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Cellular Inhibitor of Apoptosis Protein 1 ubiquitinates endonuclease G but does not affect endonuclease G-mediated cell death

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ABSTRACT

Inhibitors of Apoptosis Proteins (IAPs) are evolutionarily well conserved and have been recognized as the key negative regulators of apoptosis. Recently, the role of IAPs as E3 ligases through the Ring domain was revealed. Using proteomic analysis to explore potential target proteins of DIAP1, we identified *Drosophila* Endonuclease G (dEndoG), which is known as an effector of caspase-independent cell death. In this study, we demonstrate that human EndoG interacts with IAPs, including human cellular Inhibitor of Apoptosis Protein 1 (cIAP1). EndoG was ubiquitinated by IAPs *in vitro* and in human cell lines. Interestingly, cIAP1 was capable of ubiquitinating EndoG in the presence of wild-type and mutant Ubiquitin, in which all lysines except K63 were mutated to arginine. cIAP1 expression did not change the half-life of EndoG and cIAP1 depletion did not alter its levels. Expression of dEndoG 54310, in which the mitochondrial localization sequence was deleted, led to cell death that could not be suppressed by DIAP1 in S2 cells. Moreover, EndoG-mediated cell death induced by oxidative stress in HeLa cells was not affected by cIAP1. Therefore, these results indicate that IAPs interact and ubiquitinate EndoG via K63-mediated isopeptide linkages without affecting EndoG levels and EndoG-mediated cell death, suggesting that EndoG ubiquitination by IAPs may serve as a regulatory signal independent of proteasomal degradation.

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1. Introduction

Inhibitors of Apoptosis Proteins (IAPs) are established negative regulators of caspase-dependent cell death [1,2]. These proteins are well conserved from viruses to humans and possess one to three Baculovirus IAP Repeat (BIR) domains responsible for interacting with other proteins [3]. More than half of all IAPs contain the Really Interesting New Gene (Ring) domain, which recruits the E2 conjugating enzyme and functions as an E3 ligase by ubiquitinating substrates to target them for proteasomal degradation [4]. Their E3 ligase activities play a role in negatively regulating cell death [5,6]. Interestingly, recent studies have shown that, besides cell death, IAPs function in a wide range of cellular events through their Ring domain. For example, in *Drosophila*, DIAP1 is implicated in translational regulation [7], whereas Diap2 is involved in controlling innate immunity [8]. In mammals, cIAP1 and cIAP2 are involved in NF-kB signaling to regulate inflammation [9] and cell migration [10]. Collectively, these data suggest that IAPs play a regulatory role in cellular events by ubiquitinating substrate proteins via their Ring domain.

IAPs modify substrates via canonical ubiquitination that involves isopeptide linkages via lysine 48 of ubiquitin (Ub), which provides a signal for proteasomal degradation [2]. However, recent reports have shown that some IAPs ubiquitinate target proteins that do not undergo subsequent degradation. For instance, cIAP1 ubiquitinates RIP1via K63-mediated isopeptide linkages that are not recognized by proteasomes, but rather, function as interacting modules for recruiting TAK1-binding protein (TAB), thereby leading to NF-kB activation [10–12].

Endonuclease G (EndoG) is localized in mitochondria and translocates to the nucleus upon apoptotic stress to cleave chromosomal DNA in a caspase-independent manner [13,14]. EndoG expression correlates with the sensitivity of cells to chemical- or oxidative stress-induced cell death in cancer cells [15,16], neurons

Abbreviations: cIAP1, cellular Inhibitor of Apoptosis Protein; EndoG, Endonuclease G; CHIP, C-terminus of Hsc70-interacting protein; Hsp, heat shock protein; TB, Trypan Blue; H_2O_2, hydrogen peroxide; GFP, green fluorescent protein; CHX, cycloheximide; EDTA, ethylenediaminetetraacetic acid; DAPI, diamidino-2-phenylindole; FBS, fetal bovine serum.

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[17], and Parkinson's disease [18]. However, studies on *endoG* knock-out mice have shown contradictory results. EndoG is either essential [19] or dispensable [20,21] for embryogenesis and normal apoptosis. In fact, several reports have demonstrated that EndoG has a non-apoptotic function. Nuc1, a homolog of EndoG in *Saccharomyces cerevisiae*, has functions in survival and apoptosis depending on the type of carbon source comprising the culture medium [22]. Mammalian EndoG has also been shown to be involved in proliferation [23]. Furthermore, a recent study using EndoG-deficient mice demonstrated that EndoG plays an important role in cardiac hypertrophy [24]. Collectively, these observations suggest that EndoG plays a role in various intracellular events besides cell death.

