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Letters to the Editor

Cytoplasmic p53 is not required for PUMA-induced apoptosis

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Dear Editor,

The p53 upregulated modifier of apoptosis, PUMA, was originally identified as a gene product that was transcriptionally upregulated by p53.[1,2] Unlike thymocytes from wild-type mice, which undergo marked apoptosis following irradiation, thymocytes from mice genetically mutant for *puma* or *p53* are profoundly resistant, indicating that both PUMA and p53 are required for radiation-induced apoptosis in this cell type.[3] Thus, in thymocytes, PUMA is an essential mediator of p53-induced apoptosis.

PUMA is a proapoptotic 'BH3-only' member of the Bcl-2 family of apoptotic regulators (reviewed in Strasser[4]). Like other BH3-only proteins, PUMA induces apoptosis by binding to the prosurvival members of the Bcl-2 family, for example, Bcl-2, Bcl-x, Mcl-1, thereby relieving the inhibitory effect of the prosurvival proteins on the proapoptotic proteins, Bax and Bak. This results in activation of Bax and Bak, subsequent release of cytochrome *c* from the mitochondria, and ultimately, cell death. For example, DNA damage caused by ionizing radiation results in the accumulation and activation of p53, which upregulates *puma* transcription. Newly synthesized PUMA is then able to antagonize Bcl-2 family prosurvival proteins resulting in the activation of Bax and Bak and eventual cell death.

Although it was initially proposed that p53 acts solely in the nucleus to transactivate genes such as *puma* to induce cell death, a recent report by Chipuk *et al.*[5] proposed an additional, radically different role for p53 in PUMA-mediated apoptosis. In this model, p53 is not only needed to transactivate *puma*, it must also accumulate in the cytoplasm, where it initially binds to Bcl-x. As the level of PUMA rises, it displaces p53 from Bcl-x. Liberated p53 is then able to bind and activate Bax, thereby inducing mitochondrial outer membrane permeabilization and cytochrome *c* release leading to cell death.

According to this model, PUMA is unable to induce cell death in the absence of p53. Indeed, Chipuk *et al.*[5] stated that 'PUMA is not sufficient for apoptosis or sensitization to UV-induced apoptosis in the absence of p53,' and consistent with this, they found that *puma* expression did not cause apoptosis in HCT116 $p53^{-/-}$ cells, or sensitize them to apoptosis after exposure to UV.

To verify this model, in which cytoplasmic p53 is required for PUMA-mediated apoptosis, we used a 4-hydroxy tamoxifen (4HT)-inducible lentiviral system to express wild-type PUMA in $p53^{-/-}$ cells (Figure 1a). Using this system we generated 4HT-inducible PUMA lines of mouse embryonic fibroblasts (MEFs) and IL-3-dependent myeloid cell lines from $p53^{-/-}$ mice. Rather than confirming that p53 was necessary for the induction of cell death by PUMA, in both p53-null cell types, expression of PUMA efficiently caused cells to undergo apoptosis (Figure 1b–d). Importantly, apoptosis occurred at levels of PUMA expression that were below the level of detection by Western blotting in MEFs and in IL-3-dependent cells (data not shown and Supplementary Figure S1), and the induction of apoptosis was specific, because PUMA expression failed to induce death of *bax/bak* double knockout (DKO) MEFs and myeloid cells (data not shown).

These results are not consistent with a mechanism in which PUMA acts by liberating p53 from Bcl-x so that it can bind and activate Bax on the mitochondria, but support a model in which p53 is solely required for the transcriptional activation of *puma* following irradiation.

