

DNA damage- and stress-induced apoptosis occurs independently of PIDD

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Abstract The p53-induced protein with a death domain, PIDD, was identified as a p53 target gene whose main role is to execute apoptosis in a p53-dependent manner. To investigate the physiological role of PIDD in apoptosis, we generated PIDD-deficient mice. Here, we report that,

although PIDD expression is inducible upon DNA damage, PIDD-deficient mice undergo apoptosis normally not only in response to DNA damage, but also in response to various p53-independent stress signals and to death receptor (DR) engagement. This indicates that PIDD is not required for DNA damage-, stress-, and DR-induced apoptosis. Also, in the absence of PIDD, both caspase-2 processing and activation occur in response to DNA damage. Our findings demonstrate that PIDD does not play an essential role for all p53-mediated or p53-independent apoptotic pathways.

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Introduction

Apoptosis is a critical process in a wide range of biological and pathological processes, including embryonic development, immunity, aging, and cancer [1]. The highly complex process involves a large number of proteins with specialized functions that can be largely categorized into either the intrinsic or extrinsic pathway. The intrinsic, or mitochondrial apoptotic pathway is induced by ionizing irradiation and/or cellular stress, resulting in the release of several proteins from the mitochondrial intermembrane space into the cytoplasm. The extrinsic pathway is initiated upon engagement of a ligand to its respective death-domain (DD)-containing cell surface receptor, such as Fas/CD95 or TNF- α receptor 1 (TNFR1), thereby recruiting and activating downstream signaling molecules [2–4]. Deficiencies of the molecular components in the extrinsic Fas-pathway have been linked to autoimmune diseases such as ALPS or Canale Smith Syndrome [5, 6], indicating the importance of these molecules in immune homeostasis [7]. While

distinct, these intrinsic and extrinsic pathways collaborate in determining the ultimate fate of a cell.

The transcription factor p53 plays a central role in orchestrating the activation of death machinery components. As a multi-faceted protein, p53 is implicated in a wide range of functions, including cell cycle regulation, senescence, DNA repair, genomic instability, and apoptosis [8–10]. In fact, the list is still growing as more of p53 functions are being recognized. Of these crucial functions of p53 is its role as a transcription factor [11]. When activated, p53 translocates into the nucleus and induces expression of many target genes, which then perform specific roles in different contexts. One recently identified novel p53 transcription target gene is p53-induced protein with a death domain (PIDD) [12]; the characterization of which is the focus of our study.

PIDD was initially identified as a p53 target gene in the temperature-sensitive erythroleukemia cell line [12]. PIDD is a cytoplasmic protein whose expression is induced in a p53-dependent manner and is thought to play a role in p53-mediated apoptosis. PIDD contains a number of important signaling domains such as seven repeats of leucine-rich region (LRRs) at the N-terminus and a death domain (DD) in the C-terminus and is believed to undergo spontaneous post-translational auto-processing. Although the exact mechanism is unknown, it has been suggested that such an auto-proteolysis may occur by a nucleophilic attack, whereby the hydroxyl or thiol group attacks on the preceding peptide bond replacing it with an ester or a thioester bond through the two serine residues, S446 and S588, of PIDD [13].

Interestingly, recent studies suggest that PIDD is found in a large cytoplasmic complex with receptor-interacting protein-associated ICH-1/CED-3 homologous protein with a DD (RAIDD) and caspase-2, known as the “PIDDosome” [14, 15]. The PIDDosome forms spontaneously under physiological conditions and is thought to play a critical role in apoptosis in a caspase-2-dependent manner, although the role of caspase-2 in apoptosis remains controversial [16]. In addition, the crystal structure of its core complex reveals that seven DDs of RAIDD and five DDs of PIDD asymmetrically oligomerize, which in turn recruits seven caspase-2 molecules into proximity for dimerization and subsequent activation [17].

To investigate the physiological functions of PIDD, we have generated PIDD-deficient mice. For this study, we have specifically aimed at understanding the role of PIDD in apoptosis by evaluating the contribution of PIDD to both p53-dependent and p53-independent apoptotic pathways. Here, we report the surprising finding that PIDD is dispensable for both p53-dependent and p53-independent apoptosis *in vivo* from our analyses of fibroblasts, lymphoid cells, and oocytes derived from PIDD-deficient mice.

Results

Generation of PIDD-deficient mice

To investigate the physiological role of PIDD, we have generated PIDD-deficient mice by replacing exons 1 through 5 and a portion of exon 6 with a neomycin-resistant cassette (Fig. 1a). Homologous recombination of the PIDD mutant allele was confirmed by Southern blot analysis on genomic DNA isolated from tissue samples (Fig. 1b). To confirm the homozygous null mutation of PIDD, we performed Northern blot analysis of PIDD mRNA on total RNA isolated from γ -irradiated (5 Gy) thymocytes (Fig. 1c). The amount of pidd transcripts was decreased in PIDD heterozygous thymocytes compared to the wild-type and was undetectable in PIDD-deficient thymocytes, whereas the level of GAPDH transcripts remained unaltered for all genotypes (Fig. 1c). Similar results were observed from two independent lines of PIDD-deficient mice generated from independent ES clones.

Based on the genotyping results of 483 offspring obtained from heterozygous matings, PIDD knockout mice were born at the expected Mendelian ratio, suggesting that PIDD-deficient mice are viable and developmentally normal (Fig. 1d). Also, flow cytometric analysis showed that PIDD-deficient mice have normal immune cell subsets in various lymphoid tissues (Fig. S1).

