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**Targeted degradation of proteins localized in subcellular compartments by hybrid
small molecules**

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Running Title

Degradation of subcellular localized protein by SNIPER

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Abbreviations

ATRA, *all-trans* retinoic acid; cIAP1, cellular inhibitor of apoptosis 1; cIAP2, cellular inhibitor of apoptosis 2; CRABP, cellular retinoic acid binding protein; ER α , estrogen receptor α ; MitoLS, mitochondria localization signal; MLS, membrane localization signal; NES, nuclear export signal; NLS, nuclear localization signal; SNIPER, Specific and Non-genetic IAP-dependent Protein ERaser; TACC3, transforming acidic coiled-coil-3; UPS, ubiquitin-proteasome system; XIAP, X-linked inhibitor of apoptosis protein.

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Abstract

Development of novel small molecules that selectively degrade pathogenic proteins would provide an important advance in targeted therapy. Recently, we have devised a series of hybrid small molecules named SNIPER (Specific and Non-genetic IAP-dependent Protein ERaser) that induces the degradation of target proteins via the ubiquitin-proteasome system. To understand the localization of proteins that can be targeted by this protein knockdown technology, we examined whether SNIPERs are able to induce degradation of CRABP-II proteins localized in subcellular compartments of cells. CRABP-II is genetically fused with subcellular localization signals, and they are expressed in the cells. SNIPER(CRABP)s with different IAP-ligands, SNIPER(CRABP)-4 with bestatin and SNIPER(CRABP)-11 with MV1, induce the proteasomal degradation of wild-type, cytosolic, nuclear and membrane-localized CRABP-II proteins, whereas only SNIPER(CRABP)-11 displayed degradation activity towards the mitochondrial CRABP-II protein. The siRNA-mediated silencing of cIAP1 expression attenuated the knockdown activity of SNIPER(CRABP)s against wild-type and cytosolic CRABP-II proteins, indicating that cIAP1 is the E3 ligase responsible for degradation of these proteins. Against membrane-localized CRABP-II protein, cIAP1 is also a primary E3 ligase in the cells, but another E3 ligase distinct from cIAP2 and XIAP could also be involved in the SNIPER(CRABP)-11-induced degradation.

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However, for the degradation of nuclear and mitochondrial CRABP-II proteins, E3 ligases other than cIAP1, cIAP2 and XIAP play a role in the SNIPER-mediated protein knockdown. These results indicate that SNIPER can target cytosolic, nuclear, membrane-localized and mitochondrial proteins for degradation, but the responsible E3 ligase is different depending on the localization of the target protein.

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Introduction

Mutations that promote the expression or activity of specific proteins often drive the excessive cell proliferation associated with various diseases such as cancer (Muller and Vousden, 2013; Pylyayeva-Gupta et al., 2011). Therefore, a therapeutic strategy of inhibiting the expression or function of such oncogenic proteins is a generally useful approach to cancer treatment. A series of oncogenic kinase inhibitors, including imatinib and crizotinib, have been developed and demonstrated to be highly effective in clinic (Drenberg et al., 2013). However, cancer cells often acquire resistance by mutating amino acids in the respective kinase domains, so that novel kinase inhibitors continue to be developed in an effort to overcome this problem (Holohan et al., 2013). In addition to the chemical inhibitors, genetic knockdown by RNA interference and antisense oligonucleotides to downregulate the expression of target proteins has been widely used in research applications and become a promising therapeutic approach for the treatment of multiple diseases, including cancer (Jung et al., 2015). However, only limited success has been achieved thus far in clinical applications of siRNA drugs (Ozcan et al., 2015). This may be mainly due to their poor cellular delivery or instability under physiological conditions (Kirchhoff, 2008). Furthermore, these genetic methods are ineffective in the case of proteins with a long half-life. Therefore, novel approach to overcome these problems is needed to achieve the

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desired therapeutic breakthrough.

