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Letters to the Editor

Silencing of the transcription factor KLF6 by siRNA leads to cell cycle arrest and sensitizes cells to apoptosis induced by DNA damage

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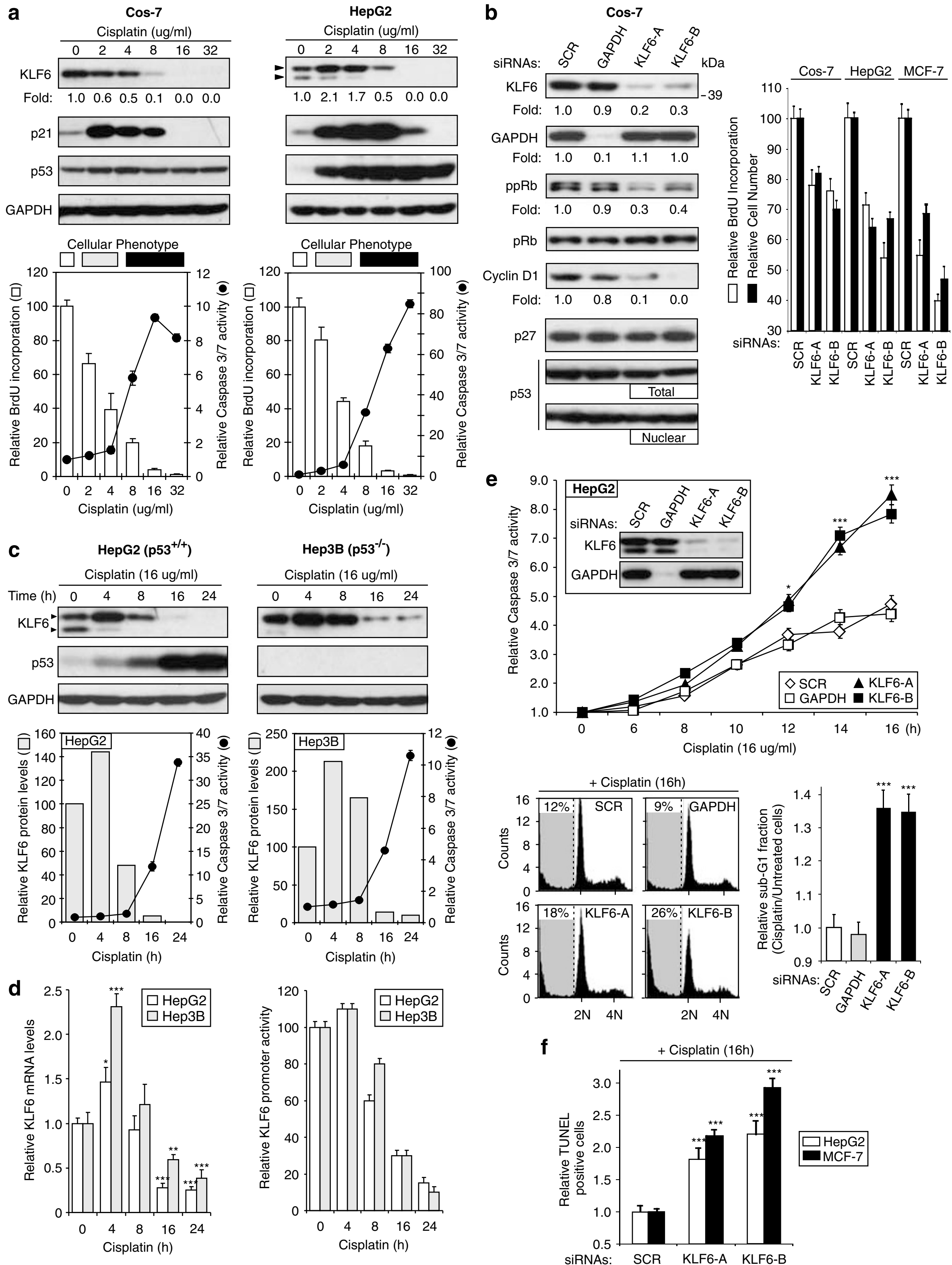
Dear Editor,

