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X-linked inhibitor of apoptosis protein mediates neddylation by itself but does not function as a NEDD8–E3 ligase for caspase-7

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ABSTRACT

X-linked inhibitor of apoptosis protein (XIAP) is a potent antagonist of caspases, and functions as a ubiquitin–E3 ligase by itself and for caspases. Recently, NEDD8, a ubiquitin-like modifier, has been suggested to be used for modification of caspase-7 mediated by XIAP. However, it is not clear whether caspase-7 is a bona fide target for NEDD8. Here we showed that no neddylation of caspase-7 but that of XIAP itself was observed under the conditions in which caspase-7 was modified with ubiquitin. These results reveal that XIAP does not function as a NEDD8–E3 ligase for caspase-7 in vivo.

Structured summary of protein interactions:
NEDD8 physically interacts with **Caspase-7** by pull down (View interaction)
XIAP physically interacts with **NEDD8** by anti-bait coimmunoprecipitation (View interaction)

1. Introduction

The inhibitor of apoptosis proteins (IAPs) are the endogenous inhibitors for apoptosis, and eight IAPs have been thus far isolated in mammals [1,2]. They share a conserved region known as the baculovirus IAP repeat (BIR) domain. On the other hand, caspase is a family of cysteine proteases that are required for cytokine maturation and apoptosis execution [3–5]. In mammals, caspases with a large prodomain including caspase-2, -8, -9, and -10 are termed as the initiator caspases of apoptosis, whereas the enzymes with a short prodomain comprising caspase-3, -6, and -7 are the effector caspases of apoptosis. The effector caspases are activated through proteolytic processing catalyzed by the initiator caspases. X-linked inhibitor of apoptosis protein (XIAP) is one of IAPs, which contains three BIR domains and a RING finger domain. The distinct regions including each BIR domain bind directly to caspase-3, -7 and caspase-9 to inhibit their protease activity. The RING domain can act as a ubiquitin–E3 ligase, leading to ubiquitination and subsequent degradation of pro-apoptotic XIAP counterparts, including cytosolic Smac/DIABLO [6,7], caspase-9 [7], caspase-7 [8], and caspase-3 [9,10], as well as XIAP itself [11].

Among the ubiquitin-like protein family, NEDD8 is most homologous to ubiquitin [12,13]. NEDD8 conjugation is mechanistically similar to ubiquitination, in that NEDD8 is activated and transferred to substrates by E1, E2, and E3 enzymes. Unlike the ubiquitin E1 enzymes, which are a single polypeptide, the NEDD8 E1 enzyme is a heterodimer composed of amyloid precursor protein–binding protein 1 (APP–BP1) and Uba3 protein. The E2 conjugating enzyme for NEDD8 is Ubc12 or Ube2f protein [14,15]. Dcn1 and Rbx1 are the NEDD8-specific E3 complex [16–18], while all other NEDD8 ligases seem to have dual activity for NEDD8 and ubiquitin. The best-studied and most abundant NEDD8 substrates are the cullin proteins, which are scaffolds for the ubiquitin E3 ligases [16,17,19,20]. Several substrates for NEDD8 have been identified such as p53, MDM2, VHL, p73, BCA3, EGFR, L11, and HIFα [21–27]. The NEDD8 conjugation effects on the function of target proteins, such as stability, transcriptional activity, and subcellular localization [21,27,28].

Recently, it was reported that XIAP works as a NEDD8–E3 ligase by itself and for caspase-7, thereby inhibits caspase-7 activity [29]. On the other hand, it was suggested that NEDD8 overexpression triggers NEDD8 activation mediated by the E1 enzymes for ubiquitin, leading to neddylation of target proteins [30]. Therefore, it is unclear whether XIAP and caspase-7 are bona fide targets for NEDD8 in vivo. Here we examined the NEDD8–E3 ligase activity of XIAP for

Abbreviations: IAPs, inhibitor of apoptosis proteins; BIR, baculovirus IAP repeat; XIAP, X-linked inhibitor of apoptosis protein; NEDD8, neural precursor cell expressed, developmentally downregulated 8; GFP, green fluorescent protein; HA, hemagglutinin

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caspase-7 in comparison with the ubiquitin–E3 ligase activity. We detected the neddylation of XIAP mediated by itself, while the efficiency was far less than ubiquitination. Consistent with these results, no neddylation of caspase-7 was observed under the conditions where caspase-7 could be modified with ubiquitin. Consequently, it is plausible that XIAP does not function as a NEDD8–E3 ligase for caspase-7 in vivo.