The ubiquitin–proteasome system (UPS) targets many intracellular proteins for degradation and thereby controls most cellular process. To reduce the pathogenic intracellular proteins, it is useful to take advantage of UPS since it is a highly specific system to target a certain protein for degradation, and the degradation occurs very quickly. Thus, we previously devised a protein knockdown system for inducing the degradation of cellular target proteins via UPS with synthetic hybrid molecules named SNIPER (Specific and Non-genetic IAP-dependent Protein ERaser) (Okuhira et al., 2011; 2013; 2016; Ito et al., 2010; 2011a; 2011b; 2012; Demizu et al., 2012; Ohoka et al., 2014). A similar approach employing hybrid molecules PROTACs (proteolysis targeting chimeras), which is capable of targeted protein knockdown in both cultured cells and a mouse model, was reported (Bondeson et al., 2015; Toure and Crews, 2016). In addition, Winter et al. demonstrated that phthalimide-conjugated ligands for the transcriptional coactivator BRD4 induced selective degradation of BRD4 protein by hijacking the cereblon E3 ubiquitin ligase complex and delayed leukemia progression in mice (Winter et al., 2015). These studies highlight the therapeutic potential of targeting the pathogenic proteins for ubiquitylation and proteasomal degradation with bifunctional chemical compounds.

The SNIPER molecules used for protein knockdown are composed of two distinct

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molecules that are chemically linked. One is a ligand for cellular inhibitor of apoptosis protein 1 (cIAP1), while the other is a ligand for the target protein. Accordingly, SNIPER is expected to crosslink the ubiquitin ligase cIAP1 and the target protein, resulting in the ubiquitylation and proteasomal degradation of the target protein. To date, successful SNIPER applications to oncogenic proteins such as cellular retinoic acid binding protein II (CRABP-II) (Okuhira et al., 2011; Ito et al., 2010; 2011a; 2012), estrogen receptor α (ER α) (Okuhira et al., 2013; 2016; Ito et al., 2011b; Demizu et al., 2012) and transforming acidic coiled-coil-3 (TACC3) (Ohoka et al., 2014) for degradation have been reported, and some of them were able to induce cell death selectively in cancer cells (Okuhira et al., 2013; Ohoka et al., 2014).

Cells possess strict regulatory mechanisms for protein localization, and many proteins are appropriately sorted and retained in the specific organelle where their function is carried out (Hung and Link, 2011). The subcellular localization of CRABP-II and ER α are mainly in the cytosol and partially in the nuclei (Delva et al., 1999; Yoshimaru et al., 2013), suggesting that SNPERs can target cytosolic proteins for degradation, which may rely on the presence of cIAP1 and proteasome in cytosol. However, it remains unknown whether SNIPERs are able to induce degradation of target proteins in other subcellular compartments, such as in the peripheral membrane, mitochondria, or proteins that are exclusively expressed in the nuclei.

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In this study, we generated cells constitutively expressing CRABP-II proteins in cytosol, nuclei, plasma membrane and mitochondria, and examined whether SNIPERs are able to target the localized CRABP-II proteins for degradation. We demonstrated that SNIPER can induce degradation of cytosolic, nuclear, membrane-localized and mitochondrial proteins in the cells.

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Materials and Methods

Reagents. SNIPER(CRABP)-4 (“SNIPER-4” in Okuhira et al., 2011), SNIPER(CRABP)-11 (“compound 6” in Ito et al., 2012) and MV1 were synthesized as described previously (Ito et al, 2010; 2012). The following reagents were purchased from the indicated supplier: M2 anti-FLAG mouse monoclonal antibody and mouse anti- β -actin antibody (SIGMA-Aldrich, St. Louis, MO); FuGENE HD (Roche, Basel, Switzerland); anti-human cIAP1 goat polyclonal antibody (1:1000; R&D systems, Minneapolis, MN); anti-human XIAP mouse monoclonal antibody (1:500; MBL, Nagoya, Japan); Lipofectamine RNAi MAX transfection reagent and Stealth Select RNAi (Thermo Fisher Scientific, Waltham, MA). The target sequences for RNAi were as follows; cIAP1 (5’-TCTAGAGCAGTTGAAGACATCTCTT-3’); cIAP2 (5’-TTTAGACTCTGAACGAATCTGCAGC-3’); XIAP (5’-CCAGAATGGTCAGTACAAAGTTGAA-3’).

DNA constructs. cDNAs encoding human CRABP-II were amplified by PCR from a JHH-5 cDNA library and cloned into a p3xFLAG-CMV-10 expression vector (SIGMA-Aldrich), and the signal sequence was subcloned into the vector as depicted in Fig. 1A. We inserted either a triplicated nuclear localization signal (NLS) derived from SV40 Large T antigen or a nuclear export signal (NES) derived from MEK2 (Mitogen activated

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kinase kinase 2) in-frame between FLAG-tag and CRABP-II to generate NLS-CRABP-II and NES-CRABP-II, respectively (Chen et al., 2012). A membrane localization signal (MLS)-conjugated FLAG-CRABP-II (MLS -CRABP-II) was generated by adding in-frame the first 23 amino acids of the p60 c-Src (Resh et al., 1999), and a mitochondria localized signal (MitoLS)-conjugated FLAG-CRABP-II (MitoLS-CRABP-II) was generated by adding in-frame the first 29 amino acids of the cytochrome c oxidase subunit 8A (Nantajit et al., 2010), both of which were fused to the amino-terminus of FLAG-CRABP-II.

