Supplementary Information for

Caspase 3-mediated stimulation of tumor cell repopulation during cancer radiotherapy

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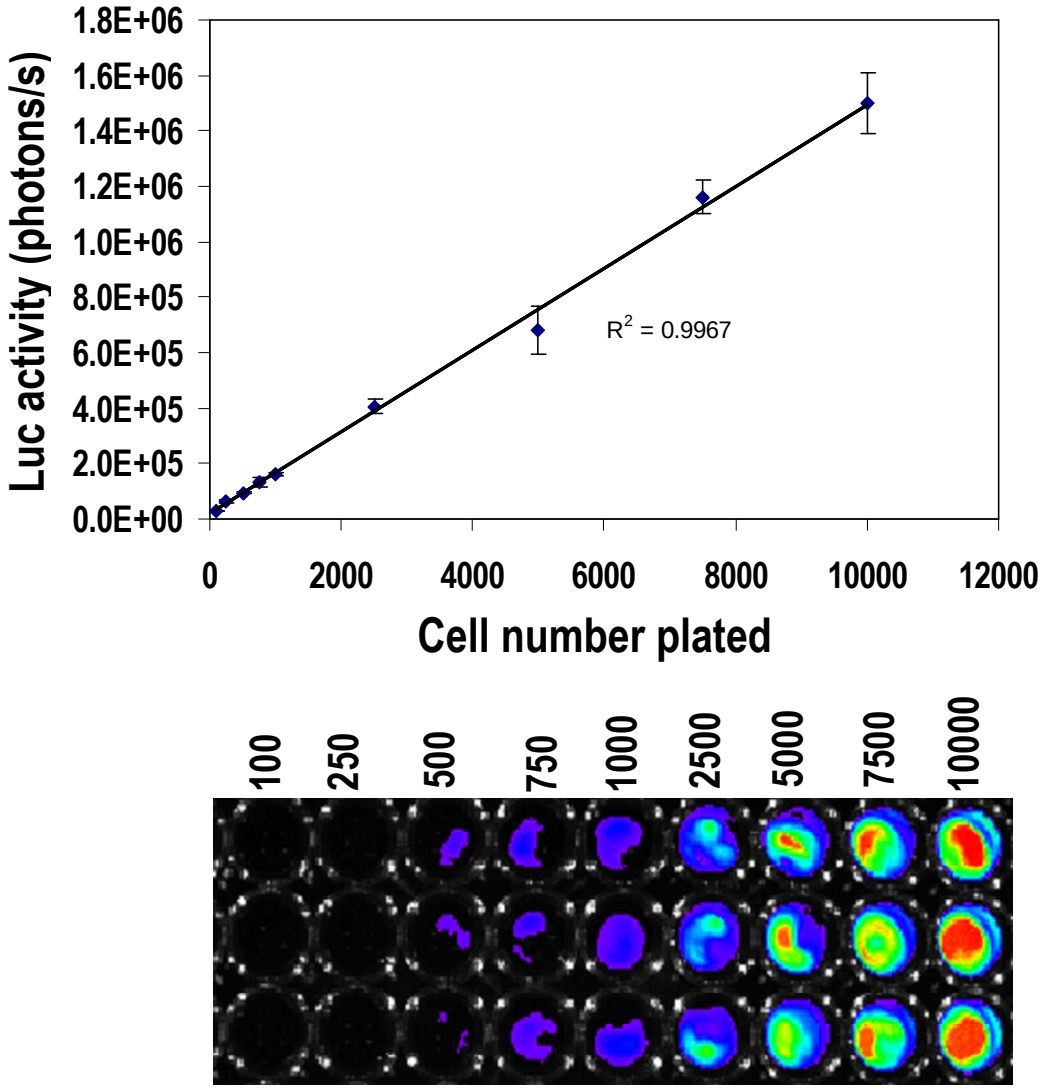
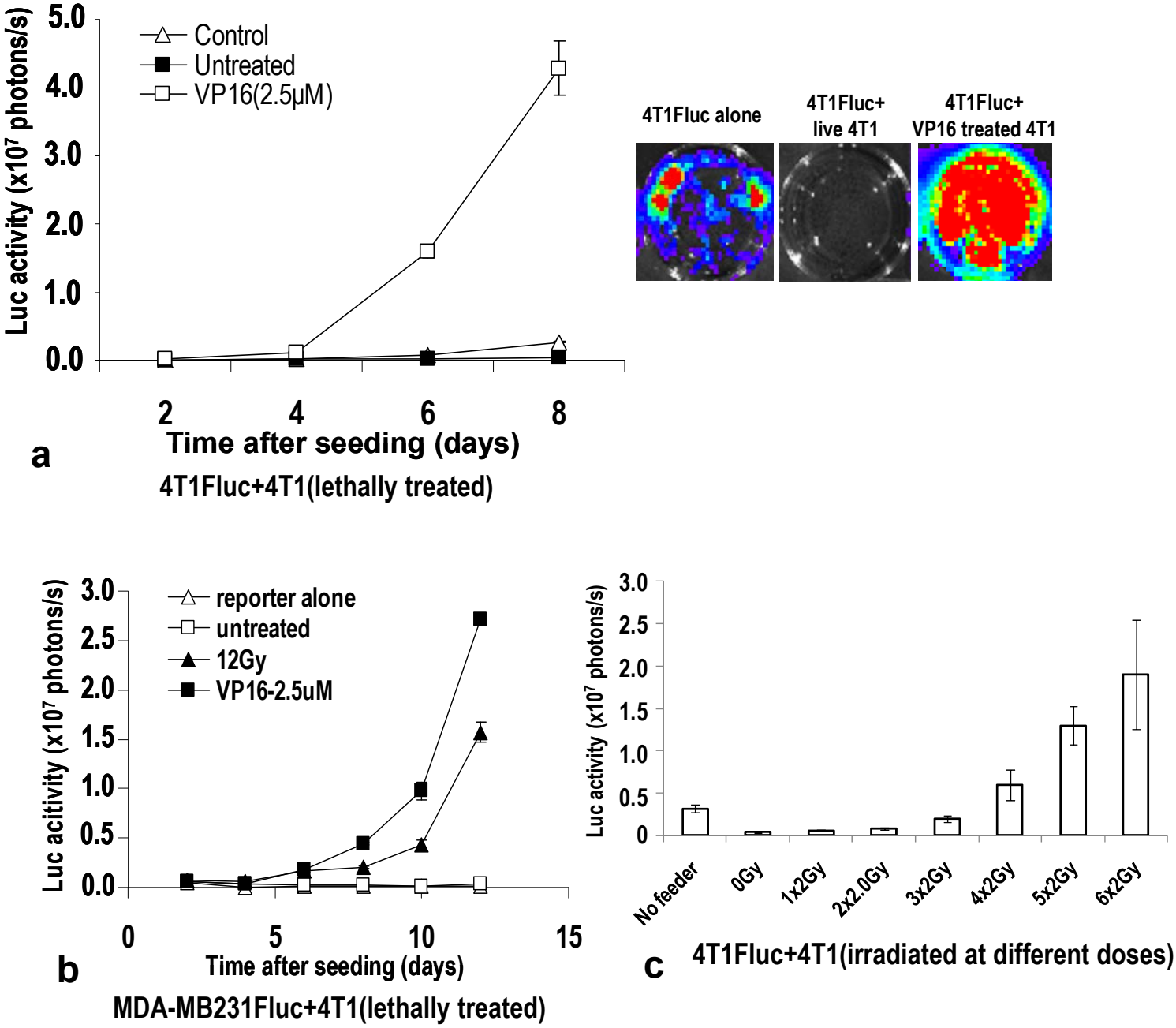
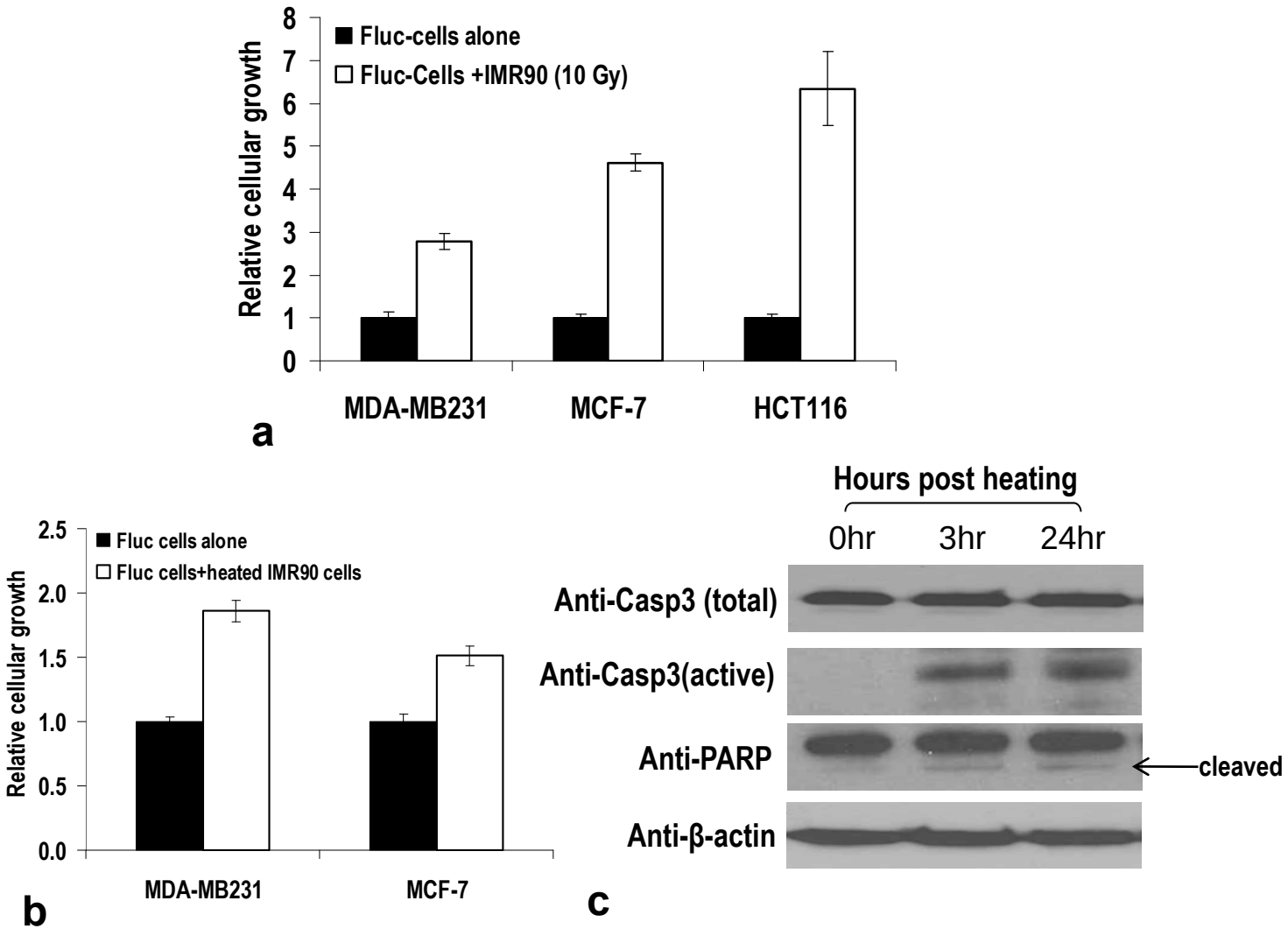
Supplementary Figures



