Abortive apoptosis and its profound effects on radiation-, chemical-, and oncogene-induced carcinogenesis

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Abstract

Traditionally apoptosis and the apoptotic machinery have been deemed as anti-carcinogenic because of their presumed roles in eliminating damaged or unwanted cells. However, recent work from our laboratory and others have shown that the established paradigm is deeply flawed. The fundamental flaw is the assumption that apoptosis, once initiated, is irreversible and invariably leads to cell death. However, there is increasing evidence that cells can survive activation of the apoptotic cascade. This new revelation about abortive apoptotic cells can dramatically change our assessment of the biological roles of apoptosis. In this brief review, we will cover some of the original studies that report the "undead" apoptotic cells and how they lead to unexpected new roles for apoptotic factors in space radiation and other stress induced genetic instability and carcinogenesis. We will also review exciting new discoveries on the association among abortive apoptosis, spontaneous DNA double strand breaks, DNA damage response, and stemness of cancer cells.

A. Apoptosis and apoptotic factors in carcinogenesis: the established paradigm

Apoptosis as a term to describe cell death first appeared in the early 1970s(1). Apoptosis refers to the process of programmed cell death that occurs in multicellular organisms. Traditionally, apoptosis is recognized as an essential biological process that is required in early developmental processes to remove unwanted or damaged cells(2). At the cellular level, apoptosis is characterized by cellular shrinkage, blisters or bubbles on the plasma membrane, chromatin condensation, and fragmentation of nuclear DNA. A major characteristic of apoptosis that distinguishes it from other forms of cell death such as necrosis or autophagy is that end-stage apoptotic cells become membrane-bound smaller cellular fragments called apoptotic bodies that can be phagocytized by scavenger cells such as macrophages. The engulfment of the apoptotic cells is thought to be mediated by the membrane lipid phosphatidylserine, which is "flipped" from the inner to outer plasma membrane(3). The engulfment of apoptotic cells by macrophages occurs before their cellular contents can be leaked out into surrounding environment, which prevents inflammation(4).

At the molecular level, apoptosis is relatively well defined(5). It can occur through either an extrinsic or an intrinsic pathway. The extrinsic pathway occurs through the engagement of external ligands to cell surface "death receptors" such as Fas, Trail, or TNF receptors. The engaged receptors then recruit downstream factors such as FADD and Casp8, which form the death-inducing signaling complex (DISC) that cleaves and activates Caspase-8. Activated Caspase-8 will then cleave and activate end-stage "executioner" caspases such as Caspase-3, -6, and -7, which subsequently cleave hundreds of cellular targets that form the basis of the observed apoptotic phenotype.

The intrinsic pathway starts from the mitochondria. Internal cellular stresses such as DNA damage or high ROS induces the permeabilization of the mitochondrial outer membrane, which leads to the release of the mitochondrial protein SMAC/DIABLO into cytosol. The leaked SMAC/DIABLO then competitively binds inhibitors of apoptosis (IAPs) and pulls them away from caspases, enabling the latter to activate. Another major factor that is leaked out of the mitochondria is cytochrome C. In the cytoplasm free cytochrome C forms a complex with Apaf-1 and Caspase-9 that is appropriately termed the "apoptosome." In the apoptosome, Caspase-9 is activated, which then cleaves and activate downstream "executioner" caspases such as Casp-3, -6, and -7. The activation of the "executioner" caspases then cleaves hundreds of downstream targets that lead to cell death. The intrinsic and extrinsic pathway can also intersect at the mitochondrial membrane. One example is the activation of Caspase-8, which cleaves the BH3-only protein BID that leads to the destabilization of the mitochondrial membrane and activation of the intrinsic pathway.

B. Existence of the "undead" apoptotic cells and a break from the established paradigm.

For many years since the advent of apoptosis as an active field of research, a paradigm was established that apoptosis is an irreversible process that, once initiated, always leads to cell death. In fact, it is still the prevailing view among most biologists today. As an example of how entrenched the idea is, apoptotic cell death is still assayed by staining for Caspase-3 cleavage or phosphatidyl serine (PS) staining in most recent scientific literature,

However, there is increasing evidence that apoptosis is a reversible process. In a study from our own group on the mechanism of the induction of pluripotent stem cells from human primary fibroblast cells, we showed that in cells transduced with reprogramming factors, both Caspase-3 and Caspase-8 are activated significantly over the course of three weeks (**Fig. 1**, luciferase imaging through the use of a reporter)(6). Some of the cells were clearly lost to cell death during the reprogramming process. However, by use of a Caspase-3 GFP reporter, it was shown that many of the cells with Caspase-3 activation actually survive. In fact, in the induced pluripotent stem cells derived from the reprogramming, it was shown that Caspase-3, the "executioner" caspase, remain active at robust levels persistently. Consistently, Casp3 activation was also observed in the H9 ES cells established from the human embryonic tissue(7) (**Fig. 2**). Our finding of the persistent activation of Caspase 3 in the ES cells was also confirmed independently by another group that showed Caspase-3 played important roles in ES cell differentiation(8).

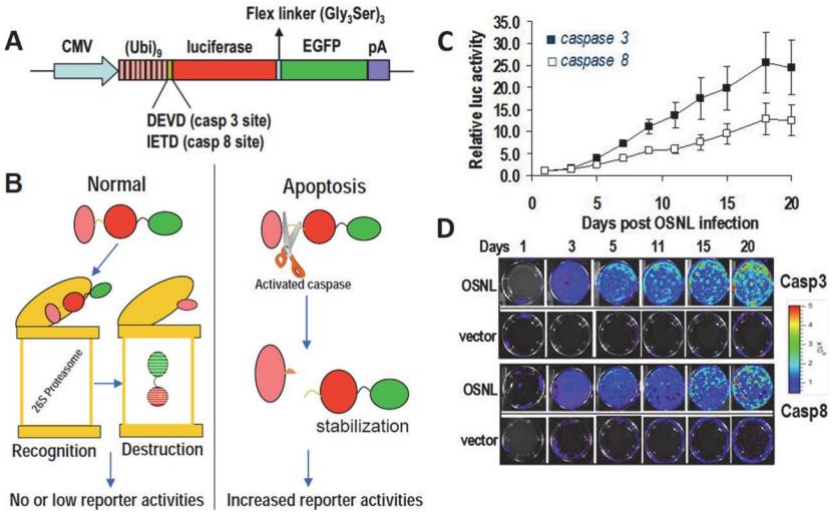

Figure 1. Caspase-3 & 8 activation during the iPSC induction process. **A**) A schematic diagram of a Caspase-3 or -8 reporter. **B**) Principle of the reporter based on proteasome-mediated protein degradation. Only in cells with Casp3 or 8 activation will the reporter (Luc-GFP fusion) be cleaved. **C & D**) Quantitative and luciferase imaging of Casp3 & 8 activation in IMR90 fibroblast cells transduced with iPSC induction factors Oct4, Sox2, Nanog, & Lin 28.

