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C-terminal binding protein-mediated transcriptional repression is regulated by X-linked inhibitor of apoptosis protein

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ABSTRACT

Inhibitors of Apoptosis Proteins (IAPs) are known as the key negative regulators of apoptosis. To explore new functions of IAPs, we sought to identify proteins that interact with Diap1 in insect S2 cells. We found that Diap1 bound to *Drosophila* C-terminal binding protein (dCtBP), which is a transcriptional co-repressor. CtBP1 also interacted with X-linked inhibitor of apoptosis protein (XIAP) in human cells. CtBPs were ubiquitinated by IAPs and targeted for proteasome-mediated degradation. Finally, the expression of CtBP1 target genes was regulated by XIAP expression. This is the first report to demonstrate that XIAP specifically regulates CtBP1, suggesting that XIAP may play a role in regulating CtBP1-mediated transcriptional repression by regulating the level of CtBP1.

1. Introduction

Inhibitors of apoptosis proteins (IAPs) are key negative regulators of apoptosis [1,2]. They are evolutionarily conserved from viruses to humans and all IAPs have 1–3 baculovirus IAP repeat (BIR) domains that bind to and inhibit caspases [3]. Another domain found in most IAPs is the really interesting new gene (RING) domain, which functions as an E3 ubiquitin ligase [4]. The RING domain directs the ubiquitination of substrates, which targets them for proteasomal degradation [5–7].

IAPs have non-apoptotic functions in addition to inhibition of apoptosis. For instance, Diap1 is implicated in translational regulation [8] and Diap2 in control of innate immunity [9] in *Drosophila*. In mammal, X-linked inhibitor of apoptosis protein (XIAP) is the most potent caspase inhibitor but also functions in tumor growth factor-β signaling [10], nuclear factor-kB signaling [11], stress-induced Jun-N-terminal kinase signaling [12], and copper homeostasis [13]. It is likely that E3 activity through the XIAP RING domain participates in non-apoptotic processes by ubiquitinating substrates and subsequent proteasome dependent degradation. Therefore, many IAPs could be involved in non-apoptotic cellular events as well as apoptosis.

C-terminal binding protein (CtBP) is a transcriptional co-repressor involved in a wide range of cellular events, including cell migration, tumor cell survival, and cellular senescence [14,15]. CtBP1 has a PXDLS-binding motif at both the N- and C-terminus responsible for binding to PXDLS motifs, which are common in many transcriptional repressors [14]. In addition, the central region of CtBP1 has an Arg-Arg-Thr (RRT)-binding motif that interacts with proteins that are not transcriptional repressors, such as the homeodomain interacting protein kinase 2 [16].

In response to changing intracellular NAD/NADH ratios, CtBP1 undergoes a conformational change that alters its affinity for target proteins, which in turn affects the expression of CtBP1 target genes [17]. CtBP1 is also regulated by post-translational modifications, such as sumoylation at K428 for nuclear localization [18] and phosphorylation at S422 upon UV irradiation, which leads to a pro-apoptotic response [19]. However the molecular mechanism of CtBP1 regulation remains unknown.

In the present study, we identified *Drosophila* CtBP (dCtBP) as a binding partner of Diap1 and tested whether the interaction between Diap1 and dCtBP also occurs in human cells. Additionally, we examined whether IAPs functioned as an E3 ligase for CtBPs. Finally, we investigated the role of XIAP in CtBP1-mediated transcriptional repression.

Abbreviations: IAP, inhibitor of apoptosis proteins; CtBP, C-terminal binding protein; RING, really interesting new gene; BIR, baculovirus IAP repeat.

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2. Materials and methods

2.1. In vitro and in vivo ubiquitination assay

Constructs encoding dCtBP (purchased from Flybase) were generated by PCR and cloned into pET-His23a (Novagen), expressed in *Escherichia coli* and purified. HeLa cell S100 lysates were prepared as previously described [20]. For *in vitro* ubiquitination assays, dCtBP-His, GST-Ubiquitin, and S100 lysates were added to the reaction mixture in the presence or absence of purified Diap1. For *in vivo* ubiquitination, 24 h after transfection HeLa cells were treated with 20 μM MG132 for 6 h and lysed in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X100, 10% glycerol, protease inhibitor cocktail). Co-IPs were performed using anti-CtBP1 and analyzed by anti-HA.