2. Materials and methods

2.1. Cell culture and transfection

HEK293T (a human embryonic kidney cell line) cells were cultured in DMEM supplemented with 10% fetal bovine serum. Transfection was performed using Lipofectamine (Invitrogen) or FuGENE HD (Promega) according to the manufacturer's instructions.

2.2. Plasmid constructions

Construction of pcasp7-Wt-GFP and the XIAP cDNA fragment cloned into the *Xho*I-*Eco*RI site of pBluescript SK(−) (Stratagene) were described elsewhere [31,32]. To generate C450A mutation in XIAP, a PCR method employing mutagenic oligonucleotide primers was used. The resulting human XIAP fragments with or without mutation were cloned into the *Xho*I-*Eco*RI site of pDsRed-Monomer-C1 (BD Biosciences) to generate pDsRed-XIAP-Wt and pDsRed-XIAP-Mu, respectively. The NEDD8 cDNA fragment was amplified from a human brain cDNA library with the primers, 5′-GGAGCAGCAATTTATCCGTG-3′ and 5′-TATGATGCCTCATTATGAGC-3′ for the 1st PCR, and 5′-TGCAGCCCCAAACTGGAAAG-3′ and 5′-AGAGGTAAAATGGAGGGTCC-3′ for the 2nd PCR. The resultant fragment was cloned into pGEM-T Easy (Promega) and sequenced. For construction of His-tagged NEDD8 expression plasmid, the NEDD8 cDNA was amplified with the primers, 5′-AGATCTGCCACCATGCTAATTAAAG-3′ and 5′-AGATCTCACTGCCTAAGACC-3′, using NEDD8 cDNA cloned in pGEM-T EASY as a template, and cloned into the *Bam*HI site of pcDNA3.1/His C (Invitrogen) to generate pcDNA-His-NEDD8. Hemagglutinin (HA)-tagged ubiquitin expression plasmid, pCGN-HA-Ub, was kindly provided by M. Nakao (Kumamoto University). To construct pCGN-HA-NEDD8, a PCR method was used with primers, 5′-CGTGCTCGTCTAGAATGCTAATTAAAGTGAAGACGCT-3′ and 5′-CGTGCTCGGGTACCTCACTGCCTAAGACCACCTC-3′, and pcDNA-His-NEDD8 as a template. The resultant fragment was cloned into the *Xba*I-*Kpn*I site of pCGN-HA.

2.3. Purification of His-tagged NEDD8

For purification of His-tagged NEDD8, cells were lysed in lysis buffer [50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 1% Nonidet P-40, 10 μg/mL phenylmethanesulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM dithiothreitol]. Lysates were incubated with Ni–NTA beads (Qiagen) for 2 h at 4 °C with constant rotation.

2.4. Immunoblot analysis

Protein samples were separated by SDS–polyacrylamide gel electrophoresis and blotted onto Immobilon polyvinylidene difluoride membrane (Millipore). Each protein was detected using primary antibodies, horseradish peroxidase-conjugated secondary antibodies, and ECL detection reagent (GE Healthcare).

2.5. Antibodies

Anti-caspase-7 monoclonal antibody (M053-3), anti-NEDD8 polyclonal antibody (PM023), and anti-XIAP monoclonal antibody (M044-3) were obtained from Medical and Biological Laboratories; anti-GFP monoclonal antibody (1814460), anti-HA monoclonal antibody (1666606), and anti-HA rat monoclonal antibody (1867423) were from Roche; anti-DsRed polyclonal antibody (632496) was from Clontech; anti-GFP polyclonal antibody (A6455) used for immunoprecipitation was from Molecular Probes.

2.6. Immunoprecipitation

For immunoprecipitation experiments, cells were lysed in lysis buffer [50 mM Tris–HCl (pH 7.5). 150 mM NaCl, 20 mM NaF, 1% Nonidet P-40, 10 μg/mL phenylmethanesulfonyl fluoride, 5 mM EDTA, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM dithiothreitol, 10 μM MG132, 10 mM *N*-ethylmaleimide]. Lysates were incubated with anti-GFP polyclonal antibody or anti-XIAP monoclonal antibody together with 5% (vol/vol) protein G Sepharose (GE Healthcare) for 2 h at 4 °C. Cell lysates and immunoprecipitates were separated by SDS–PAGE and immunoblotted with the indicated antibodies.