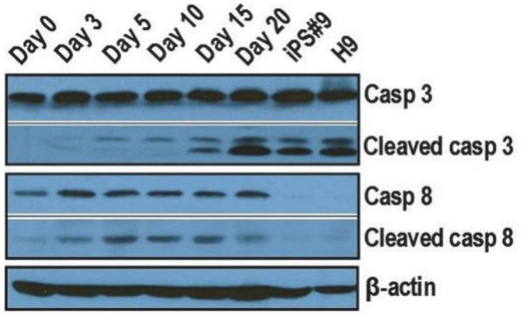

Figure 2. Western blot analysis of Casp3 &8 in IMR90 cells transduced with Oct4, Sox2, Nanog, Lin28. Shown are data from different time points after gene transduction into the cells as well from a iPSC clone (#9) and the H9 ES cell line obtained from Dr. James Thomson of University of Wisconsin.

Non-lethal caspase activation was not only restricted to ES or iPS cells. In a study carried out by Tang et al (9), it was shown among various primary and transformed cells treated with ethanol, even those that exhibited markers of late stage apoptosis such as apparent morphological changes and DNA fragmentation could still recover if ethanol was removed from the culture media. Such cellular resurrection from almost certain death has been termed "Anastasia" by the authors.

In another study conducted by Ichim *et al* the authors found that human and mouse cells exposed to various stressers such as staurosporin, ABT737(a BCL2 inhibitor), and TNFa/cyclohexamide could

survive with limited mitochondria membrane permeabiliation (limited MOMP) and caspase activation (10).

Despite the above observations, one can still argue that all the observed phenomena of cells surviving caspase activations occur under very artificial conditions, mostly in cell culture. That point was addressed elegantly by a recent study from the Montel group (11). In that study, by use of a reporter (CasExpress) that can track the activation of the Caspase-3 *in vivo*, it was demonstrated clearly that during development in Drosophila, there were widespread and diverse patterns of cellular survival. Those data thus confirmed the relevance of sublethal caspase activation in a living organism.
More recently, work from our group showed that cancer cells have spontaneously activated Caspase 3/7 and those cells with low level caspase activation suffered no ill consequences at all in terms of survival and growth (12).

What about the roles of caspases in radiation induced cell death? Radiation has long been known to induce apoptosis in many cell types such as lymphocytes (13). However, its role in inducing cell death in other cell types, including epithelial cells from which most human cancers originate, have remained controversial(14). In order to examine if Caspase-3 activation plays any roles in radiation induced cell death, we carried out experiments to examine the fate of irradiated mammary epithelial cells (MCF10A) with different levels Caspase-3/7 activation by use of a recombinant Casp3/7-GFP reporter(15). MCF10A cells were irradiated with different doses of x-rays. Twenty-four hours later, the cells were sorted individually into 96-well plates according to their Casp3/7 activities. After two weeks of culture, the plates with individually seeded MCF10A cells were then examined and counted to determine the numbers of colony formation. To our great surprise, even among those cells with relatively high levels of Casp3 activation, the cells could still form colonies at robust levels (**Fig. 3**).
In a more recent experiment, our lab shows that over-expression of the oncogene *MYC*, which has been known to induce apoptosis in untransformed cells (16), can also activate Casp3/7 without killing MCF10A cells (17).

Taken together, these findings indicate that the established concept of Caspase3 activation invariably leading to cell death is simply wrong.

C. Consequences of abortive apoptosis: increasing genetic instability

An established paradigm in cancer biology is that apoptosis is a process for organisms to get rid of damaged cells. Cells with DNA damage from intrinsic and extrinsic stresses, including simulated space radiation, activate the cellular DNA damage response (DDR) (18). ATM(19, 20) is a central player in DDR which causes cell cycle arrest (e.g. through the p53/p21 axis) and repairs the damaged DNA (e.g. DNA-PKcs/DNA Ligase IV axis). Cells with damage that cannot be repaired activate their apoptotic machinery and die. In that ideal scenario, apoptosis plays the "good cop" role of suppressing genetic instability. However, on careful examination of that paradigm, it is obvious that

