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Letter to the Editor

Ser58 of mouse p53 is the homologue of human Ser46 and is phosphorylated by HIPK2 in apoptosis

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Dear Editor,

The tumor suppressor p53 is known to function in the prevention of genetic instability and in the activation of apoptosis. To mediate these functions, the constitutively expressed latent form of p53 is activated by a series of post-translational modifications that occur at the N- and C-terminal regions.[1,2] Among the post-translational modifications, phosphorylation of human p53 (hp53) at Ser46 was identified as a specific modification involved in apoptotic response.[3] Compared to other N-terminal phosphorylation sites, nearly 100% conserved in different species, the region encompassing Ser46 has low level of amino-acid (aa) identity questioning the existence of a Ser46 homologue site in mice.[4] Evidence indicates that some species-dependent differences exist in the post-translational modification of p53. However, the crucial role of hp53-Ser46 in apoptotic regulation and the widespread use of mouse models in biological and preclinical studies requires a direct evaluation of this issue, rather than relying only on sequence homology.

The major bioinformatics tools currently available for proteome-wide identification of phosphorylation sites are NetPhos (http://www.cbs.dtu.dk/services/NetPhos) and its extension DISPHOS (http://www.ist.temple.edu/DISPHOS), two neural-network-based predictors, and Scansite (http://scansite.mit.edu), a motif-based service. Unfortunately, for p53 the prediction accuracy of these programs is largely unsatisfying as can be assessed by comparing the computationally predicted with the experimentally identified phospho-sites. Recently, we employed the recurrence quantification analysis (RQA), a nonlinear technique fruitfully applied to several different fields including protein sequence (http://homepages.luc.edu/~cwebber and http://www2.phys.rush.edu/JZbilut/physiozbi.html), to successfully discriminate, on a purely sequence basis, between experimentally characterized DNA-contact defective and conformational p53 mutants.[5] Thus, we applied RQA to identify the putative ortholog of hp53-Ser46 (Supplementary Information 1S).

To experimentally test whether the theoretically predicted Ser58 site of mouse p53 (mp53) is functionally homologous to hp53-Ser46, we first generated antibody (Ab) to specifically recognize phospho-Ser58 (Supplementary Information 2S). To determine whether mp53-Ser58 is phosphorylated *in vivo* and whether this phosphorylation is similar to that of hp53-Ser46, we irradiated the wtp53-carrying F9 murine cells with increasing doses of UV and tested p53 accumulation and phosphorylation by Western blotting, 24 h after irradiation. Accumulation of p53, phosphorylation of Ser18, and Ser389 (the mouse homologues of human Ser15 and Ser392) were detected in total cell extracts (TCEs) at each dose. In contrast, Ser58 was phosphorylated only at doses able to significantly promote cell death, as assessed by cell viability (Figure 1a). In addition, time course analysis of p53 expression and phosphorylation of F9 cells treated with apoptotic doses of UV or adriamycin (ADR) (Supplementary Information, Figure 2S-A and 2S-B) showed kinetics of Ser58 phosphorylation very similar to that previously reported for Ser46 in human cells subjected to similar stressing conditions.[3] Finally, the ALLN-stabilized p53[1] was detected by the anti-total p53 Ab but not by the phospho-specific Abs, supporting the specificity of our anti-Ser58^P Ab (Supplementary Information Figure 2S-A).

The homeodomain-interacting protein kinase 2 (HIPK2) is a Ser/Thr kinase that physically and functionally interacts with p53 and activates its apoptotic function by phosphorylating hp53-Ser46.[6,7] *In vitro* kinase assay showed that recombinant HIPK2 phosphorylates similarly hp53-Ser46 and mp53-Ser58 (Supplementary Information, Figure 2S-C). To test this activity *in vivo*, p53-null H1299 cells were transfected with expression vectors coding for wild-type hp53 or mp53 or their relative hp53S46A or mp53S58A mutants in the presence of co-transfected EGFP-HIPK2. Human and mouse p53s were immunoprecipitated with anti-total p53 Ab, resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and reacted with anti-Ser46^P or anti-Ser58^P-specific Abs, respectively. As shown in Figure 1b, only the wild-type proteins were recognized by their relative phospho-specific Abs, showing that HIPK2 overexpression induces mp53-Ser58 phosphorylation *in vivo*. Comparable results were obtained with mp53 or mp53S58A mutant co-transfection with EGFP-HIPK2 in mouse fibroblasts (MFs) from mdm2/p53 double knockout (dKO) mice to avoid differences in p53 protein expression owing to potential differences in p53 sensitivity to MDM2-mediated degradation (data not shown).

