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LETTER TO THE EDITOR

Loss of ID3 in pancreatic cancer cells increases DNA damage without impairing MDC1 recruitment to the nuclear foci

Dear Editor,

Pancreatic cancer is one of the most aggressive human cancers and is predicted to become the second leading cause of cancer-related death by the year 2030 [1, 2]. In the United States, approximately 60,000 newly diagnosed cases and 48,000 deaths were estimated to occur in 2021 [3]. Because early screening is very difficult and most patients are diagnosed with advanced disease, curing pancreatic cancer patients still faces huge challenges. Accordingly, a comprehensive understanding of the pathogenesis of pancreatic cancer is required to promote the development of efficient treatment modalities.

Inhibitor of DNA-binding/differentiation-3 (ID3) is a helix-loop-helix (HLH)-containing protein that belongs to the ID protein family [4–7]. ID3 lacks a DNA-binding domain but can bind to other HLH proteins and target transcription factors to further inhibit their binding to DNA [7, 8]. It has been widely reported that ID3 works individually or cooperatively with other ID family members in tumor-mediated angiogenesis, or to promote cell proliferation, invasion and stemness regulation in many types of human cancers, including pancreatic cancer [6, 9-13].

Lee and colleagues [8] reported an ID3-dependent pathway recruiting the mediator of DNA damage checkpoint protein 1 (MDC1) to sites of DNA damage and demonstrated that the interaction between ID3 and MDC1 plays a key role in DNA damage response (DDR). Moreover, other proteins in the ID family lack the Ser 65 motifs in the ID3 HLH domain critical for this function, suggesting DNA repair is a non-redundant function of ID3 [8]. The goal of our study is to investigate whether ID3 also participates in the repair process of pancreatic cancer cells with DNA damage and to determine whether this affects their sensitivity to DNA damaging agents.

To begin, we examined the expression profile of ID3 in eight different standard pancreatic cancer cell lines by immunoblot. As shown in Figure 1A, highest expression of ID3 was detected in MIA PaCa-2 and PANC-1 cells, followed by Capan-1 cells. The remaining cell lines had low to no expression of ID3, demonstrating that this protein may not be critical for pancreatic cancer cell survival. To determine whether reduced expression of ID3 impairs DDR, we treated PANC-1 and MIA PaCa-2 cells with cisplatin, a commonly used chemotherapeutic agent known to induce DNA-DNA and DNA-protein cross-links. PANC-1 and MIA PaCa-2 cells were exposed to various concentrations and durations of cisplatin treatment then immunoblot of PAGE separated cell lysates was performed to assess the relative abundance of ID3 (as above) and phospho-Histone H2AX (γ-H2AX). As expected, increases in γ-H2AX, a DNA damage marker, were associated with increased cisplatin dose and treatment duration (Figure 1B). Treatment with 12.5 μmol/L of cisplatin for as little as 2 hours was sufficient to induce DNA damage in PANC-1 cells, while treatment for 5 hours was required in MIA PaCa-2 cells.

ID3 expression was unaffected by cisplatin treatment except when cells were exposed to the high dose of 50 μmol/L cisplatin for at least 8 hours. Interestingly, this resulted in a reproducible decrease in ID3 protein in both cell lines without any changes in the GAPDH loading control. We did not investigate the mechanism for this decrease further. Subsequent experiments were carried out using lower doses and durations of cisplatin treatment.

To confirm that ID3 plays a role in the DDR process in pancreatic cancer cells, we next established stable cell lines with ID3 knockdown (KD) through lentivirus-based short-hairpin RNA (shRNA) expression system. PANC-1 and MIA PaCa-2 cells were each transduced with two different shRNAs. As shown in Figure 1C, we found that the two cell lines had different sensitivity to the two shRNAs. In MIA PaCa-2 cells, shRNA#1 (shID3#1) transduction

