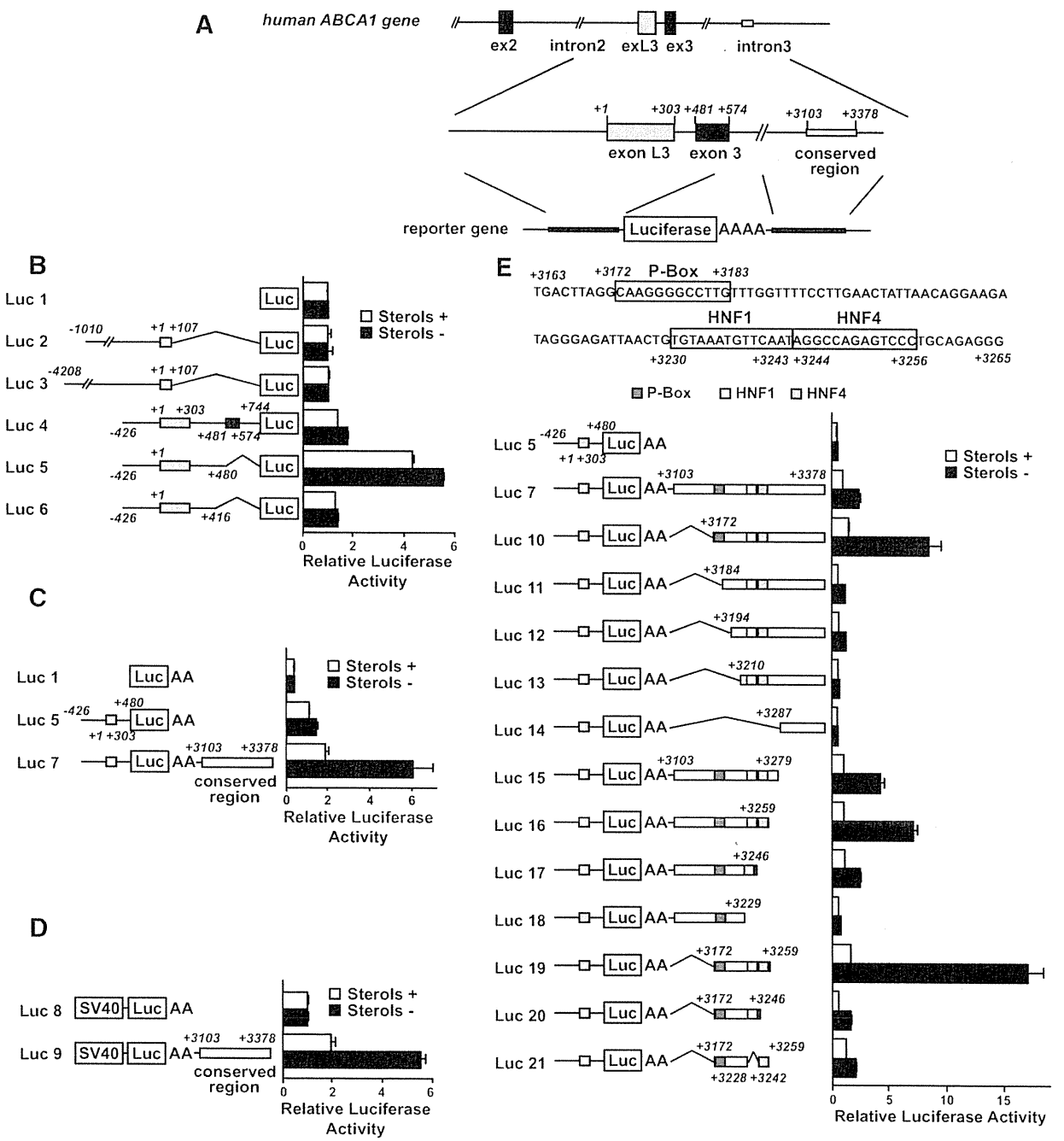

Figure 4. Identification of type L3 promoter and its cholesterol-responsive enhancer elements. **A**, Genomic structure of the promoter region of type L3 transcript (**top** and **middle rows**) and the structure of the luciferase (Luc) reporter constructs (**bottom row**). The transcription start site of type L3 is indicated as +1. The exons (ex) are represented by thick boxes. **B** to **E**, JHH-5 cells were transfected with the indicated reporter plasmids and control plasmid control renilla luciferase plasmid-SV40. After 30 hours, cells were treated with sterols (10 μg/mL cholesterol plus 1 μg/mL 25-hydroxycholesterol: sterols+) or compactin (50 μmol/L, with 40 μmol/L mevalonate: sterols−) for 16 hours. The firefly luciferase activity in cell lysates was measured and normalized with *Renilla* luciferase activity. The data represent means±SD (n=3). **E**, **top panel**, Putative sterol-response element sequence in intron 3. P-Box indicates a palindromic sequence box; hepatocyte nuclear factor (HNF) 1, a putative HNF1-binding site; HNF4, a putative HNF4-binding site.

contains a palindromic sequence box (P-Box) (Figure 4E, upper scheme). In a similar fashion, 3′-end deletions up to +3260 enhanced the promoter activity and the response to sterol depletion (Luc16), whereas deletion up to +3247 (Luc17) markedly decreased the response, and further deletion up to +3230 (Luc18) completely abolished these effects. Accordingly, a reporter containing the region from +3172 to +3259 showed a strong response to sterol depletion (Figure 4E, Luc19). Within this responsive region, deletion of either the 3′-end up to +3247 or the region between +3229 and +3241 significantly repressed the sterol-mediated response (Figure 4E, Luc20, Luc21). These results suggest that the P-box and the 2