Cell culture, Transfection and Treatment with compounds. Human fibrosarcoma HT1080 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum and 100 µg/ml of kanamycin. Transfections were carried out using FuGENE HD for cDNAs and Lipofectamine RNAi MAX for siRNAs according to the manufacturer's instructions. 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 SNIPER(CRABP)s or vehicle (DMSO) for 6 h, then lysed in lysis buffer (1% SDS, 0.1 M Tris-HCl (pH 7.0), 10% glycerol) and boiled for 10 min. The protein concentration was measured by the BCA protein assay kit (Thermo Fisher Scientific) and an equal amount of protein lysate was separated by SDS-PAGE, transferred to a PVDF membrane and Western blotted using the appropriate

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antibody. Protein signals were detected using SuperSignal West Dura Maximum Sensitivity Substrate (Thermo Fisher Scientific). All the Western blot analysis were repeated at least three times from separate experiments and the densitometric analysis of proteins was carried out using ImageQuant TL software (GE Healthcare, Little Chalfont, UK). The representative blots were shown.

Immunofluorescence. FLAG-CRABP-II expressing cells were fixed with chilled methanol for 15 min at -20 °C and permeabilized with 0.25% Triton X-100 for 5 min. The cells were subjected to immunofluorescence staining with anti-FLAG (M2) antibodies (10 µg/ml) for 1 h at room temperature followed by incubation with Alexa Fluor 488-conjugated anti-mouse IgG (2 µg/ml) and Hoechst 33342 (2 µg/ml) for 1 h. Fluorescent images were obtained using a BZ-9000 (Keyence, Osaka, Japan) and the mean fluorescence intensity was calculated using the Keyence BZ-9000 analysis software.

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Results

Establishment of cell lines constitutively expressing organelle-localized CRABP-II.

We generated a series of CRABP-II constructs containing subcellular localization signals as illustrated in Fig. 1A and established cell lines constitutively expressing these CRABP-II proteins (Fig. 1B and 1C). The CRABP-II protein expression patterns were visualized using an anti-FLAG antibody and Alexa Fluor 488-conjugated secondary antibody under fluorescence microscopy to determine the subcellular localization of the CRABP-II proteins. As shown in Fig. 1B, the wild-type CRABP-II protein was diffusely expressed throughout the cells (WT), whereas the NLS-CRABP-II protein was found exclusively within the nuclei (Nuclei). In contrast, the NES-CRABP-II protein was excluded from the nuclei (Cytosol). The MLS-CRABP-II expressing cells exhibited fluorescence at the cell surface (Membrane), suggesting that the MLS-CRABP-II protein was myristoylated and anchored to the plasma membrane from the cytosol. The MitoLS-CRABP-II protein co-localized with a mitochondrial specific dye MitoTracker, indicating the mitochondrial localization of this protein (Fig. 1C, Mitochondria). The distribution of the CRABP-II proteins to the subcellular fractions was confirmed by biochemical cell fractionation. Taken together, these organelle-localized CRABP-II proteins were sorted successfully to the expected subcellular compartments.

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SNIPER(CRABP)-4 and -11 targets cytosolic, nuclear and membrane-localized

CRABP-II proteins for proteasomal degradation. To investigate whether SNIPERs can induce degradation of CRABP-II proteins localized in subcellular compartments, cells constitutively expressing the localized CRABP-II proteins were treated with SNIPER(CRABP)s and the expression of the CRABP-II protein was examined by Western blot analysis. We used two SNIPER(CRABP)s for the degradation experiments. One was SNIPER(CRABP)-4, a hybrid molecule consisting of *all-trans* retinoic acid (ATRA) as a ligand for CRABP-II and bestatin methyl amide as a ligand for cIAP1 (Okuhira et al., 2011). The other was SNIPER(CRABP)-11, a hybrid molecule consisting of ATRA and MV1, a pan-antagonist of cIAP1, cIAP2 and XIAP (Ito et al., 2012) (Fig. 2A). In the wild-type CRABP-II expressing cells, SNIPER(CRABP)-11 reduced both the cIAP1 and CRABP-II protein levels, whereas SNIPER(CRABP)-4 reduced the CRABP-II protein but had a rather smaller effect on the protein level of cIAP1 (Fig. 2B, WT). The reduction of the wild-type CRABP-II and cIAP1 by SNIPER(CRABP)s was abrogated by treatment with the proteasomal inhibitor MG132, suggesting that SNIPER(CRABP)s induce the proteasomal degradation of both proteins, as we previously reported (Okuhira et al., 2011; Ito et al., 2012).