3. Results and discussion

It was reported that XIAP works as a NEDD8–E3 ligase by itself and for caspase-7 [29]. However, the elevation of free NEDD8 level relative to ubiquitin was suggested to trigger neddylation by ubiquitination enzymes [30]. Therefore, we set out to confirm whether XIAP targets caspase-7 for neddylation in cells. After coexpression of caspase-7 and XIAP in the presence of His-tagged NEDD8, conjugated proteins were affinity purified using nickel columns (Fig. 1). Although the expression level of His-tagged NEDD8 was much lower than that of endogenous NEDD8, neddylated proteins were detected in cell lysates (Fig. 1, NEDD8 blot in lower panel), and the neddylation was enhanced by the presence of XIAP (compare lane 2 with lane 3). Consistently, the level of neddylation in the presence of XIAP after affinity purification was higher than that in the absence of XIAP (Fig. 1, NEDD8 blot in upper panel, compare lane 2 with lane 3), suggesting that XIAP could function as NEDD8–E3 ligases for some proteins. Under these conditions, the presence of neddylated caspase-7 was assessed by immunoblot analysis of the eluates. Modified caspase-7-GFP was detected in addition to caspase-7-GFP band in the presence of XIAP (Fig. 1, casp7 blot in upper panel, lane 3), while the non-modified caspase-7-GFP bands which seemed to bind non-specifically to nickel columns was detected (lanes 1–3), and the modest modified caspase-7-GFP bands were also detected in the absence of XIAP (lane 2). Under these conditions in which caspase-7 and XIAP were overexpressed in the presence of His-NEDD8 and conjugated proteins were affinity purified using nickel columns, we confirmed that neddylation of caspase-7 was mediated by XIAP as reported previously [29].

Since the increase in the free NEDD8 to ubiquitin ratio triggers activation of NEDD8 by the ubiquitin–E1 enzymes [30], we set out to compare the E3 ligase activity of XIAP for ubiquitin and NEDD8 in cells (Fig. 2). Ubiquitin and NEDD8 tagged with HA were constructed in pCGN plasmid to drive the expression of each protein under the control of cytomegalovirus promoter, thereby the levels of exogenously expressed HA-ubiquitin and HA-NEDD8 were expected to be at equivalent levels. To compare the expression levels of HA-ubiquitin and HA-NEDD8, the expression plasmids, pCGN-HA-Ub and pCGN-HA-NEDD8, were transiently transfected into 293T cells, and HA-tagged ubiquitin and NEDD8 were detected with anti-HA antibody (Fig. 2A). Although a band corresponding to monomer HA-ubiquitin, but not monomer HA-NEDD8, was detected, multiple bands with higher molecular weight were seen (lanes 2 and 3), suggesting that HA-ubiquitin and HA-NEDD8 were