Krüppel-like factor 6 (KLF6) belongs to a large family of mammalian Sp1-like/KLF transcription factors that play critical roles in regulating key cellular functions ranging from differentiation to proliferation and apoptosis.[1–5] KLF6 is an evolutionarily conserved and ubiquitously expressed protein that was originally identified as an activator of pregnancy-specific genes.[6–8] Initial evidence has implicated KLF6 as a tumor suppressor gene, which was found to be the subject of frequent somatic mutations in certain carcinomas.[9,10] Given its potential role as a tumor suppressor gene, much effort has gone into determining altered KLF6 genotypes. Results have been rather controversial since a number of studies established that genetic alterations of KLF6 were infrequently observed in distinct types of human cancers (see Lievre *et al.*[11] and references therein). More significantly, a recent study in mice indicated that targeted inactivation of KLF6 was lethal and severely impaired the proliferation rate of embryonic stem cells.[12] The phenotype observed in $KLF6^{-/-}$ mice and stem cells strongly suggests that endogenous KLF6 is required for cell cycle progression, thereby contrasting with its potential tumor-suppressive activity. In view of the central role of KLF6 as a key factor in regulating cell growth, we aimed at elucidating the impact of endogenous KLF6 on cell proliferation and apoptosis by successfully applying approaches that employ DNA-damaging drugs and RNA interference. The effects of DNA-damaging agents on cells that undergo cell cycle arrest or apoptosis depending on the extent of the DNA insult, provided a useful experimental system to test whether KLF6 protein levels were modified during these events (Figure 1a). Cells were exposed to different concentrations of DNA-damaging agents and cell cycle arrest and apoptosis were determined by BrdU incorporation and caspase 3/7 measurements, respectively. Also, the protein levels of $p21^{cip1}$ and p53 were employed to monitor the magnitude of the cellular response to DNA damage. Interestingly, KLF6 protein levels were modified only marginally during cell cycle arrest induced with low doses of DNA-damaging agents (Figure 1a and Supplementary Figure S1). In addition, a striking downregulation of KLF6 was found in cells treated with high doses of DNA-damaging drugs that are compatible with apoptosis induction (see below).

The observation that cells maintained KLF6 expression during the cytostatic effect of the DNA-damage response led us to investigate whether KLF6 could be involved in cell cycle control. Therefore, to determine the impact of endogenous KLF6 on cell cycle progression, RNA interference was applied to knock down KLF6 in mammalian cells and the expression of key cell cycle-related proteins together with cell proliferation were analyzed (Figure 1b). Two KLF6-specific siRNAs were employed that cause a more than 70% reduction of the endogenous KLF6 protein in Cos-7 cells. Notably, the expression levels of the hyperphosphorylated retinoblastoma protein (ppRb) and cyclin D1 were decreased in KLF6-deficient cells, while the cyclin-dependent kinases CDK4, CDK6 and the CDK inhibitor p27 remained unchanged (Figure 1b, left; data not shown). Also, p53 was analyzed and its protein levels or nuclear accumulation was not modified. Downregulation of ppRb and cyclin D1 is interesting since it might reflect that cells are impaired in their proliferation rate. To test this idea, DNA synthesis as an indication of cell cycle progression was determined by BrdU-incorporation assays. Interestingly, specific loss of KLF6 induced cell cycle arrest, while the apoptotic status remained unaffected (Figure 1b, right; data not shown). To extend these results, similar experiments were performed in hepatocarcinoma (HepG2) and breast cancer (MCF-7)-derived cell lines (Figure 1b, right and Supplementary Figure S2). Results clearly showed that KLF6 loss by siRNAs A and B decreased BrdU incorporation, which was also reflected as a reduction of cell numbers in all cell lines analyzed. In conclusion, our results that KLF6 loss induced cell cycle arrest in distinct cell lines position this transcription factor as an important positive regulator of cell proliferation. In light of other reports, our data were rather unexpected since it has been shown that ectopic expression of KLF6 led to cell cycle arrest by increasing $p21^{cip1}$.[9,13] A possible explanation is that KLF6 might trigger either cell proliferation or cell cycle arrest depending on its protein expression levels, via distinct occupancy of target gene promoters. In addition, KLF6 could also exert its growth promoting activity by regulating other target genes that remain to be identified. Collectively, our data that siRNA-mediated loss of KLF6 led to cell cycle arrest are in full agreement with recent reports demonstrating that KLF6 knockdown impaired cell proliferation in hepatocarcinoma cells[14] and that $KLF6^{-/-}$ embryonic stem cells proliferate more slowly than their wild-type counterparts.[12]