Thus far, the mechanism underlying the regulation of EndoG protein is poorly understood. EndoG has been shown to be ubiquitinated for proteasomal degradation [25], and we have reported that the co-chaperone E3 ligase CHIP is involved in regulating EndoG expression levels [16]. Considering that the activities of multifunctional proteins are controlled via different modifications, EndoG is likely modified in various ways.

In this study, we identified dEndoG as an interacting partner of DIAP1 and demonstrated that this interaction also occurs in human cells. Additionally, we examined whether IAPs function as an E3 ligase for EndoG ubiquitination. Finally, we investigated whether IAPs play a regulatory role in EndoG-mediated cell death.

2. Materials and methods

2.1. Cell culture and transfection

Schneider cells (S2 cells) were maintained at 27 °C in Schneider's *Drosophila* medium (Welgene) containing 10% FBS (Welgene). The *dendoG* or *DIAP1* gene was cloned into pMT/V5-His A (pMT, Invitrogen) for expression in S2 cells. S2 cells (6×10^6) were transfected with Genecarrier-1 (Epochbiolabs) according to the manufacturer's instructions. Gene expression was induced with 50 μM $CuSO_4$ for 24 h. HEK293T cells were cultured in DMEM (Welgene) supplemented with 10% FBS (Welgene). Transfection was performed using PEI (Sigma–Aldrich) or Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. HeLa cells were transfected with 100 pmol cIAP1 siRNA (Dhamacon, a combination of #1 and #2), siEndoG (Dhamacon), or scrambled RNA (scRNA, Genolution) using Lipofectamine 2000 (Invitrogen). After 48 h, cells were harvested and lysed for Western blot (WB) analysis. The siRNA sequences used were as follows: cIAP-specific, #1: UCGCAAUGAUGAUGUCAAA, #2: GAAUGAAAGGCC AAGAGUU, EndoG-specific, AAGAGCCGCGAGUCGUACGUG.

2.2. Antibodies and co-immunoprecipitation

Antibodies against EndoG (Pro-Sci), cIAP1 (R&D Systems), Myc (Millipore), GFP (Millipore), HA (Covance), GST (Santa Cruz Biotechnology), and actin (Bethyl) were used for WB or co-immunoprecipitation (co-IP). For co-IPs, lysates were incubated with the indicated antibodies at 4 °C, and immunocomplexes recovered with protein-A Sepharose (Sigma–Aldrich) were washed three times with IP-Wash buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100) and analyzed by WB with the indicated antibodies.

2.3. In vitro and in vivo ubiquitination assays

The *dendoG* gene was cloned into pET-His 23a (Novagen) and purified according to the manufacturer's instructions. For the *in vitro* ubiquitination assay, dEndoG-6His, GST-Ubiquitin (GST-Ub), and S100 [26] were added to the reaction mixture with or without purified DIAP1. Reactions were stopped by adding SDS sample buffer and analyzed by WB with anti-His antibody (QIAGEN). HEK293T cells were transfected for *in vivo* ubiquitination. At 24 h post-transfection, cells were treated with 20 μM MG132 for 6 h and then lysed in buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, and protease inhibitor cocktail. Co-IPs were performed using anti-GFP antibody followed by WB analysis using anti-HA antibody.

2.4. Protein half-life

HEK293T cells were co-transfected with GFP-EndoG and an empty vector or 6Myc-cIAP1. At 24 h post-transfection, cells were treated with 100 μg/ml cyclohexamide (CHX) and harvested at the indicated time points. Cells were lysed with RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 0.1% SDS, 1% Triton X-100, 0.5% and sodium-deoxycholate), and then analyzed by WB with the indicated antibodies.

2.5. GST pull-down assay

DIAP1 on glutathione-agarose beads (GE Healthcare) were incubated with *in vitro* translated dEndoG WT and dEndoG 54310 in lysis buffer for 2 h at 4 °C in a rotator. The glutathione beads were washed four times and analyzed by WB with anti-His antibody.

2.6. S2 cell survival assay

pMT, pMT-dEndoG WT, or pMT-dEndoG 54310 was co-transfected with pMT-LacZ into S2 cells and gene expression was induced by 50 μM $CuSO_4$ for 24 h. After 24 h, cells were stained with a β-gal staining kit (Invitrogen). Cells stained blue were measured and the values presented represent the mean ± standard deviation (SD) of three independent experiments.

2.7. Cell death assay

HeLa cells were transfected with scRNA or the indicated specific siRNA for 48 h before treatment with 1 mM H_2O_2 for 4 h. Cell death was assessed by Trypan Blue staining, and WB analysis was performed using anti-EndoG, anti-cIAP1, and anti-actin antibodies.