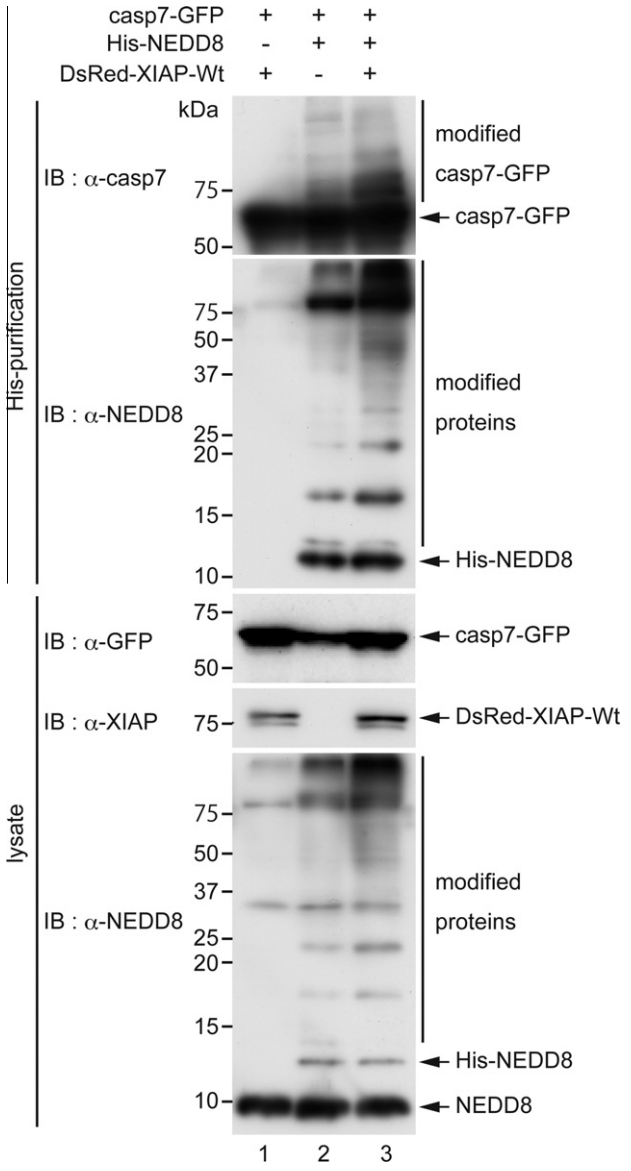

Fig. 1. Detection of neddylated caspase-7 after purification of His-NEDD8. 293T cells were transfected with pcasp7-Wt-GFP, pcDNA-His-NEDD8, and pDsRed-XIAP-Wt as indicated. After incubation for 30 h, lysates were purified with nickel column for His-NEDD8 proteins. The input lysates (lower panels) and the purified His-tagged proteins (upper panels) were fractionated by SDS–PAGE and immunoblotted with antibodies as indicated.

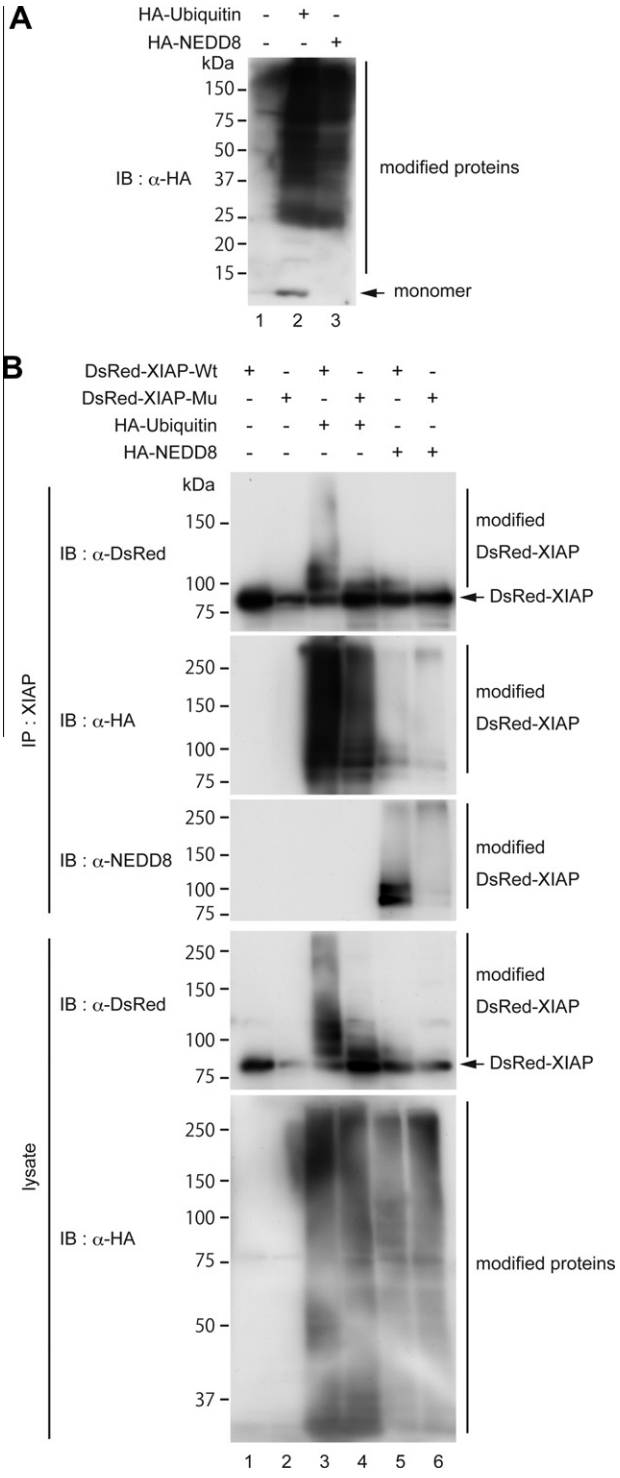

Fig. 2. Modification of XIAP with ubiquitin and NEDD8. (A) 293T cells were transfected with pCGN-HA-Ub or pCGN-HA-NEDD8 as indicated. After incubation for 24 h, lysates were fractionated by SDS–PAGE and immunoblotted with anti-HA antibody. (B) 293T cells were transfected with pDsRed-XIAP-Wt, pDsRed-XIAP-Mu, pCGN-HA-Ub, and pCGN-HA-NEDD8 as indicated. DsRed-XIAP-Mu contained a C450A mutation that abrogates its E3 activity. After incubation for 30 h, lysates were immunoprecipitated with anti-XIAP antibody. The input lysates (lower panels) and the immunoprecipitates (upper panels) were fractionated by SDS–PAGE and immunoblotted with antibodies as indicated.