2.2. Antibodies and immunoprecipitation

Anti-V5 (Invitrogen), anti-GFP (Santa Cruz Biotechnology), anti-CtBP1 (BD Biosciences), anti-XIAP (BD Biosciences), anti-Myc (Upstate), anti-HA (Covans), anti-tetraHis (QIAGEN), anti-Bax (Cell Signaling Technology), and anti-actin (Sigma) were used for western blot (WB) or co-immunoprecipitation (IP). For IP, lysates were mixed with the indicated antibody overnight at 4 °C and immunocomplexes were recovered by protein-A Sepharose (Sigma). Sepharose beads were washed with wash buffer (20 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X100, protease inhibitor cocktail) three times and analyzed by WB.

2.3. Luciferase assay

The promoter region of E-cadherin was obtained by PCR from HeLa genomic DNA (QIAGEN) and inserted into the pGL3-luciferase expression vector (Promega). At 24 h after transfection, luciferase activity was measured using the dual-luciferase reporter assay kit (Promega). Light production was measured for 10 s in a luminometer (Promega). The luciferase activity values of samples were normalized to the activity of Renilla luciferase (pRL-TK, Promega).

2.4. RT-PCR analysis

Total RNA was isolated using TRI reagent® (Molecular Research Center, Inc.) according to the manufacturer's instructions. Synthesis of cDNA was carried out using M-MuLV reverse transcriptase (MP Biomedicals). RT-PCR was performed using a Maxime PCR PreMix kit (iNtRON) according to the manufacturer's instructions. PCR primers were chosen using the PRIMER3 program (http://frodo.wi.mit.edu/).

2.5. Protein half-life

Human embryonic kidney (HEK) 293 cells were transfected with empty vector or 6Myc-XIAP. At 24 h after transfection, cells were treated with 200 μg/ml cyclohexamide and then harvested 0, 4, 8, and 16 h following treatment. Cells were lysed with lysis buffer, and each sample was subjected to WB.

2.6. siRNA and 2-deoxyglucose treatment

HEK 293 cells were transfected with 100 pmol XIAP specific siRNA or a scrambled RNA (Ambion). After 24 h, cells were treated with 10 mM 2-deoxyglucose (2-DG) for 6 h. Total RNAs were prepared and subjected to RT-PCR for sirt1 and GAPDH mRNAs. PCR products were then subjected to electrophoresis on 2% agarose gels followed by staining with ethidium bromide.

3. Results and discussion

3.1. dCtBP binds to Diap1 and this interaction is conserved from insects to humans

To explore new functions of IAP, we sought to identify proteins that interact with Diap1. We performed immunoaffinity purification of Diap1 and used mass spectrometry to identify co-purified proteins. Double epitope tagged-9MycTEV6HisDiap1 was stably expressed in S2 cells, and the purified complexes were used for multidimensional protein identification [MuDPIT; [21,22]]. dCtBP was identified as a Diap1-interacting protein by MudPIT analysis. To confirm the interaction between dCtBP and Diap1, we co-transfected GFP-Diap1 and dCtBP-V5 into S2 cells and carried out co-IP. GFP-Diap1, but not GFP alone (negative control), was detected in co-IPs with anti-V5 for dCtBP (Fig. 1A). A Diap1 mutant that lacks E3 ligase activity (Diap1th6) due to a mutation in the RING domain also bound to dCtBP, indicating that Diap1 E3 activity was not required for interaction with dCtBP. Given that both CtBP and IAP are evolutionally conserved, and sequence comparison of *Drosophila ctbp* with human *CTBP1* revealed approximately 80% sequence homology [14], we predicted that CtBP and Diap1 also associate in human cells. We therefore examined whether CtBP1 interacts with human IAPs. As shown in Fig. 1B, among the four IAPs tested, XIAP showed strong binding to CtBP1, but cIAP1 and other IAPs showed slight or no binding to CtBP1. This suggested that the interaction between dCtBP and Diap1 observed in insect cells is conserved in human cells as well.

Next we mapped the region of CtBP1 that directly interacts with XIAP, because XIAP does not have typical motifs for CtBP1 binding, such as the PXDLS or RRT motifs typically found in CtBP1-interacting proteins. Full length CtBP1 or various truncated CtBP1 mutants (Fig. S1a) were examined for their interaction with XIAP. The CtBP1 truncation mutants that contained the RRT-binding region (Myc/His-CtBP1/1-369, Myc/His-CtBP1/141-440, and Myc/His-CtBP1/141-300 in Figs. 1C and S1a) bound to XIAP, but no XIAP binding was observed in CtBP1 truncation mutants containing only the PXDLS-binding region (Myc/His-CtBP1/1-140 and Myc/His-CtBP1/301-440 in Figs. 1C and S1a). Our results indicate that the RRT-binding region of CtBP1 is responsible for the interaction with XIAP, whereas the two PXDLS-binding regions in CtBP1 are not involved.