As mentioned before, KLF6 protein was clearly down-regulated to undetectable levels in cells treated with high

doses of DNA-damaging drugs that are compatible with apoptosis induction (Figure 1a). Results also revealed a direct association between high levels of stabilized p53 and increased caspase 3/7 activities during the course of the treatment with distinct DNA-damaging drugs (Figure 1a and Supplementary Figure S1). The striking correlation between



p53 stabilization, apoptosis induction and KLF6 reduction led us to investigate whether p53 might be directly involved in KLF6 downregulation. Therefore, p53 and KLF6 protein levels were examined in p53 wild-type (HepG2) or p53 null (Hep3B) cells after treatment with cisplatin (Figure 1c, left and right, respectively). Similar KLF6 expression kinetics were observed in both cell lines indicating that during cisplatin-induced apoptosis, the downregulation of endogenous KLF6 was p53-independent. Our data further indicated that KLF6 downregulation after DNA damage-induced apoptosis was due, at least in part, to decreased levels of its mRNA, which was accompanied by a strong reduction of its promoter activity in a p53-independent manner, while KLF6 protein stability remained unchanged (Figure 1d, left and right, respectively and Supplementary Figure S3). Interestingly, DNA-damaging agents (cisplatin) and other cytotoxic drugs (flavopiridol) may cause global transcriptional inhibition and this mechanism seems to be relevant in the regulation of short-lived proteins like $p21^{cip1}$, MDM2 and also KLF6 (Vichi *et al.*[15] and Demidenko *et al.*[16]; Supplementary Figure S3).

Downregulation of KLF6 upon DNA damage has been previously described.[17,18] However, the potential impact of KLF6 on apoptosis regulation has not yet been analyzed and so far this is the first report indicating that endogenous KLF6 might play a role in the underlying mechanisms of apoptosis triggered by DNA-damaging drugs. To further explore this possibility, we set out to efficiently knock down endogenous KLF6 in HepG2 cells where apoptosis was induced by DNA damage in a particular time frame as determined by caspase 3/7 activities (Figure 1e, top). Interestingly, KLF6 loss sensitized HepG2 cells to cisplatin-induced apoptosis in a time-dependent manner, indicating an antiapoptotic function for endogenous KLF6. To extend these results, a similar KLF6 knockdown experiment was conducted and DNA fragmentation indicative of cell death was analyzed by flow cytometry and TUNEL assays (Figure 1e, bottom and f, respectively). Significantly, these data clearly demonstrated that KLF6 loss by siRNAs increased the sub-G_1 apoptotic cell population, which in turn correlated directly with nuclear DNA fragmentation. In addition, controlled overexpression of KLF6 protected HeLa cells from cisplatin-induced apoptosis (Supplementary information, Supplementary Figure S4).

At this stage of analysis, it is most likely that KLF6 is operating as an antiapoptotic factor, yet further studies are necessary to determine how KLF6 exerts its activity on apoptosis regulation. Also, in this context, it was recently reported that KLF6 protects hepatocellular carcinoma-derived cells from apoptosis *in vitro*, thus highlighting the potential role of KLF6 as an antiapoptotic factor.[14]

Taken together, our results are interesting for several reasons: (i) the role of KLF6 as a positive regulator of cell cycle progression and also as an antiapoptotic factor may have a significant impact in determining cell numbers in mammals since KLF6 is a ubiquitous factor that is expressed early during development and in adult tissues. (ii) $KLF6^{-/-}$ stem cells and KLF6-deficient cells by siRNA exhibit an overlapping phenotype, which was further characterized in this work since endogenous KLF6 loss inhibited cell proliferation irrespectively of the cell type analyzed. (iii) DNA damage as a therapeutical anticancer tool often accelerates cell death mechanisms as does the inactivation of the potential antiapoptotic factor KLF6 in human cells.