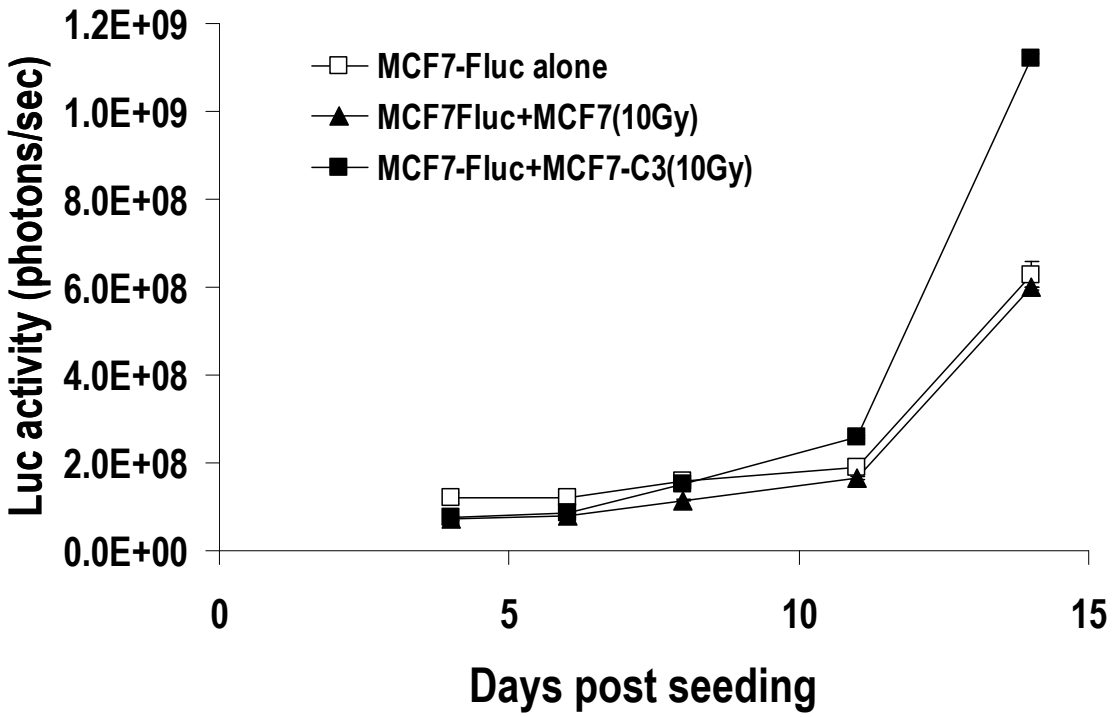
Supplementary Figure 1. Linearity of measured bioluminescence vs 4T1Fluc cell number. 4T1Fluc cells were plated in 96 well dishes in triplicates in different numbers. They were then imaged by use of the IVIS200 imaging system. Top panel, luc activity plotted against cell number. There is clearly a very good linearity (R^2=0.99). Lower panel, a luciferase image of the plated 4T1Fluc cells.



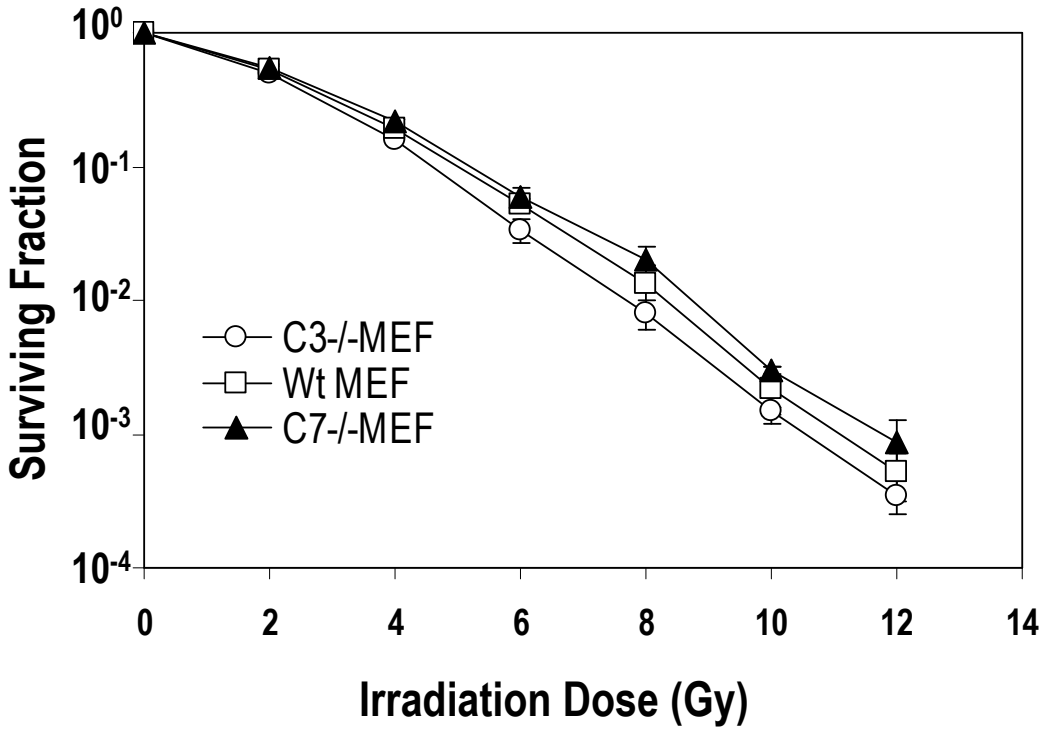
Supplementary Figure 2. Growth stimulation from cells dying from other cytotoxic treatments. **a**) About $2x10^5$ lethally treated (2.5 μM VP-16 or 12 Gy x-rays) cells were plated into each well of 12-well plates. Twenty-four hrs later, about 1000 Fluc-labeled 4T1 (top left panel) or MDA-MB231 (lower panel) cells were plated onto the treated 4T1 cells and monitored for growth through non-invasive bioluminescence imaging. The right panel shows representative images of 4T1-Fluc cells plated alone or onto non-treated or VP-16 treated 4T1 cells. **b**). Similar experiments were done with MDA-M231Fluc cells as "reporter" and lethally irradiated 4T1 cells as "feeder". **c**) Similar experiments were conducted with 500 4T1Fluc cells $+2x10^5$ 4T1 cells irradiated with fractionated radiation at different doses over the course of 1 week. Data shown were results at 7 days after the last irradiation.



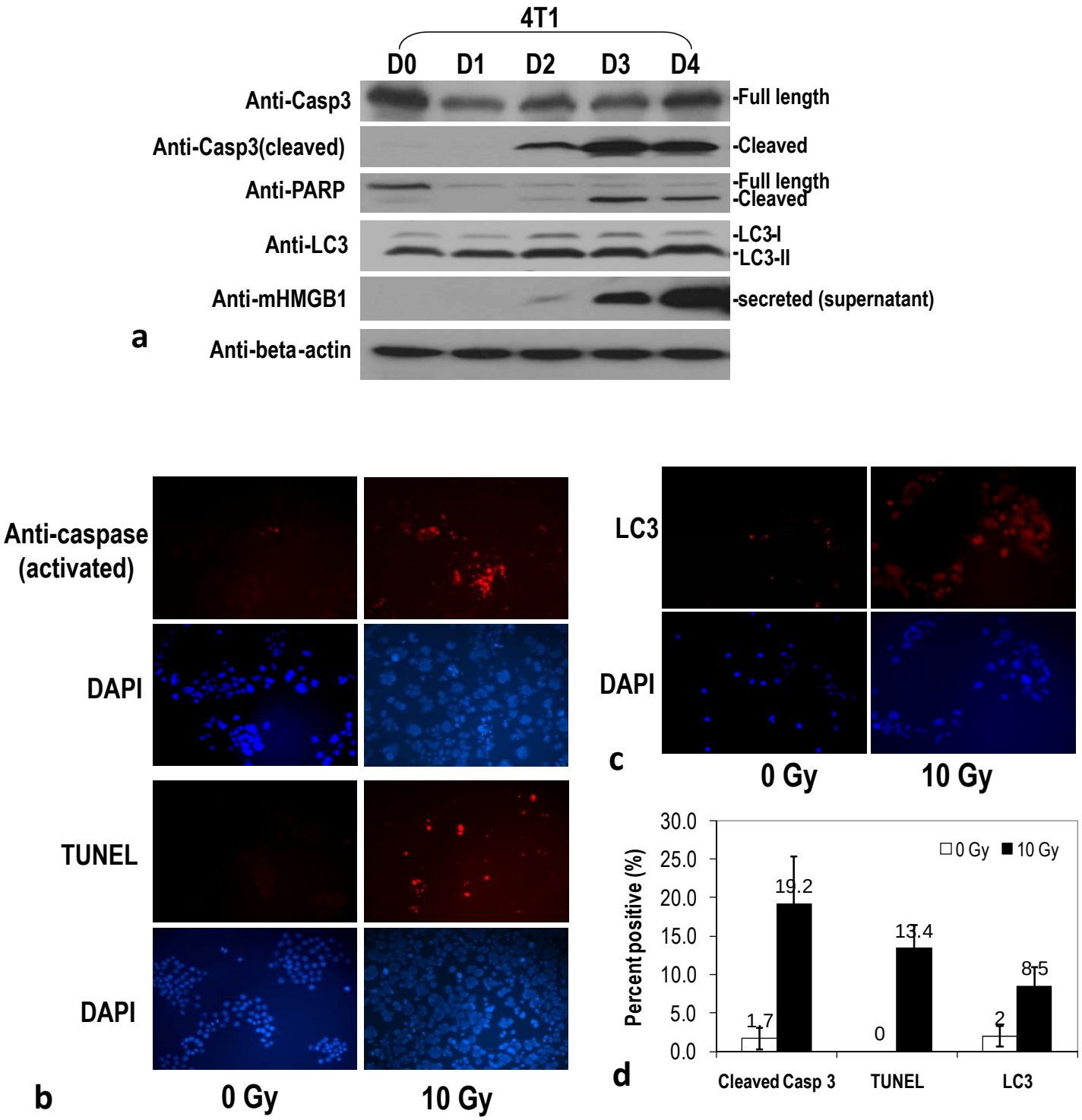
Supplementary Figure 3. Growth of various Fluc-labeled human tumor cells (about 1000 each) plated onto human IMR-90 fibroblast cells. IMR-90 cells(about $2x10^5$) were lethally treated with x-rays (10 Gy) (**a**) or heated (43°C for 30 minutes) (**c**). Western blot analyses (**c**) show caspase 3 cleavage and corresponding cleavage of PARP in heated cells, both evidence of caspase 3 activation.