Several reports have demonstrated that binding of CtBP1 to various transcriptional regulators is determined by the intracellular NAD/NADH ratio [14,17] and that post-translational modification of CtBP1, especially phosphorylation, destabilizes CtBP1 levels due to proteasome degradation [19]. Thus, we examined whether these modifications are required for the interaction between CtBP1 and XIAP. We mutated the residues responsible for NAD(H)-binding (G183V), phosphorylation (S422A), and sumoylation (K428R); these CtBP1 mutants showed greater or equal interaction with XIAP than did wild-type (WT) CtBP1, especially the NAD(H)-binding mutant (G183V), which had the strongest binding (Fig. 1D). It has been reported that expression of the NAD(H)-binding mutant is very low [23], even though it forms a dimer just like WT. Thus, this mutant is expressed but rapidly degraded, and as such the amount of CtBP1G183V in lysates was low compared to WT CtBP1 or other mutants (Fig. 1D). We expected to see lowered or no binding of XIAP to the S422A mutant because phosphorylation on CtBP1 was suggested to be necessary for destabilization [19], but our results showed that the S422A mutant bound to XIAP better than WT CtBP1. Together, our results indicate that post-translational modification of CtBP1 is not required for the interaction with XIAP.

Next we investigated which region of XIAP interacts with CtBP1. We expected that the BIR domains, but not the RING domain,