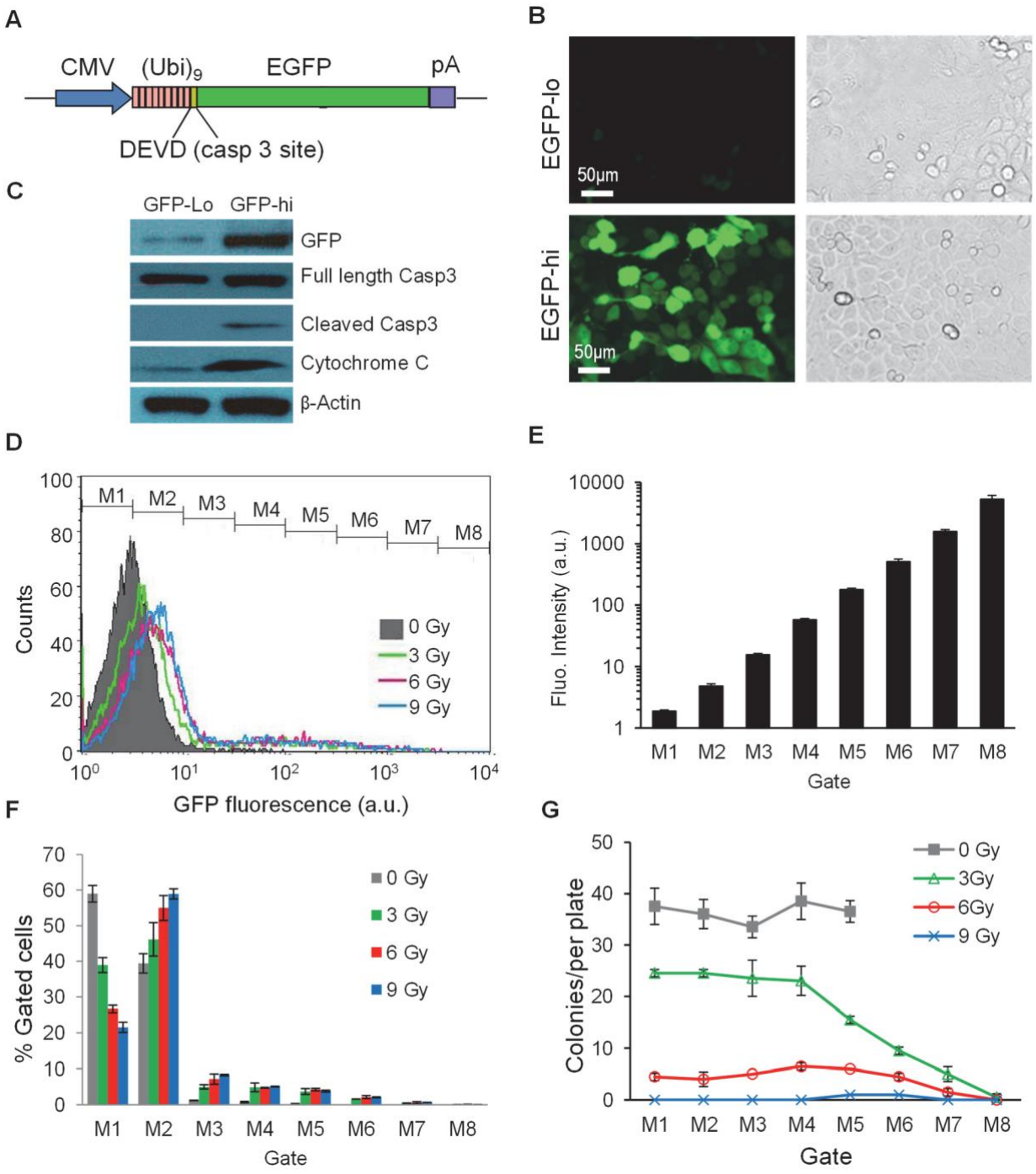

Figure 3. Subethal activation of caspases 3 in MCF10A cells exposed to x-rays. **A)**. Diagram of the caspase 3 reporter gene. $(Ub)_9$, a nine-ubiquitin polyubiqutin domain that serves as the proteasome recognition signal that causes the rapid degradation of the reporter protein.**B)**. Irraidated MCF10A cells with low (top panels) and high (lower panels) caspase 3 reporter activities after separation by a FACS sorter based on GFP expression levels. **C).** Western blot analysis of caspase 3 cleavage and activation in cells with high and low Casp3EGFP reporter activities.**D).** Flow cytometry profiles of MCF10A-Casp3EGFP reporter activities in cells exposed to different doses of x-rays. Cells were analyzed 4 days after irradiation. Cells were gated into 8 different groups for colony forming assays according to their fluorescence intensity levels (M1-M8). **E).** The mean (geo) GFP fluorescence intensities of gated MCF10A cells. **F).** Distribution of MCF10A cells in each gate after different irradiation doses. For each radiation dose, M1+M2+…+M8=100%. **G).** Colony forming abilities of cells from different gates. Cell from each fluorescence gate were flow-sorted into individual wells of 96-well plate at 1 cell/well. Three weeks later, the numbers of MCF10A colonies on each plate were counted and plotted. Error bars represent standard deviation. All values are derived from the average of triplicate experiments.

a key foundation of the whole paradigm is the assumption that cells that activate the apoptotic program will carry it through and go into certain death. However, as described in the previous section, such an assumption is incorrect in many circumstances, including when cells are exposed to ionizing radiation. How will this new revelation influence our assessment of the roles of apoptosis in stress induced genetic instability?

From a theoretical point of view, it could be a game changer. This is because the apoptotic machinery, once activated, is programmed not only to cleave and inactivate hundreds of protein targets, but also to systematically digest nuclear DNA by use of several nucleases. In fact, DNA fragmentation and "ladder formation" from genomic DNA detected by gel electrophoresis is a hallmark of apoptosis (21-24). Based on the fact that even late stage apoptotic cells could survive (9), it is theoretically possible that surviving cells could activate the apoptotic nucleases and partially damage genomic DNA. Indeed, several studies have confirmed this hypothesis. In an earlier study, Tang *et al* showed that cells exposed to alcohol could initiate apoptosis and survive with DNA damage induced by the apoptotic DNA fragmentation process (9). In another study, Ichim *et al* showed that cells exposed to different stress signals such as a Bcl2 inhibitor (ABT737), staurosporin, or TNF-α/cyclohexamide could survive with extensive DNA double strands breaks as indicated by strong γH2AX staining in surviving cells (10). A key aspect of these observations is that DNA damage can occur de novo and persistently after the initial insult.

In terms of radiation, our own study showed that cells exposed to low doses of simulated space radiation, such as 0.1-0.5 Gy of ^{56}Fe ions, could also survive activation of the apoptotic caspases, as described earlier (15). Most strikingly, the cells that survived irradiation with ^{56}Fe ions contain significantly elevated γH2AX foci for an extended period of time, as long as 3 months after radiation exposure (15). Furthermore, if Caspase 3 was inhibited by either an shRNA or a dominant/negative Casp3, the persistent genetic instability induced by radiation was almost completely gone (**Fig.4**). Those results are very significant and highly relevant to the field of radiation biology, especially space radiation biology. The relevancy comes from the fact that persistent genetic instability has long been established as a key aspect of radiation-induced biological effects in mammalian cells(25-27). However, a convincing mechanism has not been presented despite many years of study. Our study suggests that radiation-induced persistent genetic instability can be caused by sublethal activation of caspases and downstream apoptotic DNase that occurs through limited mitochondrial membrane leakage. Such a mechanism, if proven generally applicable to other cell types/biological