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Figure 1 (**a**) Cells were treated for 24 h with different amounts of cisplatin as indicated. Western blots for KLF6, $p21^{cip1}$, p53 and GAPDH are shown (top panels). BrdU incorporation and caspase 3/7 activities were determined. Data represent the average with error bars from three independent experiments performed in triplicates. Cellular phenotypes i.e., proliferating, growth-arrested and apoptotic cells are indicated by horizontal bars in white, gray and black, respectively. (**b**) (Left) Cos-7 cells were transfected with scrambled (SCR), GAPDH or KLF6-specific siRNAs (KLF6-A and KLF6-B) as indicated on top. Protein expression levels were calculated considering the values of SCR siRNA-transfected cells as 1. (Right) Cell proliferation was determined in cells treated as in (**b**, left) and Supplementary Figure S2. Cell proliferation and total cell numbers were determined by BrdU incorporation and trypan blue staining, respectively. In all cases, $P<0.001$; $n=3$, one-way ANOVA; Bonferroni. (**c**) Cells were treated with cisplatin as indicated and caspase 3/7 activities were determined. Western blots for KLF6, p53 and GAPDH are shown. (**d**) (Left) qRT–PCR analysis of KLF6 mRNA levels in HepG2 and Hep3B cells after treatment with cisplatin for the specified times. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $n=3$, one-way ANOVA; Tukey–Kramer. (**Right**) Cells were transiently transfected with reporter vectors containing the human KLF6 promoter (pROM6[8]) in combination with a Renilla luciferase vector as control. Average values from at least three independent experiments performed in triplicates with S.D. bars are shown. (**e**) (Top) RNAi for KLF6 in HepG2 cells as revealed by Western blot (inset). siRNA-transfected cells were incubated for 60 h and then treated with cisplatin as indicated. Caspase 3/7 activities were determined and plotted relative to untreated cells ($^{***}P<0.001$ and $^*P<0.05$; $n=4$, one-way ANOVA; Tukey–Kramer). (Bottom) Cells treated as before (**e**, top) were analyzed after 16 h of cisplatin treatment by flow cytometry stained with propidium iodide. The gray strips mark the sub-G_1 region. 2N and 4N represent the DNA content of G_1 and G_2, respectively. The graph shows the sub-G_1 DNA content in samples treated with cisplatin and that were quantified and plotted relative to untreated cells ($^{***}P<0.001$; $n=3$, one-way ANOVA; Bonferroni). (**f**) TUNEL assays were performed in cells that were transfected with siRNAs as indicated and grown for 60 h followed by addition of cisplatin (16 μg/ml) for 16 h. Data represent the average of two experiments with $\pm$S.D. ($^{***}P<0.001$; $^{**}P<0.01$; $n=3$, one-way ANOVA; Bonferroni)

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Unexpected role of the phosphate carrier in mitochondrial fragmentation