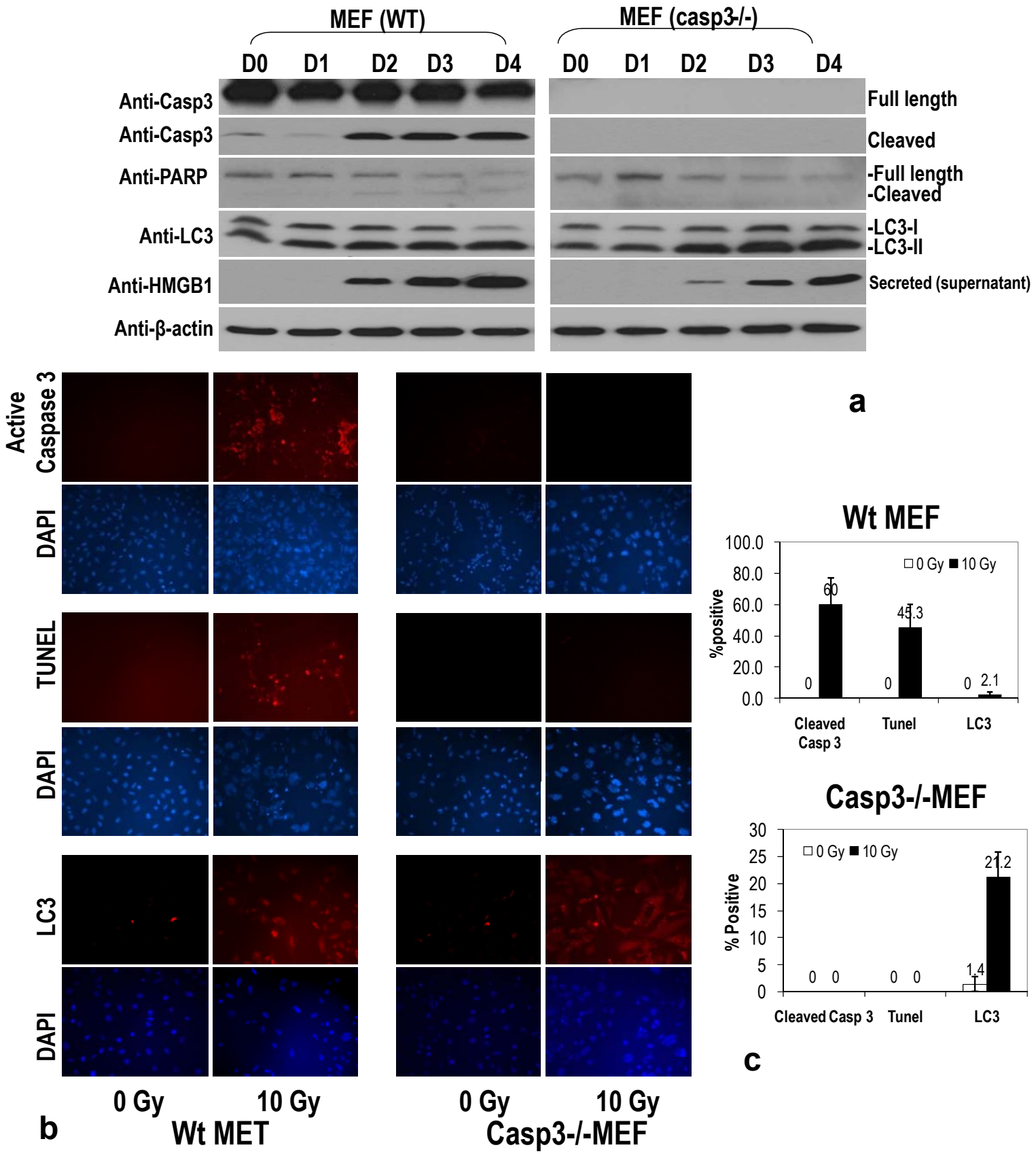
Supplementary Figure 4. Growth of Fluc-labeled MCF-7Fluc cells (about 1000) when plated either alone, with lethally irradiated parental MCF-7 cells (which does not express caspase 3), or with MCF7-C3, which express an exogenous caspase 3 gene. Lethally irradiated cells numbered around $2x10^5$.



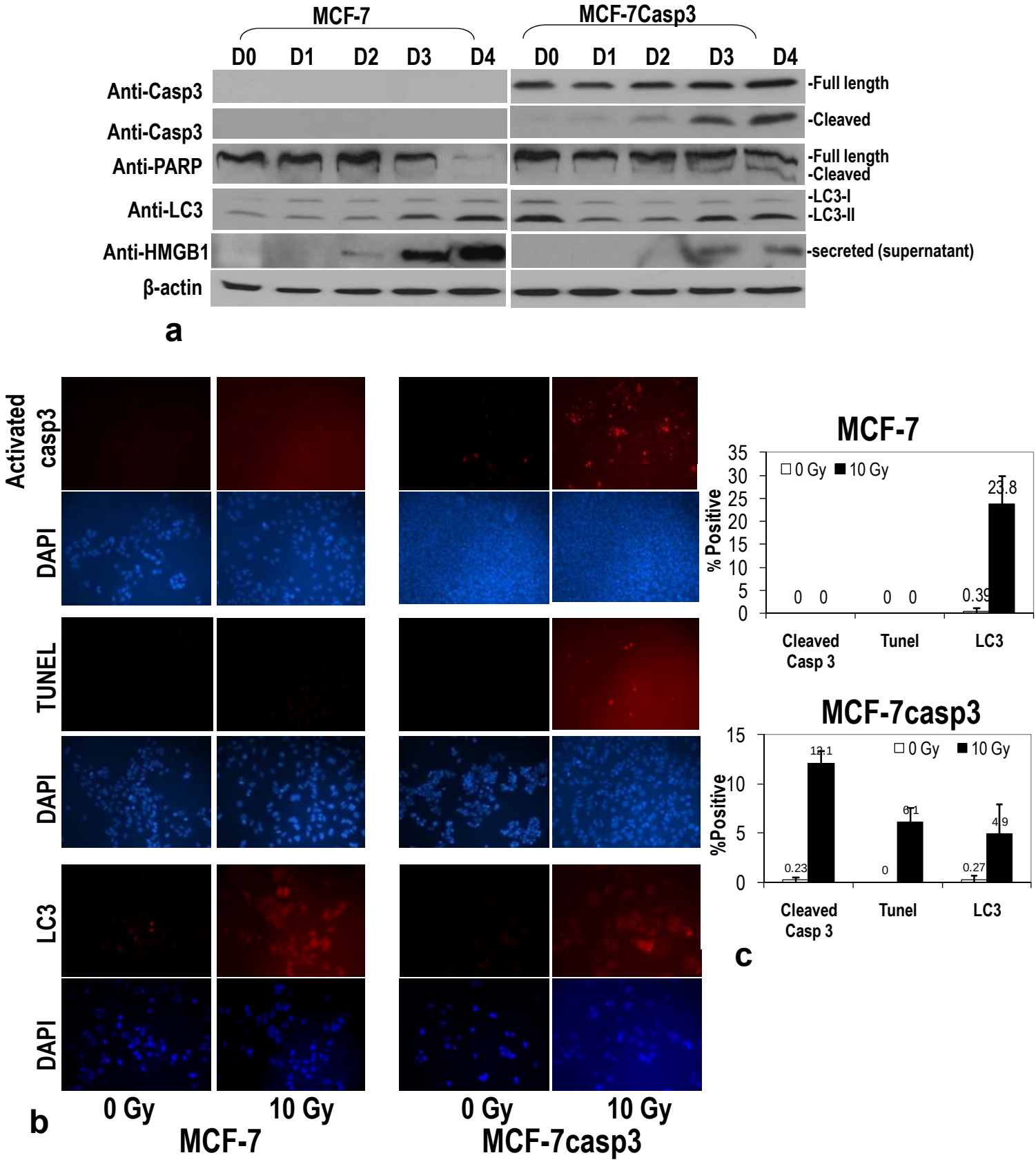
Supplementary Figure 5. Clonogenic survival of wild type and caspase 3 or caspase 7 deficient MEF cells. Cells were irradiated with different doses of x-rays and then plated into 10-cm Petri dishes at various densities. After 10-12 days, colonies that emerged in the Petri dishes were counted (the criteria for a colony was that it contained more than 50 cells). Each data point is the average of data from three replicate dishes. Data were normalized against non-irradiated controls. The error bar represents standard error of the mean.



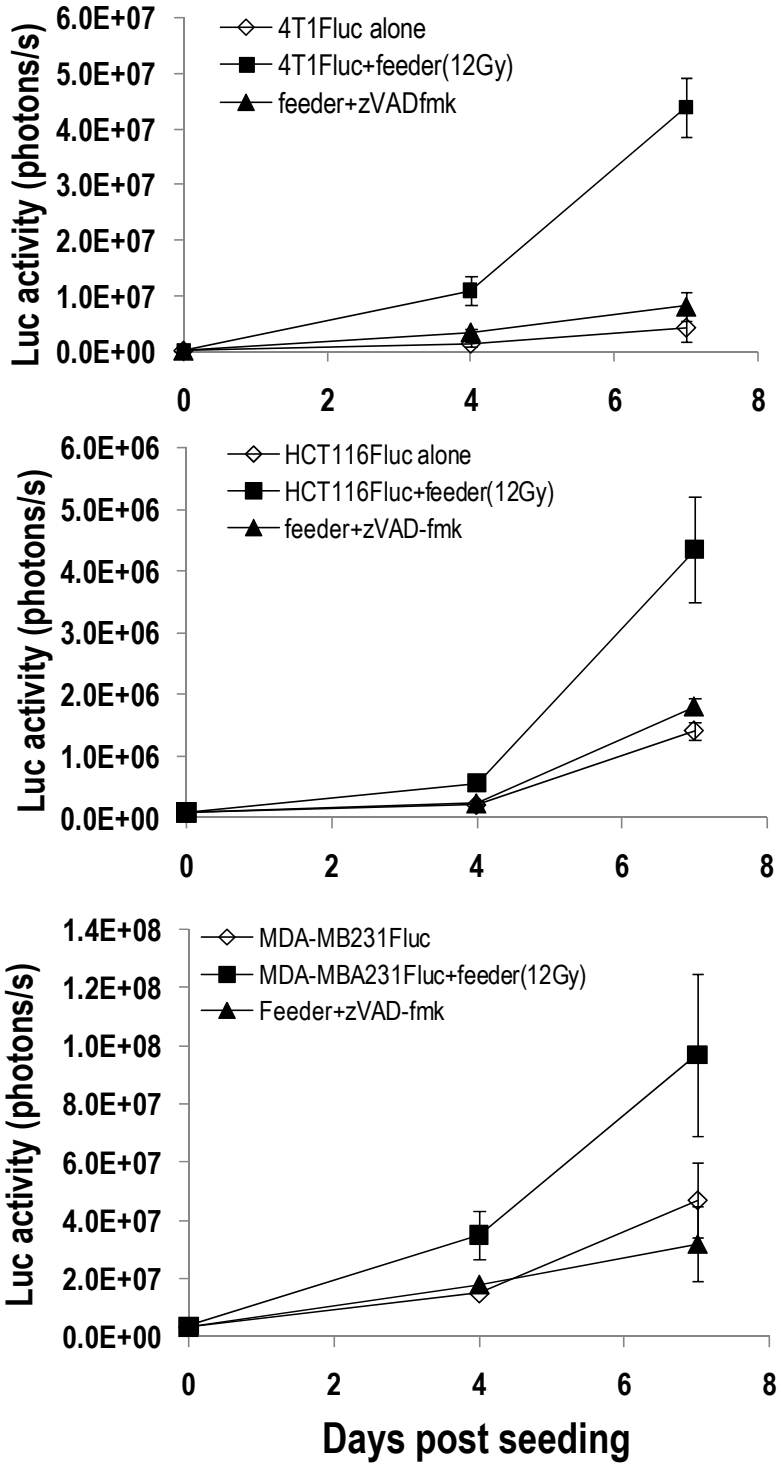
Supplementary Figure 6. Modes of cell death in 4T1 cells 4 days after 10Gy irradiation. **a**). Western blot analysis of markers for apoptosis (cleaved caspase 3& PARP), autophagy (increased LC3-II), and necrosis (HMGB1 secretion into supernatant). **b**). Immunofluorescence staining of activated caspase 3 (top panels) and TUNEL(lower panels) after 0 Gy (left panels) or 10 Gy (right panels) of x-rays. DAPI (lower panels) was used to stain for nuclei of cells. **c**). Immunofluorescence staining of control and irradiated (10 Gy) cells for the autophagic marker LC3.**d**). Quantitative data on the fraction of cells going through different modes of cell death. For LC3, Only those cells with perinuclear staining were counted. Error bars represent SEM (n=3).