used for protein modification immediately after expression. The levels of modified proteins with ubiquitin were much higher than that with NEDD8 (compare lane 2 with lane 3). This difference may suggest that the total enzyme activities for ubiquitination is higher than those for neddylation, or that ubiquitin is more stable than NEDD8. Under these conditions, we compared the E3 ligase activities of XIAP for ubiquitin and NEDD8 (Fig. 2B). Although modified DsRed-XIAP bands were detected in addition to DsRed-XIAP when coexpressed with HA-ubiquitin (DsRed blot in lower panel, lane 3), modified DsRed-XIAP bands with HA-NEDD8 were obscure (lane 5). To further confirm whether DsRed-XIAP was neddylated in cells, we purified DsRed-XIAP by immunoprecipitation with anti-XIAP antibody, and detected neddylated proteins with anti-NEDD8 antibody (NEDD8 blot in upper panel). The modified bands corresponding to neddylated DsRed-XIAP-Wt were seen, and the bands disappeared by the RING finger mutation, C450A, of XIAP that abolishes E3 ligase activity [9] (compare lane 5 with lane 6). These results strongly suggested that XIAP could function as a NEDD8–E3

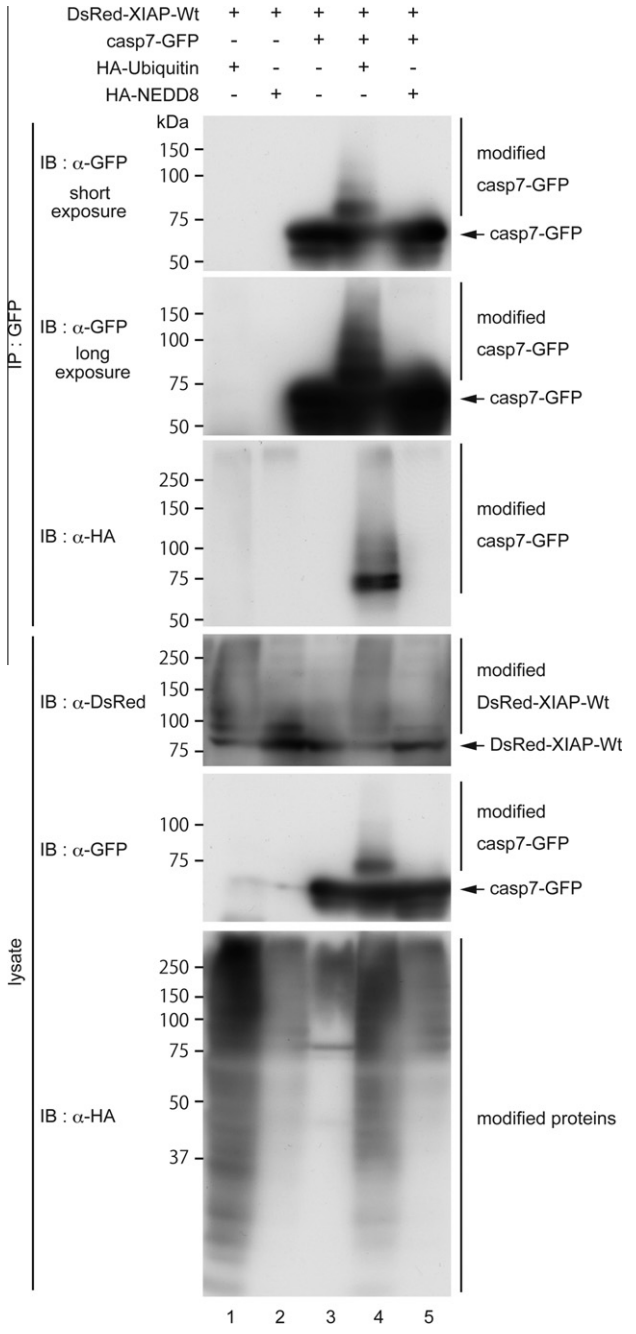

Fig. 3. Modification of caspase-7 with ubiquitin, but not NEDD8, mediated by XIAP. 293T cells were transfected with pDsRed-XIAP-Wt, pcasp7-Wt-GFP, pCGN-HA-Ub, and pCGN-HA-NEDD8 as indicated. After incubation for 30 h, lysates were immunoprecipitated with anti-GFP antibody. The input lysates (lower panels) and the immunoprecipitates (upper panels) were fractionated by SDS–PAGE and immunoblotted with antibodies as indicated.

ligase by itself under the conditions in which XIAP was overexpressed with HA-NEDD8.