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Dear Editor,

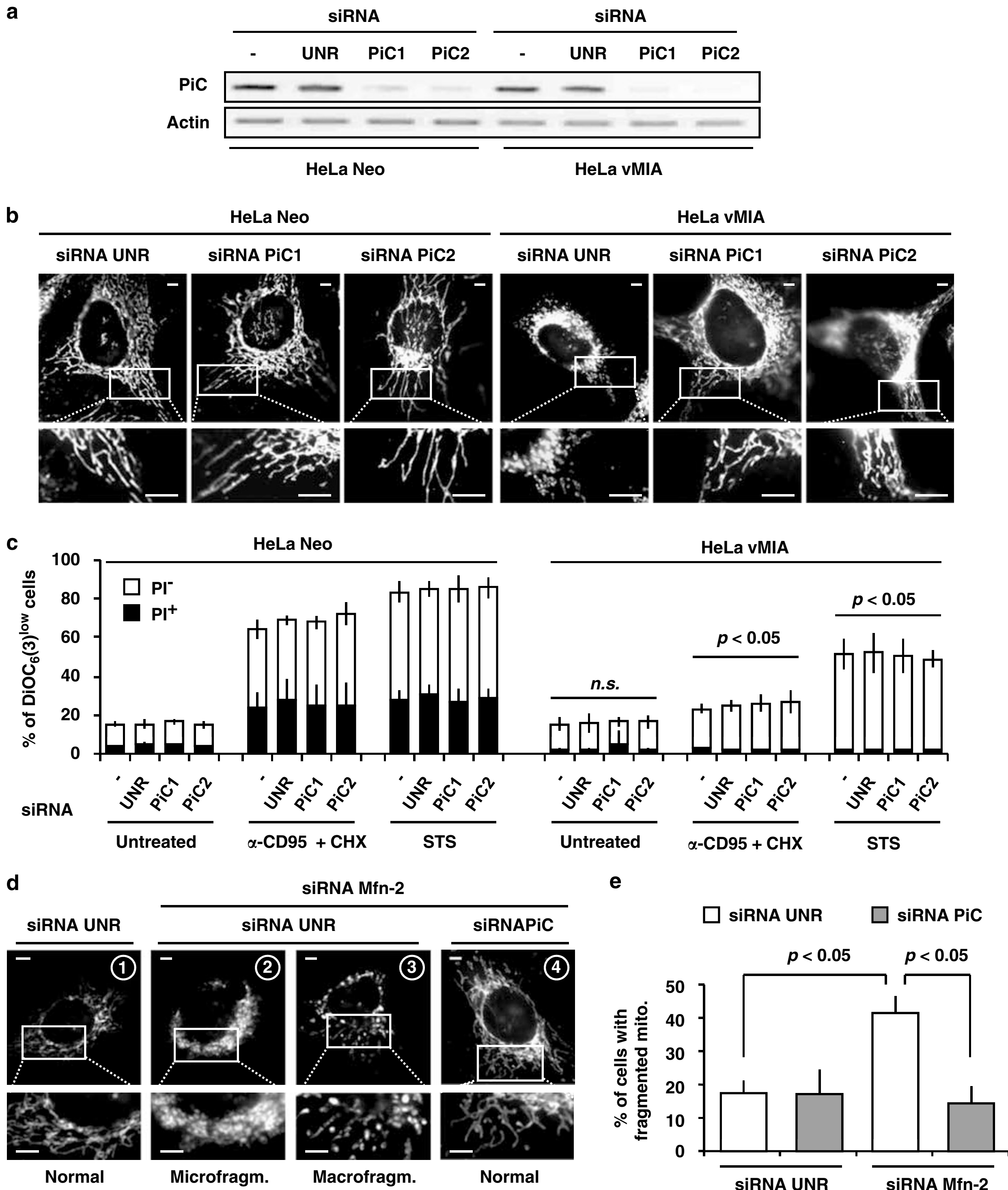
Mitochondrial carriers constitute a family of hexa-transmembrane proteins that participate in the transport of metabolites across the inner mitochondrial membrane.[1] Several members of this family have also been proposed to participate in the apoptotic processes. For instance, distinct isoforms of the adenine nucleotide translocase have been involved in the regulation (either positive or negative) of mitochondrial membrane permeabilization.[2–5] Similarly, the mitochondrial carrier homolog 2 (Mtch2) may mediate Bcl-2-dependent apoptosis inhibition.[6]

Recently, we and others have found that yet another protein from the mitochondrial carrier family, the inorganic phosphate carrier (PiC), may participate in the regulation of mitochondrial membrane permeabilization. A genome-wide cDNA screen identified PiC as one of the few proteins that, if overexpressed, is able to efficiently trigger the intrinsic pathway of apoptosis.[7] PiC was also found to functionally interact with the cytomegalovirus (CMV)-encoded protein vMIA (i.e. viral mitochondrial inhibitor of apoptosis).[8,9] vMIA inhibits apoptosis by recruiting Bax to mitochondria, thereby provoking its inactivation.[10,11] In addition, vMIA is responsible for the early cytopathic effect associated with CMV infection,[8] independently from its interaction with Bax.[8,12] Moreover, vMIA inhibits the PiC-mediated transport of inorganic phosphate across the inner mitochondrial membrane, thereby reducing ATP synthesis (which is strictly dependent on phosphate import). In turn, this results in a disorganization of the actin cytoskeleton and rounding up of the cells, which characterize the CMV-mediated early cytopathic effect.[8]