DNA damage-induced apoptosis is PIDD-independent

One of the important roles of p53 is to act as a transcriptional activator for its target genes in response to genotoxic stress. PIDD has been reported as a p53 target gene that plays a role in DNA damage-induced apoptosis. To determine whether PIDD deficiency has an impact on p53-dependent apoptosis, we exposed wild-type and PIDD-deficient cells to various DNA damage inducers, including etoposide, 5-fluorouracil (5-FU), and *cis*-platinum (II) diamminedichloride (*cis*platin) for various time points. Viability over time was determined by Annexin V staining followed by flow cytometric analysis. We observed that PIDD^{-/-} and RAIDD^{-/-} thymocytes were similarly sensitive to apoptosis induced by the above treatments as those from wild-type littermates (Fig. 2a), while those from p53^{-/-} mice were partially resistant to the treatments. We also exposed wild-type and PIDD-deficient thymocytes (Fig. 2b) and splenocytes (data not shown) to various doses of γ -irradiation and H₂O₂ for 16 h and observed similar results. These results suggest that the lack of PIDD did not rescue cell death due to DNA double-strand breaks. By comparison, the level of apoptosis was significantly lower in p53-null T cells stimulated with p53-dependent apoptotic insults even 36 h after stimulation, serving as positive

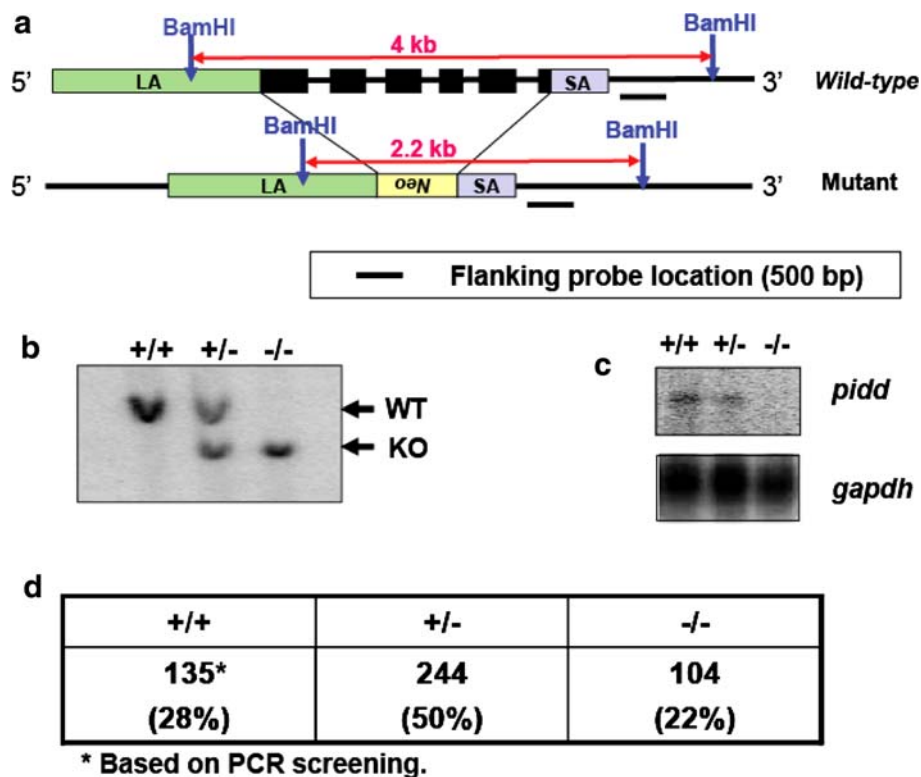


Fig. 1 Generation of PIDD-deficient mice. **a** Targeting strategy of the *PIDD* locus. The mouse *PIDD* gene was disrupted by the insertion of neomycin resistant gene. *PIDD*^{+/-} ES cells were generated by homologous recombination using a targeting vector in which exons 1 through 5 and a part of exon 6 were replaced by a NEO cassette in the reverse orientation. **b** Southern blot analysis of recombination of the *PIDD* gene. Genomic DNA samples were prepared from the tail DNA

of *PIDD*^{+/+}, *PIDD*^{+/-}, and *PIDD*^{-/-} mice. **c** Northern blot analysis of *PIDD* mRNA on total RNA isolated from γ -irradiated (5 Gy) thymocytes. *Gapdh* probe was used as a loading control. **d** Genotyping ratio of offspring from the *PIDD* heterozygous mating. PCR results show that *PIDD*-deficient mice are viable and are born at the expected Mendelian ratio

control (Fig. 2c). We have also tested the processing of caspase-3 in the thymocytes with or without exposure to γ -irradiation (Fig. 2d). The level of the cleaved caspase-3, indicated by the intracellular fluorescence intensity, increased to a similar extent in the both *PIDD*-deficient and wild-type thymocytes upon irradiation. On the other hand, the signals in the caspase-3-deficient thymocytes remained lower throughout the treatment. Therefore, the absence of *PIDD* neither rendered cells resistant to multiple p53-dependent death stimuli, nor induced further processing of the executioner caspase, caspase-3.

Whole-body irradiation and TUNEL staining of *PIDD*-deficient mice

To further examine whether *PIDD* deficiency has a role in ionizing radiation (IR)-induced cell death *in vivo*, we performed whole-body γ -irradiation on wild-type and *PIDD*-deficient mice. We then isolated radiosensitive organs, including spleen, thymus, intestine, and ovary, 6 h after irradiation and performed *in situ* terminal deoxynucleotidyl

transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) assay to determine the level of apoptosis (Fig. 3). TUNEL-positive apoptotic cells showed the punctate brown staining in the nuclei as indicated by the arrow (top right panel) and appeared specifically in splenic white pulps, thymic cortices, small intestinal villi, and ovarian follicles. The intensity and the localization of TUNEL positive cells in the *PIDD*-deficient mice were similar to those of wild-type, indicating that the detectable apoptotic responses were similar for both genotypes.