3. Results and discussion

3.1. EndoG interacts with IAP in Drosophila and human cells

Previously, we performed multidimensional protein identification analysis to identify proteins that interact with DIAP1 in insect S2 cells [7,27]. dEndoG was pulled out as one protein that associates with DIAP1. To confirm this interaction, we performed GST pull-down assays using *in vitro* translated dEndoG WT-6His and purified GST-DIAP1 or GST proteins. dEndoG WT bound to GST-DIAP1, but not the GST bead alone, indicating that dEndoG and DIAP1 physically interact (Fig. 1A). EndoG is synthesized as a pro-form in the cytosol. The mature form is produced in mitochondria once the N-terminal mitochondrial localization sequence (MLS) is cleaved upon apoptosis induction [14]. To test whether mature EndoG also interacts with DIAP1, we performed a GST pull-down assay using an EndoG mutant (dEndoG 54310) in which the MLS is deleted. Our data demonstrate that the extent of DIAP1 binding was similar between dEndoG 54310 and dEndoG WT (Fig. 1A), indicating that the MLS is not involved in the interaction between dEndoG and DIAP1.

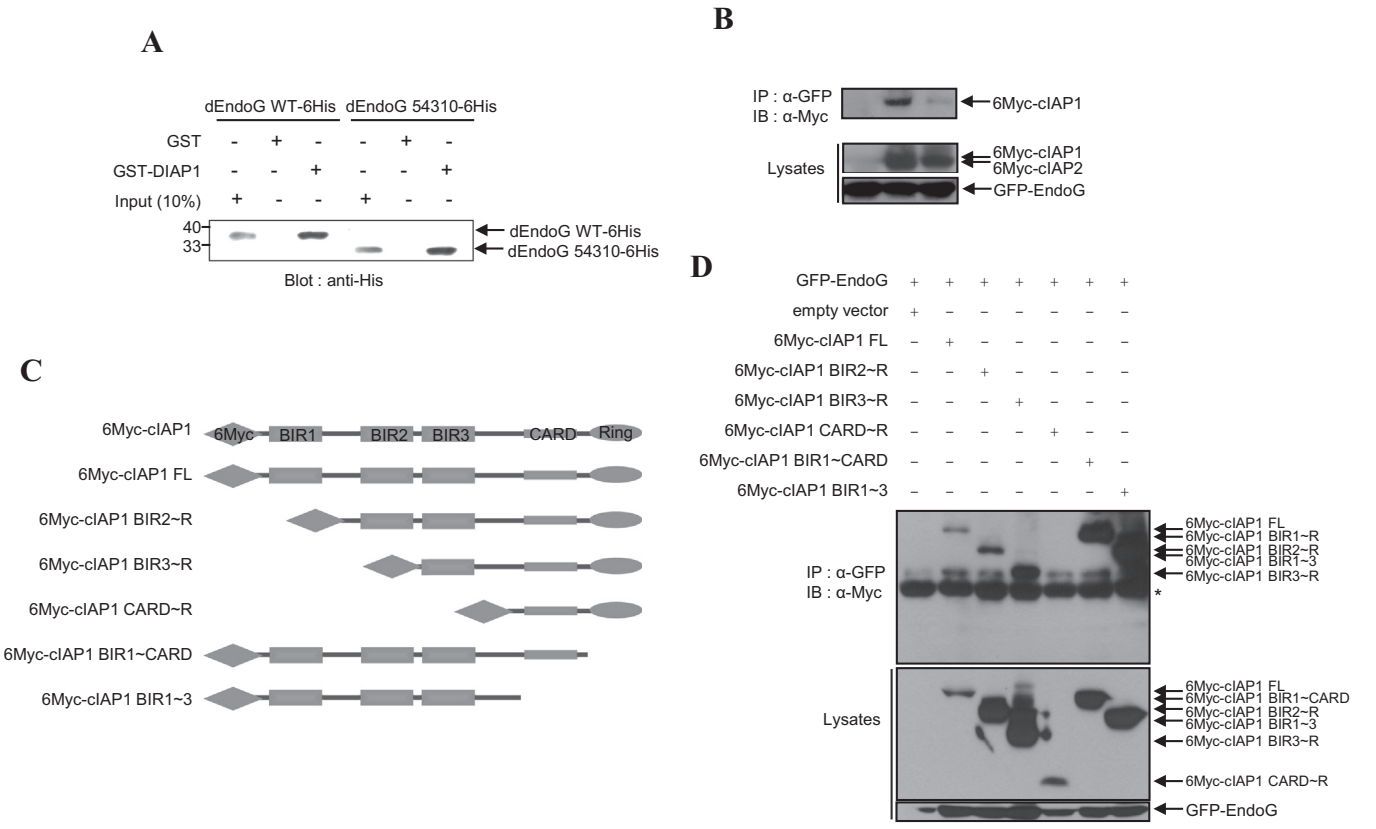

Fig. 1. EndoG and IAPs interact in *Drosophila* and human cells. (A) dEndoG interacts with DIAP1. *In vitro* translated dEndoG WT-6His and dEndoG 54310-6His through T_NT Quick Coupled Transcription/Translation Systems was incubated with GST and GST-DIAP1 on glutathione-agarose beads. After incubation, the beads were analyzed by WB using anti-His antibody. (B) cIAP1 interacted with EndoG. HEK293T cells were co-transfected with GFP-EndoG and 6Myc-cIAP1 or 6Myc-cIAP2. At 24 h after transfection, lysates were prepared for immunoprecipitation with anti-GFP antibody, subjected to SDS–PAGE, and then analyzed by Western blot using an anti-Myc antibody. (C) Schematic diagram of cIAP1 WT and various cIAP1 deletion mutants used in this study. (D) HEK293T cells were co-transfected with GFP-EndoG and 6Myc-cIAP1 WT along with one of the cIAP1 deletion mutants shown in (C). At 24 h post-transfection, lysates were prepared for immunoprecipitation with anti-GFP antibody followed by WB analysis with anti-Myc antibody. *IgG heavy chain.

Because IAP and EndoG are both evolutionarily well-conserved [7,14], we examined whether IAPs and EndoG interact in human cells. Among human IAPs, cIAP1 and cIAP2 are involved in a diversity of cellular events through the E3 ligase activity of the Ring domain [10]. However, XIAP, the most potent inhibitor of apoptosis, binds directly via BIR domains to inhibit caspases [28,29]. Since EndoG is an effector of caspase-independent cell death, we examined whether it interacts with cIAP1 and cIAP2. Co-IP showed that cIAP1, but not cIAP2, interacts with EndoG