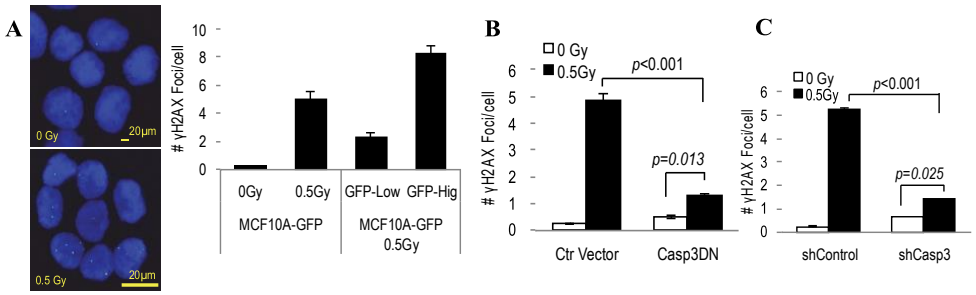

Figure 4. Key roles of caspase 3 in ^{56}Fe ions induced DNA damage. **A**). Radiation induced γH2AX foci formation (left panel) two weeks post irradiation and the correlation of γH2AX formation with caspase 3 levels (EGFP high and low groups flow-sorted based on caspase reporter shown in Fig.1, right panel). **B**). Suppression of HZE radiation induced γH2AX foci formation in MCF10A cells by dominant negative caspase 3 (Casp3DN) expression. **C**). Suppression of radiation induced γH2AX foci formation by shRNA mediated caspase 3 down-regulation.

systems beyond those tested in our study, would provide a plausible mechanistic explanation for a long-standing issue in radiation biology.

D. Consequence of abortive apoptosis: Facilitating malignant transformation.

One of the key tenets of currently established paradigm on carcinogenesis is that apoptosis is multicellular organisms' key pathway to get rid of damaged or faulty cells. Thus evasion of apoptosis is deemed a hallmark of cancer cells (28). Implicit in the paradigm is that the apoptotic machinery, especially the "executioner" caspases like Caspase-3, is tumor-suppressive. Given the strong evidence accumulating pointing to Casp3 as a facilitator of genetic instability rather than a suppressor, a key question that begs for an answer is whether Casp3 is also facilitator of carcinogenesis.

Two studies addressed this question head on. In one study, Ichim et al showed that limited MOMP not only causes significantly more DNA double strand breaks when the cells were exposed to pro-apoptotic stress, but also facilitated the malignant transformation of the exposed cells(10).

In another study, Liu et al showed that persistent Casp3 activation also played a key role in facilitating malignant transformation of MCF10A cells exposed to simulated space radiation (15). The functional importance of Casp3 is demonstrated by the fact that soft agar growth from MCF10A cells exposed to low dose ^{56}Fe ions were significantly attenuated when Casp3 were inhibited by either shRNA or a dominant negative Casp3. Strikingly, if the irradiated cells were injected into nude mice, tumor growth was only seen in the control group (**Fig. 5**).

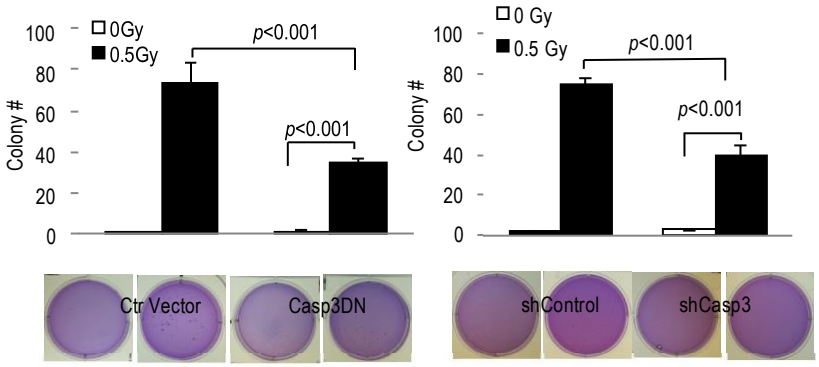

Figure 5. Key role for caspase 3 in facilitating ^{56}Fe ions induced oncogenic transformation as determined by soft agar colony formation. Left panels, data from MCF10A cells transduced with a dominant negative version of capase 3(Casp3DN). Right panels, data from MCF10A cells transduced with an shRNA minigene against caspase 3.

In a further experiment, Liu et al also demonstrated the importance of Casp3 in facilitating chemical carcinogenesis. By use of the well-established DMBA + TPA two stage carcinogenesis model, the authors showed that Caspase-3 deficient mice had significantly less, not more, skin tumor induction when compared with genetically identical mice (**Fig. 6**). A summary diagram of how abortive apoptosis facilitate radiation- and chemical-induced carcinogenesis is shown in **Fig. 7**.

MYC is one of the first oncogenes identified. However, its mechanism of action has long puzzled cancer biologists. It is known to be a powerful transcriptional regulator able to turn on thousands of genes. It is also known to be a powerful oncogene implicated in many malignancies (29, 30). Early on, it was shown that Myc can induce apoptosis in a number of normal, non-transformed cells (16). On the other hand, the powerful ability of Myc to transform is associated with its ability to induce genomic instability (31). At the mechanistic level, Myc-induced genetic instability has been attributed to production of reactive oxygen species (32). However, the evidence is controversial (33). Thus the mechanism for Myc-induced genetic instability remains unknown. In a recent study by Cartwright *et al*, it was demonstrated that exogenous expression of Myc in MCF10A cells induced activation to Caspase-3, similar to cells exposed to radiation (17). Moreover, most of those cells with Casp3 activation did not die and could actually form colonies. In fact it is in those cells with high Caspase 3 activities Myc induced limited MOMP, as evidenced by cytochrome C leakage, Caspase-3 activation, and genetic instability. Furthermore, genetic instability is correlated with endonuclease G migration into the nucleus from the mitochondria where it normally resides. The importance