In the cells expressing cytosolic, nuclear or membrane-localized CRABP-II proteins, the activities of SNIPER(CRABP)-4 and -11 on CRABP-II protein degradation were quite

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similar to that observed in the wild-type CRABP-II expressing cells. The treatment with MG132 also completely blocked the reduction of CRABP-II (Fig. 2B, Cytosol, Nuclei, Membrane). These results indicate that cytosolic, nuclear and membrane-localized CRABP-II proteins can be effectively targeted by SNIPER(CRABP)s and degraded via the proteasome pathway.

SNIPER(CRABP)-11, but not SNIPER(CRABP)-4, induces the degradation of mitochondrial CRABP-II protein. In the cells expressing mitochondrial CRABP-II protein, SNIPER(CRABP)-11 induced both CRABP-II and cIAP1 degradation, which was completely inhibited by MG132 (Fig. 3A and 3B, SN-11). However, SNIPER(CRABP)-4 had no significant effect on the level of mitochondrial CRABP-II protein (Fig. 3A, SN-4). This observation was confirmed by immunofluorescence analysis showing that SNIPER(CRABP)-4 significantly decreased the fluorescence intensity of the wild-type but not mitochondrial CRABP-II protein, while SNIPER(CRABP)-11 nearly abolished the fluorescence of both CRABP-II proteins (Fig. 3C). These results show that mitochondrial CRABP-II protein can be targeted for degradation via the proteasome pathway by using SNIPER(CRABP)-11, but not SNIPER(CRABP)-4.

cIAP1, but not XIAP, mediates the degradation of the wild-type, cytosolic and membrane-localized CRABP-II. Next, we examined whether cIAP1 is involved in the

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degradation of the organelle-localized CRABP-II proteins induced by SNIPER(CRABP)s. When cells were depleted of cIAP1 by siRNA, the degradation of wild-type and cytosolic CRABP-II proteins by SNIPER(CRABP)s were markedly suppressed, indicating that cIAP1 plays an essential role in the degradation of these proteins as a primary E3 ubiquitin ligase (Fig. 4, WT and Cytosol, cIAP1 siRNA). The degradation of membrane-localized CRABP-II protein by SNIPER(CRABP)-4 was also inhibited by the silencing of cIAP1, but the degradation by SNIPER(CRABP)-11 was only partially blocked (Fig. 4, Membrane, cIAP1 siRNA). This result suggests that an additional E3 ligase could be involved in the degradation of the membrane-localized CRABP-II induced by SNIPER(CRABP)-11. Since MV1 also interacts with XIAP, a close family member of cIAP1 (Varfolomeev et al., 2007), we examined whether XIAP is involved in the degradation of CRABP-II proteins by SNIPER(CRABP)s. The silencing of XIAP expression by siRNA did not block the degradation of wild-type, cytosolic and membrane-localized CRABP-II (Fig. 4, XIAP siRNA), suggesting that XIAP does not play a role in the degradation of CRABP-II proteins localized in these subcellular compartments.

Neither cIAP1 nor XIAP mediates the degradation of the nuclear and mitochondrial CRABP-II proteins. In contrast to the WT and cytosolic CRABP-II proteins, siRNA-mediated silencing of cIAP1 expression hardly affected the degradation of nuclear