We have recently reported that, similar to Bax, vMIA can stimulate the fragmentation of the mitochondrial network.[13] This is intriguing and counterintuitive because vMIA is a strong inhibitor of apoptosis,[9] while mitochondrial fragmentation is often associated with apoptosis induction.[14] Since vMIA-mediated mitochondrial fragmentation occurs even in Bax-depleted cells,[8] this effect must be independent of the vMIA-mediated recruitment of Bax to the outer mitochondrial membrane. We therefore sought whether the depletion of PiC with two distinct small interfering RNAs (siRNAs) (Figure 1a; Supplementary Materials and Methods) might affect the

Figure 1 (**a**–**c**) Effect of the depletion of the mitochondrial phosphate carrier (PiC) on mitochondrial morphology and vMIA activity. (**a**) Efficacy of the siRNA-mediated downregulation of PiC. PiC was depleted by using two distinct siRNAs (see Supplementary Materials and Methods) in HeLa control cells (HeLa Neo) and in cells overexpressing vMIA (HeLa vMIA), and the abundance of the mRNA transcript was assessed by RT–PCR 24 h post-transfection, as compared to non-transfected cells (−) and cells transfected with an irrelevant siRNA (UNR). (**b**) Impact of vMIA and PiC on mitochondrial dynamics. Forty-eight hours after siRNA transfection, cells were fixed and stained with an antibody recognizing the core 2 subunit of complex III of the respiratory chain (red fluorescence). Representative images are shown (scale bars represent 2 μm). (**c**) Influence of PiC on vMIA-mediated apoptosis suppression. PiC was depleted by means of two distinct siRNAs in HeLa Neo and HeLa vMIA cells. Twenty-four hours later, cells were treated for additional 6 h with 500 ng/ml anti-CD95 monoclonal antibody (α-CD95) plus 100 μg/ml cycloheximide (CHX) or, alternatively, with 500 nM staurosporine (STS) and stained with the $\Delta\Psi_m$-sensitive dye DiOC$_6$(3) as well as the membrane-impermeant dye propidium iodide (PI) for the cytofluorometry-assisted quantification of dying and dead cells. White and black columns indicate the percentage of cells exhibiting the loss of $\Delta\Psi_m$ (DiOC$_6$(3)^{low}) alone (PI$^-$) or together with ruptured plasma membranes (PI$^+$), respectively (mean ± STE; $n = 3$). Statistical significance was assessed by means of paired Student's *t*-tests comparing HeLa Neo and HeLa vMIA cells subjected to the same siRNA transfection and pro-apoptotic stimulation (NS = non-statistically significant). (**d** and **e**) Effect of PiC knockdown on mitochondrial fragmentation induced by the depletion of mitofusin 2 (Mfn-2). HeLa Neo cells were co-transfected with an irrelevant siRNA (UNR) or with siRNAs for the depletion of Mfn-2 and PiC in the indicated combinations for 72 h, followed by staining with MitoTracker® Orange for the assessment of mitochondrial morphology. (**d**) Representative pictures (at different magnifications) of cells exhibiting an intact mitochondrial network (siRNA UNR, siRNA Mfn-2 + PiC; panels 1 and 4, respectively) or micro- *versus* macrofragmentation of the organelles (siRNA Mfn-2 + UNR; panels 3 and 4, respectively). Scale bars represent 2 μm. (**e**) Mitochondrial fragmentation induced by the depletion of Mfn-2 in the presence and in the absence of PiC. For each condition, the percentage of cells displaying macrofragmented mitochondria was assessed in a population of at least 300 cells (mean ± STE; $n = 3$). Paired Student's *t*-tests were performed to assess the statistical significance of the indicated comparisons