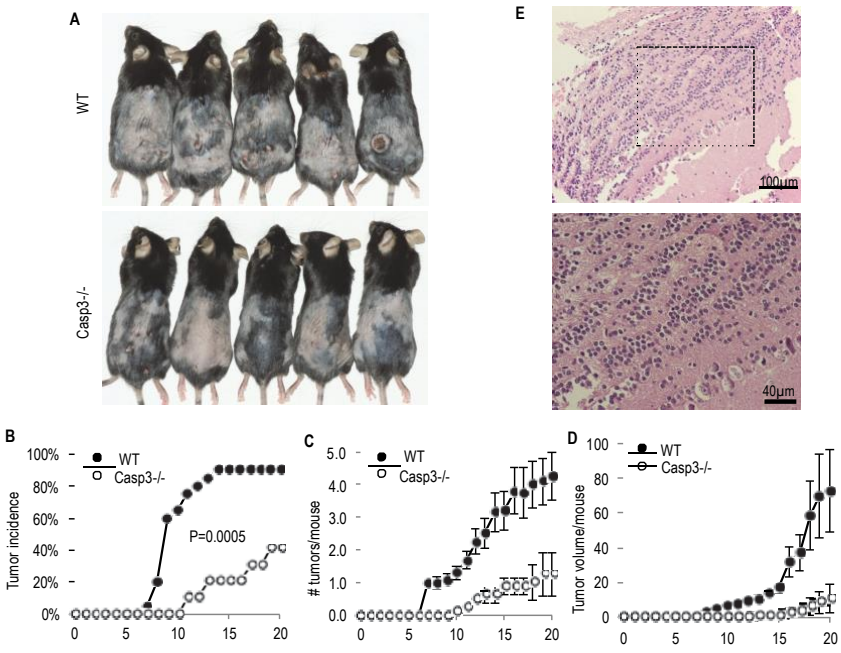

Figure 6. Chemical skin carcinogenesis in wild type and caspase 3 deficient C57BL/6 mice. About twenty wild type and 10 caspase-3 deficient mice were treated DMBA (100 μg per mouse) for once and TPA at 2.5μg per mouse twice weekly for 20 weeks. Tumor induction in the skin were followed on a weekly basis. **A.** Representative tumor formation in mice at 20 weeks post initial chemical administration. **B.** Incidence of papillomas in wild type and casp3-/- mice. **C.** Number of tumors per mouse in wild type and casp3-/- mice. **D.** Average tumor volume per mouse in wild type and casp3-/- mice. **E**. H&E staining of a typical papilloma from our experiment.

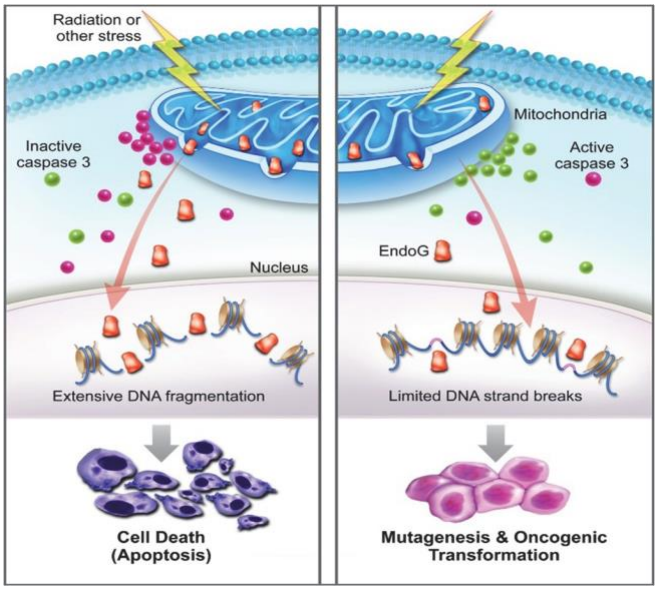

Figure 7. A schematic diagram illustrating how abortive apoptosis facilitates stress induced genetic instability and oncogenic transformation. Left panel shows the conventional scenario where mitochondrial permeability changes lead to activation of Casp3 and leakage of endonuclease G that kills the host cells. Right panel, on the other hand, shows partial leakage and survival of the cells with secondary genetic damage and oncogenic transformation (adapted from Liu *et al*, Molecular

of mitochondria leakage, Casp3 activation, and endoG nuclear migration was demonstrated by the fact that inhibition of Casp3 or endoG significantly attenuated both Myc induced genetic instability and oncogenic transformation (**Fig. 8**).

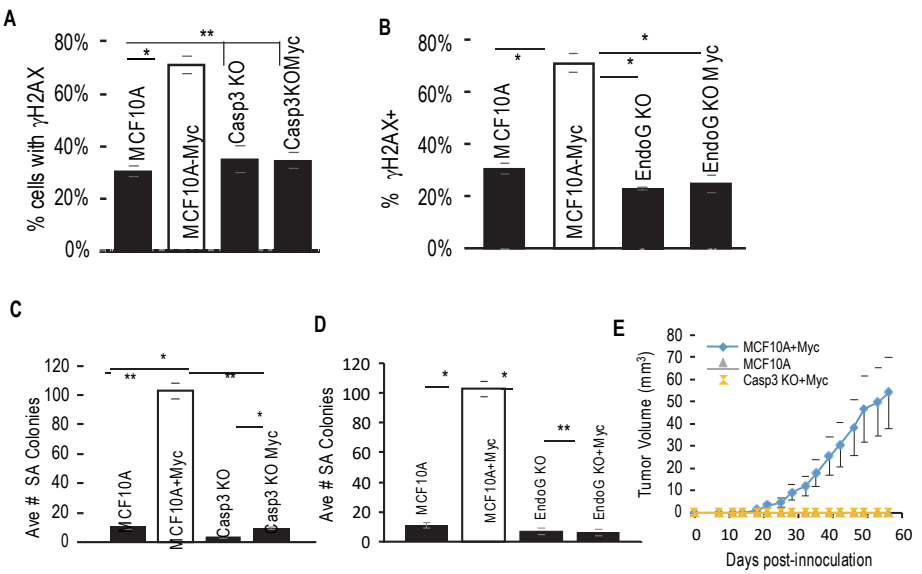

Fig 8. Requirement for Casp3 in Myc-induced DNA damage and transformation. A) Critical roles of Casp3 in Myc induced γH2AX foci in MCF10A cells. **B**) Critical roles of endoG in Myc induced γH2AX foci in MCF10A cells. **C**) Essential role of Casp3 in Myc induced transformation of MCF10A cells as assessed by soft agar colony growth. **D**). Essential roles of endoG in Myc-induced transformation of MCF10A cells. E) Tumor growth from control, Myc-overexpressing, and Casp3KO cells with Myc over-expressiong in nude mice. Notice that only wild type MCF10A cells transduced Myc were able to form tumors The error bars in B, D, and E represent standard error of the mean (SEM). * Indicates p value < 0.001, ** indicates p value << $1e^{-5}$, *** indicates a P value >0.1. Student's t-test was used to calculate the p-values in B & C. N>3.