PIDD-deficient cells undergo apoptosis in response to a variety of stress signals

Although our findings show that *PIDD* is not required for p53-dependent apoptosis, *PIDD* may be involved in apoptosis induced by other stress factors independently of p53 status. In fact, a recent study suggests that cell death induced by cytoskeletal disruption is mediated by caspase-2 and involves the *PIDDosome* [18]. In order to explore the possibility that *PIDD* may have an apoptotic role in

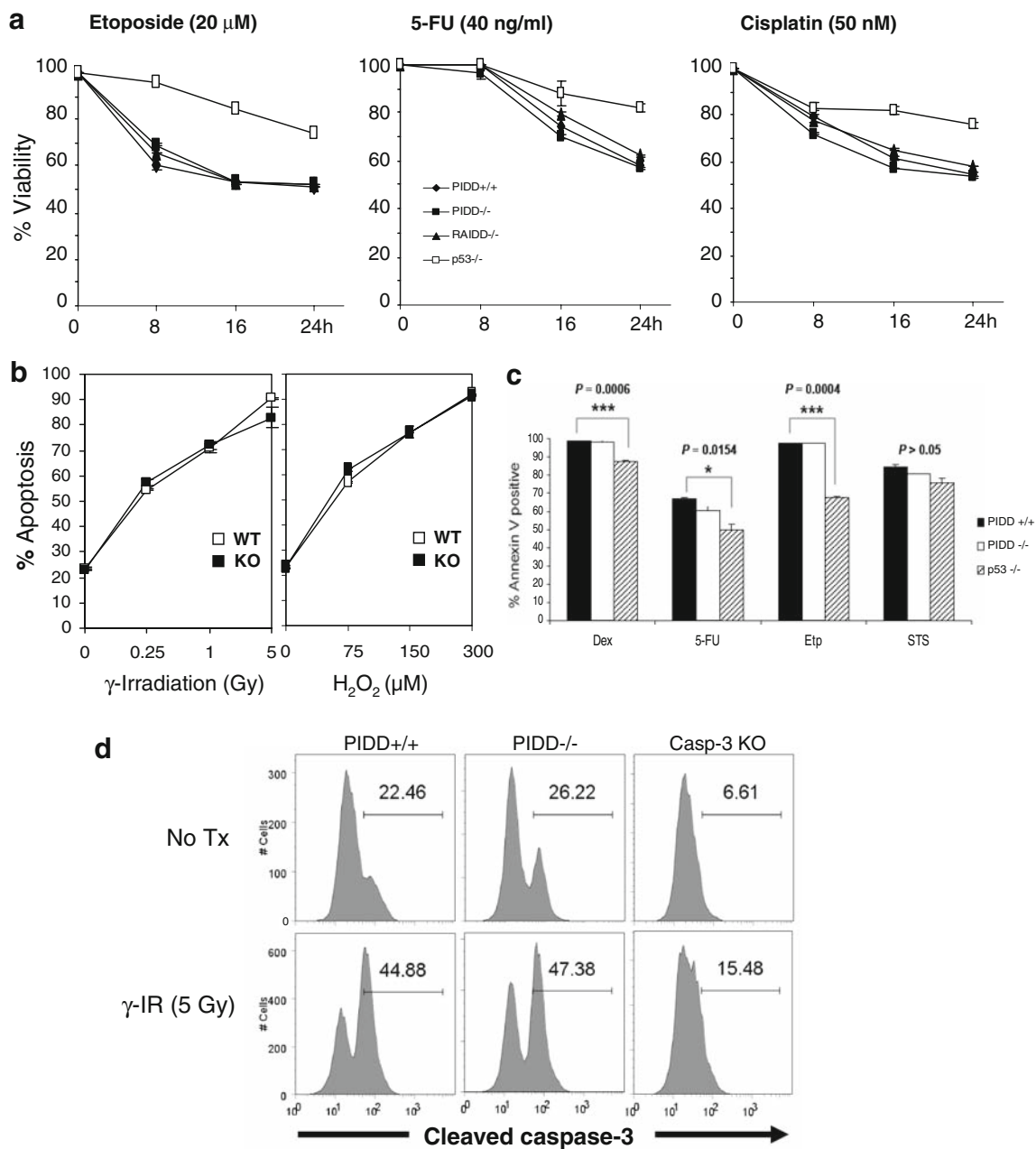


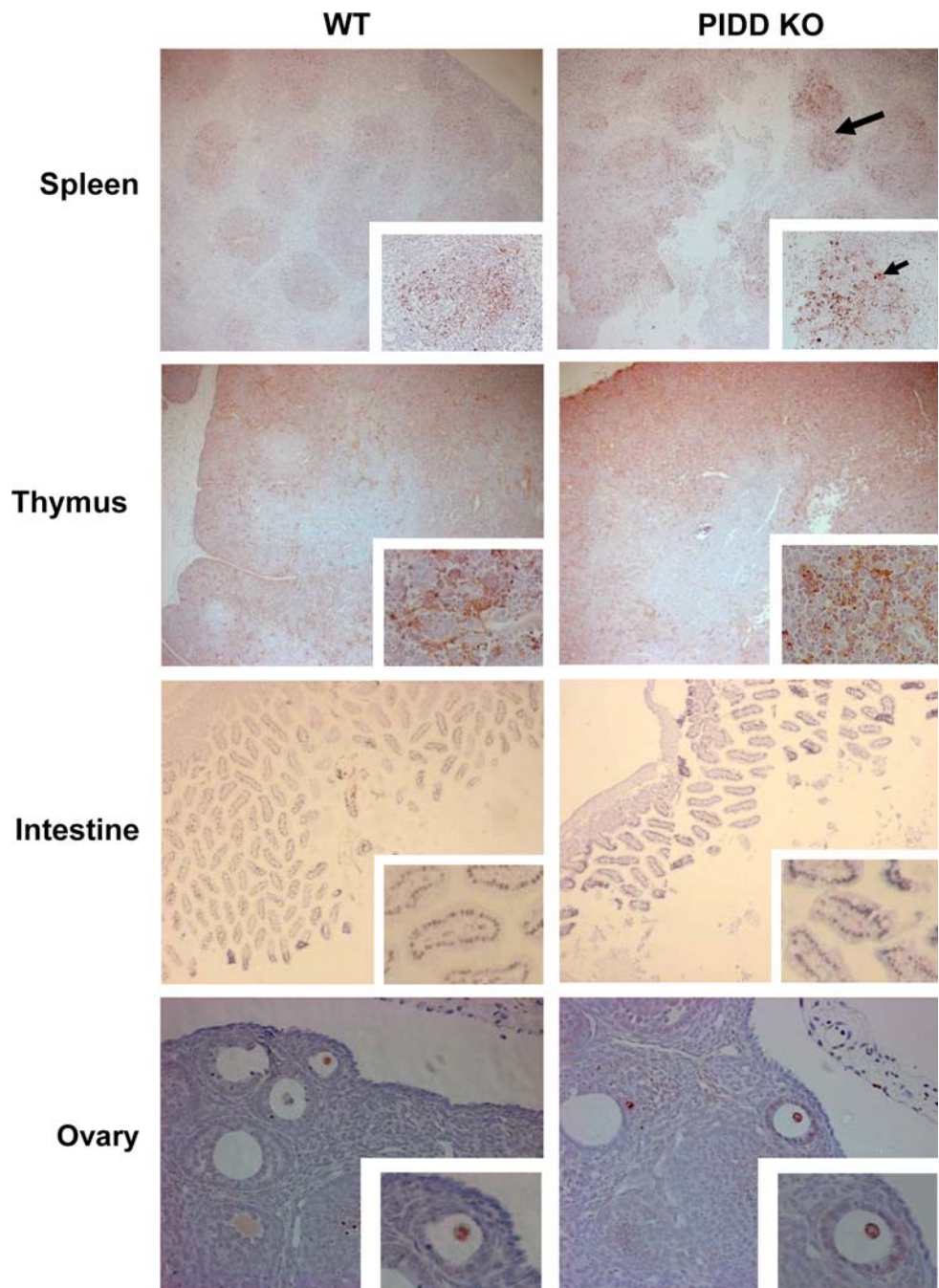
Fig. 2 PIDD-deficiency does not alter sensitivity to p53-mediated apoptosis. **a** Thymocytes isolated from PIDD+/+, PIDD-/-, RAIDD-/-, and p53-/- mice were treated with etoposide (20 μM), 5-fluorouracil (40 ng/ml), and cisplatin (50 nM). The percentage of viability was determined by flow cytometry on the population negative for Annexin V and propidium iodide (PI) staining 8, 16, and 24 h after the treatment. The values represent the mean \pm SD of triplicates from one representative of at least three independent experiments. **b** The percentage of apoptosis was determined by Annexin V staining on thymocytes exposed to increasing doses of γ -irradiation (0–5 Gy) and H_2O_2 (0–300 μM)