To evaluate the functional relevance of HIPK2-induced mp53-Ser58 phosphorylation, we constructed recombinant adenoviruses carrying HIPK2 (AdHIPK2) or the HIPK2-K221R mutant (AdK221R) and infected MFs from p53+/+ mice. p53Ser58 phosphorylation and TUNEL positivity were detected only in wild-type HIPK2 overexpressing cells (Figure 1c). Comparable results were obtained by retroviral

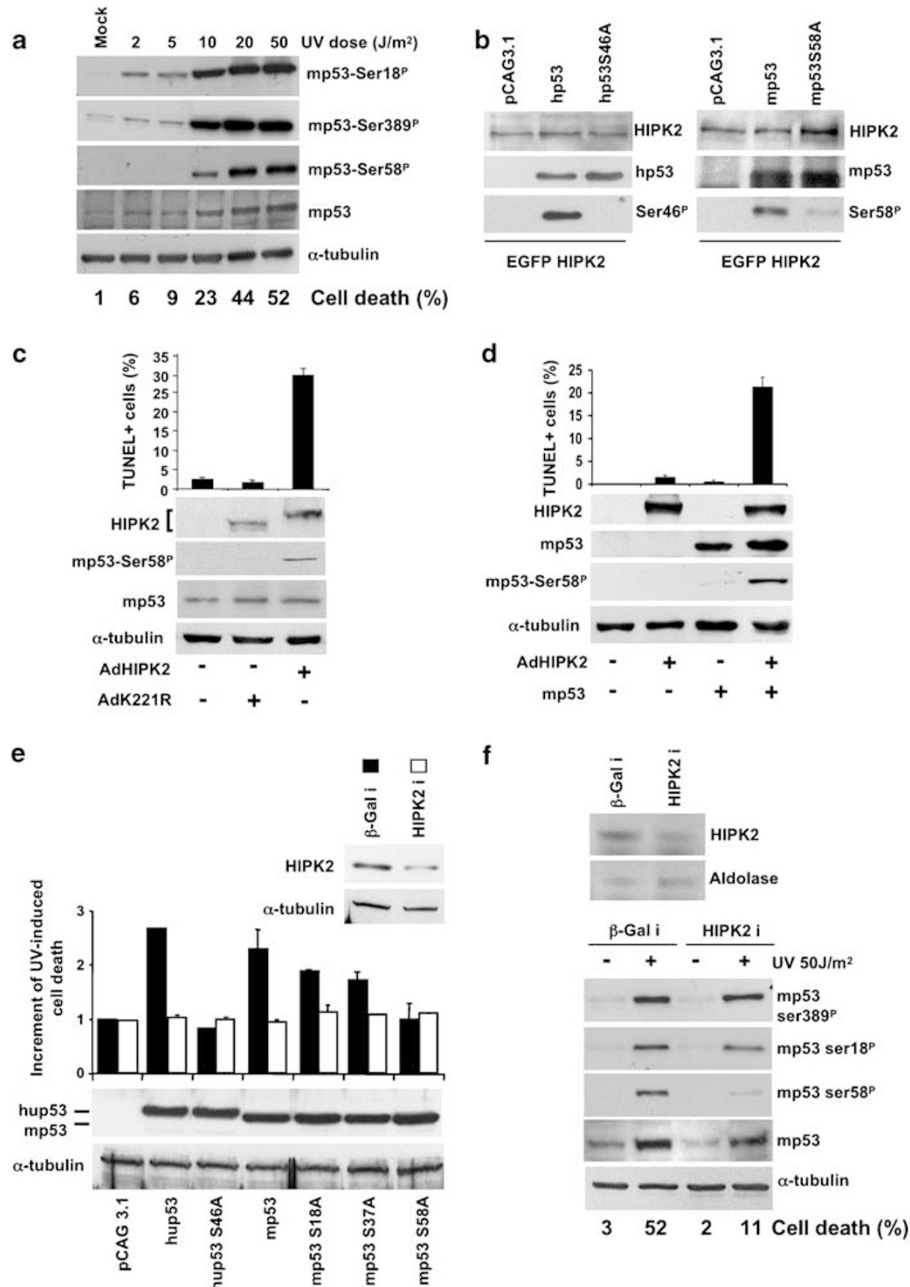

Figure 1 Ser58 is the mouse homologue of human p53-Ser46. (**a**) F9 cells were irradiated with the indicated doses of UV. Immunoblot of p53 phosphorylation at Ser18, Ser389, and Ser58 and total p53 protein was performed on TCEs. α-Tubulin was used as loading control (l.c.). The percent of dead cells at each indicated dose of UV was determined 24 h post-irradiation by trypan blue exclusion and indicated below the blots. (**b**) p53-null H1299 cells were transfected with expression vectors coding for wild-type hp53 or mp53 proteins or their relative hp53S46A or mp53S58A mutants in the presence of co-transfected EGFP-HIPK2. Human and mouse p53 proteins were immunoprecipitated with anti-total p53 Ab. Immunoblot analysis of phosphorylation of hp53-Ser46, mp53-Ser58 were performed with the phosphospecific Abs. Immunoblot with antibody to p53 shows equal amount of immunoprecipitated proteins. (**c**) Apoptotic cell death (percentage TUNEL staining) in MFs from p53 $+/+$ mice. The cells were infected with recombinant adenoviruses carrying HIPK2 (AdHIPK2) or the HIPK2-K221R mutant (AdK221R) that were constructed by using the pAdEasy1 (E1/E3 minus) vector system (Stratagene). Immunoblot analysis of the transduced HIPK2 proteins as well as of total and Ser58 phosphorylated p53 are shown below. α-tubulin was used as l.c. (**d**) Apoptotic cell death (percentage TUNEL staining) in MFs from p53$-/-$ mice infected with AdHIPK2 with or without transfection of mp53. The expression levels of transduced HIPK2 and p53 proteins and of Ser58 phosphorylation are shown below. α-Tubulin was used as l.c. (**e**) Impairment of apoptosis by Ser58 substitution. Stably transfected H1299 control (β-Gali) and HIPK2-depleted (HIPK2i) cells were analyzed for their HIPK2 protein expression (upper panel). Next, both cell