Taken together, it is pretty clear that abortive apoptosis, which involves the sublethal activation of Caspase 3 and endoG nuclear migration, play critical roles in facilitating radiation-, chemical-, and oncogene- induced malignant transformation of mammalian cells. That is certainly dramatic departure from the established paradigm.

E. Non-genetic effects of abortive apoptosis: self-inflicted DNA double strand breaks and stemness of cancer cells

In addition to their unexpected roles in promoting genetic instability and carcinogenesis, our laboratory recently discovered that caspases and their downstream factors also play previously unrecognized roles in established cancer cells. Liu *et al* discovered that many cancer cell lines possess limited MOMP spontaneously in the absence of any external stress (12). The occurrence of limited MOMP is evidenced by extensive cytoplasmic staining of cytochrome C. This is in sharp contrast to untransformed cells, where cytochrome C staining mostly stays within the mitochondria (**Fig. 9A&B**). As expected, cytochrome C leakage into the cytplasm also led to Caspase 3 activation, which was demonstrated by low level Caspase 3 cleavage in several cancer cell lines examined. Those data were very surprising given that the tumor cells examined were never exposed to any stress and were proliferating and apparently healthy. Consistently Casp3 activation and EndoG migration were correlated with increased γH2AX foci, which indicate increased induction of DNA double strand breaks. Further evidence for incurrence of spontaneous DNA damage is persistent activation of DDR, as evidenced by detection of phosphorylated ATM both by western blot analysis and immunofluorescence staining (12).

While the spontaneous induction of DNA DSBs and activation of DNA damage response in some cancers were certainly surprising, were they functionally relevant for tumor biology? In order to answer this question, Liu et al used the CRISPR/Cas9 technology of gene editing to knockout Casp3/6/7(the executioner caspases), EndoG/CAD (the two most important apoptotic endonucleases), and ATM. Consistent with sublethal caspase activation being a source of DNA damage, Casp3/6/7 triple knockout cells had more than a 50% reduction in γH2 AX foci (**Fig. 9C**). A similar reduction was observed for cancer cells with EndoG/CAD double knockout (**Fig.9D**). More strikingly, when MDA-MB231 cells with Casp3/6/7 triple knockout were injected into nude mice, they formed tumors at a significantly reduced rate when compared with parental cells (**Fig. 10A**). Equally surprising was the observation that MDA-MB231 cells with EndoG/CAD double knockout also grew at significantly reduced rate when compared with control cells (**Fig. 10B**). This latter result strongly suggests that the DNA double strand breaks were actually facilitating tumor cell growth, rather than inducing cell cycle arrest. This further raised the question of the role of DNA damage response in tumor biology. Based on results from Casp3/6/7 knockout and CAD/EnddG knockout, we would predict that ATM activation plays an important role in facilitating tumor growth. Our results with ATM knockout MDA-MB231 cells show

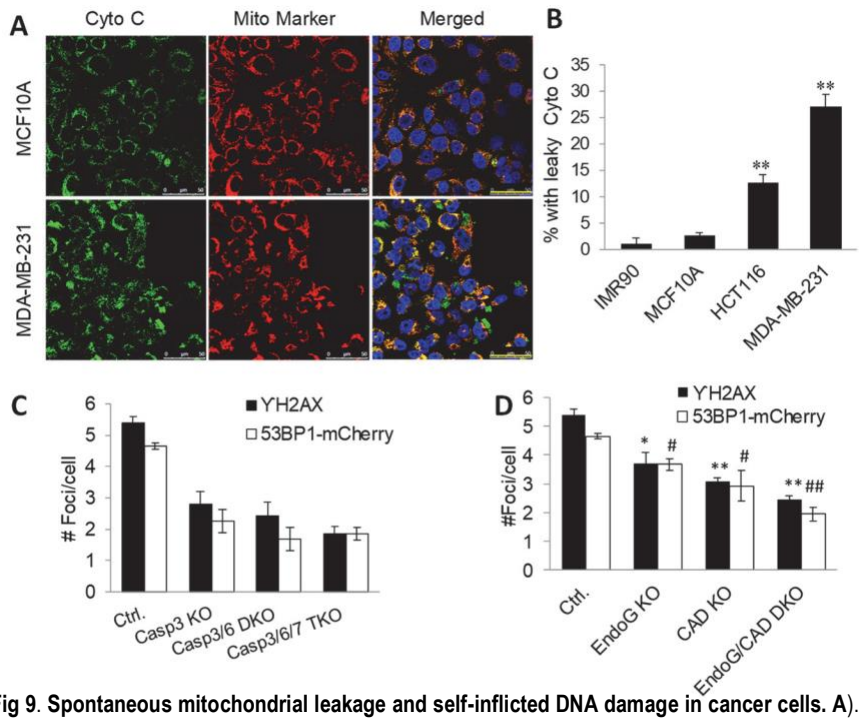

Fig 9. **Spontaneous mitochondrial leakage and self-inflicted DNA damage in cancer cells. A**). Confocal microscope imaging of endogenous cytochrome C in MCF10A (top panels) and MDA-MB-231 (lower panels) cells. A mito marker antibody was used to stain for mitochondria. Scale bars represent 20 μm. **B**). Percentage of cells with 'leaky" extra-mitochondrial expression of cytochrome c. Error bars represent standard error of the mean (SEM). **, p<0.001, when comparing the tumor cells vs IMR90 primary fibroblast cells, Student's t-test, n=3. **C**) Average number of γH2AX and 53BP1 foci in control and *CASP3, -6, -7* single, double, and triple knockout MDA-MB-231 cells. **D**) Average number of γH2AX and 53BP1 foci in control, *ENDOG* knockout (*ENDOG* KO), *CAD* knockout (*CAD* KO), and *ENDOG* and *CAD* double knockout (*ENDOG*/*CAD* DKO) MDA-MB-231 cells.