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Abbreviations: DDR, DNA damage response; DMEM, Dulbecco's Modified Eagle Medium; DSB, double-strand break; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; HLH, helix-loop-helix; ID3, Inhibitor of DNA-binding/differentiation-3; IR, ionizing radiation; KD, knockdown; MDC1, mediator of DNA damage checkpoint protein 1; SD, standard deviation; SEM, standard error of the mean; shRNA, short-hairpin RNA; γ-H2AX, phospho-Histone H2AX
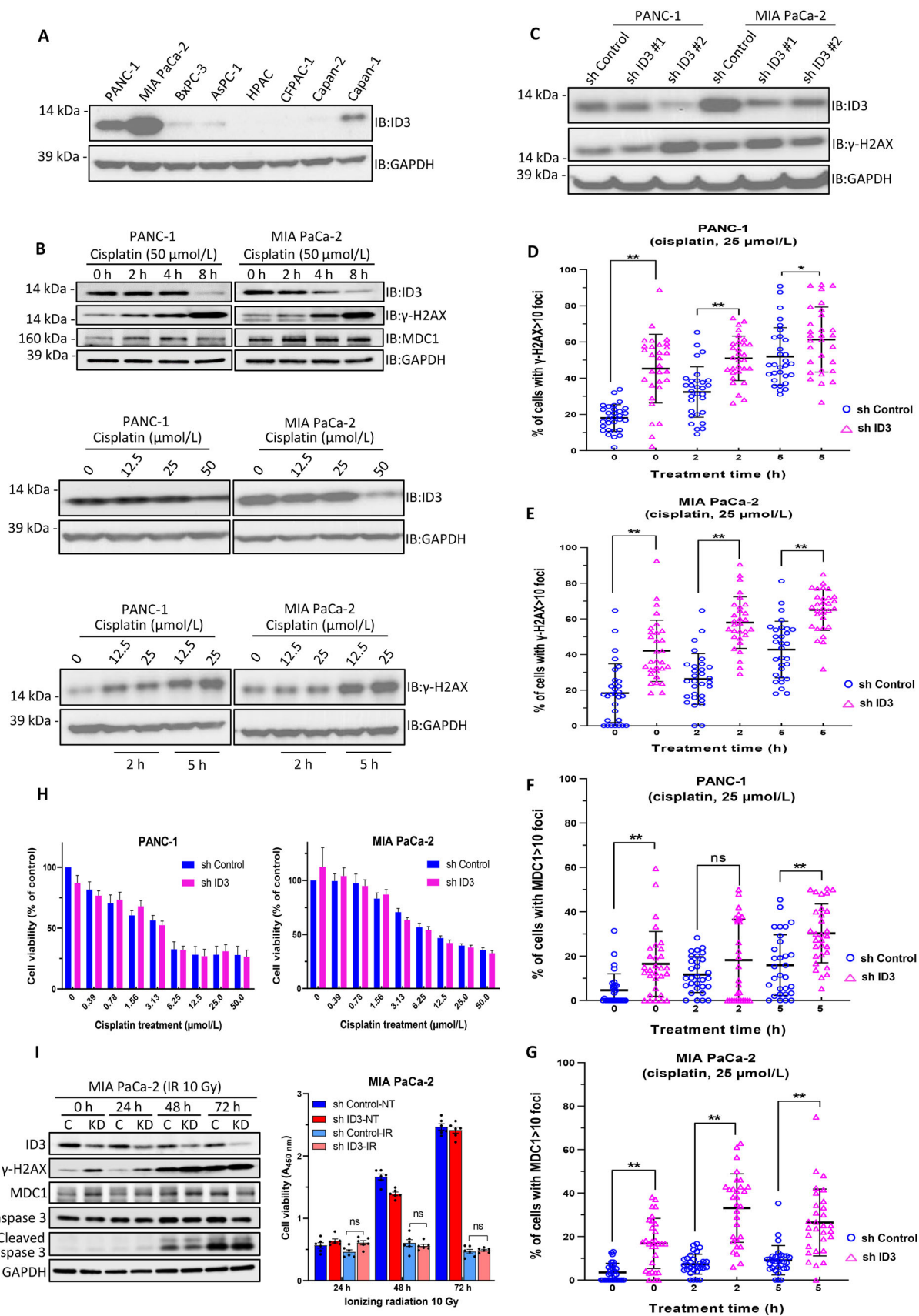
FIGURE 1 ID3 KD provokes increased DNA damage and MDC1 nuclear foci but does not increase sensitivity to platinum or ionizing radiation. (A) Expression of ID3 in the indicated pancreatic cancer cell lines visualized by Western blot. PANC-1 and MIA PaCa-2 cells show the highest ID3 expression. (B) PANC-1 and MIA PaCa-2 cells were treated with cisplatin at the specified concentrations and durations

produced better ID3 KD efficiency, while PANC-1 cells were more sensitive to shRNA#2 (shID3#2). Cells with the largest decrease in ID3 expression had greater increases in γ-H2AX consistent with prior observations of Lee *et al.* [8] that ID3 is important in the DDR. To further quantitate the effect of ID3 loss on DNA damage, a confocal microscopy-based observation was carried out. Consistent with our immunoblot data, the number of γ-H2AX nuclear foci in ID3 KD cells was significantly increased compared with the corresponding parental cells (Figure 1D and E; Supplementary Figure 1A-C) at baseline (0 h). Following cisplatin treatment, ID3 KD cells continued to have a statistically significant increase in γ-H2AX. Previously, Lee *et al.* [8] described an ID3-dependent mechanism for recruiting MDC1 to DNA repair complexes in response to DNA damage caused by ionizing radiation. Disruption of the direct interaction between ID3 and MDC1, including KD of ID3, decreased MDC1 accumulation at sites of DNA double-strand breaks (DSBs) resulting in decreased MDC1-positive foci in the nucleus. Conversely, in our DNA damage model, more MDC1 foci were observed in ID3 KD cells compared to control cells both at baseline and at 5 hours following cisplatin treatment (Figure 1F and G; Supplementary Figure 1D-F). MDC1 foci increased in concert with increasing DNA damage caused by cisplatin. These data indicate that MDC1 recruitment to sites of DNA damage is not impaired by loss of ID3 in these cells and suggest another mechanism could be responsible for the increased γ-H2AX signal that results from KD of ID3 in these models.