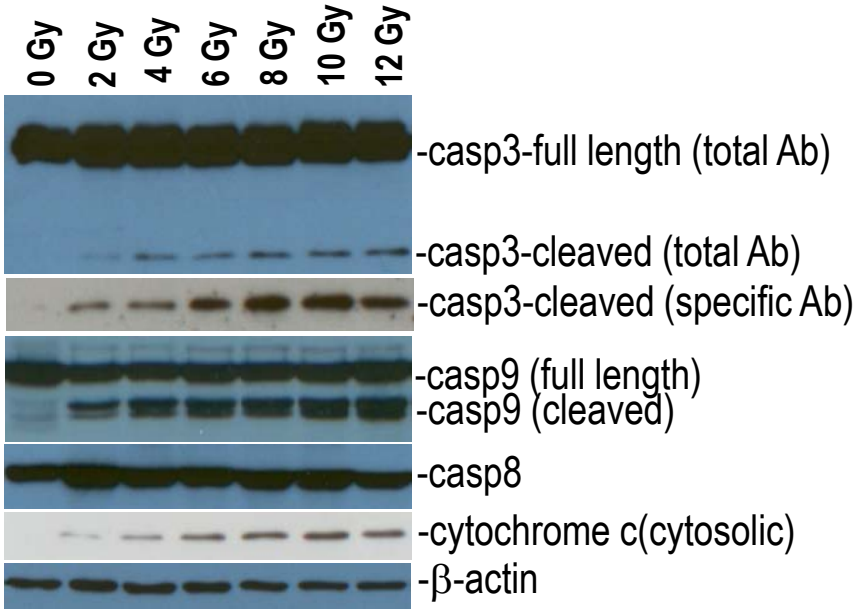
Supplementary Figure 7. Modes of cell death in wild type and caspase 3 deficient MEF cells after 10Gy irradiation. **a**). Western blot analysis of markers for apoptosis (cleaved caspase 3& PARP), autophagy (increased LC3-II), and necrosis (HMGB1 secretion into supernatant). **b**). Immunofluorescence staining of activated caspase 3, TUNEL, and LC3 in control and irradiated (10 Gy) wild type and casp3-/- MEF cells . **c**). Quantitative data on the extent of marker staining in wild type as well as casp3-/- MEF cells. For LC3, only those with perinuclear staining were counted. Error bars represent SEM (n=3).



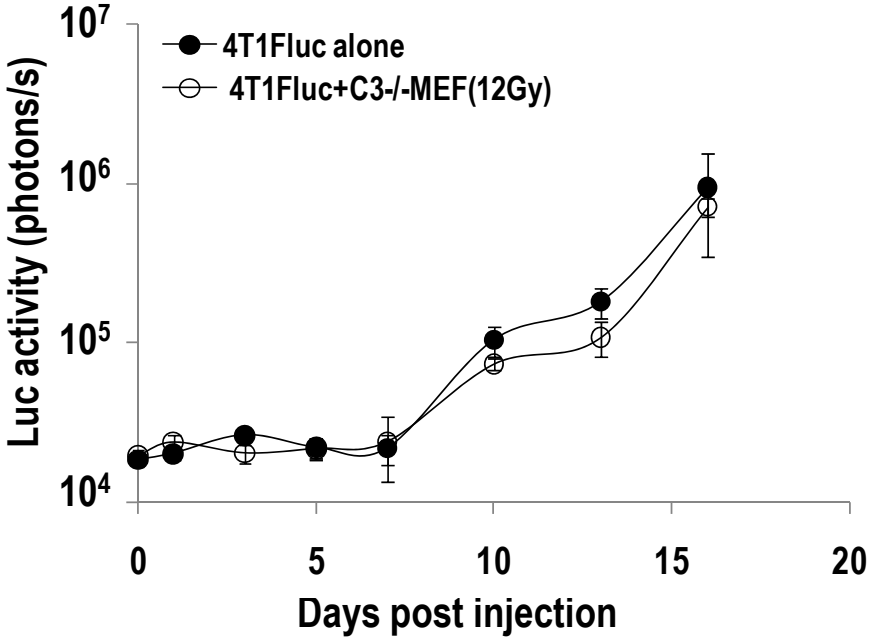
Supplementary Figure 8. Modes of cell death in wild type and MCF-7 and MCF-7casp3 cells after 10Gy irradiation. **a**). Western blot analysis of markers for apoptosis (cleaved caspase 3& PARP), autophagy (increased LC3-II), and necrosis (HMGB1 secretion into supernatant). **b**). Immunofluorescence staining of activated caspase 3, TUNEL, and LC3 after 0 or 10 Gy of x-rays in MCF-7 or MCF-7casp3 cells. DAPI staining was used to stain for nuclei of cells. **c**).Quantitative data on the extent of marker staining. For LC3, only those with perinuclear staining were counted. Error bar represents SEM (n=3).



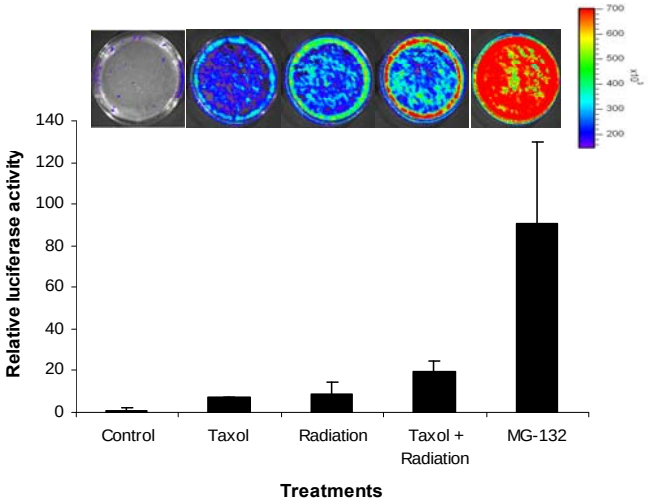
Supplementary Figure 9. The effect of a pan-caspase inhibitor z-VAD-fmk on dying cell stimulated, luc-labeled tumor cell growth. About 1000 Fluc cells were plated either alone, with non-irradiated, same type but lethally irradiated tumor cells, or with lethally irradiated tumor cells plus a pan-caspase inhibitor z-VAD-fmk (at 8μM).

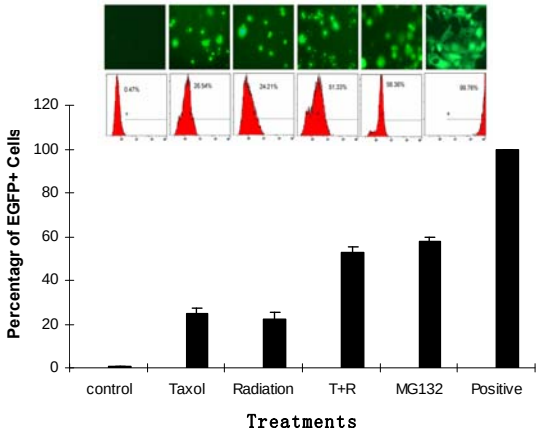


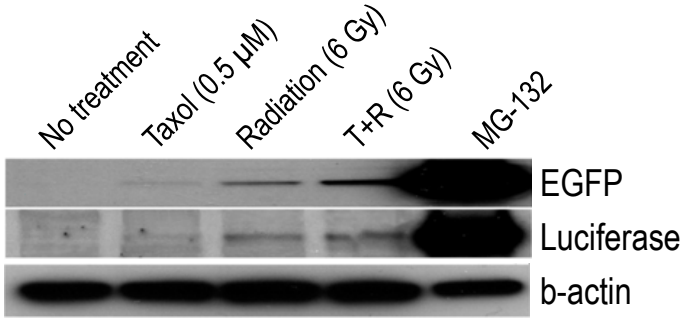
Supplementary Figure 10. Dose-dependent caspase 3 activation in wild type MEF cells. MEF cells were exposed with X-rays at different doses. Twenty-four hours later, cells were collected and cellular lysates were collected (see supplementary Methods section for more details). The lysates were analyzed through western blot analysis for various apoptosis related proteins. Two antibodies were used for caspase 3. One (top panel) detects the full length as well as the cleaved, activated fragments. The other one (second panel from the top) only detects the cleaved fragment. There is a clear dose-response relationship between caspase 3, cytochrome c, caspase 8 activation and radiation exposure.



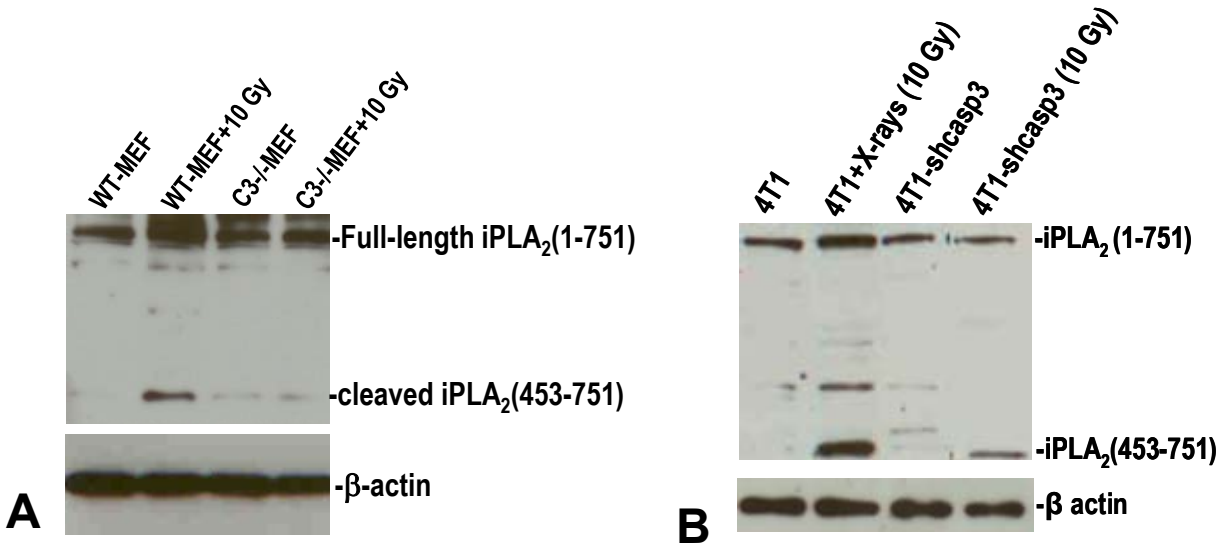
Supplementary Figure 11. Lack of growth stimulating effect of irradiated caspase-deficient MEF cells. About 1000 4T1 Fluc cells were injected subcutaneously into the right hind leg with $2.5x10^5$ C3-deficient, irradiated MEF cells. As controls, about 1000 4T1Fluc cells were injected alone in the contra-lateral left hind legs. The growth of the Fluc labeled cells were then monitored through bioluminescence imaging. The difference between two groups were insignificant ($P>0.05$, $n=5$) for most of the data points.