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CRABP-II by SNIPER(CRABP)-4 and -11 (Fig. 5, Nuclei, cIAP1 siRNA). XIAP silencing neither affected the degradation of nuclear CRABP-II protein (Fig. 5, Nuclei, XIAP siRNA). These results suggest that cIAP1 and XIAP are not required for the SNIPER-mediated degradation of the CRABP-II proteins in the nuclei. Similarly, the silencing of cIAP1 and XIAP expression by siRNA did not block the degradation of CRABP-II localized in the mitochondria by SNIPER(CRABP)-11 (Fig. 5, Mitochondria, SN-11), though XIAP silencing minimally induced the degradation of mitochondrial CRABP-II proteins by SNIPER(CRABP)-4. This result indicates that cIAP1 and XIAP are not involved in the degradation of mitochondrial CRABP-II. Since MV1 also interacts with cIAP2 (Varfolomeev et al., 2007), we further examined the effect of triple silencing of the cIAP1/cIAP2/XIAP expression by siRNA, but found no significant effect on the degradation of CRABP-II proteins localized in the nuclei and mitochondria by SNIPER(CRABP)-4 and -11 (Supplementary Fig. 1). These results suggest that cIAP1/cIAP2/XIAP do not play a role in the SNIPER(CRABP)-induced degradation of CRABP-II proteins localized in nuclei and mitochondria.

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Discussion

We have been developing a series of SNIPERs and determining the molecular mechanism of SNIPER-dependent target protein degradation with the goal of generating therapeutic agents against multiple diseases, including cancer. To date, successful SNIPER applications against endogenous oncogenic proteins including CRABP-II (Okuhira et al., 2011; Ito et al., 2010; 2011a; 2012), ER α (Okuhira et al., 2013; 2016; Ito et al., 2011b; Demizu et al., 2012) and TACC3 (Ohoka et al., 2014) for degradation have been developed, and some of them were able to induce cell death selectively in cancer cells (Okuhira et al., 2013; Ohoka et al., 2014).

At the beginning of the investigation, we developed SNIPER(CRABP)-2, which is a hybrid molecule of a bestatin methyl ester and ATRA, and revealed that it induced the degradation of both the endogenous and exogenous CRABP-II protein in the cells (Ito et al., 2010). Next, we developed SNIPER(CRABP)-4 with a bestatin methyl amide as a ligand (Fig. 2A), which exhibited more selective degradation activity towards CRABP-II than SNIPER(CRABP)-2. SNIPER(CRABP)-4 also displayed a longer duration to reduce the protein expression than SNIPER(CRABP)-2, which might be explained by the superior chemical and biological stability of an amide-bond as compared with an ester-bond (Okuhira et al., 2011; Sekine et al., 2008). We also developed SNIPER(CRABP)-11, bearing a pan IAP-antagonist MV1 as a

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ligand (Fig. 2A), which degraded CRABP-II protein at a lower concentration than the bestatin-based SNIPER(CRABP)s (Ito et al., 2012).

In this study, to investigate whether SNIPERs can induce degradation of proteins localized in subcellular compartments, we generated cell lines constitutively expressing FLAG-CRABP-II proteins in cytosol, nuclei, peripheral membrane and mitochondria, and examined the degradation of CRABP-II proteins by SNIPER(CRABP)-4 and -11 treatment. The results demonstrated the following. 1) The CRABP-II proteins expressed in the cytosol and anchored to the plasma membrane were degraded by both SNIPER(CRABP)-4 and -11 in a proteasome- and cIAP1-dependent manner, but additional E3 ligase could also be involved in the SNIPER(CRABP)-11-induced degradation of membrane-localized CRABP-II protein. 2) Nuclear CRABP-II protein was degraded by both SNIPER(CRABP)-4 and -11 in a proteasome-dependent manner, but this degradation did not depend on cIAP1 expression. 3) Mitochondrial CRABP-II protein was targeted for degradation by SNIPER(CRABP)-11, but not by SNIPER(CRABP)-4, and the SNIPER(CRABP)-11-dependent degradation of the mitochondrial CRABP-II does not require cIAP1 expression. These findings suggest that SNIPER(CRABP)s recruit cIAP1 for ubiquitylation of cytosolic and membrane-localized CRABP-II proteins, but they also recruit different E3 ligases for ubiquitylation of nuclear and mitochondrial CRABP-II proteins. In

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addition, the data also show the potential advantage of the MV1-based SNIPER(CRABP)-11 over the bestatin-based SNIPER(CRABP)-4 to target a broad range of proteins for degradation.