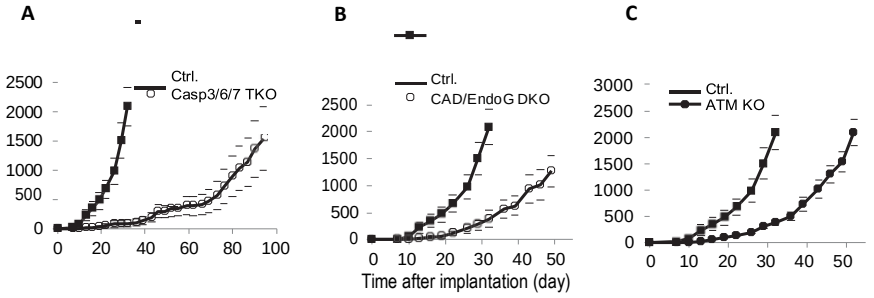

Fig. 10. The effect of deficiencies in cell death and DNA repair factors on tumor formation in MDA-MB231 cells. **A**) Tumor formation from vector-transfected (Ctrl.) and *CASP3/6/7 TKO* MDA-MB-231 cells injected subcutaneously ($2x10^5$ /per injection) into nude mice. **B**) Tumorigenic abilities of vector-transfected and *EndoG/CAD DKO* MDA-MB-231 cells in nude mice. The error bars represent SEM. **C**) Tumorigenic abilities of vector-transduced, *ATMKO* MDA-MB-231 cells in nude mice. Error bars represent SEM, n=6.

that prediction to be true. Indeed, MDA-MB231 cells with ATM gene knockout form tumors at a significantly slower rate than the parental control (**Fig. 10C**).

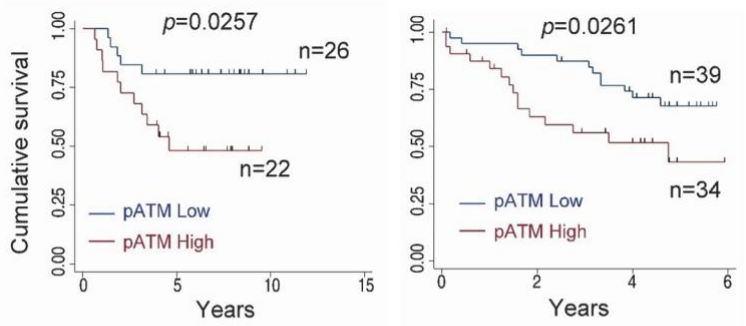

Fig. 11. Prognostic value of phosphorylated ATM (pATM). A) Kaplan-Meier survival analysis of a cohort of breast cancer patients. Plotted is patient survival for those with high or low levels of pATM in their pre-treatment tumor samples. p=0.0257, n=48, log-rank test. **B**) Kaplan-Meier survival analysis of a cohort of colon cancer patients. Plotted is patient survival for those with high or low levels of pATM in their pre-treatment tumor samples. p=0.0261, n=73, log-rank test.

Since ATM is a central player in the cellular DNA damage response charged with the task of sensing DNA double strand breaks and recruiting other DNA repair factors to repair the damage, it has been traditionally assigned the role of a tumor suppressor. However, our data clearly show that in established tumor cells, it has a significant, tumor facilitative role. What is the molecular mechanism underlying its pro-tumor growth roles? By use of gene editing techniques to knockout ATM in breast cancer and glioma cells, Liu *et al* show that DDR and ATM play critical roles in activating the NF-κB and Stat3 transcription factors, promoting the expression of stemness genes such as CD133, Oct4 to enhance the tumorigenicty of cancer cells. The clinical relevance of the pathway is demonstrated by significantly worse prognoses for patients with activated ATM (**Fig. 11**).

Taken together, our work in established cancer cells provides strong proof for a pro-tumoral role for limited MOMP-Casp3-endoG-DSB-ATM axis (**Fig.12**).

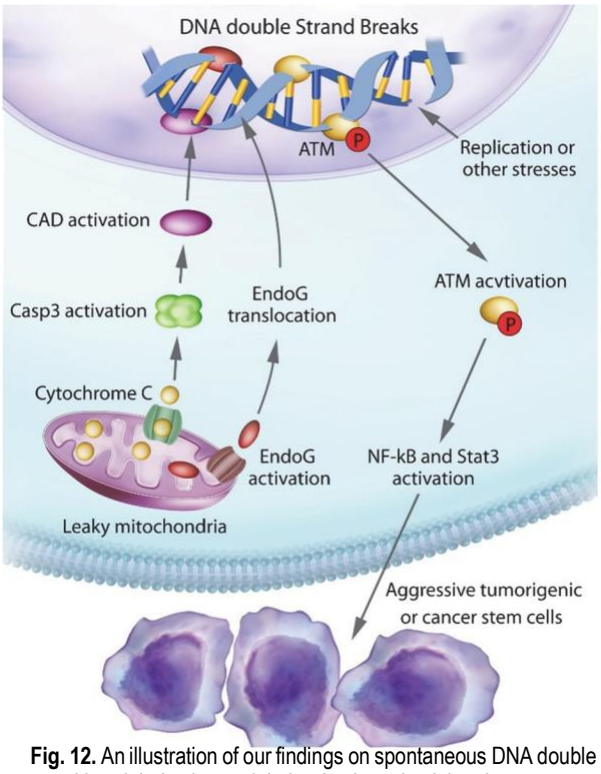

Fig. 12. An illustration of our findings on spontaneous DNA double strand break induction and their roles in maintaining the stemness and tumorigenicity of cancer cells.

F. Summary

Molecular factors regulating cell death and DNA repair play central roles in cellular response to external stressors such as radiation. Thus understanding the precise molecular mechanisms governing their actions is critical in assessing their contributions to radiation induced carcinogenesis or other biological effects. In the past 5- 10 years, the work from our lab and others have significantly changed our views on the biological roles those factors. In addition to upending the established paradigm on those factors, some unexpected novel roles, such as promoting DNA double strand breaks and stemness of cancer cells have also been discovered. It is to be expected additional new roles of these important factors will be unveiled in future.