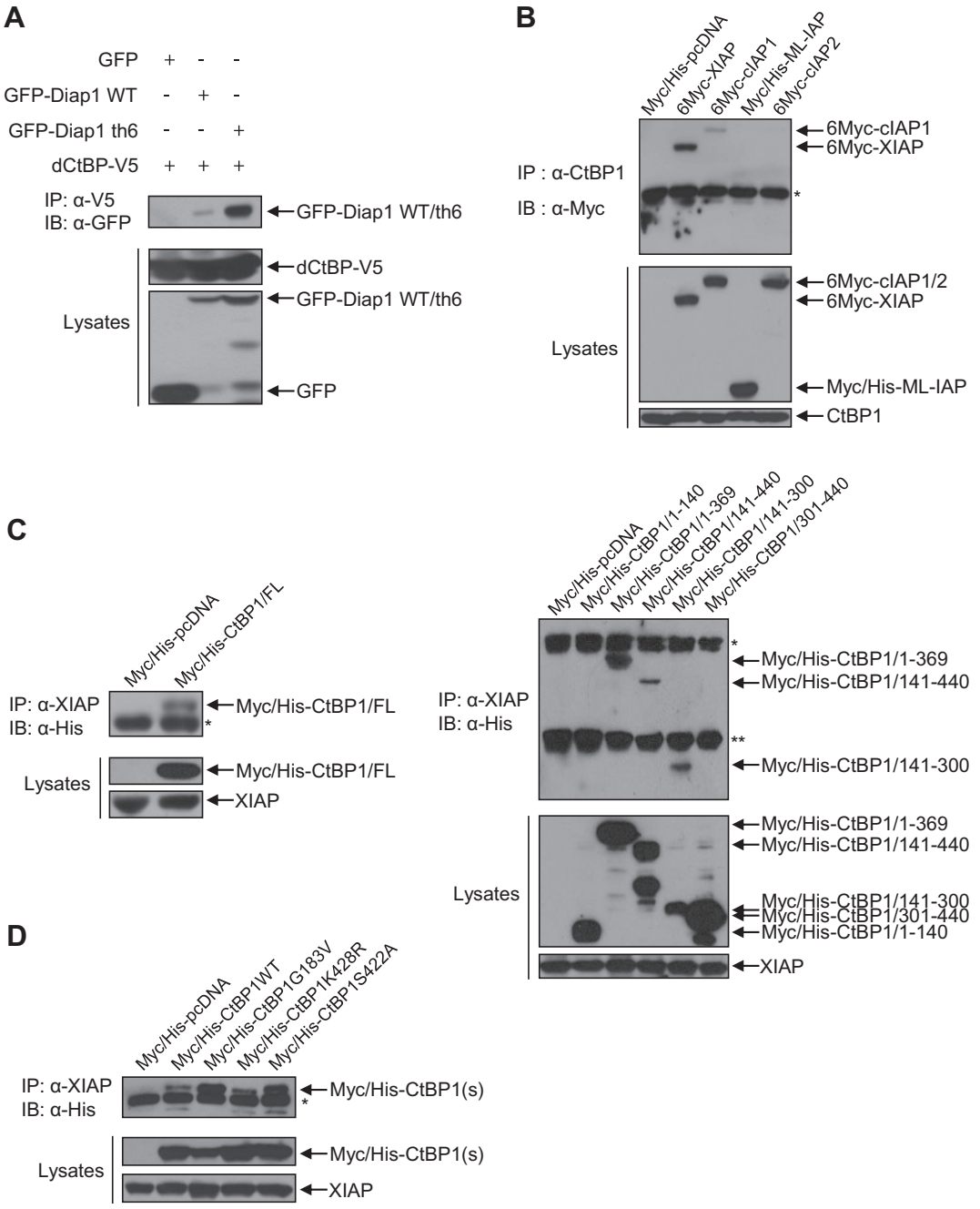

Fig. 1. dCtBP interacts with Diap1 in insect S2 cells and HEK 293 human cells. (A) Interaction of Diap1 with dCtBP in S2 cells. Both Diap1WT and the RING domain mutant (th6) bind to dCtBP. (B) Interaction of XIAP with CtBP1 in HEK 293 cells. Co-IP was carried out with anti-CtBP1 antibodies and WB with anti-Myc. *Indicates IgG heavy chain. (C) Interaction of XIAP with various CtBP1 domain constructs (shown in Fig. S1a). HEK 293 cells were transfected with Myc/His-pcDNA or Myc/His-CtBP1 constructs respectively (left, CtBP1 WT; right, various constructs). (D) Comparison of XIAP binding of CtBP1 modification mutants. Myc/His-pcDNA, Myc/His-CtBP1 WT, Myc/His-CtBP1G183V, Myc/His-CtBP1K428R, and Myc/ His-CtBP1S422A (shown in S1a) were used. *Indicates IgG heavy chain and **indicates IgG light chain.

would interact with XIAP because the RING domain primarily interacts with E2 [4], and a number of reports have shown that XIAP interacts with proteins via BIR1–3 domains to fulfill both apoptotic or non-apoptotic functions. For instance, XIAP interacts with caspase 3 and 7 via BIR2 [3], apoptosis-inducing factor (AIF) via BIR2 [24], and Murr1 via BIR3 [13]. We generated BIR1-3 deletion mutants (Fig. S1b) and examined their interaction with endogenous CtBP1 in HEK 293 cells. Full-length XIAP and BIR-deletion mutants except for 6Myc-XIAP/351-497, in which all three BIR domains were deleted, bound to endogenous CtBP1 (Fig. S1c), indicating that BIR3 is responsible for the interaction with CtBP1. Together, these results demonstrate that IAP and CtBP interact in both insect and mammalian cells, that the RRT-binding region of CtBP1 is responsible for binding to XIAP, and that BIR3 of XIAP is responsible for binding CtBP1.

3.2. CtBP1 is regulated by XIAP

To determine the functional importance of the interaction between IAP and CtBP, we tested whether IAPs ubiquitinated CtBP and modulated its stability, using an *in vitro* ubiquitination assay. High molecular weight (ubiquitinated) bands were detected in

the presence of Diap1 and the intensity of the bands increased in a time-dependent manner (Fig. 2A), indicating that dCtBP is ubiquitinated specifically by Diap1. Next, we examined whether dCtBP was ubiquitinated *in vivo* by Diap1. S2 cells were co-transfected with GST-tagged ubiquitin (GST-Ub) and WT Diap1 or the Diap1th6 mutant, and then treated with the proteasome inhibitor MG132, to test whether ubiquitinated dCtBP was degraded by the proteasome. We found that dCtBP was ubiquitinated by Diap1WT but not by the Diap1th6 E3 ligase mutant (Fig. S2a), suggesting that the RING domain of Diap1 is critical for ubiquitination of dCtBP. Additionally, treatment with MG132 led to an accumulation of high molecular weight ubiquitinated dCtBP, indicating that ubiquitinated dCtBP was targeted for proteasome-mediated degradation (Fig. S2a).