Intriguingly, against the mitochondrial CRABP-II protein, SNIPER(CRABP)-11, but not SNIPER(CRABP)-4, showed an activity to degrade the protein (Fig. 3). This may be attributed to the differences in accessibility of the SNIPER(CRABP)s to the mitochondria, which depends on the hydrophobic properties of SNIPER(CRABP)s that are likely affect its ability to pass through the mitochondrial membrane. Since SNIPER(CRABP)-11, but not SNIPER(CRABP)-4, induces degradation of cIAP1, and the reduction of cIAP1 leads to the activation of caspases that is often coupled with depolarization and the breakdown of mitochondrial membrane (Gilmore et al., 2001), we tested the mitochondrial membrane integrity in the SNIPER(CRABP)-treated cells. However, SNIPER(CRABP)-4 and -11 had no effect on the fluorescence intensity of the MitoPT probe or the morphology of the mitochondria (Supplementary Fig. 2), suggesting that the SNIPER(CRABP)-11-induced degradation of CRABP-II in mitochondria was not caused by either the breakdown or hyperpermeability of the mitochondrial membrane. Another possibility is that although both SNIPER(CRABP)-4 and -11 can penetrate into mitochondria, only SNIPER(CRABP)-11 can recruit the E3 ligase for ubiquitylation of mitochondrial CRABP-II protein. Currently, we

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have not identified the E3 ligases responsible for ubiquitylation of the nuclear and mitochondrial CRABP-II proteins. Further research is needed to identify the E3 ligases and to elucidate the molecular mechanisms of protein knockdown in these cellular compartments.

Compared with genetic knockdown by RNA interference or gene targeting, the protein knockdown by SNIPERs has certain advantages. (a) SNIPERs are small molecules easily delivered into cells, which is especially advantageous for medical applications. (b) The degradation of the target protein begins to occur soon after the addition of SNIPERs, and therefore the protein knockdown is achieved in a matter of several hours, which is much sooner than the genetic methods that usually require a couple of days to downregulate the expression. In addition to these advantages, SNIPERs are able to target a variety of proteins for degradation by replacing the target-recognizing ligand moiety. It is also suggested that the protein knockdown induced by SNIPERs has the potential to become a complementary technology to RNA interference, and if combined together, it may be possible to downregulate a target protein more rapidly and robustly. This is especially the case against a long-lived protein that is insufficiently downregulated by RNA interference alone. Consequently, the protein knockdown by SNIPERs affords a new means of developing novel molecular target drugs against wide variety of diseases including cancer.

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Authorship contributions

Participated in research design: Okuhira and Naito.

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Contributed new reagents or analytic tools: Shoda, Demizu, Ito, Ishikawa, Hashimoto, and Kurihara.

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Wrote or contributed to the writing of the manuscript: Okuhira and Naito.

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Footnote

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Figure Legends

Fig. 1. Expression of CRABP-II protein in subcellular compartments. (A)

FLAG-CRABP-II constructs for localized expression. (B) Fluorescence images of the cells expressing wild-type, nuclear, cytosolic and plasma membrane-localized CRABP-II proteins. CRABP-II expression was visualized with anti-FLAG and Alexa Fluor 488-conjugated secondary antibody. Nuclei were stained with Hoechst 33342. Bar: 100 μm . (C) Fluorescent images of the cells expressing mitochondria-localized CRABP-II protein. Mitochondria were stained with MitoTracker Red. Bar: 30 μm .

Fig. 2. SNIPER(CRABP)-4 and -11 target the nuclear, cytosolic and membrane-localized CRABP-II proteins for proteasomal degradation. (A) Chemical structures of SNIPER(CRABP)-4 and -11. (B) Cells were treated with the indicated concentrations of SNIPER(CRABP)-4 or -11 for 6 h in the presence or absence of 10 μM MG132. The representative immunoblots of the cell lysates stained with the indicated antibodies are shown. Data are the mean \pm SD of 3 independent experiments; asterisks indicate $P < 0.05$ compared to DMSO-treated cells (black bars).

Fig. 3. SNIPER(CRABP)-11, but not SNIPER(CRABP)-4, induces the degradation of the

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mitochondrial CRABP-II protein. (A) Cells were treated with the indicated concentration of SNIPER(CRABP)-4 or -11 for 6 h. (B) Cells were treated with SNIPER(CRABP)-11 for 6 h in the presence or absence of 10 μ M MG132. Data are the mean \pm SD of ≥ 3 independent experiments; asterisks indicate $P < 0.05$ compared to DMSO-treated cells (black bars). (C) Fluorescent images of the cells expressing wild-type and mitochondrial CRABP-II proteins treated with 1 μ M of SNIPER(CRABP)-4 or 0.3 μ M of SNIPER(CRABP)-11 for 6 h. Bar: 100 μ m. Quantification of FLAG-CRABP-II mean intensity is shown on the bottom. Data are the mean \pm SD of ≥ 3 independent experiments; asterisks indicate $P < 0.05$ compared to DMSO-treated cells (black bars).