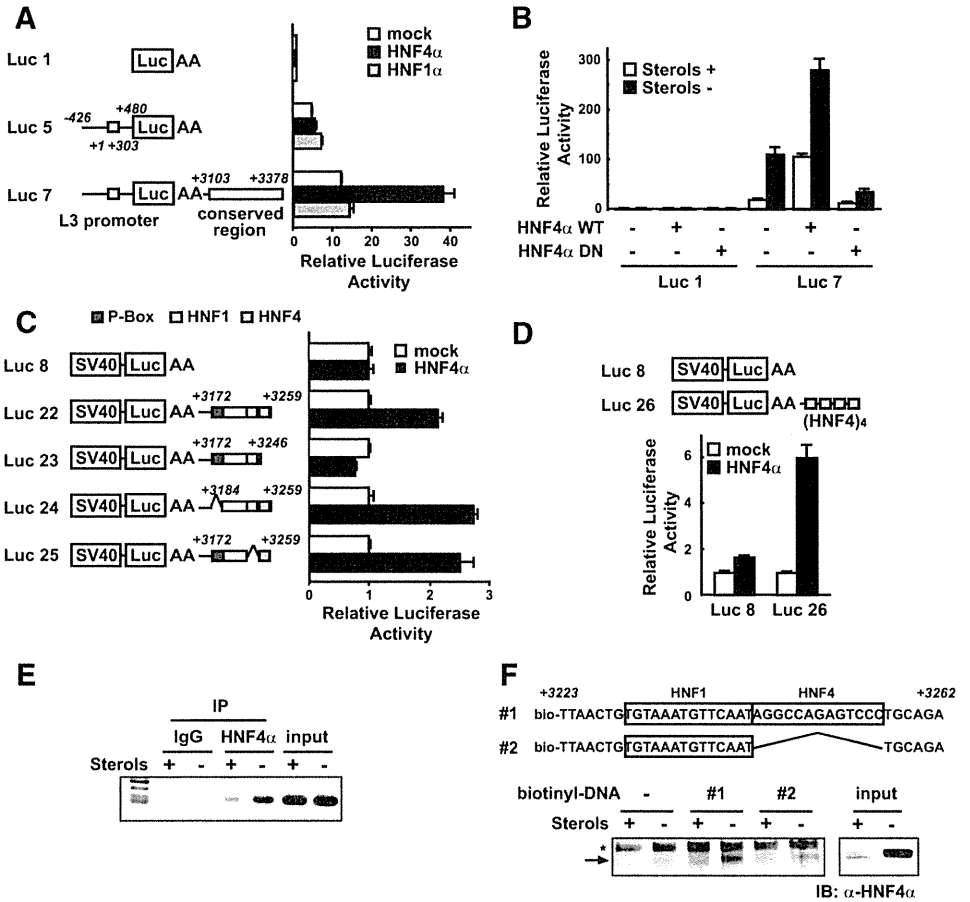

Figure 5. Hepatocyte nuclear factor (HNF) 4α regulates ATP-binding cassette transporter A1 (ABCA1) type L3 promoter-enhancer activity by binding to the distal enhancer element. **A** to **D**, JHH-5 (**A**, **B**, and **D**) or HepG2 (**C**) cells were transfected with the indicated reporter plasmids and control plasmid control renilla luciferase plasmid-SV40 in the absence or presence of HNF4α or HNF1α expression vectors. **A**, **C**, and **D**, After 48 hours, the firefly luciferase (Luc) activity in cell lysates was measured and normalized with *Renilla* luciferase activity. **B**, After 30 hours of transfection, the cells were treated under sterols+ or sterols− conditions as in Figure 2C for 16 hours. DN indicates dominant negative. Data represent means±SD (n=3). SV40 indicates SV40 promoter. **E** and **F**, JHH-5 cells were treated under sterols+ or sterols− conditions for 16 hours. **E**, Cells were subjected to chromatin immunoprecipitation (IP) assay using the indicated antibodies or control IgG. The immunoprecipitated region containing HNF4α-response elements within the intron 3 enhancer region was amplified with polymerase chain reaction (PCR) using specific primers as described in Materials and Methods. The amplified PCR products were subjected to electrophoresis in a 2% agarose gel. Input indicates that genomes extracted from 2% of total lysates were amplified with PCR. **F**, The cell lysates were subjected to avidin biotin–conjugated DNA assays using 2 biotin-conjugated nucleotides (#1 or #2) as indicated in the **top panel** and, together with an aliquot of the lysates (input), immunoblotted (IB) with anti-HNF4α antibody. The **arrow** and **asterisk** indicate HNF4α protein and nonspecific bands, respectively.

elements (+3247 to +3259, +3229 to +3241) are required for the sterol-mediated response in the distal enhancer region in *ABCA1* intron 3. A homology search detected consensus binding sequences for various transcription factors, suggesting that these elements (+3230/+3243 and +3224/+3256) could be binding sites for liver-enriched transcription factors such as HNF1 and HNF4, respectively (http://tfbind.ims.u-tokyo.ac.jp/; Figure 4E, upper scheme).

HNF4α Regulates *Type L3* Promoter Activity Through Binding to the Distal Enhancer Elements

The role of liver-specific transcription factors HNF1α and HNF4α in the sterol-responsive distal enhancer activity within *ABCA1* intron 3 was investigated. Whereas HNF4α expression effectively augmented *type L3* promoter-enhancer activity in a distal enhancer region-dependent manner, HNF1α produced only minimal enhancement (Figure 5A). Expression of a dominant negative form of HNF4α lacking the AF-2 domain repressed its promoter-enhancing activity, especially under cholesterol-depleted conditions (Figure 5B). HNF4α expression also augmented the activity of a *SV40* promoter-driven reporter gene containing the *ABCA1* enhancer region (Figure 5C, Luc22) but did not activate a construct that lacked the putative HNF4-binding site (Luc23). Deletion of the P-box (Luc24) or putative HNF1 site (Luc25) did not affect this HNF4α-mediated augmentation. In addition, the activity of a reporter gene containing 4 tandem repeats of the HNF4-response element was enhanced by HNF4α expression (Luc26). These results indicate that the HNF4-element, but not the P-box nor putative HNF1-binding site, is critical for HNF4α-mediated activation of ABCA1 expression.