fragmentation of the mitochondrial network promoted by vMIA. The transient (not shown) or stable transfection with vMIA clearly caused a major fragmentation of the mitochondrial network from a tubular to a dot-like appearance in several cell lines, including HeLa cervical carcinoma cells (Figure 1b), HCT116 colon carcinoma cells and human fibroblasts (not shown). This fragmentation was prevented by the downregulation of PiC by means of two distinct siRNAs (Figure 1b). As a note, PiC depletion did not influence *per se* mitochondrial morphology (Figure 1b). In spite of this major – and unexpected – influence of PiC on mitochondrial dynamics, PiC depletion did not affect the antiapoptotic activity of vMIA. Expression of vMIA protected HeLa cells against mitochondrial dysfunction and cell death induced by staurosporine (a general kinase inhibitor that promotes the intrinsic pathway of apoptosis) or by crosslinking the death receptor CD95/Fas



(a trigger for the extrinsic apoptotic pathway), even when PiC was depleted (Figure 1c). Hence, PiC is required for the mitochondrial fragmentation caused by vMIA, yet has no role in vMIA-mediated apoptosis inhibition. These data underline the notion that mitochondrial fragmentation and initiation of the mitochondrial pathway of apoptosis do not correlate in an obligatory fashion.[13,15] This holds true also at a molecular level, as recently demonstrated for the protein hFis1.[16] Notably, hFis1 (that normally promotes both apoptosis and mitochondrial fragmentation) loses selectively its lethal effects (not the ability to fragment mitochondria) in cells lacking the proapoptotic modulators Bax and Bak, or upon different mutations in the intermembrane region.[16]

Next, we investigated whether the contribution of PiC to mitochondrial fragmentation would be restricted to this particular system of fragmentation (i.e. that stimulated by vMIA) or whether PiC might play a general role in this process. Mitofusin 2 (Mfn-2) is a GTPase required for mitochondrial fusion the absence which has been previously shown to promote mitochondrial fragmentation.[17] HeLa Neo cells transfected with an irrelevant siRNA exhibited a multi-branched, reticular mitochondrial network (Figure 1d, panel 1). On the contrary, mitochondria from cells depleted for Mfn-2 appeared either with a finely punctuated perinuclear pattern (microfragmentation; Figure 1d, panel 2) or as large dots dispersed throughout the cytoplasm (macrofragmentation; Figure 1d, panel 3). Again, this effect was abolished by the simultaneous knockdown of PiC, which still failed to affect mitochondrial morphology *per se* (Figure 1d, panel 4; Figure 1e). The same effect was observed also in a non-small cell lung cancer cell line (A549), which exhibited only macro- (and not micro-) fragmented mitochondria following the knockdown of Mfn-2 (Supplementary Figure 1). Hence, PiC may play a rather general role in mitochondrial dynamics. At this point, the exact mechanism by which PiC participates in mitochondrial fragmentation is unknown. Although it appears plausible that mitochondrial fission requires PiC at some point, it remains to be established whether PiC directly contributes to the fission machinery at the inner mitochondrial membrane, or whether the metabolic alterations induced by PiC depletion (and a consequent phosphate deficiency within the mitochondrial matrix) indirectly impact on the equilibrium of fusion and fission events. Previous reports indicate that changes in energy substrate availability induce deep structural modifications of the mitochondrial network, aimed to accommodate increased amount of the respiratory chain components.[18] In this regard, PiC downregulation might (at least theoretically) counteract mitochondrial fragmentation via a similar effect, yet it seems very improbable that the lack of PiC would favor, instead of inhibiting (by limiting the availability of inorganic phosphate for ATP synthesis), oxidative phosphorylation.[18]

Irrespective of these unresolved issues, it appears clear that PiC can profoundly influence mitochondrial dynamics. Although there is overwhelming correlative evidence, in several species, that mitochondrial fragmentation correlates with mitochondrial outer membrane permeabilization,[19] the present report illustrates that this correlation is not absolute. Thus, the antiapoptotic protein vMIA induces mitochondrial fragmentation in a process that is clearly unrelated to its apoptosis-regulatory function.

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