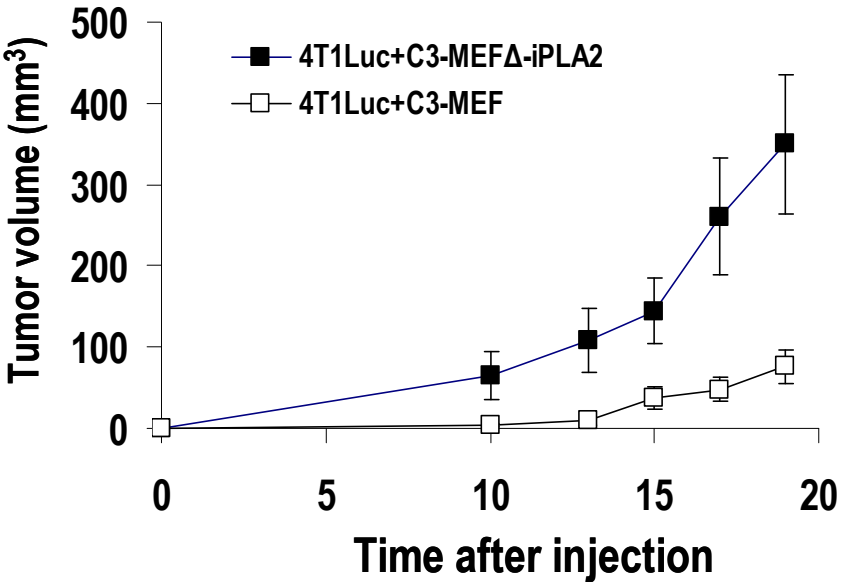




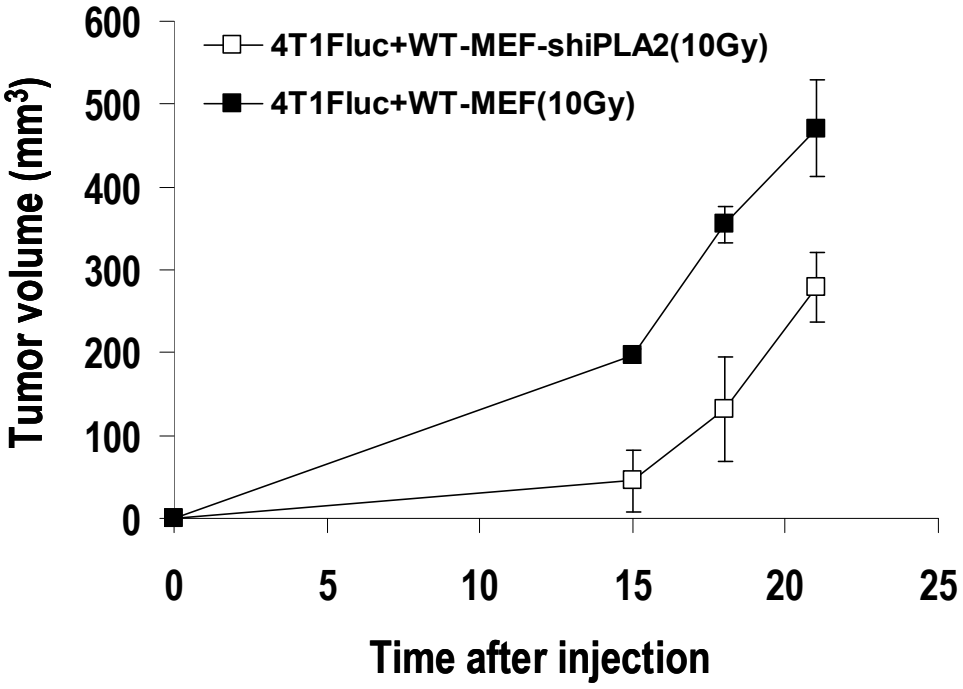
Supplementary Figure 12. Validation of the caspase 3 reporter. 4T1 tumor cells with the casp3 reporter was treated with radiation (6Gy) or taxol (0.5 μM), or the proteasome inhibitor MG132 (10uM), agents known to induced apoptosis in 4T1 cells. Top left panel, results from luc imaging. Top right panel, results from flow cytometry analysis of EGFP. Lower panel, western blot analysis of reporter proteins showing increased reporter activities with increasing doses of cytotoxic treatments 24 hrs after treatment. MG-132 is a proteasome inhibitor that prevents proteasome-mediated protein degradation.



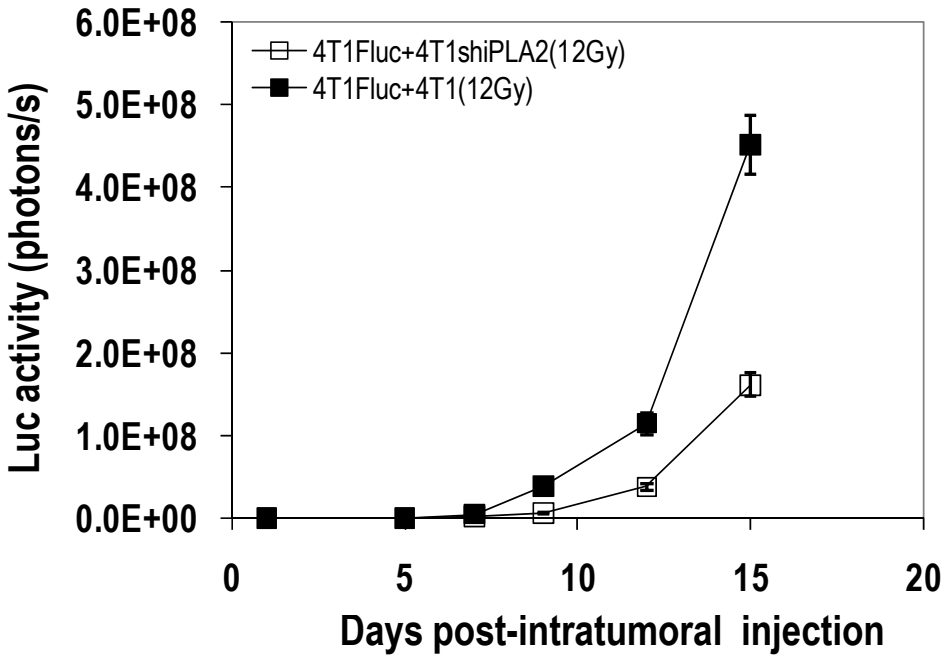
Supplementary Figure 13. Western blot analysis. **Panel A**, $iPLA_2$ analysis in MEF cells. Wild type, casp3-/- and casp7-/- MEF cells were irradiated and cultured for 24 hrs. They were then lysed for western blot analysis for $iPLA_2$. An antibody obtained from Caymen Chemicals (Ann Arbor, MI) was used in the analysis. **Panel B.** Radiation-induced, caspase-mediated $iPLA_2$ in 4T1 cells. Parental as well as casp3 shRNA knockdown cells were used.



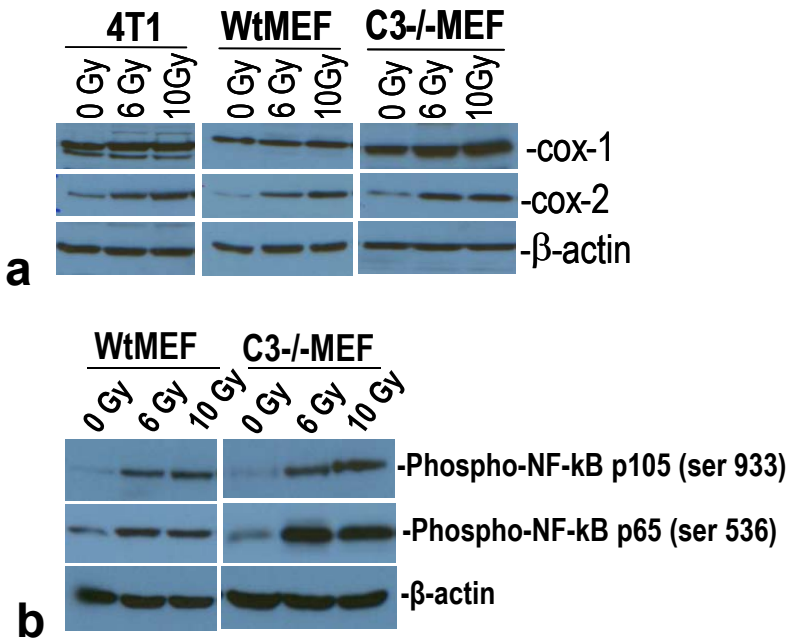
Supplementary Figure 14. Tumor growth from a small number (1000) of 4T1Fluc cells injected together with lethally irradiated parental caspase 3-deficient (C3-) MEF cells or C3-MEF cells stably transduced with a gene encoding a truncated, constitutively active murine ΔiPLA2 protein. Tumor dimensions were measured with a caliper and converted to volume using the formula $V=(1/2)\ (width)^2*(length)$



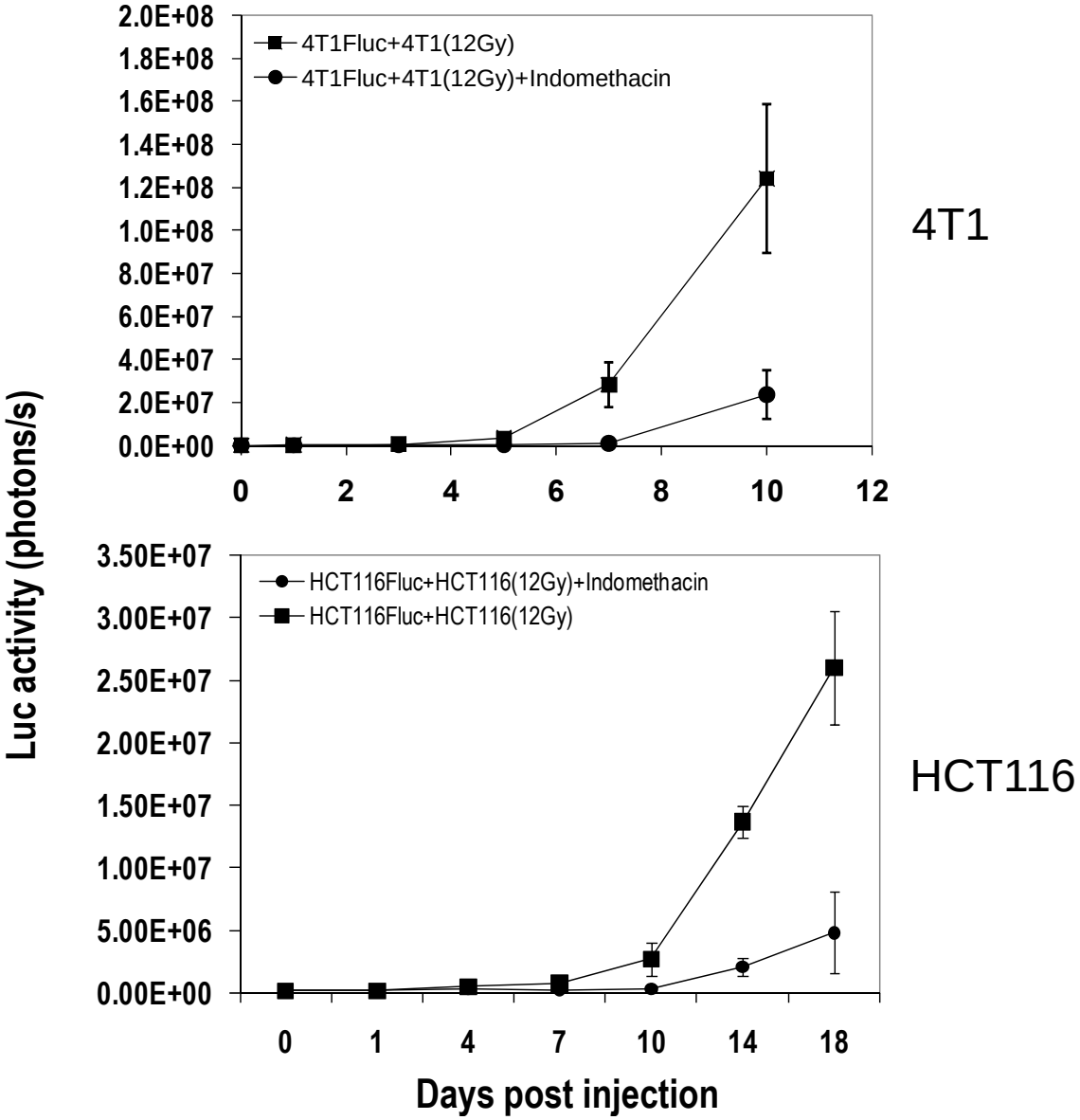
Supplementary Figure 15. Tumor growth from a small number (1000) of 4T1Fluc cells injected together with wild type MEF cells or MEF cells transduced with a shRNA gene targeted against the murine $iPLA_2$ gene.