Although the ubiquitinated bands of DsRed-XIAP were detected when wild type XIAP was expressed with HA-ubiquitin, these modifications were efficiently inhibited by the C450A mutant of XIAP (Fig. 2B, DsRed blot in upper panel, compare lane 3 with lane 4), confirming the self-ubiquitination activity of XIAP as reported previously [11]. Unexpectedly, the multiple modified bands of DsRed-XIAP-Mu were also detected when XIAP was overexpressed with HA-ubiquitin, however to a lesser extent than DsRed-XIAP-Wt (HA blot in upper panel, compare lane 3 with lane 4). Since it was reported that the RING domain of cIAP1 allowed it to bind directly to the RING of XIAP, causing its ubiquitination and degradation by the proteasome [33], the modified bands of DsRed-XIAP-Mu may be mediated by cIAP1. As compared with the ubiquitinated bands of DsRed-XIAP-Wt, the neddylated bands of DsRed-XIAP-Wt were hardly detected under the same conditions in which HA-NEDD8 was coexpressed with DsRed-XIAP-Wt (DsRed and HA blots in upper panel, compare lane 5 with lane 3). These results suggested that the activity of NEDD8–E3 ligase of XIAP by itself was much lower than that of ubiquitin–E3 ligase.

To examine whether XIAP targets caspase-7 for neddylation, DsRed-XIAP-Wt and caspase-7-GFP were overexpressed in combination with HA-NEDD8 (Fig. 3). Although modified proteins of caspase-7-GFP were detected with anti-GFP antibody in whole cell lysate when HA-ubiquitin was coexpressed, there is no modified band with HA-NEDD8 (Fig. 3, GFP blot in lower panel, compare lane 4 with lane 5), suggesting that XIAP functions as a ubiquitin–E3 ligase, but may not a NEDD8–E3 ligase, for caspase-7. To further confirm these results, we purified caspase-7-GFP by immunoprecipitation with anti-GFP antibody, and detected modified proteins with anti-GFP and anti-HA antibodies. Modification of caspase-7-GFP was observed when HA-ubiquitin, but not HA-NEDD8, was coexpressed with DsRed-XIAP-Wt (GFP and HA blots in upper panel, compare lane 4 with lane 5). These results indicated that XIAP does not function as a NEDD8–E3 ligase for caspase-7 in vivo.

We observed the modification of caspase-7 with His-NEDD8 by XIAP (Fig. 1), while we showed that XIAP did not modify caspase-7 with HA-NEDD8 (Fig. 3). This discrepancy may be explained by less purification efficiency of His-NEDD8 using nickel columns, because the non-modified caspase-7-GFP band was detected despite the presence of His-NEDD8 after purification by nickel columns (Fig. 1, casp7 blot in upper panel). These results suggested that nickel columns have a non-specific binding to some proteins other than His-tagged proteins, and modified caspase-7-GFP bands may contain ubiquitinated caspase-7-GFP proteins. Therefore, we concluded that XIAP does not function as a NEDD8–E3 ligase for caspase-7 from the data described in Fig. 3.

Broemer et al. [29] identified three NEDD8-specific isopeptidases that, when knocked down, prevented apoptosis by using systemic in vivo RNAi analysis in *Drosophila*. In addition, they proved that null mutants of one of these genes, NEDD8-specific deneddylase 1, suppressed cell death induced by IAP antagonists, Reaper (Rpr) and Head involution defective (Hid). The report strongly suggested that the protein modifications with NEDD8 play essential roles in the regulation of apoptosis. Furthermore, it was also suggested that *Drosophila* and mammalian IAPs could function as NEDD8–E3 ligases by itself and for caspases as well as ubiquitin–E3 ligases. Although our results described here do not deny the significance of neddylation in the apoptosis regulation in *Drosophila*, careful examinations will be needed to explore the NEDD8–E3 ligase activities of IAPs.

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