Next, we examined whether CtBP1 is regulated by IAPs in human cells. HEK 293 cells were transfected with 6Myc-XIAP or 6Myc-cIAP1, which both bound to CtBP1 (Fig. 1B), along with HA-Ub, and then treated with MG132. We found that CtBP1 was ubiquitinated by XIAP, but not by cIAP1, and high molecular weight ubiquitinated CtBP1 accumulated after MG132 treatment, indicating that XIAP-mediated ubiquitination of CtBP1 targets the protein for proteasome degradation (Fig. S2b). Further, CtBP1 was ubiquitinated by XIAP WT, but not by the XIAP RING domain mutant (Fig. 2B), indicating that XIAP E3 activity was responsible for the ubiquitination of CtBP1. Next, we analyzed the half-life of CtBP1 after treatment of cells with cycloheximide treatment. As shown in Fig. 2C, the CtBP1 half-life of CtBP1 in cells overexpressing XIAP was substantially shorter than in parental control cells. Taken together, these results demonstrate that steady-state levels of CtBP1 were regulated by a XIAP-dependent ubiquitin–proteasome pathway.

3.3. CtBP1-mediated transcriptional repression is de-repressed by expression of XIAP

Our results raised the possibility that XIAP plays a role in regulating CtBP1-dependent transcriptional repression. To test this hypothesis, we used an E-cadherin-luciferase reporter construct to examine CtBP1 transcriptional repression activity. E-cadherin is essential for cell adhesion and is a known target of CtBP1 [25]. We generated an E-cadherin-promoter driven luciferase reporter construct (pEcad-reporter), and *ctbp*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were co-transfected with pEcad-reporter, Myc/His-CtBP1, and increasing amounts of XIAP WT or the XIAP RING domain mutant. We used *ctbp*$^{-/-}$ MEFs to eliminate background activity from endogenous CtBP1. As shown in Fig. 3A, E-cadherin-luciferase activity was repressed by CtBP1, but addition of increasing amounts of XIAP increased luciferase levels up to the control level, indicating that the CtBP1-mediated repression of target genes can be suppressed by XIAP expression. In contrast, expression of the XIAP RING domain mutant or cIAP1 did not significantly change luciferase expression levels. Thus, the de-repression of the E-cadherin–luciferase activity by XIAP was likely from the regulation of CtBP1 by XIAP.

Next we examined whether E-cadherin mRNA transcription is affected by the regulation of CtBP1 by XIAP. RT-PCR analysis was performed on total RNA extracted from *ctbp*$^{-/-}$ MEFs transfected with Myc/His-CtBP1 alone, or together with 6Myc-XIAP. E-cadherin mRNA levels in *ctbp*$^{-/-}$ MEFs that expressed exogenous CtBP1 were observed to be less than half that of control cells, but were restored almost to control levels upon co-expression of XIAP (Fig. 3B). Because CtBP1 has been reported to repress the expression of a variety of genes involved in cell adhesion, cell survival, and apoptosis [15], we wondered whether the expression of other target genes might be regulated by XIAP. Thus, we tested the expression of keratin-8, another epithelial cell specific gene, and p21, a cell survival gene, upon CtBP1 and XIAP co-expression in *ctbp*$^{-/-}$ MEFs. The repressed mRNA levels of keratin-8 or p21 by CtBP1 expression were restored by XIAP co-expression (Fig. 3B), suggesting that transcription of CtBP1 target genes may be determined by the intracellular CtBP1 level as regulated by XIAP in certain physiological condition.

The pro-apoptotic gene *Bax* is induced by cell death stimuli and its transcription is negatively regulated by CtBP1 [26]. Therefore,