for 16 h. *Error bars* represent mean \pm SD of triplicates of at least three independent experiments. Data without *error bars* have an SD too small to visualize. **c** Percentage of Annexin V positive population for PIDD+/+, PIDD-/-, and p53-/- thymocytes 36 h after treatment with dexamethasone (1 μM), 5-fluorouracil (50 $\mu\text{g}/\text{ml}$), etoposide (10 μM), and staurosporine (1 μM). (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). **d** Histograms showing the intracellular staining of cleaved caspase-3 by flow cytometry after γ -irradiation with 5 Gy. The *number* in each plot represents the percentage of cleaved caspase-3

response to other stress inducers, PIDD-deficient cells were exposed to a variety of stress-inducing agents, including staurosporine, sorbitol, and taxol. We observed that PIDD-

deficient cells could undergo apoptosis at a normal level in response to the above treatments, indicating that PIDD is not required for apoptosis induced by these stress signals

Fig. 3 PIDD-deficient mice are sensitive to whole-body irradiation. TUNEL staining of spleen, thymus, intestine, and ovary from whole-body irradiated control and PIDD-deficient mice after 5 Gy (for spleen and thymus) and 8 Gy (for intestine and ovary) of γ -irradiation (^{137}Cs). Organs were collected 6 h post irradiation. Higher magnification is shown in the *lower right box*. Data shown are representative of three independent experiments