Although our experiments were in MEFs and IL-3-dependent myeloid cell lines, whereas Chipuk *et al.* examined HCT116 cells, the differences in requirement for p53 are not likely to be due to cell type, because other investigators have looked at the effects of PUMA expression in other p53 WT, mutant and null cell lines. For example, the Vousden and Vogelstein laboratories reported that PUMA expression was sufficient to cause apoptosis in several *p53* mutant cell lines.[1,2] Furthermore, although Chipuk *et al.*[5] also claimed that PUMA was unable to cause death in $p53^{+/+}$ HCT116 cells, Yu *et al.*[2] found that PUMA could efficiently kill $p53^{+/+}$ HCT116 cells. Because Chipuk *et al.* did not report either the efficiency of transfection nor the level of PUMA expressed in either HCT116 $p53^{+/+}$ or $p53^{-/-}$ cells, it is possible that PUMA did not cause apoptosis in their hands because too few cells were transfected, or the amount of PUMA expressed was insufficient.

Our results demonstrate that PUMA can cause apoptosis independently of p53 in both *p53* null fibroblasts and in *p53* null IL-3-dependent myeloid cell lines, and are consistent with earlier observations both in WT and $p53^{-/-}$ HCT116 cells and in several other p53 mutant cell lines.[1,2]

Several studies using cells from *puma*-deficient mice have also implicated PUMA in forms of apoptosis known to be p53-independent, namely that caused by cytokine withdrawal or treatment with dexamethasone.[3,6–9] Collectively these results indicate that PUMA can cause apoptosis in the absence of p53. Although we did not investigate whether cytosolic p53 can directly activate Bax to induce cell death, it is clear that once *puma* is transactivated by p53, there is no requirement for cytoplasmic p53 for PUMA to cause apoptosis.

