into a mouse. In the future, other such studies are likely to demonstrate new functions for caspase-mediated proteolysis in apoptosis and other aspects of cellular physiology.

Although it has been unequivocally demonstrated that suppressing apoptosis is critical for tumorigenesis, it remains unclear whether a sustained suppression is required for tumour maintenance (Fig. 1). Conversely, it is not known whether the continued expression of an oncogene is required for tumour maintenance when apoptosis has been irreversibly impaired. Gerard Evan (UCSF Cancer Center, San Francisco, CA) discussed the results of an inducible *in vivo* transgenic tumour model developed in his laboratory to characterize the apoptotic and oncogenic properties of Myc. In this system, a Myc-estrogen receptor (ER) transgene is driven by the murine insulin promoter, resulting in a fusion protein expressed only in pancreatic islet β cells. Activation of Myc by administration of the estrogen analogue 4-hydroxy tamoxifen (OHT) resulted in β cell proliferation, cell death, and subsequent islet involution and collapse within days. Interestingly, the β cells regenerated normally after OHT withdrawal. When Myc-ER transgenic mice were crossed to a Bcl- X_L transgenic mouse, Myc activation by OHT resulted in dramatic islet expansion and the formation of invasive adenomas. Again, the effects of Myc were reversible, as OHT withdrawal caused adenoma regression. These studies suggest that Myc may be necessary to maintain tumour volume, perhaps by upregulating the vascular supply. When OHT treatment was maintained continuously, carcinomas appeared within 2–3 weeks. This

suggests that, at least in mice, inappropriate expression of Myc and Bcl- X_L is sufficient for tumorigenesis. Furthermore, the continued expression of oncogenic Myc may be necessary to maintain the transformed state, even in a background of suppressed apoptosis¹².

The tumour suppressor p53 serves both as a guardian against genomic instability and an activator of apoptosis. Therefore, it is clinically important to understand how losing either of these functions may contribute to tumorigenesis. Andrei Gudkov (Cleveland Clinic Foundation, Cleveland, OH) reported that overexpression of Bcl-2 obviates any growth advantage gained from losing p53, suggesting that it is the loss of p53 pro-apoptotic activity which is critical for initial cell transformation. In contrast, overexpression of Bcl-2 reduces the metastatic potential of cells in comparison with p53-null cells. This suggests that another function of p53, separable from the induction of apoptosis, is important for metastasis. Interestingly, Gudkov's group found that the greater propensity of p53-null cells to metastasize is masked when they must 'compete' with Bcl-2-overexpressing cells *in vivo*. These results provide a possible explanation for the puzzling finding that Bcl-2 overexpression is a favourable prognostic marker in some human cancers, such as breast cancer¹³. In theory, higher levels of Bcl-2 in tumours may reduce the selection pressure for p53 loss, and may therefore reduce the genomic instability that accompanies p53 loss. According to this model, retaining p53 would decrease the chance of metastasis, and thus predict a favourable clinical outcome.

Meeting co-organizer Scott Lowe (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) addressed similar issues on the role of apoptosis in cancer in the meeting's closing talk. Lowe's laboratory has developed a powerful system for dissecting the critical steps in B cell lymphomagenesis *in vivo*¹⁴. In this system, E μ -Myc lymphoma cells are isolated from mice and genetically modified by *in vitro* retroviral transduction of a specific gene. Subsequently, control and derivative lymphomas are re-introduced into matched mice and the course of the disease, with or without therapy, is monitored. Lowe reported that, in this system, the resistance to apoptosis conferred by Bcl-2 mimics the loss of p53 during the early stages of tumorigenesis. The finding that the pro-apoptotic function of p53 is most important for blocking tumorigenesis agrees with the results of the Gudkov group. However, Lowe reported that the advantage of p53 loss in later stages of tumour progression cannot be replaced by molecules that block apoptosis, such as Bcl-2. *In vivo*, lymphomas that overexpress Bcl-2 will still, with some frequency, sponta-

neously lose p53 expression. Importantly, such a loss of p53-dependent checkpoints confers resistance to anti-cancer drugs, even relative to Bcl-2-overexpressing cells, indicating that the non-apoptotic functions of p53 may be most important for the response to chemotherapy. Consistent with this hypothesis — and with Gudkov's results — Lowe's group used tumour transplant experiments to show that mice receiving Bcl-2-overexpressing lymphomas responded better to chemotherapy and had higher survival rates than mice whose lymphomas were p53-null. Clearly, these findings have profound clinical significance for the treatment of human cancers.

Apoptosis and cancer treatment

One of the important translational goals of apoptosis research is to develop new and better cancer treatments. Ralph Schwall of Genentech Inc. discussed new results with TRAIL (TNF-related apoptosis-inducing ligand)/Apo2, a death receptor ligand that holds promise as a novel cancer therapeutic¹⁵. TRAIL efficiently kills a diverse range of tumour-derived cells in an National Cancer Institute panel screen. Interestingly, in some cell lines that are resistant to TRAIL killing, insensitivity was attributable to the absence of functional Bax, which had been lost as a result of a mismatch repair defect in those cells. Schwall and colleagues also found that using TRAIL in concert with the DNA-damaging agents etoposide or CPT-11 resulted in more effective killing, which may be caused by increased levels of the pro-apoptotic proteins DR5 and Bak in response to genotoxic stress. Thus, DNA damaging agents may help mitigate the requirement for a Bax-dependent amplification loop in TRAIL-induced death, explaining the ability of TRAIL and etoposide to kill synergistically, even in Bax-deficient cells.