in HEK293T cells (Fig. 1B). Next, we mapped the region of cIAP1 that interacts with EndoG. IAPs have two to three N-terminal BIR domains that interact with other proteins. We generated 6Myc-cIAP1 WT and various deletion mutants (Fig. 1C) and performed co-IPs with GFP-EndoG. The results show that cIAP1 BIR3∼R bound to EndoG similar to cIAP1 WT while cIAP1 CARD∼R, which lacks a BIR domain, did not bind (Fig. 1D), indicating that the BIR domains of cIAP1, especially BIR3, are responsible for EndoG binding. cIAP1 has been shown to interact with several proteins, such as TRAF2 and Smac, via BIR3 [30]. Altogether, our results demonstrate that EndoG interacts with IAPs *in Drosophila* and humans.

3.2. cIAP1 ubiquitinates EndoG but does not regulate EndoG levels

To examine whether DIAP1 ubiquitinates dEndoG, we performed *in vitro* ubiquitination assays using purified dEndoG WT-6His or dEndoG 54310-6His with and without DIAP1. WB analysis demonstrated ubiquitination of dEndoG WT and dEndoG 54310 in the presence of DIAP1 that increased in a time-dependent manner (Fig. 2A). These data suggest that dEndoG was ubiquitinated specifically by DIAP1.

Next, we examined whether EndoG is ubiquitinated by cIAP1 in human cell lines. HEK293T cells were co-transfected with GFP-EndoG and 6Myc-cIAP1 WT or 6Myc-cIAP1 RM, which lacks E3 ligase activity, and then treated with the proteasome inhibitor MG132. We found that EndoG was ubiquitinated by cIAP1 WT, but not by cIAP1 RM (Fig. 2B), indicating that cIAP1 functions as an E3 ligase for EndoG ubiquitination through its Ring domain in human cells. Next, we compared the half-life of EndoG in HEK293T cells expressing either control vector or 6Myc-cIAP1 following CHX treatment. Interestingly, the half-life of EndoG in cells overexpressing cIAP1 did not change compared to control cells (Fig. 3C). Furthermore, knockdown of cIAP1 expression with sicIAP1 did not affect endogenous EndoG steady-state levels (Fig. 2D), suggesting that cIAP1-mediated ubiquitination may not target EndoG for proteasomal degradation.

Ub possesses seven lysine residues, each of which can be used for isopeptide linkage formation. Reports have shown that cIAP1 ubiquitinates RIP1 and RIP3 via K63-mediated isopeptide linkages that are not recognized by the proteasome but rather provide an interaction module for forming the TAK1/TAB2/TAB3 complex [11,12]. Therefore, we tested whether cIAP1 can ubiquitinate EndoG via K63-mediated isopeptide linkages. To accomplish this, we co-expressed GFP-EndoG and 6Myc-cIAP1 along with HA-Ub WT or HA-Ub R63K, in which all lysines except K63 were mutated to arginine. A higher level of EndoG ubiquitination was observed with the Ub R63K mutant compared with wild-type (Fig. 2E). These