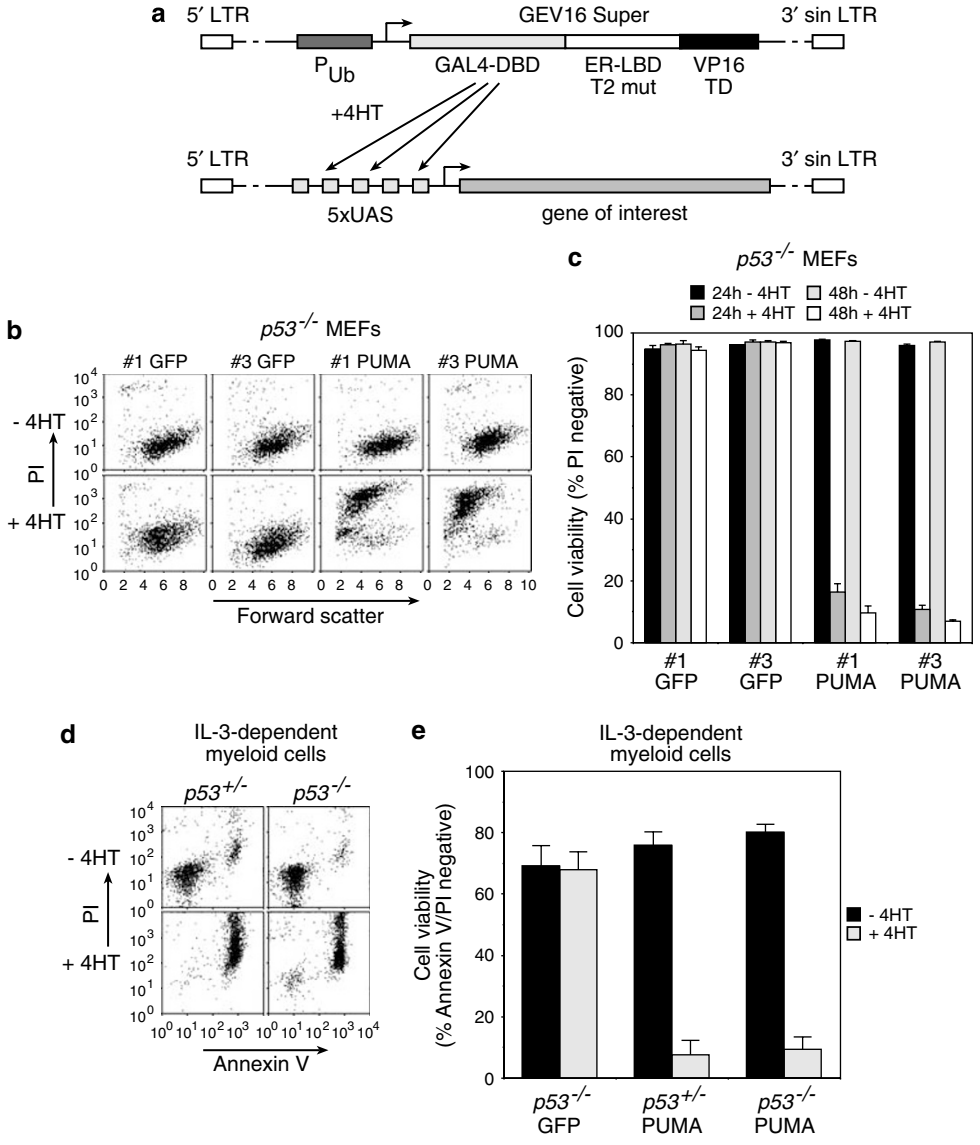

Figure 1 PUMA causes death of p53$^{-/-}$ fibroblast and IL-3-dependent myeloid cells. (**a**) Schematic representation of the 4-hydroxy tamoxifen (4HT)-inducible lentiviral expression system. The ubiquitin promoter (P_{Ub}) constitutively drives expression of the GEV16 transcription factor. In the absence of hormone, GEV16 is retained in the cytoplasm, but in the presence of 4-hydroxytamoxifen it translocates to the nucleus where the GAL4-DNA binding domain (DBD) directs DNA binding to GAL4 upstream activating sequences (UAS) whereby the VP16 transactivation domain (TD) upregulates gene transcription. The GEV16 and UAS constructs as well as hygromycin (GEV16 plasmid) or puromycin (UAS plasmid) resistance genes, for generating stable inducible cell lines, are contained within the lentiviral 5′-long terminal repeats (LTR) and 3′-self-inactivating (sin) LTR. To make stable lines lentiviruses are first generated separately in 293T cells. Viral supernatants are filtered, mixed 1 : 1 in the presence of polybrene and added to target cells. After 24–48 h hygromycin and puromycin are added to select cells infected with both viruses. (**b**) 4HT-inducible PUMA or GFP *p53*$^{-/-}$ MEFs were generated by lentiviral infection. Expression of PUMA or GFP was induced where indicated (+) with 100 nM 4HT. After 24 h, flow cytometry was used to measure plasma membrane integrity by propidium iodide (PI) exclusion. Expression of PUMA resulted in loss of MEF viability whereas induction of GFP had no effect. The numbers 1 and 3 refer to MEFs derived from independent *p53*$^{-/-}$ embryos. (**c**) Cumulative data for the experiments described in (**b**) as determined at 24 and 48 h ± 4HT. The data is presented as mean ± S.E.M from three independent experiments. (**d**) PUMA expression was induced by 1 μM 4HT in IL-3-dependent *p53*$^{+/-}$ and *p53*$^{-/-}$ cells. Cell viability was assessed after 24 h by staining with annexin V and PI. PUMA caused the same amount of cell death whether cells were heterozygous or homozygous mutant for *p53*. (**e**) Cumulative data for the experiments described in (**d**). For PUMA, cell viabilities are presented as the mean ± S.E.M. from multiple independent experiments using several clones of *p53*$^{+/-}$ ($n=5$) or *p53*$^{-/-}$ ($n=6$) cells derived from two independent embryos of each genotype. As a control, GFP was also expressed in four independent clones of *p53*$^{-/-}$ cells derived from two independent embryos. Similar results were obtained by expressing PUMA in NIH3T3 cells and independently derived WT MEFs (data not shown)

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)

Response to Callus *et al* on 'Cytoplasmic p53 is not required for PUMA-induced apoptosis'

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Dear Editor,

Mitochondrial outer membrane permeabilization (MOMP) is a critical event in the mitochondrial pathway of apoptosis, and this is effected and affected by the different members of the BCL-2 family of proteins.