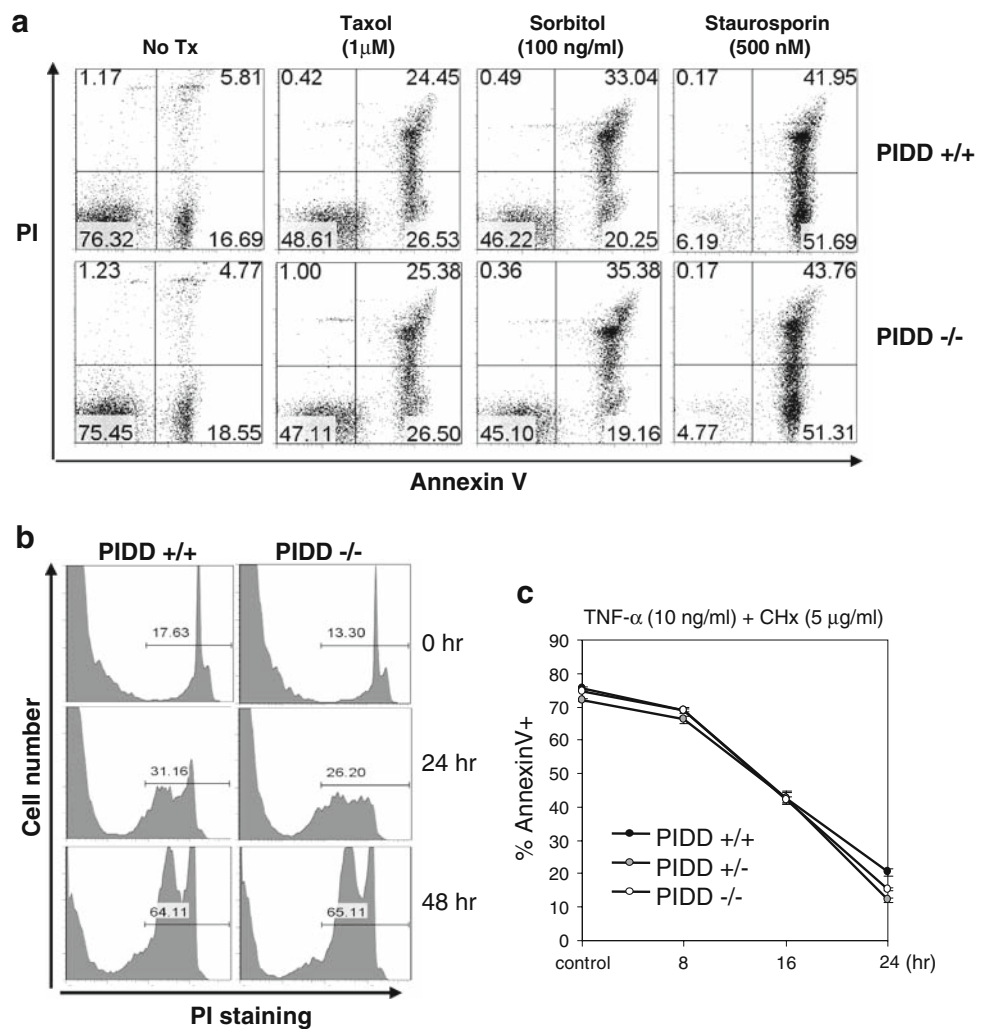
(Fig. 4a). Similarly, the kinetics of apoptosis also showed that PIDD-deficient cells undergo apoptosis normally under these conditions (data not shown).

We next investigated whether PIDD contributes to the death of activated T lymphocytes following cytokine withdrawal. Interleukin-2 (IL-2) is an important cytokine for the survival of T lymphocytes. We purified total T lymphocytes from the spleen of wild-type and PIDD-deficient mice and stimulated them with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 72 h. Activated splenic T lymphocytes were then expanded in IL-2-containing media for

48 h, prior to IL-2 withdrawal. The level of cell death was compared in PIDD-null activated T lymphocytes with the wild-type counterparts at both 24 and 48 h after IL-2 withdrawal (Fig. 4b). Wild-type and PIDD-null T lymphoblasts showed comparable levels of PI staining for both time points, demonstrating that PIDD-deficiency does not compromise cytokine withdrawal-induced cell death in activated T lymphocytes.

We also assessed if PIDD deficiency has a role in the death receptor-mediated pathway by stimulating PIDD-deficient cells with tumor necrosis factor- α (TNF- α) in the

Fig. 4 Stress-induced apoptosis can occur independently of PIDD. **a** Thymocytes treated with Taxol (1 μ M), sorbitol (100 ng/ml), and staurosporine (500 nM) for 16 h were stained with Annexin V and Propidium iodide (PI). Numbers indicate the percentage of cells in each quadrant. Similar results were obtained from at least three independent experiments performed in triplicates. **b** T lymphocytes from the spleen of wild-type and PIDD-deficient mice were isolated and expanded in the presence of PMA (50 ng/ml) and Ionomycin (1 μ M) at 0, 24, and 48 h after IL-2 withdrawal. The numbers in the histogram indicate the percentages of PI positive population. **c** TNF- α (10 ng/ml) and cycloheximide (5 μ g/ml) treatment of thymocytes from PIDD+/+, PIDD+/-, and PIDD-/- mice. Values represent the mean \pm SD of triplicates and control indicates the spontaneous apoptosis at the time of stimulation. Results represent one of the three independent experiments



presence of cycloheximide (CH \times) to block de novo protein synthesis. However, we did not observe a significant difference between wild-type and PIDD-deficient thymocytes (Fig. 4c) as well as splenocytes (data not shown), indicating that PIDD is not obligatory in TNF-receptor-mediated apoptosis.

PIDD is not required for caspase-2 processing and caspase-2-mediated cell death

Caspases are a family of evolutionarily conserved intracellular cysteine proteases and their role in apoptosis has been extensively studied. Caspase-2 is one of the most highly conserved members, yet its role in apoptosis is still controversial [16]. It has recently been proposed that the cytosolic complex PIDDosome may mediate caspase-2 processing in DNA damage-induced apoptosis. In agreement with this, a report suggested that PIDD-mediated apoptosis is RAIDD-dependent in vitro [15]. To test whether PIDD and/or RAIDD, the two components of the PIDDosome complex, are required for the DNA damage-

induced processing of caspase-2, we stimulated PIDD-deficient, RAIDD-deficient, and PIDD/RAIDD double-deficient (DKO) thymocytes with etoposide and compared the level of cleaved caspase-2 with wild-type cells (Fig. 5a). Western blotting of caspase-2 showed the accumulation of the p32 and p19 subunits in both PIDD- and RAIDD-deficient thymocytes comparable to the wild-type cells in response to etoposide (20 μ M) treatment, suggesting that neither PIDD nor RAIDD are required for caspase-2 processing in response to DNA damage.

To evaluate if other DNA damage inducers also result in normal caspase-2 processing in PIDD-deficient cells, we exposed wild-type and PIDD-deficient splenocytes and thymocytes to γ -irradiation (Fig. 5b). Similar to the etoposide treatment, caspase-2 processing occurred normally in the absence of PIDD in both types of cells as indicated by the presence of its p32 and p19 processed forms.