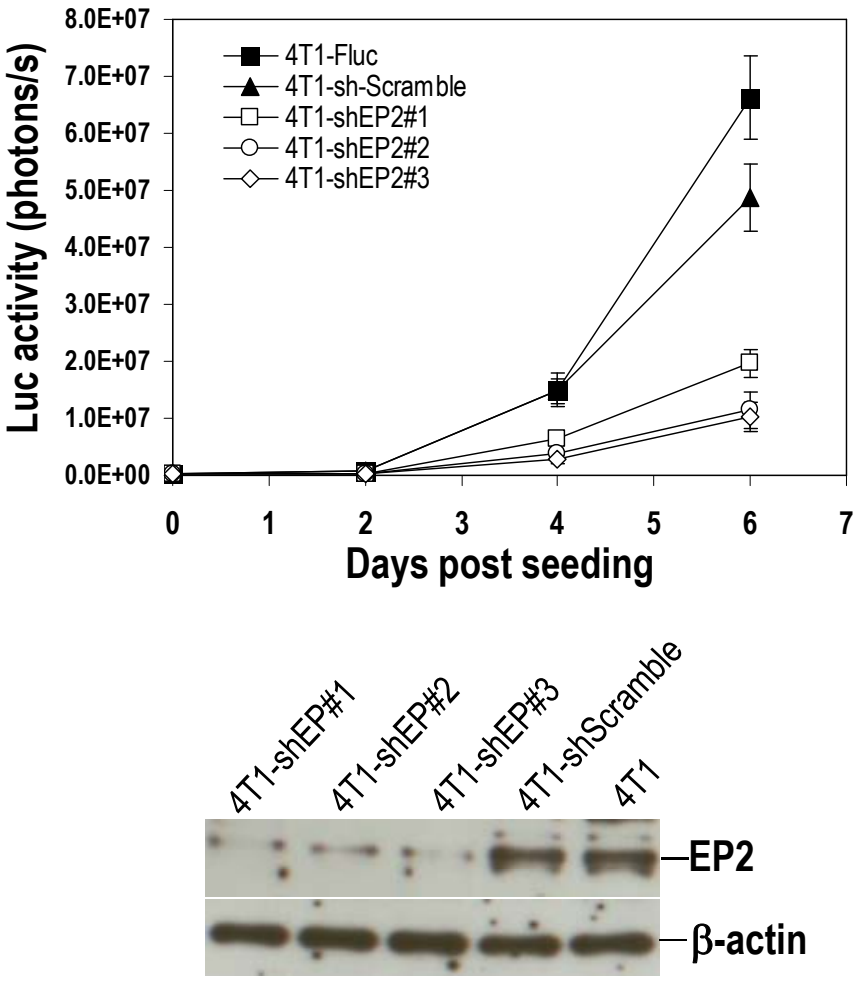
Supplementary Figure 16. The effect of stromal iPLA2 on growth of a small number of surviving tumor cells. About 1000 4TFluc cells were injected into irradiated (2x10Gy) tumors established either from the parental 4T1 or the modified 4T1shiPLA2 cells. The growth of the Fluc labeled tumor cells were then followed through bioluminscence imaging. The difference were significant from day 9 ($P<0.01$, $n=5$, t test).



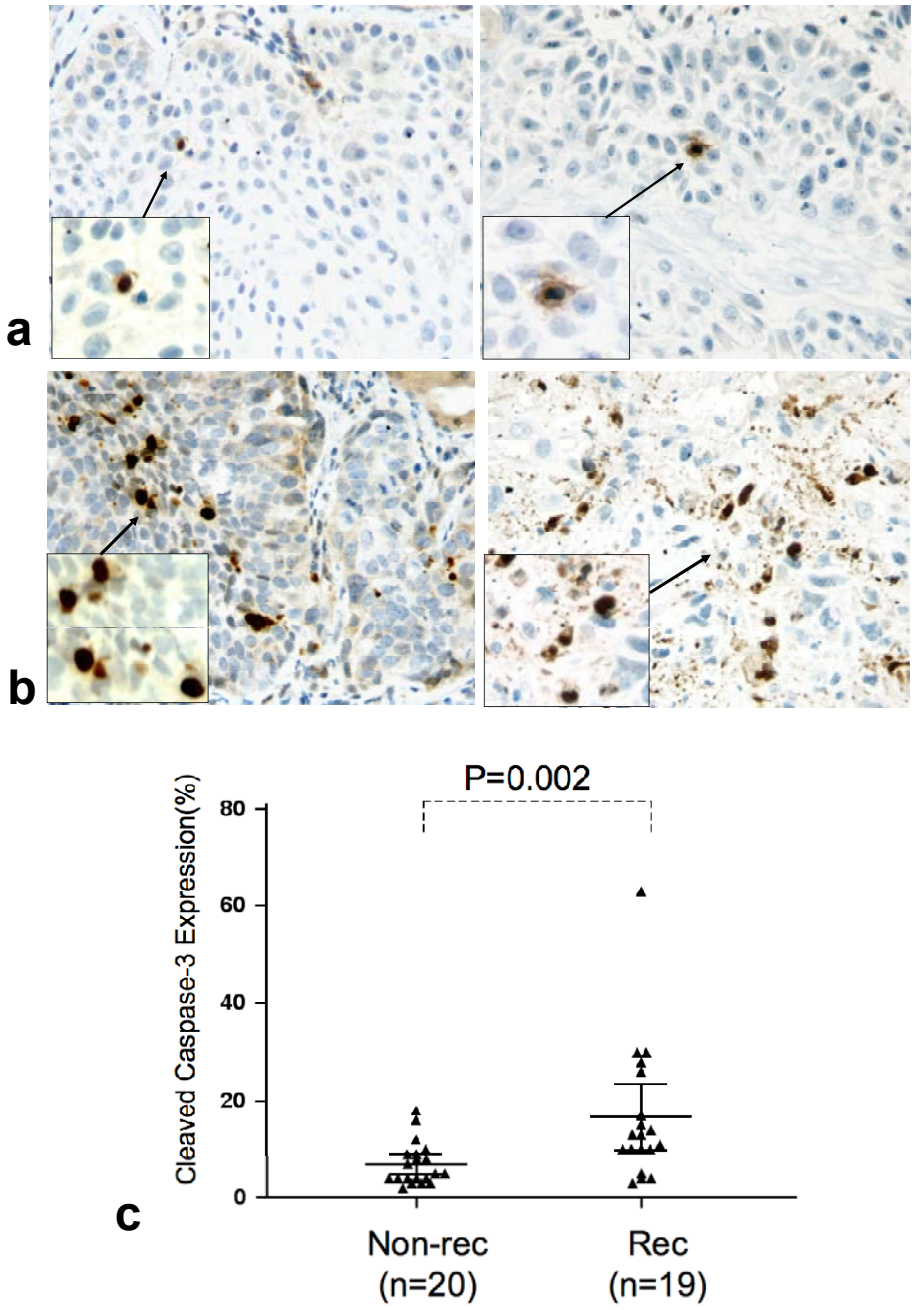
Supplementary Figure 17. Western blot analysis of Cox1&2 and NF-kB. **a**). Western blot analysis of Cox 1&2 expression. About $2x10^6$ exponentially growing 4T1, wtMEF and casp3-/- MEF were irradiated with 6 or 10 Gy of x-rays. Twenty-four hrs later, they were lysed and analyzed for Cox and Cox2 expression by western blot analysis. **b**). NF-kB activation after radiation. About $2x10^6$ exponentially growing wt and casp3-/- MEF cells were exposed to x-rays and incubated for 24 hrs. The cells were then lysed and analysed for phosphorylated p105 NF-kB and phosphorylated p65 NF-kB expression, which were indicative of NF-kB activation.



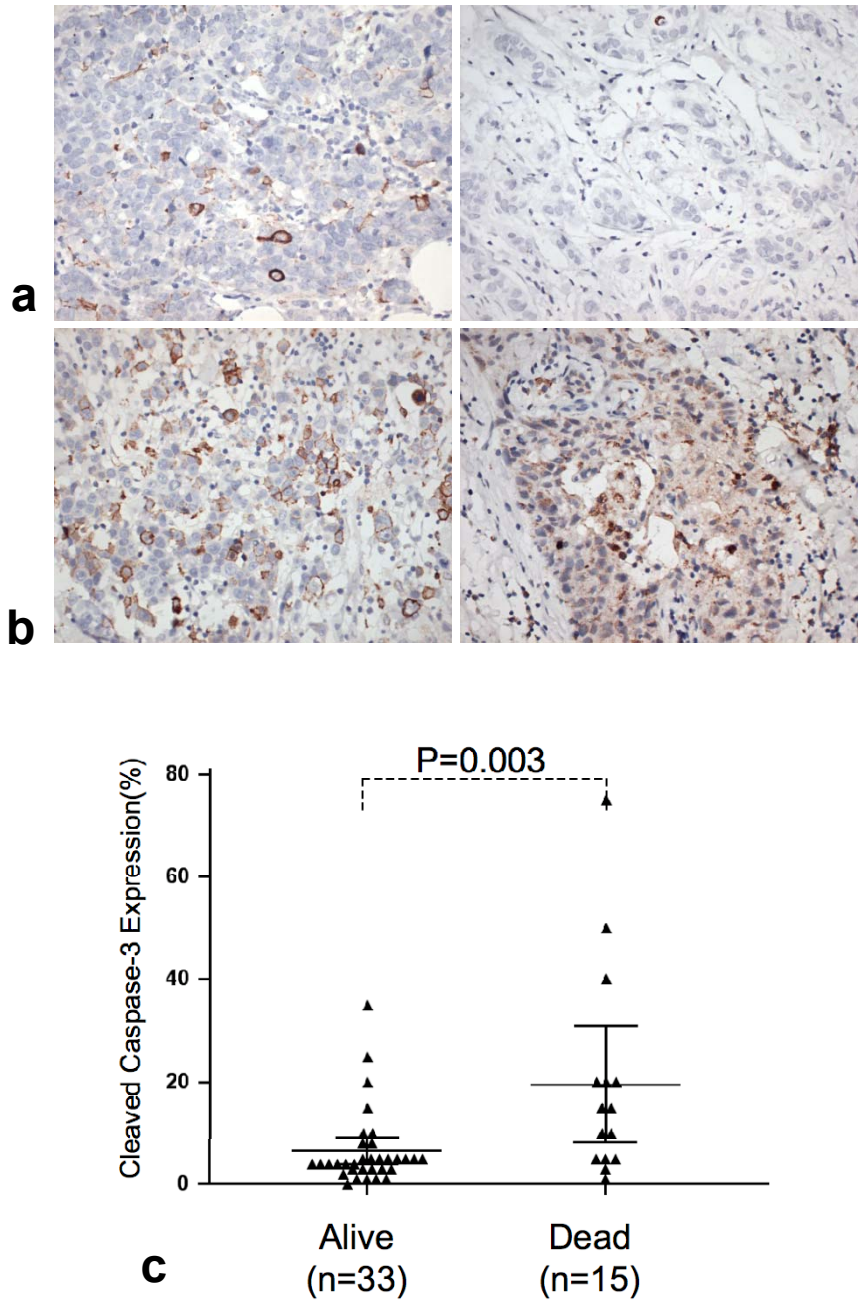
Supplementary Figure 18. Growth of intratumorally injected Fluc cells. Nude mice with 4T1(top panel), and HCT116 (lower panel) tumors established in the hind legs were irradiated with 12Gy x-rays. Indomethacin were administered to the mice at 3mg/Kg on a daily basis for 7 days after irradiation. Twenty-four hours later, about 1000 Fluc-labeled 4T1 or HCT116 cells were injected into the irradiated tumors, respectively. They were then followed by bioluminescence imaging. The error bars represent standard error of the mean (n=5).



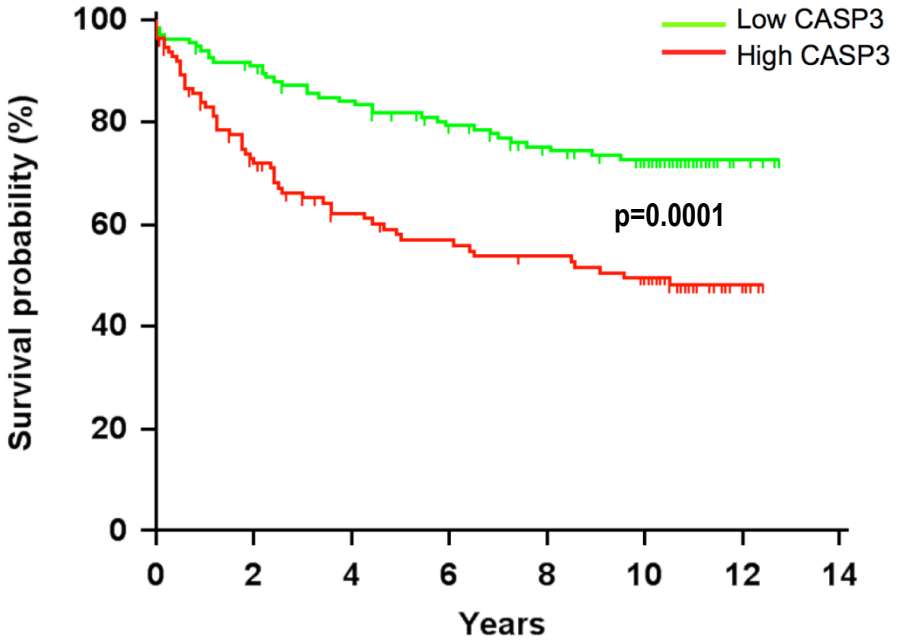
Supplementary Figure 19. The role of EP2, a PGE2 receptor, in mediating the growth-promoting effect of dying cells. 4T1-Fluc cells were tranduced with an lentiviral vector encoding a shRNA gene against EP2. Different clones were then selected based on western blots. Clones that showed significant knockdown were selected for further experiments. Top panel, about 1000 Fluc labeled 4T1 or 4T1-EP2 knockdown cells were plated onto about $2x10^5$ irradiated unlabeled 4T1 cells. Growth of the Fluc labeled cells were then followed through bioluminecence imaging. Lower panel, results of western blot analysis of EP2 for clones used in the growth experiment.