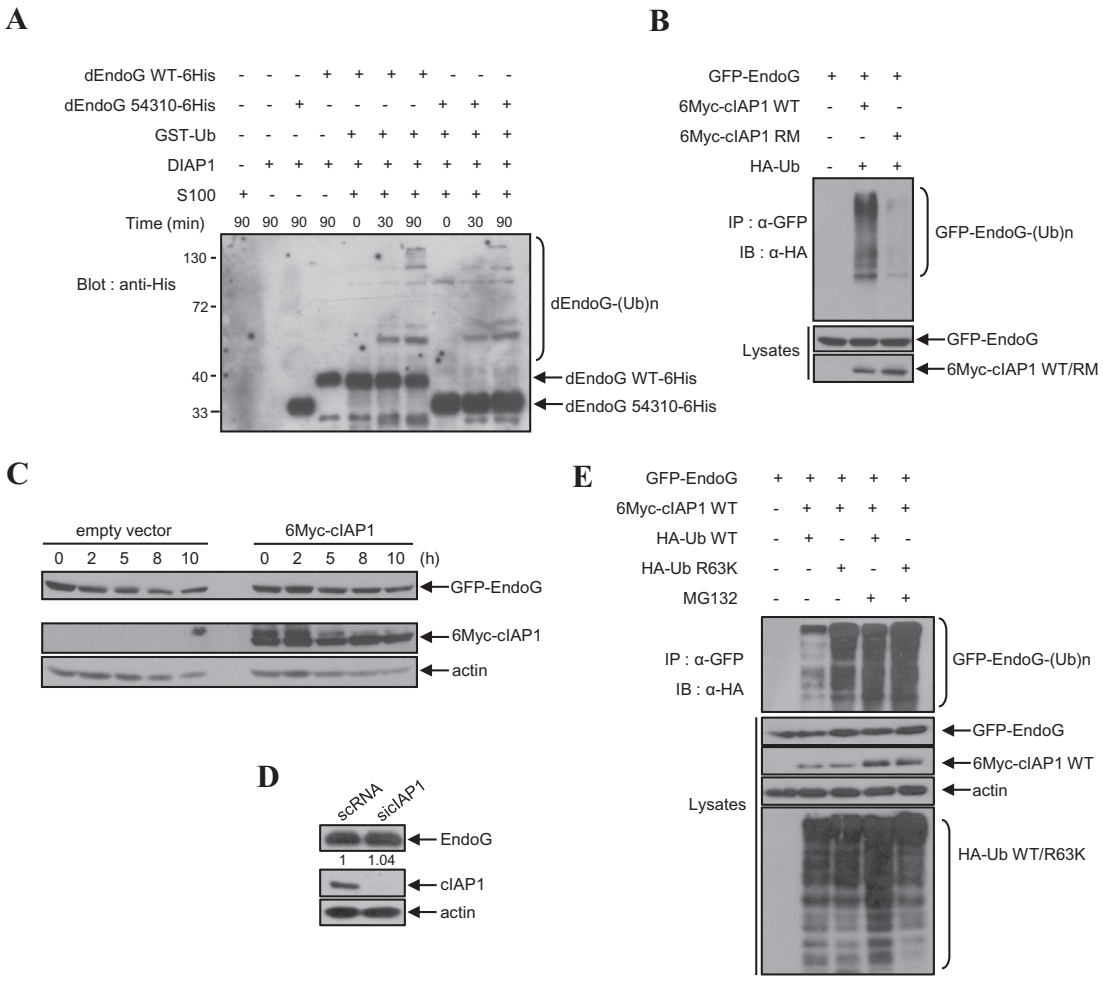

Fig. 2. cIAP1-mediated ubiquitination of EndoG does not regulate its expression. (A) dEndoG was ubiquitinated by DIAP1. *In vitro* ubiquitination of dEndoG was analyzed by WB using an anti-His antibody. (B) cIAP1 WT, but not cIAP1 RM, ubiquitinated EndoG. HEK293T cells were co-transfected with GFP-EndoG and 6Myc-cIAP1 WT or 6Myc-cIAP1 RM with HA-Ub. At 24 h post-transfection, MG132 (20 μM) was added. *In vivo* ubiquitination was performed as described in the Section 2. (C) The half-life of EndoG. HEK293T cells were transfected with GFP-EndoG and empty vector or 6Myc-cIAP1. At 24 h post-transfection, the cells were treated with 100 μg/ml of CHX and harvested at the indicated time points. Lysates were analyzed by WB using anti-GFP, anti-Myc, and anti-actin antibodies. (D) HeLa cells were transfected with scRNA or sicIAP1 for 48 h. WB was performed using anti-EndoG, anti-cIAP1, and anti-actin antibodies. The numbers indicate the relative expression of EndoG to actin. (E) EndoG is ubiquitinated by the Ub R63K mutant. HEK293T cells were co-transfected with constructs expressing GFP-EndoG and HA-Ub WT or HA-Ub R63K. At 24 h post-transfection, 20 μM MG132 was added and the cells were incubated for an additional 6 h before lysates were prepared for Western blot analysis. *IgG heavy chain.