G. References cited

1. Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26**, 239-257
2. Strasser, A., O'Connor, L., and Dixit, V. M. (2000) Apoptosis signaling. *Annu Rev Biochem* **69**, 217-245
3. Fadok, V. A., Bratton, D. L., Frasch, S. C., Warner, M. L., and Henson, P. M. (1998) The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. *Cell Death Differ* **5**, 551-562
4. Fadok, V. A., Bratton, D. L., Konowal, A., Freed, P. W., Westcott, J. Y., and Henson, P. M. (1998) Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest* **101**, 890-898
5. Elmore, S. (2007) Apoptosis: a review of programmed cell death. *Toxicol Pathol* **35**, 495-516
6. Li, F., He, Z., Shen, J., Huang, Q., Li, W., Liu, X., He, Y., Wolf, F., and Li, C. Y. (2010) Apoptotic caspases regulate induction of iPSCs from human fibroblasts. *Cell Stem Cell* **7**, 508-520
7. Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., and Jones, J. M. (1998) Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145-1147
8. Fujita, J., Crane, A. M., Souza, M. K., Dejosez, M., Kyba, M., Flavell, R. A., Thomson, J. A., and Zwaka, T. P. (2008) Caspase activity mediates the differentiation of embryonic stem cells. *Cell Stem Cell* **2**, 595-601
9. Tang, H. L., Tang, H. M., Mak, K. H., Hu, S., Wang, S. S., Wong, K. M., Wong, C. S., Wu, H. Y., Law, **H.** T., Liu, K., Talbot, C. C., Jr., Lau, W. K., Montell, D. J., and Fung, M. C. (2012) Cell survival, DNA damage, and oncogenic transformation after a transient and reversible apoptotic response. *Mol Biol Cell* **23**, 2240-2252
10. Ichim, G., Lopez, J., Ahmed, S. U., Muthalagu, N., Giampazolias, E., Delgado, M. E., Haller, M., Riley, J. S., Mason, S. M., Athineos, D., Parsons, M. J., van de Kooij, B., Bouchier-Hayes, L., Chalmers, A. J., Rooswinkel, R. W., Oberst, A., Blyth, K., Rehm, M., Murphy, D. J., and Tait, S. W. (2015) Limited mitochondrial permeabilization causes DNA damage and genomic instability in the absence of cell death. *Mol Cell* **57**, 860-872
11. Ding, A. X., Sun, G., Argaw, Y. G., Wong, J. O., Easwaran, S., and Montell, D. J. (2016) CasExpress reveals widespread and diverse patterns of cell survival of caspase-3 activation during development in vivo. *Elife* **5**
12. Liu, X., Li, F., Huang, Q., Zhang, Z., Zhou, L., Deng, Y., Zhou, M., Fleenor, D. E., Wang, H., Kastan, M. B., and Li, C. Y. (2017) Self-inflicted DNA double-strand breaks sustain tumorigenicity and stemness of cancer cells. *Cell Res* **27**, 764-783
13. Tamura, T., Ishihara, M., Lamphier, M. S., Tanaka, N., Oishi, I., Aizawa, S., Matsuyama, T., Mak, T. W., Taki, S., and Taniguchi, T. (1995) An IRF-1-dependent pathway of DNA damage-induced apoptosis in mitogen-activated T lymphocytes. *Nature* **376**, 596-599
14. Brown, J. M., and Wouters, B. G. (1999) Apoptosis, p53, and tumor cell sensitivity to anticancer agents. *Cancer Res* **59**, 1391-1399
15. Liu, X., He, Y., Li, F., Huang, Q., Kato, T. A., Hall, R. P., and Li, C. Y. (2015) Caspase-3 promotes genetic instability and carcinogenesis. *Mol Cell* **58**, 284-296
16. Evan, G. I., Wyllie, A. H., Gilbert, C. S., Littlewood, T. D., Land, H., Brooks, M., Waters, C. M., Penn, L. Z., and Hancock, D. C. (1992) Induction of apoptosis in fibroblasts by c-myc protein. *Cell* **69**, 119-128
17. Cartwright, I. M., Liu, X., Zhou, M., Li, F., and Li, C. Y. (2017) Essential roles of Caspase-3 in facilitating Myc-induced genetic instability and carcinogenesis. *Elife* **6**
18. Zhou, B. B., and Elledge, S. J. (2000) The DNA damage response: putting checkpoints in perspective. *Nature* **408**, 433-439

19. Savitsky, K., Sfez, S., Tagle, D. A., Ziv, Y., Sartiel, A., Collins, F. S., Shiloh, Y., and Rotman, G. (1995) The complete sequence of the coding region of the ATM gene reveals similarity to cell cycle regulators in different species. *Hum Mol Genet* **4**, 2025-2032
20. Shiloh, Y. (2003) ATM and related protein kinases: safeguarding genome integrity. *Nat Rev Cancer* **3**, 155-168
21. 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
22. 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
23. Li, L. Y., Luo, X., and Wang, X. (2001) Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature* **412**, 95-99
24. Parrish, J., Li, L., Klotz, K., Ledwich, D., Wang, X., and Xue, D. (2001) Mitochondrial endonuclease G is important for apoptosis in C. elegans. *Nature* **412**, 90-94
25. Chang, W. P., and Little, J. B. (1992) Persistently elevated frequency of spontaneous mutations in progeny of CHO clones surviving X-irradiation: association with delayed reproductive death phenotype. *Mutat Res* **270**, 191-199
26. Morgan, W. F. (2003) Non-targeted and delayed effects of exposure to ionizing radiation: II. Radiation-induced genomic instability and bystander effects in vivo, clastogenic factors and transgenerational effects. *Radiat Res* **159**, 581-596
27. Morgan, W. F. (2003) Non-targeted and delayed effects of exposure to ionizing radiation: I. Radiation-induced genomic instability and bystander effects in vitro. *Radiat Res* **159**, 567-580
28. Hanahan, D., and Weinberg, R. A. (2011) Hallmarks of cancer: the next generation. *Cell* **144**, 646-674
29. Dang, C. V. (2012) MYC on the path to cancer. *Cell* **149**, 22-35
30. Pelengaris, S., Khan, M., and Evan, G. (2002) c-MYC: more than just a matter of life and death. *Nat Rev Cancer* **2**, 764-776
31. Karlsson, A., Deb-Basu, D., Cherry, A., Turner, S., Ford, J., and Felsher, D. W. (2003) Defective double-strand DNA break repair and chromosomal translocations by MYC overexpression. *Proc Natl Acad Sci U S A* **100**, 9974-9979
32. Vafa, O., Wade, M., Kern, S., Beeche, M., Pandita, T. K., Hampton, G. M., and Wahl, G. M. (2002) c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: a mechanism for oncogene-induced genetic instability. *Mol Cell* **9**, 1031-1044
33. Ray, S., Atkuri, K. R., Deb-Basu, D., Adler, A. S., Chang, H. Y., Herzenberg, L. A., and Felsher, D. W. (2006) MYC can induce DNA breaks in vivo and in vitro independent of reactive oxygen species. *Cancer Res* **66**, 6598-6605