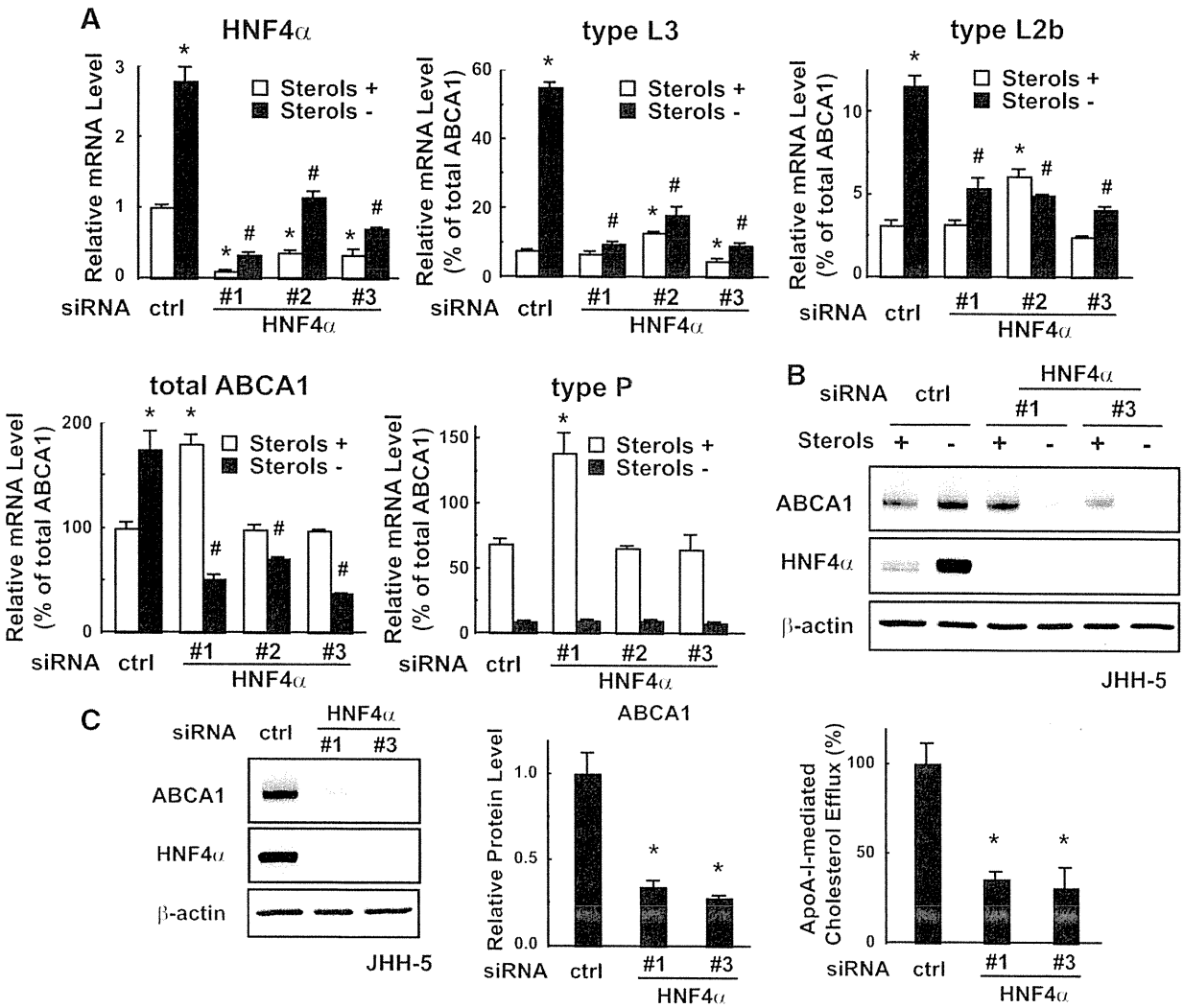

Figure 6. Hepatocyte nuclear factor (HNF) 4α regulates ATP-binding cassette transporter A1 (ABCA1) expression in human hepatoma cells by inducing liver-specific ABCA1 L3 and L2b mRNA in response to sterol depletion. **A** to **C**, JHH-5 cells were transfected with either short interfering RNA (siRNA) against HNF4α (#1–#3) or a negative control (ctrl) siRNA. After 32 hours, the cells were treated under sterols + or sterols − (**A** and **B**) or sterol − (**C**) conditions for 16 hours. **A**, Quantitative real-time reverse transcription–polymerase chain reaction analysis of total and variant ABCA1 and HNF4α mRNA expression. Data were normalized with 18S rRNA and represent means ± SD (n = 3). *$P < 0.05$ vs sterol + control, #$P < 0.05$ vs sterol − control. One of the HNF4α siRNAs (#1) induced a moderate increase in type P mRNA, probably because of an off-target effect. **B** and **C**, Immunoblot analysis for ABCA1, HNF-4α and the loading control β-actin. **C**, Cholesterol efflux to the media was determined as described in Materials and Methods. The data represent means ± SD (n = 3) *$P < 0.05$ vs control siRNA-transfected cells.

We performed a chromatin immunoprecipitation assay to investigate whether endogenous HNF4α protein associates with chromatin via this putative HNF4-binding site. Clear bands were detected when chromatin from cholesterol-depleted JHH-5 cells was immunoprecipitated with antibodies against HNF4α, indicating that a tight association of endogenous HNF4α protein with the conserved intron 3 region was induced by cholesterol depletion (Figure 5E). Additionally, an in vitro DNA binding assay confirmed the association of HNF4α with this site. Endogenous HNF4α in lysates of cholesterol-depleted cells reacted with biotin-conjugated oligodeoxynucleotides corresponding to the *ABCA1* region from +3223 to +3262 that includes the HNF4-response element (#1), but not oligonucleotides that lacked this element (#2) (Figure 5F). Furthermore, electrophoretic mobility shift assay also confirmed the association of HNF4α with this binding site (Figure VIII in the online-only Data Supplement). These results indicate that the putative HNF4-binding site on *ABCA1* intron 3 is authentic.

HNF4α Is Required for Expression of Liver-Specific *ABCA1* mRNA and Protein in Response to Sterol Depletion

The role of HNF4α in liver-type *ABCA1* mRNA variant expression was investigated using siRNA knockdown. In JHH-5 cells, 3 siRNAs against HNF4α effectively reduced *HNF4α* mRNA and protein expression (Figure 6A and 6B). HNF4α knockdown resulted in the reduced expression of

type L3 and also *type L2b* but not *type P* transcripts under sterol-depleted conditions (Figure 6A, black bars). Consistently, expression of total *ABCA1* mRNA and protein was reduced by HNF4α knockdown under sterol-depleted conditions (Figure 6A and 6B). Reduction of type L3 and total *ABCA1* mRNA was also confirmed by Northern blot (Figure V in the online-only Data Supplement). In the presence of cholesterol, however, HNF4α knockdown did not affect the expression of *ABCA1* transcripts (Figure 6A, white bars), although 1 siRNA (#1) induced *type P* and total *ABCA1* mRNA expression probably because of an off-target effect. Similar results were obtained with another human hepatic cell line Hep3B (Figure IXA and IXB in the online-only Data Supplement). In agreement with these results, HNF4α knockdown in JHH-5 and Hep3B cells reduced apoA-I-mediated cholesterol efflux under cholesterol-depleted conditions (Figure 6C; Figure IXC in the online-only Data Supplement). Conversely, forced expression of HNF4α increased the level of *type L3* and *L2b* transcripts, leading to a significant increase in total *ABCA1* mRNA expression and apoA-I-mediated cholesterol efflux (Figure X in the online-only Data Supplement). We also confirmed that HNF4α knockdown markedly reduced the *type L3* promoter-enhancer activity (Figure XIIA in the online-only Data Supplement) and the HNF4-binding site-dependent intron 3 enhancer activity (Figure XIIB in the online-only Data Supplement) in sterol depleted cells but did not efficiently modulate the expression of *LXRα*, *LXRβ*, and *micro-RNA-33*, which are regulatory genes of *ABCA1* transcript (Figure XI in the online-only Data Supplement). These results indicate that HNF4α is required for liver-specific *ABCA1* transcript (*L3* and *L2b*) expression and liver-type response of *ABCA1* mRNA and protein expression on cholesterol depletion.

Discussion

The liver is the major source of apoA-I, and hepatic ABCA1 is responsible for producing the majority of plasma HDL.[5,6] Hepatic ABCA1 has a specific role in producing early nascent HDL particles,[6] and its genetic regulation is distinct from extrahepatic ABCA1.[14] In this study, we investigated the regulation of ABCA1 expression in human liver cells and discovered a liver-specific regulatory system in humans. Among the liver-specific variants (*types L2a/b*, *L3*, *L4a/b*), *type L3* is a novel *ABCA1* transcript containing a human-specific exon L3 and is a major transcript accounting for ≈25% of total *ABCA1* mRNA in human liver.