populations were transfected with expression vectors coding for wild-type hp53 or mp53 proteins or their Ser to Ala mutants. Each transfected population was either mock- or UV-irradiated at 50 J/m^2. Percent of cell death was determined by trypan blue exclusion and the increment of UV-induced death was calculated relative to the same transfected population mock-irradiated (middle panel). Representative expression of transduced p53 proteins are shown (lower panel). α-Tubulin was used as l.c. (**f**) HIPK2-depleted F9 cells and their relative β-Gali controls were analyzed for mRNA expression levels. Aldolase was used as a control. Both stably transfected populations were UV-irradiated at 50 J/m^2. Immunoblot analyses were performed for p53 phosphorylation at Ser18, Ser389, Ser58, and for total p53 protein. α-Tubulin was used as l.c. Percent of cell death determined by trypan blue exclusion 24 h post-irradiation are reported

infection of the wtp53-carrying 32D hemopoietic cells (Supplementary Information, Figure 2S-D).

To confirm that HIPK2-induced apoptosis is p53-dependent, MFs from p53−/− mice were infected with AdHIPK2 with or without transfected mp53. As shown by TUNEL analysis, only coexpression of HIPK2 and p53 induced Ser58 phosphorylation and apoptosis (Figure 1d).

Next, we tested whether substitution of Ser58 interferes with UV-induced, HIPK2-mediated apoptosis. Control (β-Gali) and HIPK2-depleted (HIPK2i) H1299 cells (Figure 1e, upper panel) were transfected with a series of different mp53 Ser to Ala mutants and subsequently irradiated with UV. Increased apoptosis was observed in the control cells transfected with wtp53 or with the two control mutants, mp53S18A and mp53S37A. In contrast, no increase of apoptosis was observed upon UV irradiation of mp53S58A mutant-transfected control cells (Figure 1e, black bars) or when a similar experiment was performed in the HIPK2i cells (Figure 1e, white bars). Comparable results were obtained in mdm2/p53 dKO MFs co-transfected with the HIPK2-carrying vector and the different mp53s (data not shown). These results indicate that HIPK2-mediated phosphorylation of Ser58 is required to increase apoptosis and further support the homology between hp53-Ser46 and mp53-Ser58.