results indicate that cIAP1 ubiquitinates EndoG via K63-mediated isopeptide linkages and may explain why EndoG levels were unaffected by cIAP1-mediated ubiquitination (Fig. 2C and D). Taken together, these results demonstrate that cIAP1 acts as an E3 ligase that ubiquitinates EndoG via K63-mediated isopeptide linkages, and that this modification does not regulate the steady-state levels of EndoG protein.

3.3. Expression of dEndoG reduced S2 cell survival

Our results raised the possibility that IAP could play a regulatory role in EndoG-derived cell death. EndoG expression triggers cell death in *Caenorhabditis elegans*, mouse, or human cell lines [13,14,16]. However, this role has yet to be investigated in insect cells. To examine whether dEndoG triggers cell death in insect cells, we co-expressed dEndoG and pMT-LacZ into S2 cells and evaluated cell survival by measuring β-galactosidase activity [31]. The data demonstrate that expression of dEndoG WT reduced cell survival slightly by approximately 15%. However, the survival rate of dEndoG 54310-expressing cells was reduced approximately 40%. Expression of dEndoG 54310 H155A, which contained an active site mutation, restored cell survival to nearly the levels of dEndoG WT (Fig. 3A). Treatment with a pan-caspase inhibitor, Z-VAD-FMK, did not suppress dEndoG WT- or 54310-mediated cell death (Fig. 3B), indicating that caspase activity is not involved in EndoG-mediated apoptosis in S2 cells. Collectively, these results indicate that dEndoG expression can trigger cell death in insect cell lines without additional apoptotic stimuli. Moreover, this dEndoG-driven cell death is dependent on the DNase activity of dEndoG.

Based on these results, we examined how dEndoG 54310 expression led to greater cell death than the wild-type protein. EndoG translocates from mitochondria to the nucleus in response to apoptotic stimuli to cleave chromosomal DNA. Because we only expressed dEndoG without additional apoptotic stimuli, we hypothesized that differential intracellular localization of dEndoG WT and dEndoG 54310 may result in the different cell survival frequencies observed. Therefore, we expressed pMT-dEndoG WT-V5 or pMT-dEndo G54310-V5 in S2 cells and visualized their localization by immunostaining with anti-V5 antibody. The confocal images revealed that dEndoG WT proteins were mostly localized in the cytosol as reported, but dEndoG 54310 proteins were observed in the nucleus and cytosol (Fig. 3C). These data suggest that newly synthesized dEndoG 54310 proteins bypass the mitochondria and may be easily imported into the nucleus without