The ABCA1 protein translated from *type L3* transcript lacks the amino-terminal 21 amino acids, because the first ATG codon resides in the 3′-end of exon L3 (Figure III in the online-only Data Supplement). Although early studies showed that an ABCA1 expression construct lacking the 60 amino-terminal amino acids does not produce a functional protein in cells,[24] specific siRNAs against *type L3* efficiently reduced ABCA1 protein and cholesterol efflux. Moreover, transfection of L3-ABCA1 expression vector into JHH-5 cells augmented ABCA1 protein and cholesterol efflux activity, demonstrating that the L3-derived ABCA1 variant protein is stable and functionally active.

HNF4α Regulates Hepatic *ABCA1* Gene Expression in Humans

When cellular cholesterol is depleted, the liver-specific *type L3* and *L2b* transcripts are induced and positively regulate ABCA1 protein expression (Figure XIII in the online-only Data Supplement). We previously discovered that SREBP-2, a transcription factor activated on cellular cholesterol depletion,[25] upregulates the rodent liver-type (*type L*) *ABCA1* promoter.[14] However, the SREBP-2 binding element (sterol regulatory element) in the rat promoter is not conserved in humans (Figure XIVA in the online-only Data Supplement), and the human *type L2* promoter containing this region is not activated by cholesterol depletion (Figure XIVB in the online-only Data Supplement). In humans, we found a critical role of the liver-enriched transcription factor HNF4α in hepatic ABCA1 expression. Knockdown of HNF4α abolished inductions of *L3* and *L2b* mRNA and, conversely, forced expression of HNF4α in JHH-5 cells increased them. Altered *L3* and *L2b* mRNA levels led to substantial changes in total *ABCA1* mRNA and protein expression and in cholesterol efflux activity. Thus, HNF4α is required for the induction of liver-specific *ABCA1* transcripts, including types *L3* and *L2b*, and thereby modulates ABCA1 expression and HDL generation.

We identified a regulatory enhancer element for *L3* expression lying within intron 3 of the human *ABCA1* gene, to which HNF4α binds and at which HNF4α augments the L3 promoter-enhancer activity in response to cholesterol depletion. Because HNF4α expression was increased on cholesterol depletion and HNF4α knockdown abolished the *L3* and *L2b* induction, it is likely that the HNF4α level or activity (via dephosphorylation[26]) determine the sterol-responsive *L3* and *L2b* expression. However, it is more likely that along with HNF4α, several transcription factor(s) cooperatively regulate the *L2* and *L3* expression, because we identified several elements (and region) responding to cholesterol depletion in the intron 3-enhancer and the L3 promoter (Figure 4E). In addition, in human JHH-5 cells, SREBP-2 knockdown partly diminished the *L3* and *L2b* induction on sterol depletion (Figure XV in the online-only Data Supplement). Thus, SREBP2 partially regulates human hepatic ABCA1 expression through distinct mechanism from that in rodents. Although decreased HNF4α expression (Figure XV in the online-only Data Supplement) may be responsible for this effect, it is possible that SREBP-2 directly (via some unknown sterol regulatory element) or indirectly (via interaction with HNF4α) regulates *L3* and *L2b* mRNA expression. Decreased *type P* mRNA expression by SREBP-2 knockdown is probably due to the decreased supply of endogenous ligands for LXR.[27]

HNF4α knockdown resulted in the reduced expression of *types L3* and *L2b* but not *type P*. Although we have confirmed that neither LXR regulating *type P* promoter nor microRNA-33 regulating *ABCA1* mRNA stability was affected by HNF4α knockdown, further investigation on chromatin-loop or transcription factors is needed to understand the mechanism.

Because the HNF4 binding site located in *ABCA1* intron 3 is conserved among mammals (Figure III in the online-only Data Supplement), HNF4α may also regulate the liver-type

ABCA1 transcript(s) in rodents. However, we could not confirm this because of the unavailability of variant-specific primers for rodent *type L* mRNA that corresponds to human *type L2a*. In addition, a rodent variant corresponding to human *type L3* may not be expressed in rodent liver because exon L3 is not conserved in rodents. Studies have shown that acute loss of HNF4α (for 6–7 days) in mouse liver reduced *ABCA1* mRNA expression,[28] although Cre-loxP knockdown did not affect the level.[29] Thus, the detailed mechanisms that regulate liver-specific *ABCA1* gene expression may differ between humans and rodents. However, hepatic *ABCA1* gene expression is ensured by stimulating liver-specific systems on cholesterol depletion, a response that is conserved among humans, mice, and rats.

HNF4α is a highly conserved member of the nuclear receptor superfamily.[26] Loss-of-function mutations in the human *HNF4α* gene cause the disorder maturity onset diabetes of the young (MODY1).[26] HNF4α is required for early liver development and expression of many liver-specific genes, including those involved in lipoprotein and cholesterol/bile acid metabolism.[26,30] For example, HNF4α regulates expression of microsomal triglyceride transfer protein, apolipoprotein B, CII, and CIII, genes involved in very-low-density lipoprotein production[31]; cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting enzyme of bile acid biosynthesis from cholesterol[26,30]; and ABCG5/ABCG8, transporters involved in cholesterol/sterol excretion from the liver.[32] Together with these genes, HNF4α-regulated *ABCA1* gene expression may cooperatively control hepatic cholesterol elimination to maintain cholesterol homeostasis. A recent study has shown that *HNF4α*-siRNA knockdown in mouse liver reduces expression of many lipid metabolism genes, including *ABCA1*, leading to decreases in HDL and very-low-density lipoprotein in plasma.[28]

Dual System Regulates Human Hepatic *ABCA1* Gene Expression in Response to Sterols

In the model of reverse cholesterol transport pathway, the liver plays a role in excreting cholesterol transported from peripheral tissues.[8] The liver is also the major site of production of HDL, and hepatic ABCA1 is responsible for maintaining plasma HDL by producing a precursor form of HDL,[5,6] which could accept cholesterol from peripheral cells. When cholesterol accumulates in the liver, the *type P ABCA1* transcript is induced by the LXR promoter.[14] However, this rise is counterbalanced by the reduced expression of *liver-type L3* and *L2a/b* transcripts (Figure XIII in the online-only Data Supplement). The dual system may prevent overshooting ABCA1 expression and retransport of cholesterol to peripheral tissues. On cholesterol depletion, *type P ABCA1* expression is reduced, but enhanced *type L3* and *L2a/b* expression may compensate for this reduction. Thus, the dual system ensures constant expression of ABCA1 and HDL production in the human liver even under fluctuating cholesterol conditions. Recent studies show that *microRNA-33* regulates hepatic ABCA1 expression and plasma HDL levels in mice[18,19] ABCA1 expression also undergoes posttranslational regulation.[33] Because hepatic ABCA1 plays the major role in maintaining plasma HDL levels,[5] it seems reasonable that hepatic ABCA1 expression would be stringently regulated by multiple sterol-responsive systems.