Given the increase in DNA damage that occurs in ID3 KD cells, we hypothesized that ID3 KD cells would be more sensitive to cisplatin than parental cells. To test this, the cells were treated with cisplatin for 48 hours, and then cell viability was assessed. We found no significant difference in the cisplatin sensitivity of ID3 KD PANC-1 or MIA PaCa-

2 (Figure 1H) cells as compared to parental cells. Contrary to our hypothesis, a decrease in ID3 did not increase cisplatin sensitivity in these cell lines. In addition, we treated control and ID3 KD MIA PaCa-2 cells with ionizing radiation and examined the effects. Again, we observed a time-dependent increase in γ-H2AX following treatment (Figure 1I). This was associated with decreased cell viability that was independent of ID3 KD status, unlike what was observed previously [8].

To summarize, we confirmed a prior report that loss of ID3 increases DNA damage, but found that ID3 was not necessary for recruiting MDC1 to sites of DNA damage in our system, unlike the mechanism proposed previously [8]. Despite ID3 KD resulting in increased DNA damage, ID3 KD cells had similar sensitivity to cisplatin and ionizing radiation as parental cells expressing ID3, contrary to our hypothesis. This is in contrast to pancreatic cancer cells bearing mutations affecting DNA DSB-associated repair system such as BRCA or PALB2 [14, 15], and suggests that loss of ID3 may not cause DSBs. The type of DNA damage provoked by ID3 should be characterized in subsequent investigations. In summary, we have identified a new role for ID3 in DNA homeostasis of pancreatic cancer cells. Further studies will be required to determine the exact mechanism which differs from that reported previously.

DECLARATIONS
ETHICS APPROVAL AND CONSENT TO PARTICIPATE
Not applicable.

CONSENT FOR PUBLICATION
Not applicable.

(h = hours), then the expression of ID3, γ-H2AX and/ or GAPDH were detected by Western blot. Cisplatin treatment increased γ-H2AX in a dose- and time-dependent fashion. Treatment with 50 μmol/ L cisplatin for 8 hours diminished total ID3. (C) Western blot was used to detect the efficiency of ID3 KD in PANC-1 and MIA PaCa-2 cells and correlation with γ-H2AX and GAPDH expression. A larger increase in γ-H2AX was associated with better KD of ID3. For A-C, representative run of at least 3 independent experiments is shown. (D-G) The parental and ID3 KD PANC-1 and MIA PaCa-2 cells treated with cisplatin as indicated. The percentage of cells containing > 10 foci of γ-H2AX (D and E) or MDC1 (F and G) is tabulated for each of 10 fields for each treatment condition from 3 independent experiments. Composite data are presented as mean $\pm$ standard deviations (SD). Foci of γ-H2AX and MDC1 were increased by cisplatin treatment and by ID3 KD. (H) Parental and ID3 KD PANC-1 and MIA PaCa-2 cells treated with serial concentrations of cisplatin and cell viability was assessed following 48 hours of drug exposure. ID3 KD did not affect cell viability following cisplatin treatment. (I) Parental and ID3 KD MIA PaCa-2 cells treated with ionizing radiation then lysed after the indicated number of hours, and assessed by immunoblot (left panel). Radiation treatment resulted in a time-dependent decrease in ID3 and an increase in γ-H2AX. An increase in γ-H2AX was more prominent in ID3 KD cells. Presence of cleaved caspase 3 beginning at 48 hours demonstrates that the radiation dose induced apoptosis. Parental and ID3 KD MIA PaCa-2 cells treated with ionizing radiation then assayed for viability after the indicated number of hours (right panel). ID3 KD did not affect cell viability following radiation treatment. NT = no treatment, IR = ionizing radiation. Representative run of 3 independent experiments is shown in H and I. In viability experiments: triplicate measurements were made for each treatment. Data are presented as the mean $\pm$ standard error of the means (SEM). Statistical significance was assessed using the student's two-tailed t-test. *, $P < 0.05$; **, $P < 0.01$; *ns*, not significant.

AUTHORS' CONTRIBUTIONS

Concept and design: CA and JX. Acquisition of data: JX and MPD. Analysis and interpretation of data: JX, MPD, and CA. Drafting of initial manuscript: JX and CA. Critical revision of manuscript: JX, MPD, and CA.

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