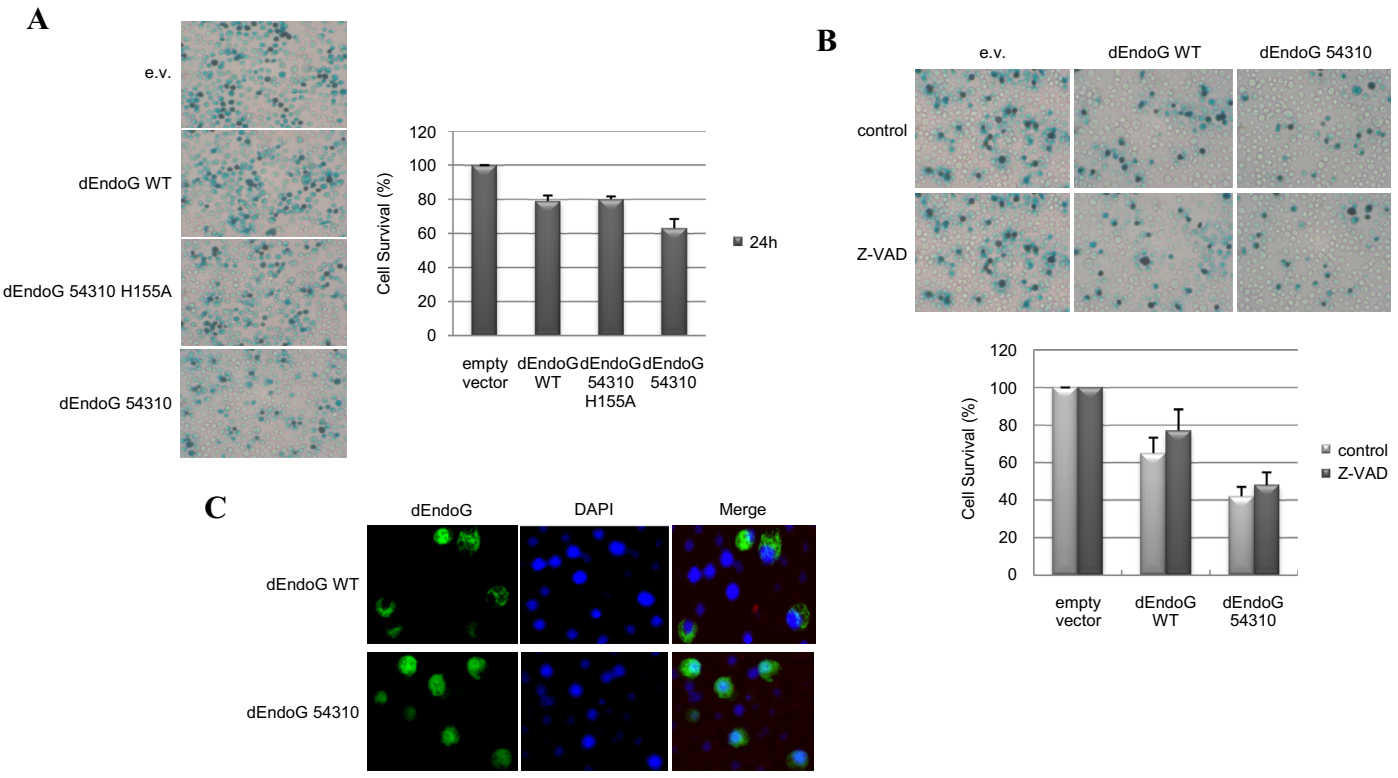

Fig. 3. Expression of dEndoG reduced S2 cell survival. (A) pMT empty vector, pMT-dEndoG WT, pMT-dEndoG 54310, or pMT-dEndoG 54310 H155A were co-transfected with pMT-lacZ into S2 cells. At 24 h post-induction with $CuSO_4$ (50 μM), cell survival was assessed by scoring β-gal activity via X-gal staining. The values represent the mean ± SD ($n = 3$). (B) Treatment with a pan-caspase inhibitor, Z-VAD-FMK, did not suppress dEndoG WT- or 54310-mediated cell death. (C) dEndoG 54310 was localized to the nucleus and cytosol. pMT-dEndoG WT or pMT-dEndoG 54310 were transfected into S2 cells and induced with 50 μM $CuSO_4$ for 24 h. The cells were stained with anti-EndoG (green) antibody and DAPI (blue) and visualized using a LSM 510 Meta confocal microscope (Carl Zeiss, Inc. Germany). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