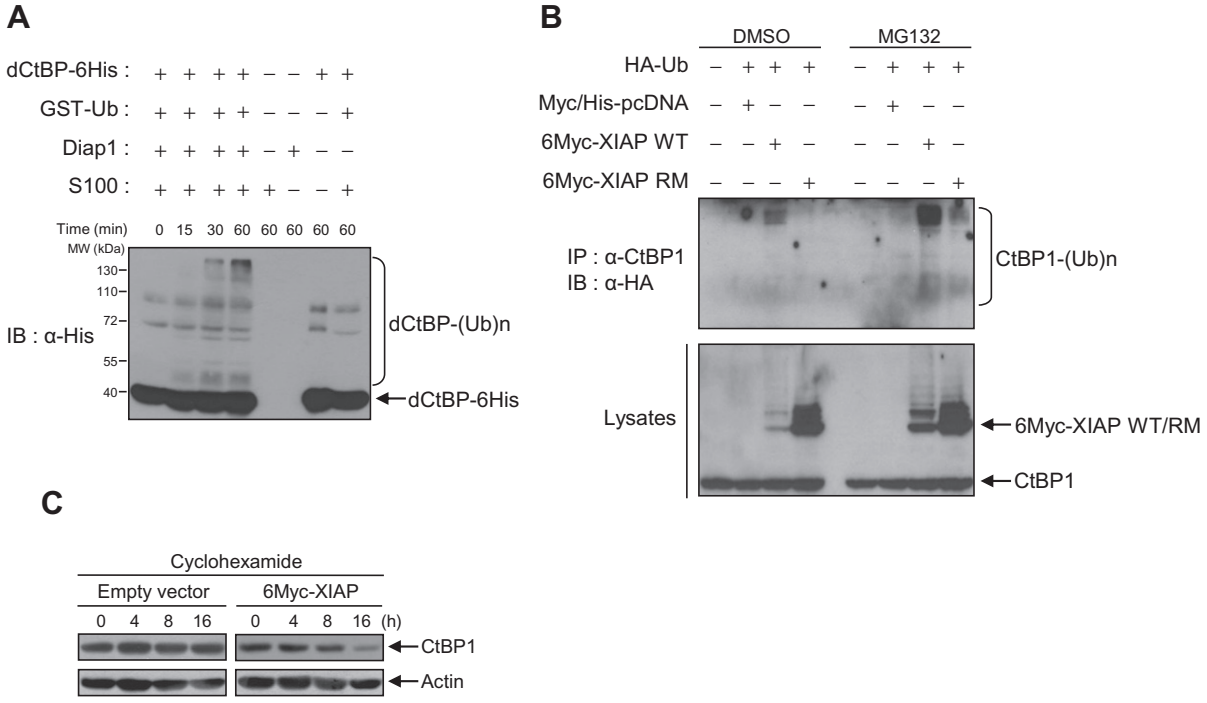

Fig. 2. CtBPs are regulated by IAPs. (A) *In vitro* ubiquitination assays were carried out in the presence or absence of purified Diap1. (B) Comparison of CtBP1 ubiquitination by WT XIAP or the RING domain mutant (RM). (C) Measure of CtBP1 half-life. CtBP1 degradation occurs more quickly when XIAP is overexpressed.