Mammalian oocytes are naturally arrested in metaphase of the second meiotic division (MII) at the time of ovulation where they remain arrested until fertilization. MII stage oocytes, when ovulated, naturally exhibit low rate of

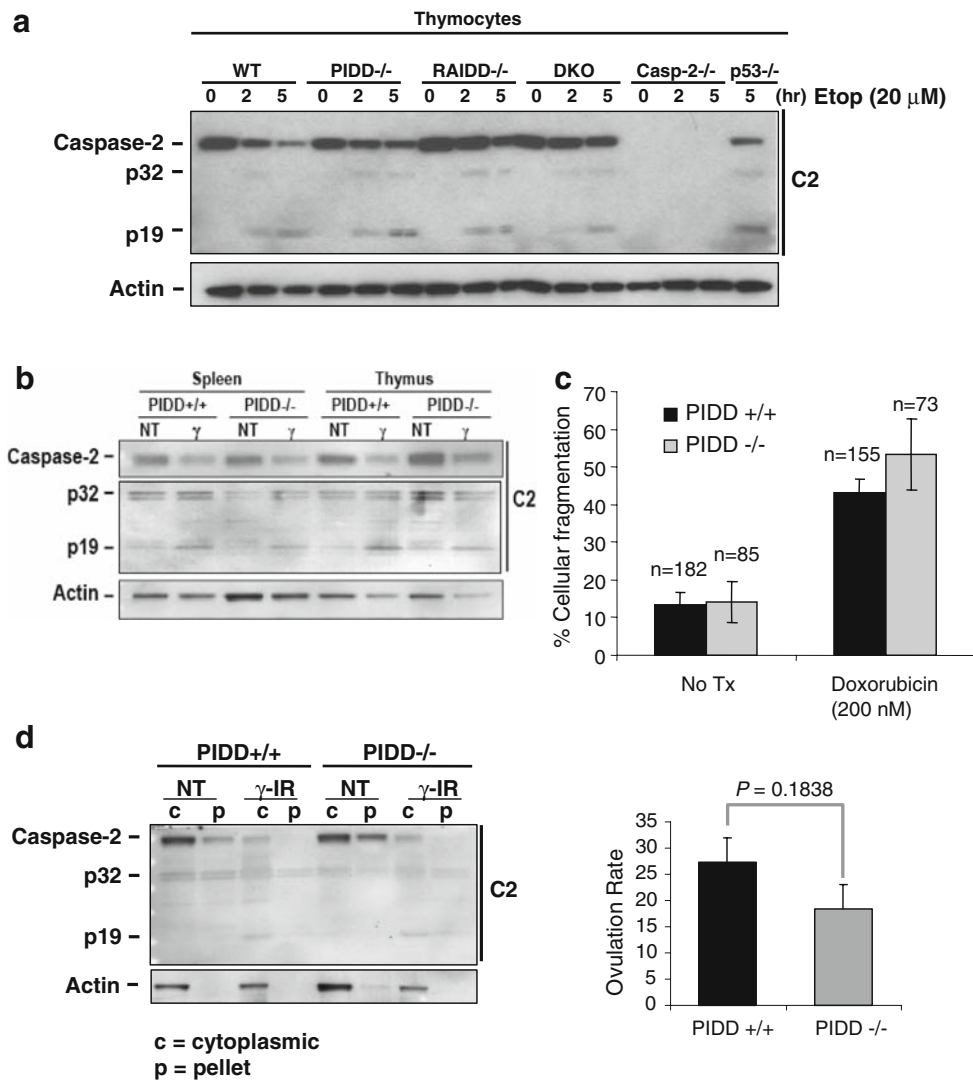


Fig. 5 Caspase-2-dependent apoptosis does not require PIDD. **a** Caspase-2 processing from the cytoplasmic cell lysates of thymocytes treated with etoposide (20 μM) from *wild-type* (WT), PIDD^{-/-}, RAIDD^{-/-}, PIDD^{-/-}RAIDD^{-/-} (DKO), and p53^{-/-} mice. **b** Western blotting of PIDD^{+/+} and PIDD^{-/-} whole-cell lysates isolated from either non-treated or γ-irradiated (5 Gy) splenocytes and thymocytes. **c** Doxorubicin-treated MII oocytes. Percentage of cellular fragmentation was determined by the morphological changes in the oocytes (*top panel*) and the ovulation rate (*bottom panel*). *n* = the total number of oocytes analyzed, where oocytes from each

female were split randomly into the control and treated group. Dead oocytes in each group were expressed as a proportion of total number per treatment. Ovulation rate per female was obtained by averaging number of obtained oocytes from *n* = 13 PIDD^{+/+} and 10 PIDD^{-/-} females. **d** Western blotting of the cytoplasmic (c) and pelleted (p) membrane organelle fractions showing caspase-2 processing in non-treated (NT) or γ-irradiated (γ-IR; 5 Gy) thymocytes after 6 h post irradiation. Actin was used as negative control for cytoplasmic fractions

spontaneous cellular fragmentation *in vitro*, but are highly susceptible to DNA damage-induced apoptosis caused by doxorubicin [19, 20]. Cellular fragmentation of oocytes has been characterized by the morphological change due to the condensation of DNA and the oocyte cytoplasm accompanied by membrane blebbing, which ultimately result in the cytoplasmic fragmentation of the dying oocytes into apoptotic bodies enclosed in the zona pellucida [20].