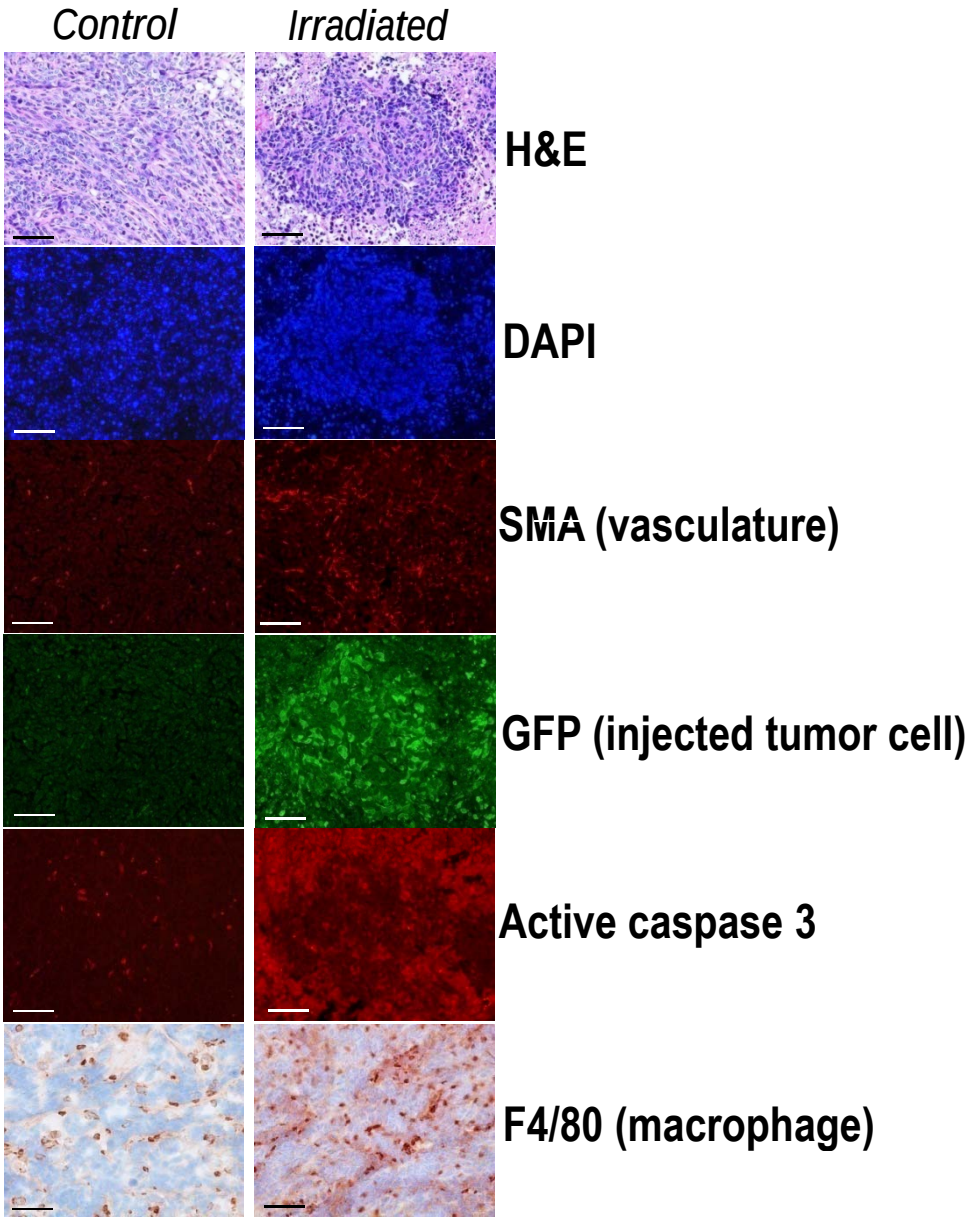
Supplementary Figure 20. IHC analysis of cleaved caspase 3 in 39 human head and neck tumor samples from Princess Margaret Hospital, Toronto, Canada. **a**) Representative IHC staining patterns for samples with low cleaved caspase 3. **b**) Representative IHC staining patterns for samples with high levels of cleaved caspase 3 .c) Distribution of caspase 3 immuno-expression in groups with no recurrence and vs recurrence. P values derived from Mann-Whitney test.



Supplementary Figure 21. IHC analysis of cleaved caspase 3 in 48 human advanced breast cancer samples from No. 1 People's Hospital, Shanghai, China. **a**) Representative IHC staining patterns for samples with low cleaved caspase 3 levels. **b**) Representative IHC staining patterns for samples with higher cleaved caspase 3 levels. **c)** Distribution of caspase 3 staining in patients who have deceased or still alive. P values derived from Mann-Whitney test.



Supplementary Figure 22. Kaplan-Meier analysis of relapse-free survival in 249 breast cancer patients from Singapore and Sweden (Ivshina et al, Cancer Research, 2006, reference 53). Data on caspase 3 mRNA levels were obtained from published microarray data set (GSE4922) in Ivshina *et al Cancer Res*. 66:10292 (2006). Log-rank test (p = 0.0001, HR=2.33, 95%CI: 1.53-3.56) were used to analyze the statistical significance between groups with high or low caspase 3 mRNA.



Supplementary Figure S23. Immunohistochemical(for F4/80) and immunofluorescence (for SMA, GFP, activated caspase) analyses of gene expression in irradiated 4T tumors injected with a small number of GFP-labeled 4T1 tumor cells. The size bars represent 100 μm.

Supplementary Notes

A key issue that needs to be addressed regarding caspase 3 is whether the mode of cell death affects the growth promoting signaling from the dying cells. To answer this question, we evaluated markers of apoptosis (activated caspase 3, TUNEL staining for DNA fragmentation), autophagy (increased accumulation of LC3-II isoform[1,2], which is essential for the formation of autophagosome), and necrosis (secreted HMGB1[3-5], a nuclear protein released into the extracellular space during necrosis) after cellular exposure to 10 Gy of x-rays. Our results indicated in any give cell type, one can see at least two forms of cell death. In murine 4T1 cells, there were indicators of apoptosis (caspase 3 activation, PARP cleavage, TUNEL staining, **Supplementary Fig. 6**), which were accompanied by the necrosis marker HMGB1 in the supernatants of cells, indicating co-existence of both types of cell death. There were also small but clear changes in the levels of autophagy marker LC3-II after radiation, accompanied by increases in perinuclear autophagosome staining (**Supplementary Fig. 6**). In wild type MEF cells, apoptosis activation (as indicated by caspase 3 activation, PARP cleavage, and TUNEL staining, **Supplementary Fig. 7**) was accompanied by increasing amount of necrosis (secreted HMGB1) and a small but clear increase in LC3-II (autophagy marker) expression, indicating co-existence of all three forms of cell death. In contrast, in caspase deficient (*Casp3*$^{-/-}$) MEF cells, there was a total absence of apoptosis markers (caspase activation, PARP cleavage, and TUNEL staining). On the other hand, there was a significant increase in LC3-II accumulation, indicating increased autophagy in these cells. Curiously, there was a clear decrease in expression of the necrosis marker HMGB1 secretion in these cells after irradiation. In human breast cancer cell line MCF-7 (**Supplementary Fig. 8**), which has no expression of caspase 3[6], exposure to radiation induced no signs of apoptosis (PARP cleavage and TUNEL staining). However, there was an increase in autophagy (as indicated by increase in LC3-II expression) as well as necrotic (as indicated by strong

induction of HMGB1 secretion) cell death. In contrast, in the MCF-7*CASP3* cell line (**Supplementary Fig. 8**), which has an exogenously expressed caspase 3 gene, radiation caused a significant increase in apoptosis (caspase 3 activation, PARP cleavage, and TUNEL staining). Induction of autophagy (as measured by LC3-II expression) appeared to be unchanged. However induction of necrosis (as measured by HMGB1 in the supernatant) appeared to be weakened. Therefore, the status of apoptotic caspase 3 affected necrotic and authophagy pathways differentially in MEF cells and MCF-7 cells. The underlying mechanism for the differential responses is not clear at present.

Taken together, our results suggest that the status of caspase 3 expression can significantly affect the ways cells die. They also suggest the cells die through more than one way after radiation exposure. Furthermore, the absence of caspase 3 will cause cells to shift their mode of death from apoptosis to necrosis with changes in autophagy status. In addition, different cells may shift their death modes in very different manners. Importantly, our results suggest that irrespective of modes of cell death, caspase 3 is important for activating the growth-promoting signals emanating from the dying cells.

Supplementary Methods

Cells and tissue culture conditions. We have used a variety of cancer and fibroblast cells in this study. Among these are the mouse breast cancer cell line 4T1, mouse fibroblast cell lines NIH3T3, human cancer cell lines MCF-7 (breast cancer line), MDA-MB231 (breast cancer line), HCT116 (colon cancer line), and human fibroblast cell strain IMR-90. All the above murine and human cancer lines are available from American Type Tissue Culture (ATCC, Manassas, VA, USA). In addition, we have obtained wild type and caspase-deficient mouse embryonic fibroblast cells from Dr. Richard Flavell of Yale University (New Haven, CT). For maintenance of the cells, Dulbecco's Eagles's Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum was used to culture the cells.

Gene transduction into the cells. To transduce various exogenous genes into target cells, our main vehicle is the lentivirus vector. We mostly used the pLEX system, a lentivector system purchased from Open Biosystems (Huntsville, AL). Genes that were cloned into this vector include: the firefly luciferase gene (Fluc) obtained commercially from Promega (Madison, Wisconsin); a truncated, activated $iPLA_2$ gene obtained from RT-PCR (see supplementary data section for details). In addition, we have used lentiviral vectors encoding shRNAs against caspase 3 or $iPLA_2$ (see subsequent sections for more details). All the lentiviral vectors were packaged into live lentiviral viruses in 293T cells following manufacturer's instructions.

Bioluminescence imaging. For imaging luciferase, we used the IVIS200 instrument from Caliper Life Sciences (Hopkinton, MA). For tissue cultured cells, we imaged luciferase signal by adding PBS or colorless OptiMEM medium (Invitrogen, Carlsbad, CA) with D-luciferin (Caliper Life Sciences,

Hopkinton, MA) at a concentration of 0.15 mg/ml. It is important to image the cells at a set time point (e.g. 10 minutes) after the administration of D-luciferin so that signals from different samples are comparable. After images are taken, we use manufacturer supplied software to process the images for quantitative data.

To monitor growth of Fluc-labeled cells in vitro, about 500 or 1000 cells were mixed together with an overwhelming number of (for example $2x10^5$) unlabeled cells either right after irradiation or 24 hrs after irradiation (or other cytotoxic treatments) the cells were then monitored at different times for up to 8 days after seeding by use of IVIS200.