Another intriguing therapeutic approach to attacking cancer cells was proposed by Craig Thompson (Department of Medicine and Cancer Biology, Abramson Family Cancer Research Institute, University of Pennsylvania, PA). Thompson's group found that the proto-oncogene AKT/PKB does not rescue cells from glucose starvation, though anti-apoptotic genes such as Bcl- X_L do¹⁶. Surprisingly, in a fasting state, cells become more sensitive to death in the presence of elevated AKT/PKB levels. This increased sensitivity may be caused by an increase in glucose metabolism that is triggered by AKT/PKB. Consistent with these data, Thompson pointed out that many types of cancers, including non-Hodgkin's lymphoma, have acquired the ability to take up glucose autonomously. Thus, treatments that target the ability of cancers to obtain glucose may provide an additional arsenal against AKT-dependent cancers.

The Future

Over the last decade, apoptosis has grown from an obscure process with an unpronounceable name to a well-accepted scientific field. During that time, many apoptotic players have been identified through genetic analysis of programmed cell death in invertebrates, biochemical studies of mammalian apoptosis and cellular characterization of apoptosis pathways. More recently, the completion of whole-genome sequencing efforts and progress in bioinformatics has dramatically increased the number of putative apoptotic players. Several apoptotic pathways are now understood in such detail that their entire molecular course, from the initial death signal to the final demise of the cell, can be precisely delineated. However, the field cannot pause to congratulate itself on its accomplishments, for we are still far from developing a 'magic bullet' capable of harnessing our knowledge of apoptotic pathways to eradicate cancers. We are hopeful that the knowledge of apoptosis and cancer that we possess today will be translated into new and more effective cancer therapies in the next decade.

We apologize to presenters of the many excellent talks and posters that were not discussed here because of space constraints. □

Or Gozani, Michael Boyce, Lina Yoo, Philip Karuman and Junying Yuan* are in the Department of Cell Biology, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115, USA

*e-mail: junying_yuan@hms.harvard.edu

1. Metzstein, M. M., Stanfield, G. M. & Horvitz, H. R. Genetics of programmed cell death in *C. elegans*: past, present and future. *Trends Genet.* **14**, 410–416 (1998).
2. Gartner, A., Milstein, S., Ahmed, S., Hodgkin, J. & Hengartner, M. O. A conserved checkpoint pathway mediates DNA damage-induced apoptosis and cell cycle arrest in *C. elegans*. *Mol. Cell* **5**, 435–443 (2000).
3. Wu, G. *et al.* Structural basis of IAP recognition by Smac/DIABLO. *Nature* **408**, 1008–1012 (2000).
4. Liu, Z. *et al.* Structural basis for binding of Smac/DIABLO to the XIAP BIR3 domain. *Nature* **408**, 1004–1008 (2000).
5. Srinivasula, S. M. *et al.* A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. *Nature* **410**, 112–116 (2001).
6. Christich, A. *et al.* The damage-responsive *Drosophila* gene *sickle* encodes a novel IAP binding protein similar to but distinct from *reaper*, *grim*, and *hid*. *Curr. Biol.* **12**, 137–140 (2002).
7. Srinivasula, S. M. *et al.* *Sickle*, a novel *Drosophila* death gene in the *reaper/hid/grim* region, encodes an IAP-inhibitory protein. *Curr. Biol.* **12**, 125–130 (2002).
8. Suzuki, Y. *et al.* A serine protease, Htra2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol. Cell* **8**, 613–621 (2001).
9. Inukai, T. *et al.* SLUG, a ccs-1-related zinc finger transcription factor gene with antiapoptotic activity, is a downstream target of the E2A-HLF oncoprotein. *Mol. Cell* **4**, 343–352 (1999).
10. Conradt, B. & Horvitz, H. R. The TRA-1A sex determination protein of *C. elegans* regulates sexually dimorphic cell deaths by repressing the *egl-1* cell death activator gene. *Cell* **98**, 317–327 (1999).
11. Paroni, G., Henderson, C., Schneider, C. & Brancolini, C. Caspase-2 can trigger cytochrome *c* release and apoptosis from the nucleus. *J. Biol. Chem.* **277**, 15147–15161 (2002).
12. Pelengaris, S., Khan, M. & Evan, G. I. Suppression of Myc-induced apoptosis in β cells exposes multiple oncogenic properties of Myc and triggers carcinogenic progression. *Cell* **109**, 321–334 (2002).
13. Briasoulis, E. *et al.* Near-absolute expression of the bcl-2 protein identifies a subgroup of stage II breast cancer patients with a most favorable outcome. Results of a clinicopathological study. *J. Exp. Clin. Cancer Res.* **20**, 341–344 (2001).
14. Schmitt, C. A. & Lowe, S. W. Bcl-2 mediates chemoresistance in matched pairs of primary E(μ)-Myc lymphomas *in vivo*. *Blood Cells Mol. Dis.* **27**, 206–216 (2001).
15. LeBlanc, H. *et al.* Tumor-cell resistance to death receptor—induced apoptosis through mutational inactivation of the proapoptotic Bcl-2 homolog Bax. *Nature Med.* **8**, 274–281 (2002).
16. Plas, D. R. & Thompson, C. B. Cell metabolism in the regulation of programmed cell death. *Trends Endocrinol. Metabolism* **13**, 75–78 (2002).

ACKNOWLEDGEMENTS

This work was supported in part by a grant from the National Cancer Institute (CA89434) to J.Y., a K08 Award (AG19245) to O.G. and a Postdoctoral Fellowship from the American Cancer Society to L.Y.