Previous studies suggest that doxorubicin mediated death of murine oocytes is dependent on caspase-2 [21]. To investigate if PIDD is required for DNA damage-induced cellular fragmentation, MII stage oocytes isolated from superovulated wild-type and PIDD^{-/-} females were collected and exposed to doxorubicin. The percentage of cellular fragmentation was determined based on oocyte morphology. We found that the fragmentation rate in the PIDD-deficient oocytes was similar to the wild-type

oocytes, indicating that PIDD deficiency does not protect MII stage oocytes from doxorubicin-mediated apoptosis (Fig. 5c). In addition, ovulation rate between wild-type and PIDD-deficient mice was insignificant ($P = 0.1838$), suggesting that PIDD is not likely to play a role in regulating the follicular recruitment at least in the young animals.

To further examine the processing of caspase-2 in different cellular compartments, we have fractionated the cell lysates into cytosolic and pellet fractions. Upon irradiation, the expression of the full-length caspase-2, p51, was decreased, whereas those of the two cleavage products, p32 and p19, increased in the cytosol, indicating that the processing of caspase-2 did not require PIDD in thymocytes (Fig. 5d) or splenocytes (Data not shown). Intriguingly, the two cleaved products of caspase-2 were detected in the pellet fraction of the γ -irradiated cell lysates from both wild-type and PIDD-deficient cells, while neither actin and Hsp70, the two cytosolic control proteins, nor the full-length caspase-2 were detected in the γ -irradiated pellet fraction. This result suggests a possibility that active caspase-2 may translocate into the membrane-bound organelle upon DNA damage induction.

Together, our results demonstrate that both p53-dependent and p53-independent apoptosis may occur independently of PIDD and that caspase-2 processing is normal in the PIDD-deficient lymphocytes.

Discussion

Our analyses of PIDD-deficient mice have indicated that PIDD is dispensable for apoptosis in response to DNA damage as well as to a variety of stress signals. While accumulating *in vitro* evidence suggests that PIDD is involved in p53-dependent apoptosis, we have not observed any defect in apoptosis due to the deficiency in PIDD. One possible explanation for such a discrepancy is that other p53 target or pro-apoptotic protein may compensate for the loss of PIDD. In fact, PIDD is one of the many p53 target genes implicated in apoptosis. Also, it is important to mention that all the previous studies were conducted on a cell culture-based system, whereas our data are based on an *in vivo* knockout mouse model derived from two independent ES clones. Our mice have also been backcrossed over 12 generations onto the C57BL/6 background.

Consistent with our findings, Shi et al. have recently reported that the PIDDosome may not be required for apoptosis while it may have a role in PK-mediated DNA repair [22]. Olsson et al. have also shown that caspase-2 activation was not significantly affected by the elimination of PIDD or RAIDD, but rather CD95 DISC formation is important for caspase-2 activation in their experimental

system [23]. Recently, Manzl et al. have also reported similar findings [24]. These studies further reinforce the observation that PIDD is not critical for the induction of apoptosis or caspase-2 activation.

As previously described, PIDD interacts with RAIDD in the cytosolic complex known as the “PIDDosome” to recruit and activate caspase-2. Caspase-2 is one of the most evolutionarily conserved members of the caspase family [25], and numerous studies over the past decade have suggested its contribution in apoptosis, particularly in germ cells, oocytes, and neurons [21, 26, 27]. However, inconsistency in the mechanism of caspase-2-mediated apoptosis makes its apoptotic role difficult to reconcile. Notably, neither RAIDD-deficient nor caspase-2-deficient mice display obvious phenotypic abnormalities [16, 21], as is the case for PIDD.

Interestingly, it has recently been reported that PIDD mRNA exists in multiple isoforms. PIDD can also be processed into a number of distinct fragments by auto-proteolytic cleavage [13, 28], each of which is believed to perform a specific role in different cellular compartments. Furthermore, a more recent study [29] indicated a possible role of PIDD in the NF- κ B-mediated survival pathway, suggesting that PIDD may be involved in “modulating” the cell survival and cell death in response to DNA damage. Such interesting findings may contribute significant complexity to the physiological functions of PIDD.

Although our data suggest that PIDD is dispensable in apoptosis, PIDD may be involved in the alternative death pathways such as autophagy and necrosis. A recent study has suggested that caspase-2 may be a crucial mediator of cell death in the *Xenopus* oocyte system. The authors reported that *Xenopus* oocytes rely on the nutrient stockpile of NADPH generated by pentose-phosphate-pathway for survival. According to Nutt et al. the inhibitory phosphorylation of caspase-2 by calcium/calmodulin-dependent protein kinase II (CaMKII) is crucial for keeping such a stockpile of nutrients, thus preventing apoptosis in the *Xenopus* oocytes [30]. At this point, it is not clear whether PIDD is directly involved in this context as no evidence for such a regulation of oocyte metabolism has been established in the mammalian system. Furthermore, p53 has been shown to be activated under the metabolic stress that results from the low glucose condition [31]. In fact, a growing line of evidence supports a link between p53 and the regulation of glucose metabolism, suggesting a possible involvement of its target genes in this process.

In conclusion, our findings based on the conditions examined in this study indicate that PIDD is neither necessary nor sufficient for DNA damage-induced, p53-mediated apoptosis as well as stress-induced p53-independent apoptosis for splenocytes, thymocytes, purified T cells, oocytes, and embryonic fibroblasts.

Materials and methods

Generation of PIDD-deficient mice

Mice were generated on the mixed 129Ola/C57BL/6 background and backcrossed on the pure C57BL/6 background for 8 or more generations. All the experiments were performed on 6–9 week-old mice and repeated on the F8 C57Bl/6 backcross generation (or greater) with littermate controls or on the F12 generation with gender and age-matched C57BL/6 wild-type mice. All mice were housed in a specific pathogen-free animal facility in the Ontario Cancer Institute (OCI) according to institutional guidelines. Targeted PIDD mice were genotyped by PCR using either the wild-type allele-specific oligonucleotides 5'-TTA TGG CTG CTC TTT GTC CCT C-3' or the targeted allele-specific oligonucleotides 5'-AGA GGC CAC TTG TGT AGC GCC AAG TGC C-3' in combination with the common oligonucleotides 5'-CGT GGC CCT GGT CCC TCC TAA AAG AC-3'.