Growth of Fluc-labeled tumor cells in vivo was followed through non-invasive bioluminescence imaging using the IVIS200 instrument (Caliper Life Sciences, Hopkinton, MA). Mice to be imaged were injected with 150 mg/kg of D-luciferin (obtained from Caliper Life Sciences) intraperitoneally in 200 μl of PBS and then anesthetized with continuous flow of isofluorane. Imaging of the mice was carried out 10 minutes later. It is important to keep the time between injection and imaging constant among different batches of mice.

Irradiation of cells and mice. X-ray irradiation of cells and mice were carried out in a RS-2000 Biological Irradiator located in the vivarium of University of Colorado Denver Anschutz Medical Campus. It was purchased from Rad Source Corporation (Atlanta, GA). The dose rate for the machine is about 1 Gy/minute.

Monitoring tumor cell growth in mice. We used different approaches to establish tumor growth than traditionally adopted by other studies. In most of our studies, we injected a very small number of Fluc-labeled tumor cells (500-1000 cells each) either alone or together with other unlabeled cells (either tumor cells or fibroblast cells) that are either irradiated or un-irradiated. Tumor growth from these cells is monitored through the quantification of bioluminescence signals emitted from labeled tumor cells by use of the IVIS200 instrument following manufacturer's instructions. In some cases, 500-1000 Fluc labeled tumor cells were injected into (7-9 mm in diameter) tumors that have been established from unlabeled tumor cells.

Measurement of arachidonic acid release. To measure arachidonic release, we plated cells in 6-well dishes at a density of 1.0-2.0x10^5 cells/well. About 1.0 μCi of [^{3}H]-archidonic acid (obtained commercially from GE Healthcare Life Sciences) was then added to the cells, which has about 1 ml of DMEM medium that was serum free and with 0.5 mg/ml of lipid-free bovine serum albumin (Sigma Chemical Co., St Louis, USA). After 16 hrs, the cells were washed with fresh medium 3X and incubated with 3 ml of DMEM medium supplemented with 5% serum. After another 5 hrs, when archidonic acid in the supernatant reaches a steady state level, the cells were exposed to 8-10 Gy of x-rays. Supernatants were then removed at 4, 24, and 48 hrs from the cells and counted with a scintillation counter for quantification of [^{3}H]-arachidonic acid.

Tumor growth delay experiments. To evaluate the function of caspase 3 in tumor radiotherapy, about 3x10^6 MCF-7 and MCF-7*CASP3* tumor cells were injected subcutaneously with matrigel were established in female nude mice implanted with estrogen pellets (1.7 mg/pellet, 60 day release formula, Innovative Research of American). When tumors reach 5-7 mm in diameter, they were exposed to x-

irradiation (2x6 Gy). The sizes of the tumors were measured every other day with a caliper. In separate experiments, B16F10 melanoma cells were injected into syngeneic C57BL/6 wild type and caspase 3 knockout (-/-) mice (Jackson Laboratory) and tumor growth experiments were conducted when tumor reach 5-7 mm in diameter.

ELISA measurement of PGE_2. To evaluate the PGE_2 secretion from cells, we plated about $2x10^5$ cells/well in 6-well dishes. Cells were cultured in DMEM medium supplemented with 2% fetal bovine serum. They were then irradiated with x-rays (8-12 Gy). Supernatant from the cells were taken right before and 24hrs after cellular irradiation. PGE_2 in the supernatants were then measured by use of an ELISA kit purchased commercially from R&D Systems (Minneapolis, MN, USA).

Molecular cloning. The pLEX lentiviral vectors system was used to deliver reporter and other genes into target cells[7]. The system was obtained commercially from Open Biosystems (Huntsville, Alabama, USA). The genes transduced through pLEX include: 1) firefly luciferase gene, which was transferred from the plasmid pGL4.31-luc2 from Promega (Madison, Wisoconsin); 2) a truncated version of the mouse calcium-independent phospholipase A_2, which was amplified through RT-PCR from murine mRNA by use of the following primers:

Forward, 5' **G ACTAGT GCCACC ATGCAG C ACCAAGGACC TCTTCGACTG-3'**
Spe I *Kozak*

Reverse, 5' **ATAAGAAT GC GGC CGC GTCCACGACCATCTTGCCCAG** 3'
Not I

We used the Pfx polymerase (Invitrogen, Carlsbad, CA) for the PCR amplification. The amplified fragment encodes aa453-679 (which is equivalent to aa514-733 of human iPLA2[8,9]) of murine $iPLA_2$ (accession# NM-016915), which has been proven to be a constitutively active caspase cleavage

product[8-10]. The fragment was cloned into PCR-Blunt and excised with Spe I and Not I restriction enzymes and cloned into the Spe I and Not I sites of a modified pLEX plasmid. The modified pLEX plasmid has an influenza hemagglutinin (HA) tag inserted between Not I and Mlu I (two of the unique restriction sites in pLEX) so that genes inserted into the NotI site can be fused with the HA tag In addition, we obtained lentiviral vectors encoding shRNA-encoding minigenes targeted against the murine iPLA$_2$ gene from Open Biosystems (Huntsville, AL). These genes were carried in the pLKO.1 lentiviral vectors system. The one that showed most efficacy had the following targeting sequence:

5' CCGG**CGTATGAAGGACGAGGTGTTT**CTCGAG**AAACACCTCGTCCTTCATACG**TTTTTTG-3'

(catalog #Rmmu534-NM-016915 from Open Biosystems, in bold red are the sense and antisense targeting sequences and in underlined blue is loop sequence).

For knocking down the PGE2 receptor EP2, we used a ready made vector from Open Biosystems (catalog #RMM3981-9594402). The target sequence for this shRNA is:

5'-CCGG**GCTTTCACTATGACCTTCTTT**CTCGAG**AAAGAAGGTCATAGTGAAAGC**TTTTT-3'

(in bold red are the sense and antisense targeting sequences and in underlined blue is the loop sequence).

We have also constructed a caspase 3 targeted, shRNA minigene-encoding lentiviral vector. The sequences of these shRNA is:

5'-CGCGTCCCCATGGGCATATGCATAATAA**TTCAAGAGAT**TATTATGCATATGCCCA**TTTTTTGGAAAT-3'** (in bold read are the sense and antisense targeting sequences and in underlined blue is loop sequence). A double stranded oligo containing the above shRNA sequence was cloned into the pLVTHM vectors, which is a lentiviral vector developed by D. Trono lab [11].

For dominant caspase 3, we mutated a key cystein in the catalytic domain of wild type murine cascapase 3 (C163→A) through site directed mutagenesis. The mutant version was then cloned into the lentiviral vector pLEX. In all cases, we followed manufacturer's instructions or published protocols to produce live, replication-deficient recombinant lentiviral vectors in 293T cells.

Western blot analysis. Cellular lysates were obtained under most circumstances using the standard RIPA buffer. For running western blot analysis, 40-60 μg/per sample was used for gel electrophoresis. Samples were usually heated to 95^{o}C for 5 minutes before loading into electrophoresis gel. See **Supplementary Table 1** for information of antibodies used in western analyses.

For cytochrome c western analysis, a digitonin-based buffer was used to lyse the cells. The buffer conisists of: 190μg/ml of digitonin (obtained from Sigma Chemical, St Louis, MO), 75 mM NaCl, 1 mM NaH_2PO_4, 8mM Na_2HPO_4, 250 mM sucrose. Treatment of cells with this buffer will lead to plasma membrane leakage of cytosolic material. After a brief spin, the supernatant were used to analyze for cytochrome c leakage into the cytosol through western blot analysis.

Key chemicals used in this study: the pan caspase inhibitor z-VAD-fmk, was obtained from EMD Bisciences (Gibbstown, NJ). Cyclooxygenase inhibitor indomethacin and a long half-life version of PGE2, 16,16-dimethyl PGE2 (dmPGE2) were both obtained from Caymen Chemical (Ann Arbor, MI).

Immunofluorescence and immunohistochemistry analysis. For immunofluorescence analyses of the following proteins: casp 3, SMA, GFP, antibodies were used. Paraffin-embedded tumor tissues were

used. For F4/80, sectioned frozen tumor tissues were used because of antibody functionality. See supplementary Table 1 for source and details of antibodies used.

IHC analysis of human cancer samples. Tumor tissue samples from two cohorts of patients were used to determine the correlation between cleaved caspase 3 levels and clinical outcomes. In the first cohort, 39 tumor samples from patients with head and neck squamous cell carcinoma (HNSCC) treated at the Princess Margaret Hospital, Toronto, Canada were examined. Patient characteristics are provided in **Table S2**. All patients underwent radiotherapy. In the second cohort, 48 tumor samples from patients with advanced breast cancer patients treated at Shanghai No. 1 People's Hospital were examined. Patient characteristics are provided in **Table S3**. In both cohorts, ethical rules established by IRB (institutional review board) were followed to obtain the clinical samples.

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Supplementary Table 1. Antibodies used in this study for western blot, IHC, and IF analysis.

Target name	Antibody source	Clone info
Caspase 3 (full length)	Cell Signaling Technology	8G10 , rabbit monoclonal
Caspase 3 (cleaved, activated)	Cell Signaling Technology	5A1E, rabbit monoclonal
Caspase 8	Cell Signaling Technology	1C12, mouse monoclonal
Caspase 9	Cell Signaling Technology	Rabbit polyclonal
Cytochrome c	Cell Signaling Technology	Rabbit polyclonal
iPLA2	Caymen chemical	Rabbit polyclonal
EP2	Caymen chemical	Rabbit polyclonal
HA epitope	Roche Applied Science	12CA5. Mouse monoclonal
SMA	Dako	1A4, mouse monoclonal
GFP	Abcam	Rabbit polyclonal (ab290)
HMGB1	R&D systems	Mouse monoclonal (115603)
LC3	Novus Biologicals	Rabbit polyclonal
F4/80	AbCam	BM80, rat monoclonal
Cox-1	Caymen chemical	Rabbit polyclonal
Cox-2	Caymen Chemical	Rabbit polyclonal
NF-kB p105 phosphorylated	Cell signaling technology	18E6, rabbit monoclonal
NF-kB p65 phosphorylated	Cell signaling technology	93H1, rabbit monoclonal

Supplementary Table 2.Clinical characteristics of the HNSCC patients

Characteristic		Number (%)
Age (years)		
	≤65	19 (49%)
	>65	20 (51%)
Sex		
	Male	30 (77%)
	Female	9 (23%)
Tumor site		
	Larynx	27 (69%)
	Hypopharynx	12 (31%)
Tumor stage		
	III	14 (36%)
	IVA	24 (61%)
	IVB	1 (3%)
Relapse		
	Local/Regional	15 (39%)
	Distant	8 (21%)
Treatment		
	Conventional conformal RT	36 (92%)
	IMRT	3 (8%)
	RT alone	26 (67%)
	CRT (70 Gy/35f/7weeks)	13 (33%)

RT: radiotherapy
IMRT: intensity-modulated radiotherapy
CRT: concurrent chemo-radiation

Supplementary table 3. Clinical characteristics of breast cancer patients.

Characteristic		Number (%)
Age (years)		
	≤50	21 (43.8%)
	>50	27 (56.2%)
Menopause*		
	Pre	26 (54.2%)
	Post	22 (45.8%)
Tumor type		
	Infiltrating ductal carcinoma	44 (91.7%)
	Infiltrating lobular cancinoma	4 (8.3%)
Tumor stage		
	I	3 (6.2%)
	IIa	13 (27%)
	IIb	13 (27%)
	IIIa	8 (16.6%)
	IIIb	1 (2%)
	IIIc	10 (20.8)
Molecular pathology		
	ER positive	27 (56.2%)
	PR positive	25 (52%)
	Her-2 positve	11 (23%)
Treatment		
	Chemotherapy	44 (91.6%)
	Tamoxifen	28 (58.3%)
	Radiotherapy	23 (47.9%)
Metastasis		18 (37.5%)
Death		15 (31.2%)

*all are female breast cancer patients who have undergone surgery
ER: estrogen receptor
PR: Progesterone receptor