Our paper by Chipuk *et al*[1] introduced the concept that complex interactions between the different types of BCL-2 proteins extends to interactions with non-BCL-2 family proteins, such as p53. Specifically, we showed that the ability of cytosolic p53 to activate the MOMP effector BAX[2] is inhibited by sequestration of cytosolic p53 by BCL-xL (as also suggested by others[3]) and, subsequently, de-repressed by displacement of cytosolic p53 by the BH3-only protein PUMA. This scenario is an extension of the ideas of Letai and Korsmeyer,[4,5] upon which we have elaborated.[6]

Callus *et al* believe that they challenge our conclusions by demonstrating that overexpression of PUMA can cause apoptosis in *p53*$^{-/-}$ cells. However, their observation is far from novel, and represents, at best, an incremental extension of the early observations on PUMA we incorporated into our hypothesis. The original descriptions of PUMA demonstrated that its ectopic overexpression triggered apoptosis in the p53-null cell line H1299[7] and in HCT116 *p53*$^{-/-}$.[8] Studies using cells from *puma* knockout animals showed roles for PUMA in forms of apoptosis that are known to be p53-independent.[9–12] In our paper[1] (Supplementary Figure 4D and E) and in another study,[13] we showed that the PUMA BH3 domain peptide sensitized cells to apoptosis induced by BID, BIM, or staurosporine, in a p53-independent manner. The model we developed was with full knowledge of these findings, and is misrepresented by Callus *et al* whose observations do not contradict our results.

Callus *et al* also imply that our experimental use of HCT116 *p53*$^{-/-}$ cells was not properly controlled, as we had not ruled out additional, undefined mutations in the apoptotic pathways of these cells. On the contrary, we demonstrated in Chipuk *et al*[1] Supplementary Figure 4D that HCT116 wild-type and *p53*$^{-/-}$ responded similarly to staurosporine treatment indicating that apoptotic signaling upstream of mitochondria is intact. Furthermore, in Supplementary Figure 4E, HCT116 wild-type, *p53*$^{-/-}$, *p21*$^{-/-}$ and *p21*$^{-/-}$/*Puma*$^{-/-}$ cells responded almost identically to the BID and BIM BH3 domain peptides, arguing against any major defects in the mitochondrial pathway of apoptosis in the cells we employed.

While ectopic overexpression of PUMA can cause apoptosis in some cell lines,[7,8] a simple search for the constitutive endogenous expression of *Puma* in normal human tissues, pathology samples and developmental stages using the NCBI UniGene EST database[14] clearly indicates that PUMA is not, itself, sufficient to trigger apoptosis in all cases. Furthermore, there are numerous common laboratory cell lines that constitutively express PUMA protein in the absence of proapoptotic treatment (e.g., K562, HT1080, A204, NIH3T3, MOLT4, and U937); and more recently, two reports of constitutive PUMA expression in mouse embryonic fibroblasts.[15,16] To understand this apparent paradox, it is necessary to consider the role of direct activator versus sensitizer/de-repressor BH3-only proteins in the control of BAX and/or BAK activation (Figure 1), as discussed in more detail elsewhere.[6] In general, cells that are transformed or otherwise stressed (e.g., by culture conditions) can become 'addicted' to the antiapoptotic members of the BCL-2 family, such that de-repression will release an activator of BAX and/or BAK to trigger MOMP and apoptosis.[5] Activators include BID, BIM, and cytosolic p53, and probably other proteins. In addition, nonprotein activators of BAX and/or BAK (e.g., heat, pH extremes) have also been described suggesting that numerous proapoptotic pathways converge on promoting an active multidomain conformation.[6,17,18] In contrast, PUMA acts as a de-repressor. While we cannot exclude that at some concentrations or under some conditions PUMA may