Cell culture

Mouse embryonic fibroblasts (MEFs) were prepared from E12.5–E14.5 embryos and primary MEFs were subsequently immortalized or transduced with E1A and Ras oncogenes. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 2 mM L-glutamine, and 0.1% β -mercaptoethanol. Thymocytes and splenocytes were isolated and cultured in the Iscove media supplemented with 10% FCS, 2 mM L-glutamine, and 0.1% β -mercaptoethanol.

Activated T cell culture

Pan T cells were purified from the whole spleen using magnet activated cell sorting (MACS) purification method (Miltenyi biotec) by negative selection and stimulated with PMA (5 ng/ml; Sigma-Aldrich) and ionomycin (1 μ M; Sigma-Aldrich) for 48–72 h. Surviving T cells were then counted and seeded at the density of 1×10^6 cells/ml in the Iscove media supplemented with 10% FCS and 2 ng/ml of mouse IL-2 (PeproTech). Viability was determined by Annexin V and propidium iodide staining for flow cytometry.

Oocyte isolation and culture

Female mice were superovulated with 5 IU of Pregnant Mare Serum Gonadotrophin (PMSF) followed by 5 IU of human chorionic gonadotropin (hCG) (both from Sigma) 48 h later. Mature oocytes were collected from the oviducts 16 h after hCG injection. Cumulus cells were removed

from the oocytes by incubating in 80 IU/ml of hyaluronidase (Sigma) for 1 min and the denuded oocytes were washed three times with HTF w/HEPES medium (Life-Global) containing 0.1% bovine serum albumin (Sigma). Oocytes were cultured in 20 μ l drops of HTF Xtra (Life-Global) culture medium under paraffin oil (Speciality Media, Phillipsburg, NJ, USA), and incubated with or without freshly prepared doxorubicin (200 nM; Alexis Biochemicals) in a humidified 37°C, 5% CO₂ incubator. Oocyte morphology was evaluated 0, 24, and 48 h after the treatment.

Southern blotting, Northern blotting

Genomic DNA samples were prepared from either ES cells or tail samples. Total RNA samples were isolated with Trizol® (Invitrogen) according to the manufacturer's protocol. 10 μ g of RNA was separated with a 1% denaturing formaldehyde agarose gel and transferred to a Hybond N+ membrane (Amersham Biosciences). cDNA probes specific for PIDD and GAPDH were labeled using the Rediprime DNA labeling system (Amersham Biosciences) and hybridized to the membrane in the buffer containing dextran sulfate. Membranes were stripped by boiling for 10 min in 0.25% SDS and incubated at 65°C for 30 min. Equal loading across the lanes was confirmed by GAPDH hybridization.

Flow cytometry

Cells from the spleen, lymph nodes, bone marrow, or thymus were depleted of erythrocytes with ACK hypotonic lysis buffer and sorted by negative selection using MACS column where necessary. Cells were then preincubated with Fc γ RII blocking antibody (BD PharMingen) for 20 min followed by the incubation with specific antibodies for 30 min on ice followed by flow cytometric analysis using a FACS Calibur (Becton Dickinson). Monoclonal antibodies were used for CD8 (eBioscience) and CD4, CD25, CD44 (BD Bioscience PharMingen; San Diego, CA). Caspase-3 intracellular staining was performed using Alexa Fluor 488 conjugated cleaved caspase-3 (Asp175) antibody (Cell Signaling).

Apoptosis assays

For the induction of cell death, cells were plated in 24 well plates at 1×10^6 cells/ml and treated for the indicated time periods with various cell death stimuli, including Dexamethasone (1 M), γ -irradiation (5 Gy), Etoposide (20 μ M), TNF- α (10 ng/ml) with CH \times (5 μ g/ml), staurosporine (500 nM), or sorbitol (100 ng/ml). All reagents used were obtained from Sigma, unless indicated otherwise. Cell

viability was determined by staining with 2 µg/ml of PI and quantified with flow cytometer. For apoptosis studies Annexin V staining (BD Pharmingen) was performed with either propidium iodide (PI) or 7-amino-actinomycin D (7-AAD). Cells and mice were irradiated with a ¹³⁷Cs source (Gammacell 40) at a dose of 1.0 Gy/min calibrated by the Radiation Division at the Department of Medical Biophysics at the Ontario Cancer Institute.

Western blotting

Cells were lysed in buffer containing 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 0.1% NP-40, 150 mM NaCl, and 1 mM EDTA, supplemented with complete protease inhibitor cocktail (Roche, Mannheim, Germany), PMSF, NaVO₄, aprotinin, and leupeptin. Lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to membranes by electroblotting. Membranes were incubated with the antibodies for caspase-2 (clone 11B4; Alexis), hsp70 (BD Pharmingen), actin (sigma-Aldrich) in TBS containing 0.1% Tween 20 (v/v) with 5% milk (w/v) overnight at 4°C. Secondary antibodies for anti-mouse, anti-rat, and anti-rabbit IgGs were purchased from Amersham Biosciences. Bound antibodies were detected by enhanced chemiluminescence (ECL plus, Amersham). Membranes were stripped either with 0.1 M glycine pH2.5 for 40 min at 55°C or with Restore Western Stripping Buffer (Pierce) at room temperature for 10 min.

TUNEL staining

In situ terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed by the histology department at the University Health Network.

Statistical analysis

Data are reported as mean ± SD. Duplicates or triplicates were performed within each individual experiment. *P*-values for statistical significance were calculated using the Student's *t*-test, and values of *P* < 0.05 were considered significant.

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Conflict of interest The authors declare no financial or commercial conflict of interest.

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