To directly test the contribution of HIPK2 in mp53-Ser58 phoshorylation *in vivo* in mouse cells, HIPK2-depleted F9 cells and their relative β-Gali controls (Figure 1f, upper panel) were irradiated with high doses of UV and analyzed for cell death and phosphorylation of the endogenous p53 (Figure 1f, lower panel). UV irradiation stabilized p53 in both cell populations and induced phosphorylation at Ser18 and Ser389. In contrast, in HIPK2-depleted cells, Ser58 phosphorylation as well as the percentage of cell death were strongly reduced, confirming that Ser58 is a target of HIPK2 and that its phosphorylation by HIPK2 contributes to UV-induced apoptosis.

The existence of six major p53 tryptic-phosphopeptides from ^{32}P-labeled mouse fibroblasts was originally reported.[8] One of these peptides (aa 28–62) contains both Ser37 and Ser58, but based on further analyses, Ser37 was favored as the site of phosphorylation. However, the experimental conditions employed[8] (i.e., ^{32}P-labeling) were subsequently shown to induce DNA damage but not apoptosis,[1] supporting the absence of phosphorylation at Ser58.

Recently, an extensive characterization of the mouse proline-rich domain and of its role in apoptosis has been reported.[9] Surprisingly, the authors observed that all of the deletion mutants screened resembled wtp53 in the amount of death induced with the exception of mutant Δ58–88 that was unable to induce cell death in all cell lines and experimental conditions tested. Even more surprisingly, mutant Δ61–88 was a strong inducer of cell death and had a wild-type phenotype in all assays performed, suggesting that some relevant events might depend on aa 58–61. Our data on Ser58 can explain these results and together with them strongly support the relevant contribution of this site in the mp53-mediated apoptotic pathway.

Skepticism on the existence of a mouse homologue of the hp53-Ser46 has been mainly due to the apparent divergence of human and mouse p53 aa sequences in the proline-rich region.[3] However, concerns have been raised on the inadequacy of making predictions about protein structure and function based on the aa code. Indeed, from an informational theory point of view, protein sequences are considered as strings just slightly apart from randomness,[10] implying that even minor modifications in the aa sequences, for instance those observed among different mammalian species, can give rise to relevant modifications of their spectra. In contrast, less stringent homology criteria, such as those taking into account the pattern of hydrophobicity along the protein chains, their clustering, and/or recurrence within the proteins have given interesting results for protein characterization (Supplementary Information 1S) Here, we applied this mathematical approach to predict the mouse homolog of hp53-Ser46. The subsequent biochemical and functional analyses confirmed the theoretic prediction despite the opposite location of a proline residue relative to the serine one. Indeed, a proline follows the hp53-Ser46 whereas precedes mp53-Ser58. Whether this sequence configuration would have an effect for the other kinases that were shown to phosphorylate hp53-Ser46 needs to be addressed. However, our data strongly support RQA as a particularly advantageous tool to identify structural and functional characteristics of proteins, including the identification of phosphorylation sites and, potentially, for investigating any kind of post-translational modification sites.

Altogether, the findings reported here indicate mp53-Ser58 as the mouse homologue of hp53-Ser46 and open the way for a greater understanding of this p53 post-translational modification, its activation process, and the control of apoptotic pathways in mouse models.

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1. Siliciano JD *et al.* (1997) Genes Dev. 11: 3471–3481.
2. Appella E and Anderson CW (2001) Eur. J. Biochem. 268: 2764–2772.

3. Oda K *et al.* (2000) Cell 102: 849–862.
4. Xu Y (2003) Cell Death Differ. 10: 400–403.
5. Porrello A *et al.* (2004) Proteins 55: 743–755.
6. Hofmann TG *et al.* (2002) Nat. Cell Biol. 4: 1–10.
7. D'Orazi G *et al.* (2002) Nat. Cell Biol. 4: 11–19.
8. Meek DW and Eckhart W (1988) Mol. Cell Biol. 8: 461–465.
9. Edwards SJ *et al.* (2003) Oncogene 22: 4517–4523.
10. Weiss O *et al.* (2000) J. Theor. Biol. 206: 79–386.

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