Regulation of Human Hepatic ABCA1 as a Novel Strategy to Prevent Atherosclerosis

Statins are widely used to treat hypercholesterolemia and raise plasma HDL cholesterol independently of reducing low-density lipoprotein cholesterol.[34] Several studies have shown that statins upregulate hepatic *ABCA1* mRNA expression in human hepatoma cells and mouse liver,[15–17] whereas statins repress peripheral *ABCA1* mRNA expression by depleting endogenous LXR ligands.[10,11] Our findings indicate that the liver-specific *ABCA1* variants, including *type L3* and *L2a/b* (or *L* in rodents), are responsible for statin-induced ABCA1 expression and may be associated with increased plasma HDL levels.

Our current study clarified for the first time a mechanism by which human hepatic ABCA1 expression is regulated. HDL cholesterol levels are inversely correlated with cardiovascular risk. Because hepatic ABCA1 has the largest impact on plasma HDL levels[5,6] and is the most promising therapeutic target, our findings provide a basis for developing novel drugs to control plasma HDL levels and prevent atherosclerosis.

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Disclosures

None.

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Specific degradation of CRABP-II via cIAP1-mediated ubiquitylation induced by hybrid molecules that crosslink cIAP1 and the target protein

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ABSTRACT

Manipulation of protein stability with small molecules is a challenge in the field of drug discovery. Here we show that cellular retinoic acid binding protein-II (CRABP-II) can be specifically degraded by a novel compound, SNIPER-4, consisting of (−)-*N*-[(2*S*,3*R*)-3-amino-2-hydroxy-4-phenyl-butyryl]-L-leucine methyl ester and all-trans retinoic acid that are ligands for cellular inhibitor of apoptosis protein 1 (cIAP1) and CRABP-II, respectively. Mechanistic analysis revealed that SNIPER-4 induces cIAP1-mediated ubiquitylation of CRABP-II, resulting in the proteasomal degradation. The protein knockdown strategy employing the structure of SNIPER-4 could be applicable to other target proteins.

1. Introduction

The ubiquitin–proteasome system (UPS) plays a crucial role in selective degradation of proteins, which is involved in the regulation of cell cycle, proliferation, differentiation and cell death [1–3]. In the UPS, the target protein is poly-ubiquitylated by the sequential action of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-ligase (E3), where E3 determines the specificity for the target protein in many cases. The poly-ubiquitylated proteins are degraded by proteasome. Inappropriate regulation of the UPS results in an accumulation or depletion of certain proteins, which results in a variety of diseases such as cancer, cachexia, neurodegenerative disorders and malformation [4–8]. To reduce a pathogenic protein in cells, it is useful to take advantage of the UPS since it is a highly specific system to target a certain protein for degradation, and the degradation occurs very quickly. However, few studies have been reported so far on the specific degradation of target proteins utilizing the UPS [9].

We previously reported that a class of small molecules, represented by (−)-*N*-[(2*S*,3*R*)-3-amino-2-hydroxy-4-phenyl-butyryl]-L-leucine methyl ester (MeBS), destabilize cellular inhibitor of apoptosis protein 1 (cIAP1), a ubiquitin-ligase (E3) belonging to IAP (inhibitor of apoptosis protein) family [10–12], and sensitize cancer cells to apoptosis induced by anti-cancer drugs and death receptor ligation [13–16]. MeBS directly interacts with cIAP1 at its BIR3 domain and induces auto-ubiquitylation of cIAP1 depending on its RING domain, resulting in the proteasomal degradation of cIAP1. Structure–activity relationship study indicated that analogs with a carboxyl-ester reduce the amount of cIAP1 even though the methyl group is substituted to other residues, and other modifications of MeBS seriously affected the activity [13]. Based on these observations, we hypothesized that the methyl group can be substituted to a ligand for a target protein without ablating the ubiquitin ligase activity of cIAP1. The hybrid molecule consisting of MeBS and the ligand would be able to cross-link cIAP1 and the target protein, to induce ubiquitylation and proteasomal degradation of the target protein. As a proof-of-concept study, we designed and synthesized a series of hybrid compounds consisting of MeBS and all-trans retinoic acid (ATRA), and found that they

Abbreviations: cIAP1, cellular inhibitor of apoptosis protein 1; MeBS, (−)-*N*-[(2*S*,3*R*)-3-amino-2-hydroxy-4-phenyl-butyryl]-L-leucine methyl ester; ATRA, all-trans retinoic acid; CRABP, cellular retinoic acid binding protein

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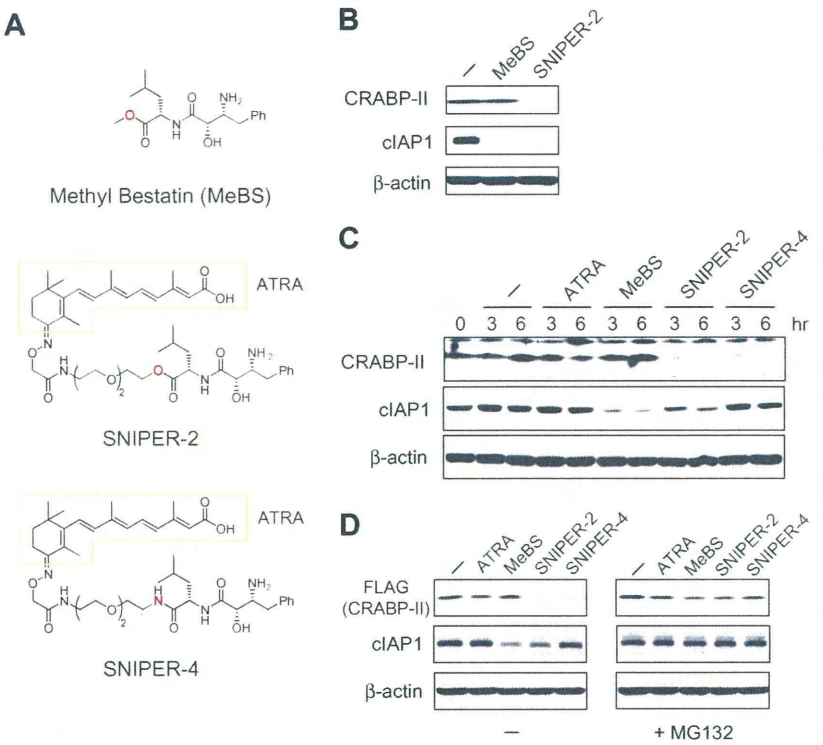

Fig. 1. Structure and protein knockdown activity of SNIPERs. (A) Structures of MeBS (top), SNIPER-2 (middle) and SNIPER-4 (bottom). (B) Reduction of endogenous CRABP-II and cIAP1 by SNIPER-2. (C) SNIPER-4 reduces endogenous CRABP-II but not cIAP1. (D) Inhibition of the SNIPER-mediated protein knockdown by a proteasome inhibitor, MG132. Human primary fibroblasts (B), HT1080 (C) or HT1080 cells expressing FLAG-CRABP-II (D) were incubated with 10 μM of indicated compounds for 6 h. Cells were pre-treated with 10 μM of MG132 for 30 min in (D). Shown are immunoblots of cell lysates stained with indicated antibodies.

induce the proteasomal degradation of CRABP-II, an ATRA-binding protein [17]. Thus, selective degradation of target protein (protein knockdown) can be attained by the hybrid molecules that cross-link the target protein and cIAP1.