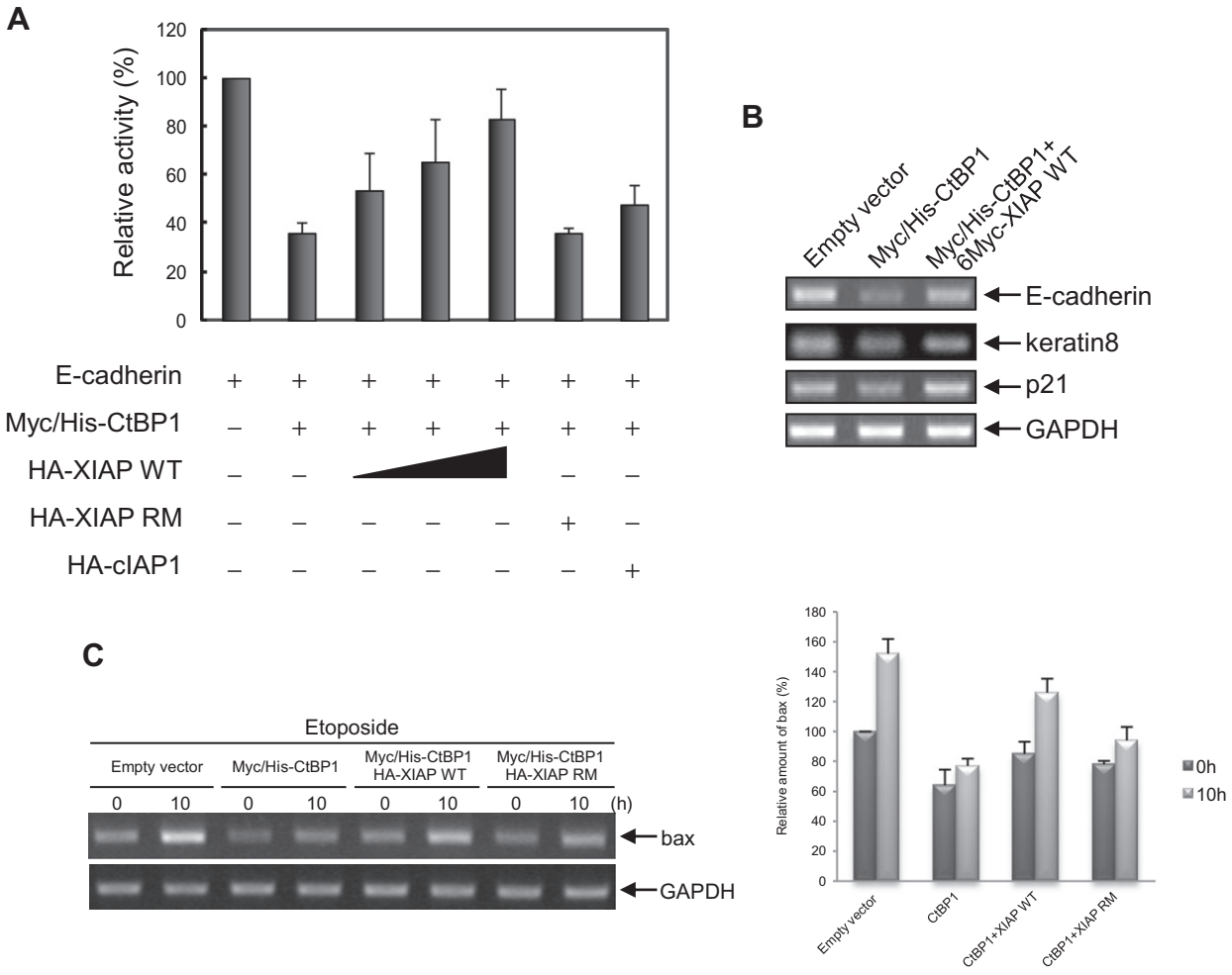

Fig. 3. CtBP1-mediated transcriptional repression is regulated by XIAP. (A) *ctbp*$^{-/-}$ MEFs were co-transfected with an E-cadherin promoter-driven luciferase reporter and increasing amounts of the HA-XIAP WT or HA-XIAP RING domain mutant (RM) or HA-cIAP1 and Myc/His-CtBP1. At 24 h after transfection, E-cadherin promoter activity was measured by a luminometer. (B) RT-PCR of the indicated genes was performed. RNAs were derived from *ctbp*$^{-/-}$ MEFs transfected with empty vector, Myc/His-CtBP1 and/or 6Myc-XIAP. (C) *ctbp*$^{-/-}$ MEFs were transfected with empty vector, Myc/His-CtBP1, HA-XIAP WT, and HA-XIAP RING domain mutant (RM). At 24 h after transfection, *ctbp*$^{-/-}$ MEFs were treated with 50 μM etoposide for 0 and 10 h and then RT-PCR was performed. RT-PCR results were quantified using the ImageJ program (NIH, Bethesda, MD). The data are means from three separate experiments.

we questioned whether CtBP1-mediated *Bax* transcription in *ctbp*$^{-/-}$ MEFs was affected by XIAP expression under conditions of cell stress. *ctbp*$^{-/-}$ MEFs were transfected with Myc/His-CtBP1 alone or together with 6Myc-XIAP, and then treated with the DNA damage reagent etoposide. *Bax* mRNA levels in control cells were increased approximately 1.5-fold upon etoposide treatment, but much lower levels (~60%) were observed in the presence of CtBP1 (Fig. 3C). However, when XIAP was co-expressed with CtBP1, *Bax* mRNA levels increased and were similar to those seen in the control on etoposide treatment (Fig. 3C). The *Bax* mRNA expression level was slightly increased by the expression of the XIAP RING domain mutant, but the induction ratio was similar to that seen in the presence of CtBP1 alone, indicating that the E3 activity of XIAP is required for the induction of *Bax* mRNA expression. Because Bax initiates apoptotic processes in mitochondria, these results suggest that XIAP might have a pro-apoptotic function through RING domain-dependent E3 ligase activity in certain physiological conditions, though XIAP is known thus far as the most potent caspase inhibitor that functions through the BIR domains in mammalian cells. Taken together, our results indicate that the RING domain-dependent E3 activity of XIAP contributes to the de-repression of CtBP1-dependent transcriptional repression, suggesting that XIAP might play a role in the transcription of CtBP1 target genes through the regulation of CtBP1.