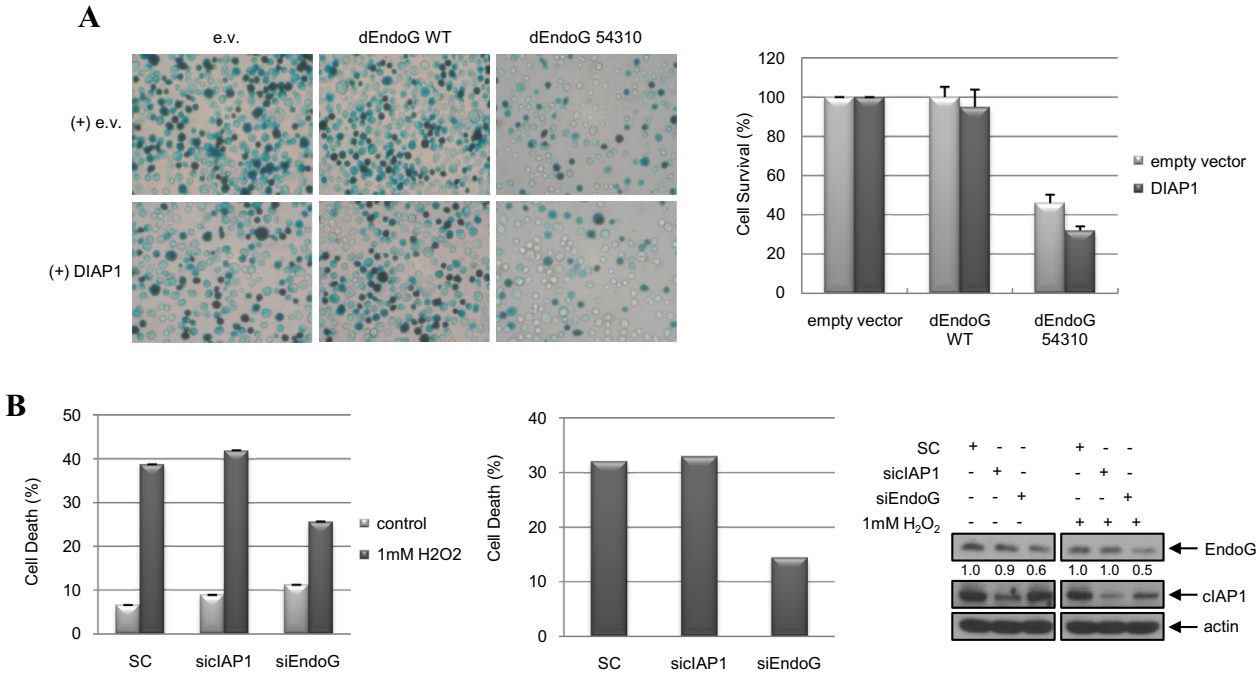

Fig. 4. cIAP1 does not affect EndoG-mediated cell death. (A) DIAP1 does not rescue the reduced cell survival of dEndoG 54310-expressing S2 cells. pMT empty vector, pMT-EndoG WT, or pMT-EndoG 54310 were co-transfected with empty vector or pMT-DIAP1 with pMT-lacZ into S2 cells. At 24 h post-induction with 50 μM of $CuSO_4$, cell survival was assessed by scoring β-gal activity via X-gal staining (left). The values represent the mean ± SD ($n = 3$) (right). (B) HeLa cells were transfected with scrambled RNA (scRNA) or siRNA against cIAP1 (siclAP1) for 48 h and treated with 1 mM H_2O_2 for 4 h. Cell death was assessed by Trypan Blue exclusion assay (left). The values represent the mean ± SD ($n = 3$). The percentage of cell death following H_2O_2 treatment was re-plotted after subtracting out the control (middle). WB analysis was performed using anti-EndoG, anti-cIAP1, and anti-actin antibodies (right). The numbers indicate relative expression of EndoG to actin.

apoptotic stimuli, participating in cell death by fragmenting chromosomal DNA.

3.4. cIAP1 does not affect EndoG-mediated cell death upon oxidative stress

Next, we assessed whether DIAP1 expression affects EndoG-mediated cell death in S2 cells. The cell survival assays showed that DIAP1 overexpression did not alter the cell survival rate of either dEndoG WT- or dEndoG 54310-expressing cells (Fig. 4A), indicating that DIAP1 does not suppress EndoG-mediated cell death in S2 cells. Previously, we showed that oxidative stress triggers EndoG-mediated cell death in human cells [16]. Therefore, we investigated whether cIAP1 affected EndoG-mediated cell death of human cells treated with H_2O_2. Depletion of EndoG expression by siEndoG reduced cell death approximately 50% compared with scrambled (sc)RNA-expressing control cells (Fig. 4B, left and middle) as reported [16]. WB analysis demonstrated a similarly reduced level of EndoG expression (Fig. 4B, right). These results indicate that intracellular levels of EnodG determine the rate of cell death during oxidative stress. When cIAP1 was depleted by sicIAP1, the rate of cell death was similar to control scRNA-expressing cells (Fig. 4B, left and middle). As expected, WB analysis showed that EndoG protein levels did not change in cIAP1-depleted cells. Altogether, these data indicate that IAPs do not regulate EndoG protein levels and subsequent EndoG-mediated cell death.

Previously, we also reported that a co-chaperone E3 ligase, CHIP, ubiquitinates EndoG for proteasomal targeting to regulate its steady-state levels [16]. Here, we demonstrated cIAP1 as another E3 ligase for EndoG. Interestingly, we found that the EndoG level was not regulated by cIAP1 despite its binding and ubiquitination, possibly because cIAP1 ubiquitinates EndoG via K63-linked isopeptide linkages. Finally, we showed that cIAP1 (and DIAP1) did not suppress EndoG-mediated cell death, suggesting that the EndoG protein level is not regulated by IAPs. In this regard, these results support our previous finding that the extent of EndoG-driven cell death correlates with the level of intracellular EndoG [16].

Our results suggest that cIAP1-mediated EndoG ubiquitination may function as a regulatory signal instead of a marker for proteasomal degradation. There are several possible functions for EndoG ubiquitinated by cIAP1. For instance, cIAP1 may change EndoG localization or its interacting partners as cIAP1-mediated K63 ubiquitination of RIP1 recruits TAB proteins and others instead of undergoing proteasomal degradation [11,12]. Alternatively, cIAP1 may mediate a vital function of EndoG other than cell death. Indeed, EndoG has been shown to play a role in cell survival in yeast [22], proliferation [23], and mitochondrial maintenance [24] in mammals. EndoG purification and characterization were initially performed using chicken erythrocyte nuclei not mitochondria [32]. Although most of the research on EndoG has been focused on its role in apoptosis, further investigation of its involvement in non-apoptotic functions is necessary to understand the role of cIAP1-mediated EndoG ubiquitination.

Acknowledgments

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