The hybrid molecule we have developed previously [17], which we named SNIPER (Specific and Non-genetic IAP-dependent Protein ERaser)-2 in this study, however, has two major downsides that would be unfavorable for protein knockdown: (1) SNIPER-2 causes a simultaneous degradation of cIAP1 with the target protein CRABP-II, which may make the protein knockdown unsustainable, and (2) SNIPER-2 contains an ester-bond which is vulnerable to hydrolysis. To overcome these issues, we here developed SNIPER-4, in which the ester-bond is substituted to amide-bond, and found that the SNIPER-4 induces specific and sustained degradation of CRABP-II without inducing cIAP1 degradation. We also investigated biochemical mechanisms by which SNIPERs target CRABP-II for degradation, and demonstrate that SNIPERs induce the ubiquitylation of CRABP-II, which is mediated by cIAP1 but not by XIAP.

2. Materials and methods

2.1. Reagents and plasmids

MeBS was kindly provided by Nippon Kayaku Co. Ltd. (Tokyo, Japan). SNIPER-2 was synthesized as described previously [17]. The synthesis and structural analysis of SNIPER-4 would be reported elsewhere (Itoh et al., manuscript in preparation). cDNA encoding human CRABP-II were amplified by PCR from JHH-5 cDNA library and cloned into a p3xFLAG-CMV-10 expression vector (SIGMA). The correct DNA sequence was confirmed. The following reagents were purchased from indicated supplier: M2 anti-FLAG mouse monoclonal antibody, M2 anti-FLAG agarose-conjugated antibody, mouse anti-β-actin antibody (SIGMA); Fugene HD, anti-HA rat monoclonal antibody (Roche); CRABP-II antibody (Abcam); anti-human cIAP1 goat polyclonal antibody (R&D systems); anti-human XIAP mouse monoclonal antibody (MBL); Lipofectamine RNAi MAX transfection reagent, Stealth Select RNAi (Invitrogen). The target sequences for cIAP1 and XIAP RNAi were as follows: cIAP1-#1 (5′-TCTAGAGCAGTTGAAGACATCTCTT-3′); cIAP1-#2 (5′-GGAAATGCTGCGGCCAACATCTTCA-3′); XIAP-#1 (5′-ACACTGGCACGAGCAGGGTTTCTTT-3′); XIAP-#2 (5′-CCAGAATGGTCAGTACAAAGTTGAA-3′).

2.2. Cell culture, transfection and treatment with compounds

Human fibrosarcoma HT1080 were maintained in RPMI 1640 medium containing 10% fetal bovine serum and 100 μg/ml of kanamycin. Transient transfections were carried out using Fugene HD according to the manufacturer's instructions. siRNA transfections were carried out by reverse transfection method with Lipofectamine RNAi MAX according to the manufacturer's instructions. In brief, 60 pmol siRNA and 3 μl of Lipofectamine RNAi MAX were incubated in 200 μl Opti-MEM medium for 20 min at room temperature in 12-well plates, and then 1 ml of cell suspension (1.5×10^5 cells) was added to the siRNA–RNAi MAX complex. HT1080 cells constitutively expressing FLAG-CRABP-II were generated and maintained in RPMI 1640 medium containing 10% fetal bovine serum and 100 μg/ml of kanamycin and 500 μg/ml of Geneticin (G418). The cells were treated with 10 μM SNIPERs or vehicle (DMSO) for 6 h unless otherwise indicated, and lysed in

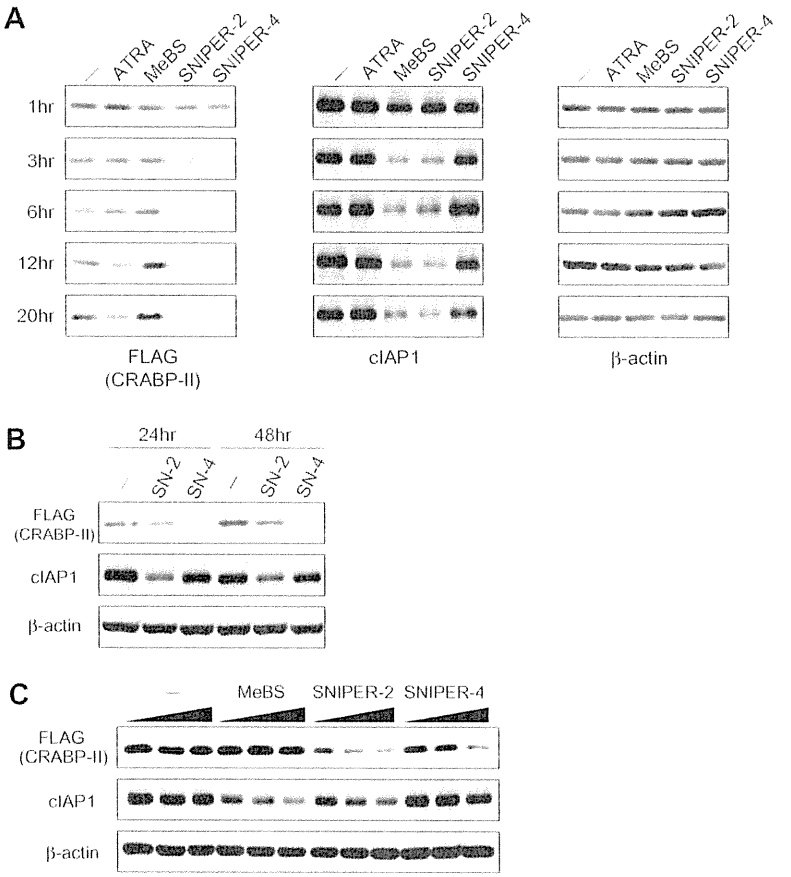

Fig. 2. (A and B) Time-course and (C) dose-dependent response of CRABP-II degradation induced by SNIPERs. HT1080 cells constitutively expressing FLAG-CRABP-II were incubated with 10 μM compounds for indicated times (A and B), or 0.3, 1 or 3 μM of compounds for 6 h (C, from the left of black triangle). Cell lysates were Western blotted with indicated antibodies.