3.4. Depletion of XIAP reduces the transcription of sirt1, the target of CtBP1

Next, we investigated the regulatory role of XIAP in the expression of CtBP1-target genes by depleting XIAP. Further, since the CtBP1-G183V mutant, which does not bind to NAD(H), strongly bound XIAP and its steady-state intracellular level was substantially lower than WT CtBP1 (Fig. 1D), we investigated if the regulation of CtBP1 by XIAP was influenced by changes in the intracellular redox state. The transcription of *sirt1* was shown to correlate with CtBP1 binding to the Hic1 repressor. The interaction between CtBP1 and Hic1 is dependent on intracellular redox status, which is determined by the NAD+/NADH ratio [27]. 2-DG blocks glycolysis and effectively changes the redox status of cultured human cell lines [27]. We therefore examined whether the transcription of *sirt1* was affected by RNAi-mediated silencing of XIAP in the presence or absence of 2-DG treatment. When we depleted XIAP using siRNA, endogenous CtBP1 levels were greater than in cells treated with a scrambled siRNA negative control (Fig. 4A),

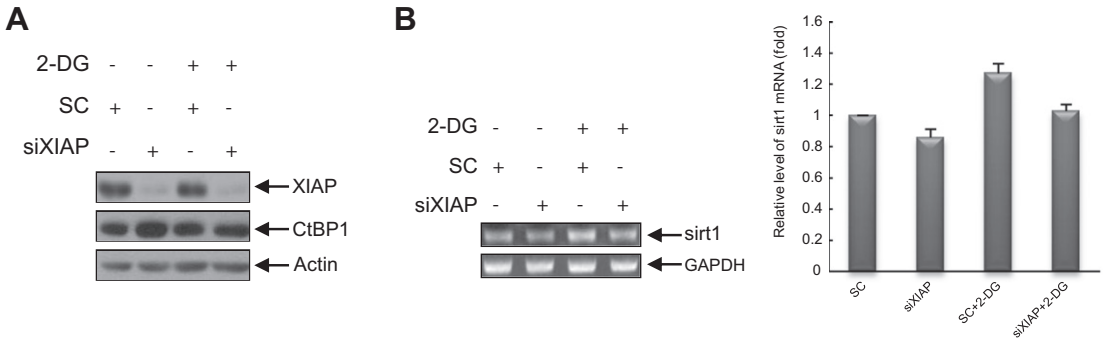

Fig. 4. Knockdown of XIAP increases CtBP1 levels leading to reduced *sirt1* transcription. (A and B) HEK 293 cells were transfected with scrambled RNA (control) and XIAP siRNA for 24 h and then exposed to 10 mM 2-deoxyglucose (-2DG) for 6 h. CtBP1 and XIAP proteins were assayed by WB and *sirt1* transcription was measured by RT-PCR. The density of the RT-PCR product was measured using the ImageJ program (NIH) and normalized against GAPDH bands. The data are means from three separate experiments.

indicating that endogenous CtBP1protein levels were regulated in XIAP-dependent manner. This confirms that CtBP1 is regulated by XIAP, as shown in Fig. 2. CtBP1 levels were still higher in XIAP-targeted siRNA-treated cells after 2-DG (Fig. 4A), suggesting that CtBP1 is regulated by XIAP to a certain extent regardless of redox changes within the cell.

We then measured the amount of *sirt1* mRNA by RT-PCR in cells transfected with either scrambled siRNA or XIAP-targeted siRNA. The production of *sirt1* mRNA was reduced by about 20% in XIAP-depleted cells compared to control cells (Fig. 4B), implying that the increased CtBP1 protein levels in XIAP-depleted cells actively participates in the transcriptional repression of *sirt1*. When cells were treated with 2-DG, *sirt1* mRNA levels increased approximately 30% compared to control cells, but the increased *sirt1* mRNA was still 20% lower in XIAP-depleted cells (Fig. 4B), similar to the reduction of *sirt1* transcription in XIAP-depleted cells in the absence of 2-DG treatment. These results suggest that XIAP regulates CtBP1 to a certain extent, regardless of intracellular redox status. However, the available data are insufficient to determine which form of CtBP1 would be a good substrate for XIAP *in vivo*.

In summary, our results demonstrate that IAP and CtBP interact in both insect and human cells, XIAP regulates the intracellular level of CtBP1 through its E3 ligase activity, and transcription of CtBP1 target genes is controlled by this regulation. Our report provides the first evidence that CtBP1 is specifically regulated by XIAP. Because CtBP1 is a member of transcriptional repression complexes, changes in intracellular CtBP1 levels by XIAP would be expected to influence the function of associated host factors, expression of target genes, and ultimately influence various biological processes. A growing number of studies have revealed that IAPs play multiple roles within cells, in addition to the inhibition of apoptosis. Our report unveils a new critical function of XIAP: the regulation of CtBP1-mediated transcriptional repression via the ubiquitination and proteasomal degradation of CtBP1.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.11.080.

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