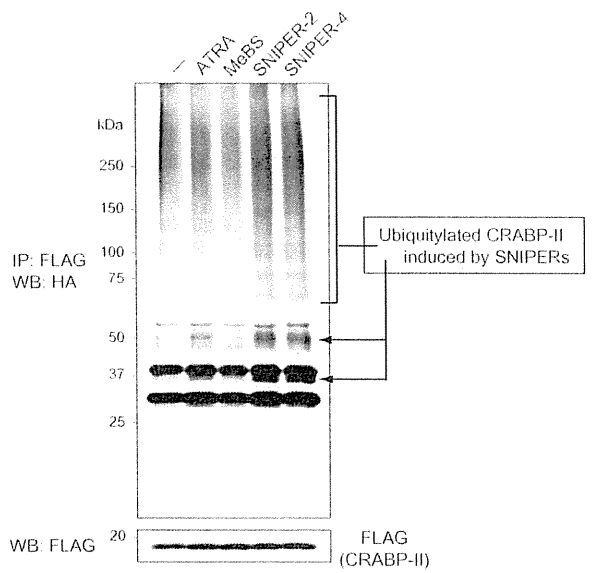

Fig. 3. Ubiquitylation of CRABP-II induced by SNIPERs. HT1080 cells expressing FLAG-CRABP-II and HA-ubiquitin were pre-treated with MG132 for 30 min, and then incubated with 10 μM of compounds for 1 h. Cells were lysed and FLAG-CRABP-II was immunoprecipitated with anti-FLAG antibody. The ubiquitylated CRABP-II was detected with anti-HA antibody.

lysis buffer (1% SDS, 0.1 M Tris–HCl (pH 7.0), 10% glycerol) and boiled for 10 min. Protein concentration was measured by BCA method (Pierce) and the equal amount of protein lysate was separated by SDS–PAGE, transferred to Hybond-P (GE Healthcare) membrane and Western blotted using appropriate antibody. Protein signals were detected using SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo scientific) or ECL™ Western Blotting Detection Reagents (GE Healthcare).

2.3. Ubiquitylation of CRABP-II

HA-ubiquitin was transiently transfected in HT1080 cells constitutively expressing FLAG-CRABP-II. Cells were lysed in lysis buffer and boiled, and the lysates were diluted 10 times with 0.1 M Tris–HCl. CRABP-II was immunoprecipitated with anti-FLAG agarose-conjugated beads. The immunoprecipitates were extensively washed with diluted lysis buffer, and analyzed by Western blotting using anti-HA antibody.

3. Results and discussion

The structures of MeBS (top), SNIPER-2 (middle) and SNIPER-4 (bottom) are shown in Fig. 1A. These SNIPERs are hybrid molecules consisting of bestatin moiety and ATRA connected by an ester-bond (SNIPER-2) or an amide-bond (SNIPER-4) spacer. SNIPER-2 induces the degradation of endogenous CRABP-II and

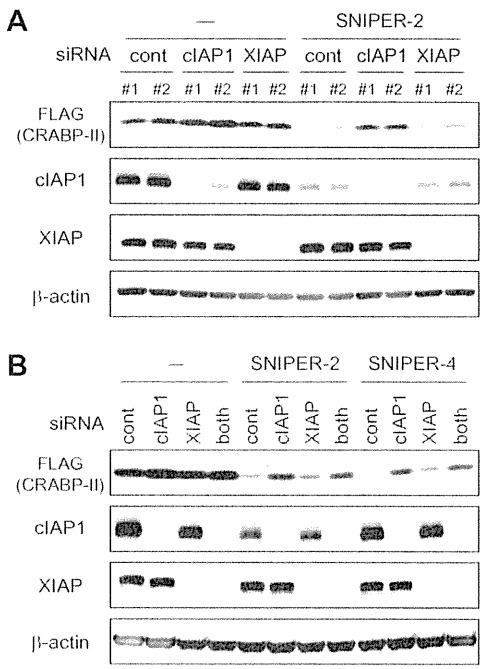

Fig. 4. Silencing of cIAP1 attenuates the SNIPER-dependent CRABP-II protein degradation. In HT1080 cells expressing FLAG-CRABP-II, the endogenous cIAP1 and XIAP were knocked down by two different siRNAs (#1 or #2) against each protein for 48 h (A). cIAP1, XIAP or both were knocked down by siRNAs (cIAP1-#1, XIAP-#2) (B). Cells were incubated with 10 μM compounds for 6 h. Shown are immunoblots of cell lysates stained with indicated antibodies.

cIAP1 in human primary fibroblasts as we reported previously (Fig. 1B) [17]. To develop a novel SNIPER that reduces CRABP-II but not cIAP1, we designed SNIPER-4 in which the ester-bond is substituted to amide-bond, because our previous study indicated that bestatin methyl amide could bind to cIAP1 without reducing it [13]. Fig. 1C shows that the SNIPER-4 reduced the level of CRABP-II but not cIAP1 in HT1080 cells as we speculated. SNIPER-2 reduced both the CRABP-II and cIAP1, while MeBS reduced cIAP1 specifically. Likewise, in HT1080 cells expressing FLAG-tagged CRABP-II, SNIPER-4 reduced FLAG-CRABP-II but not cIAP1. The combined use of MeBS and ATRA did not induce the degradation of CRABP-II (Supplementary Fig. 1). In addition, the reduction of CRABP-II by SNIPER-4 and SNIPER-2, and that of cIAP1 by MeBS and SNIPER-2, were all abrogated by a proteasome inhibitor, MG132 (Fig. 1D). These results indicate that SNIPER-2 induces proteasomal degradation of CRABP-II and cIAP1 while SNIPER-4 degrades CRABP-II specifically, and that the linking MeBS and ATRA in one molecule is required for the CRABP-II degradation.

Then, we evaluated the effect of SNIPERs on the target protein degradation, examining the reaction time-course and the treatment dose. As shown in Fig. 2A, SNIPER-2 and -4 reduce the CRABP-II at 1 h, then kept suppressing the CRABP-II expression over 20 h. On a longer time scale, the expression levels of CRABP-II at 24 or 48 h were lower in the cells treated with SNIPER-4 than with SNIPER-2 (Fig. 2B). This may be due to the maintenance of cIAP1 level in the SNIPER-4-treated cells (Fig. 2A and B), and/or the chemical stability of SNIPER-4 compared with SNIPER-2 since ester-bond is more easily hydrolyzed than amide-bond. We also tested the dose–response of SNIPERs on CRABP-II degradation. The CRABP-II was effectively reduced by over 3 μM and subtly affected by 0.3 μM or 1 μM of SNIPER-4 (Fig. 2C and Supplementary Fig. 2).

Next, we examined whether the SNIPERs induce the ubiquitylation of CRABP-II as we assumed. Lysates from the cells expressing FLAG-tagged CRABP-II and HA-tagged ubiquitin were immunoprecipitated with anti-FLAG (CRABP-II) and the immunoprecipitates were analyzed by Western blot with anti-HA (ubiquitin) to detect the ubiquitylated CRABP-II. Smear protein bands that migrate slowly in the gels increased by SNIPER-2 and SNIPER-4 (Fig. 3), which indicates the poly-ubiquitylation of CRABP-II. We further examined whether cIAP1 is the ubiquitin ligase responsible for the degradation of CRABP-II in the SNIPER-treated cells. Silencing cIAP1 expression by siRNAs significantly suppressed the SNIPER-mediated CRBAP-II degradation, whereas silencing XIAP, a close family member of cIAP1, did not (Fig. 4A and B). Expression of cIAP2, another close family member, is hardly detected in HT1080 cells (data not shown). These results indicate that cIAP1 is the primary ubiquitin ligase for CRABP-II.

Fig. 5 shows the schema of the protein knockdown by MeBS, SNIPER-2 and SNIPER-4. MeBS interacts with BIR3 domain of cIAP1 to induce auto-ubiquitylation mediated by its RING domain for proteasomal degradation (Fig. 5, top). SNIPER-2 with ester-bond cross-links cIAP1 and CRABP-II, and induces ubiquitylation of both proteins, resulting in the degradation of cIAP1 and CRABP-II by proteasome (Fig. 5, middle). SNIPER-4 with amide-bond also cross-links cIAP1 and CRABP-II, but it specifically induces proteasomal degradation of CRABP-II but not cIAP1 (Fig. 5, bottom). Thus, the amide-type SNIPER-4 would be more useful than the ester-type SNIPER-2 for protein knockdown in terms of the specificity and prolonging the duration of the degradation process.

The reason why SNIPER-4 does not degrade cIAP1 is not fully understood. We previously reported that bestatin-methyl ester (MeBS) induced proteasomal degradation of cIAP1, whereas bestatin-methyl amide (BE-04) did not (13). Therefore, we hypothesize that BE-04 and SNIPER-4 with amide-bond would not induce auto-ubiquitylation of cIAP1, or if any it is not enough for efficient proteasomal degradation. Probably, the robustness and/or kinetics of the auto-ubiquitylation would be much lower than that induced by MeBS and SNIPER-2 with ester-bond, and therefore, de-ubiquitylation of cIAP1 quickly occurs to prevent degradation.

To study physiological functions of certain proteins, genetic knockdown by RNA interference or genetic knockout by gene targeting was commonly applied for suppressing the expression. Comparing with such genetic methods, the protein knockdown by SNIPERs has several advantages: (i) SNIPERs are small molecules and easily delivered into cells, which is especially advantageous for medical application in future. (ii) The degradation of the target protein begins soon after the addition of SNIPERs, and therefore the protein knockdown is attained in several hours. We suppose that the protein knockdown by SNIPERs could be a complementary technology to RNA interference, and if combined it may be possible to downregulate a target protein more rapidly and robustly. This is especially the case for a long-lived protein that is insufficiently downregulated by RNA interference alone.

The protein knockdown by SNIPERs depends on cIAP1-mediated ubiquitylation of the target protein. The cIAP1 is ubiquitously expressed in a variety of tissues and cells [18], indicating that the protein knockdown by SNIPERs would be attained in most tissues and cells expressing cIAP1. Since cIAP1 is involved in NFκB signaling in some cells, the effect of SNIPERs on normal cell function should be carefully investigated. Structurally, SNIPERs could target other proteins for degradation if ATRA is substituted to ligands for other target proteins. We propose the amide-type SNIPER could be applicable to downregulate pathogenic proteins for therapeutic purposes.

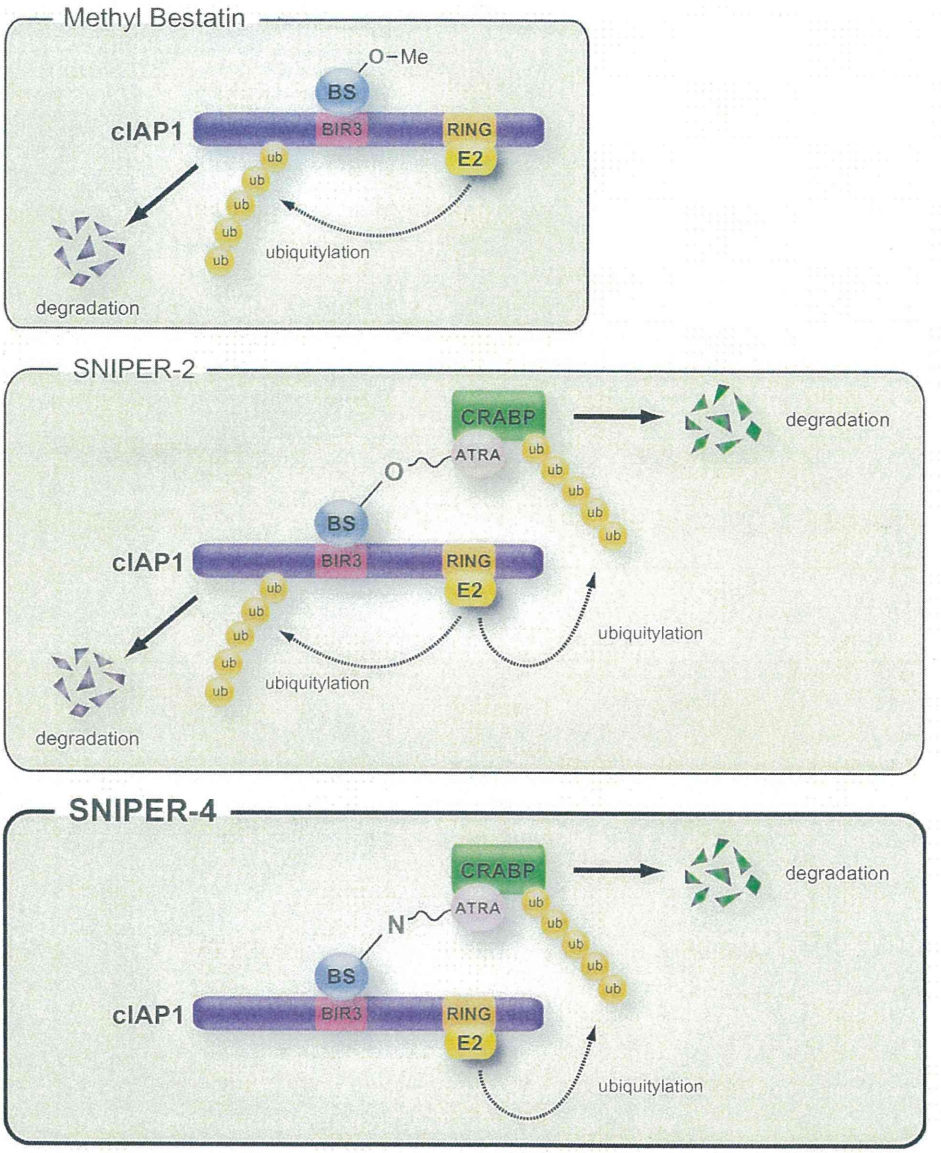

Fig. 5. Scheme of the protein knockdown by MeBS (top), SNIPER-2 (middle) and SNIPER-4 (bottom). See text for the explanation.

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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.febslet.2011.03.019.

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