Fig. 4. cIAP1 but not XIAP mediates the SNIPER(CRABP)-induced degradation of wild-type, cytosolic and membrane-localized CRABP-II proteins. cIAP1 or XIAP was depleted by siRNA for 48 h, then cells were treated with SNIPER(CRABP)-4 or -11 for 6 h. The representative immunoblots of the cell lysates stained with the indicated antibodies are shown. Data are the mean \pm SD of ≥ 3 independent experiments; asterisks indicate $P < 0.05$ compared to DMSO-treated cells (black bars).

Fig. 5. Neither cIAP1 nor XIAP is required for the SNIPER(CRABP)-induced degradation

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of nuclear and mitochondrial CRABP-II proteins. cIAP or XIAP was depleted by siRNA for 48 h, and then cells were treated with SNIPER(CRABP)-4 or -11 for 6 h. The representative immunoblots of the cell lysates stained with the indicated antibodies are shown. Data are the mean \pm SD of ≥ 3 independent experiments; asterisks indicate $P < 0.05$ compared to DMSO-treated cells (black bars).

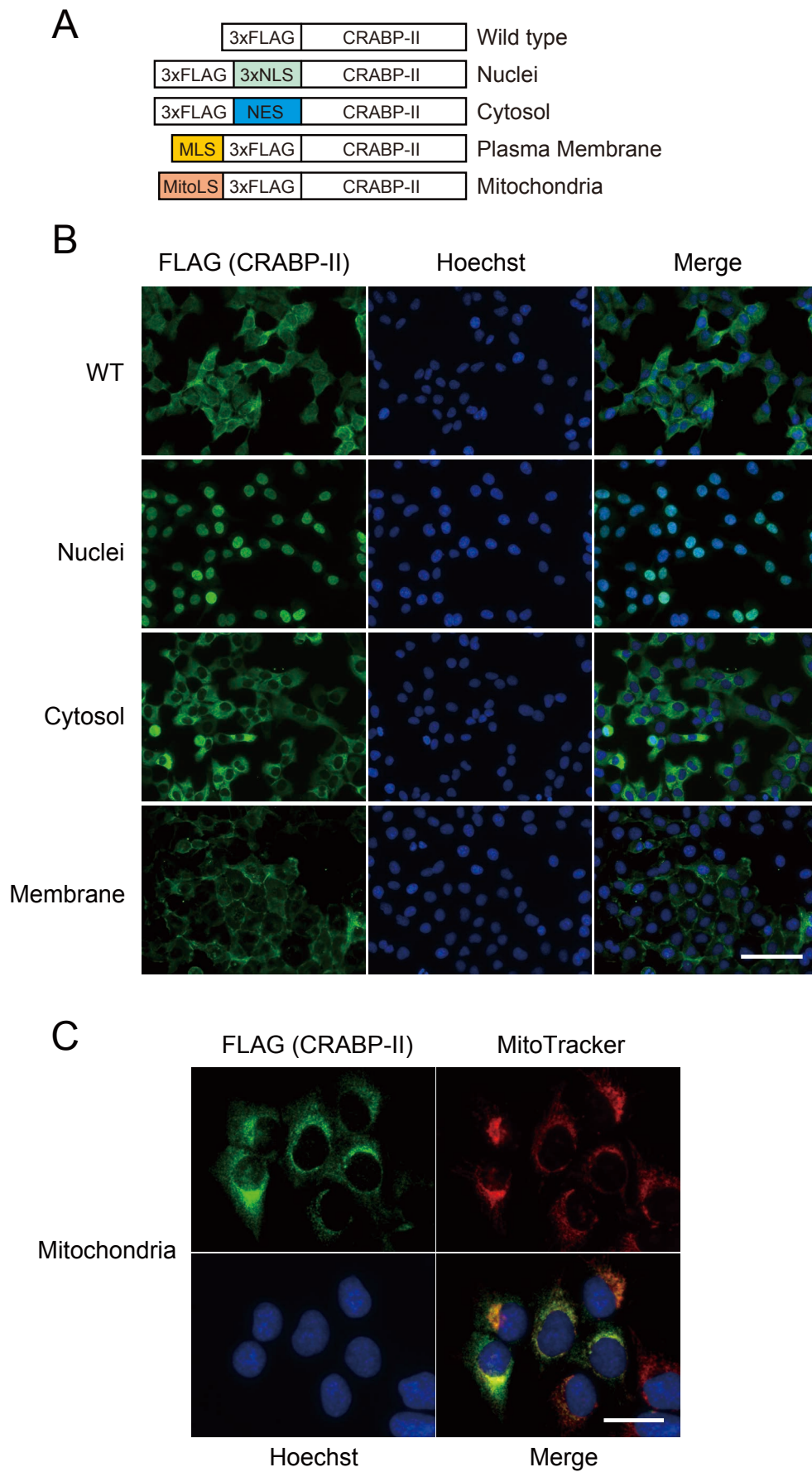


Fig.1

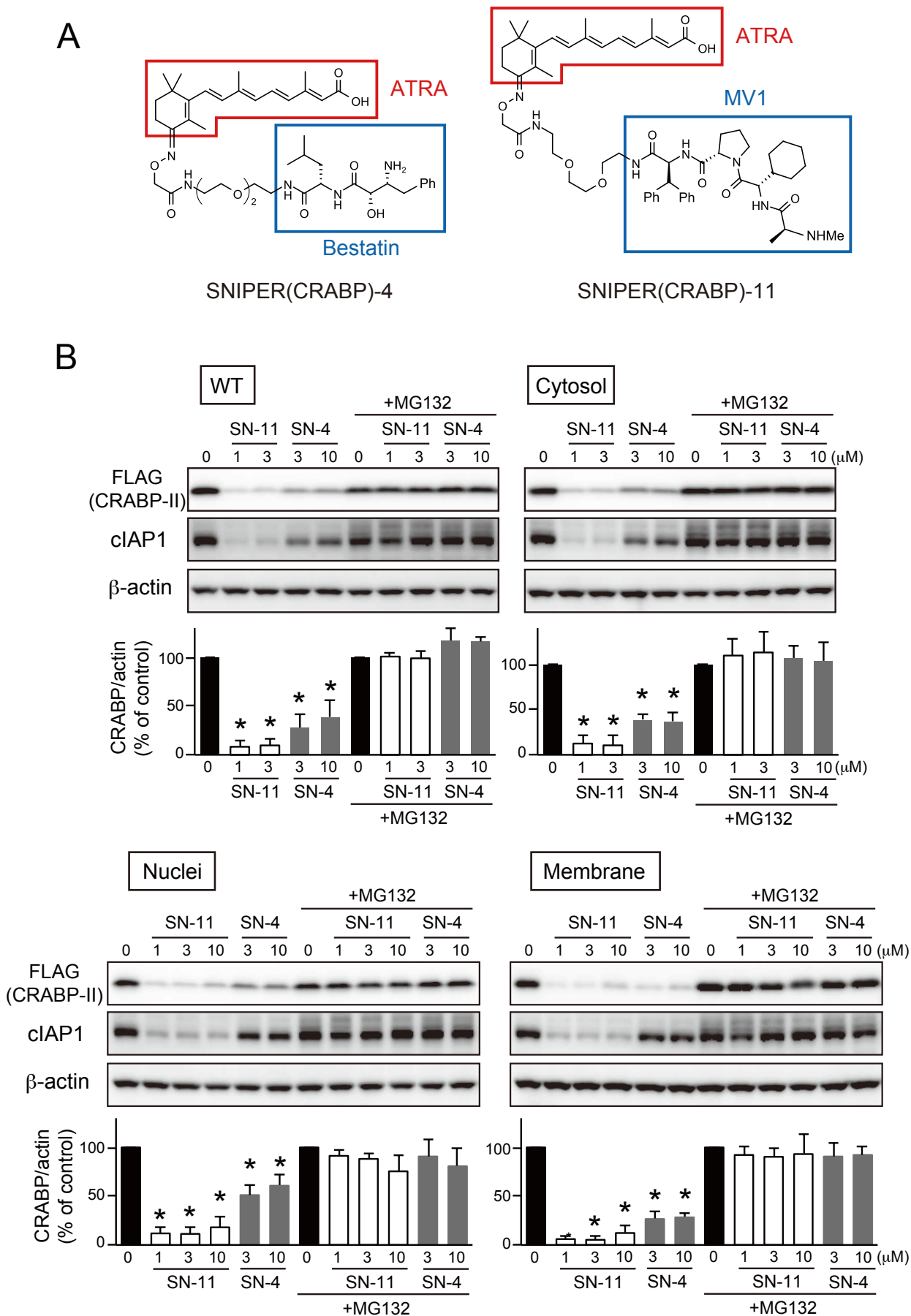


Fig.2

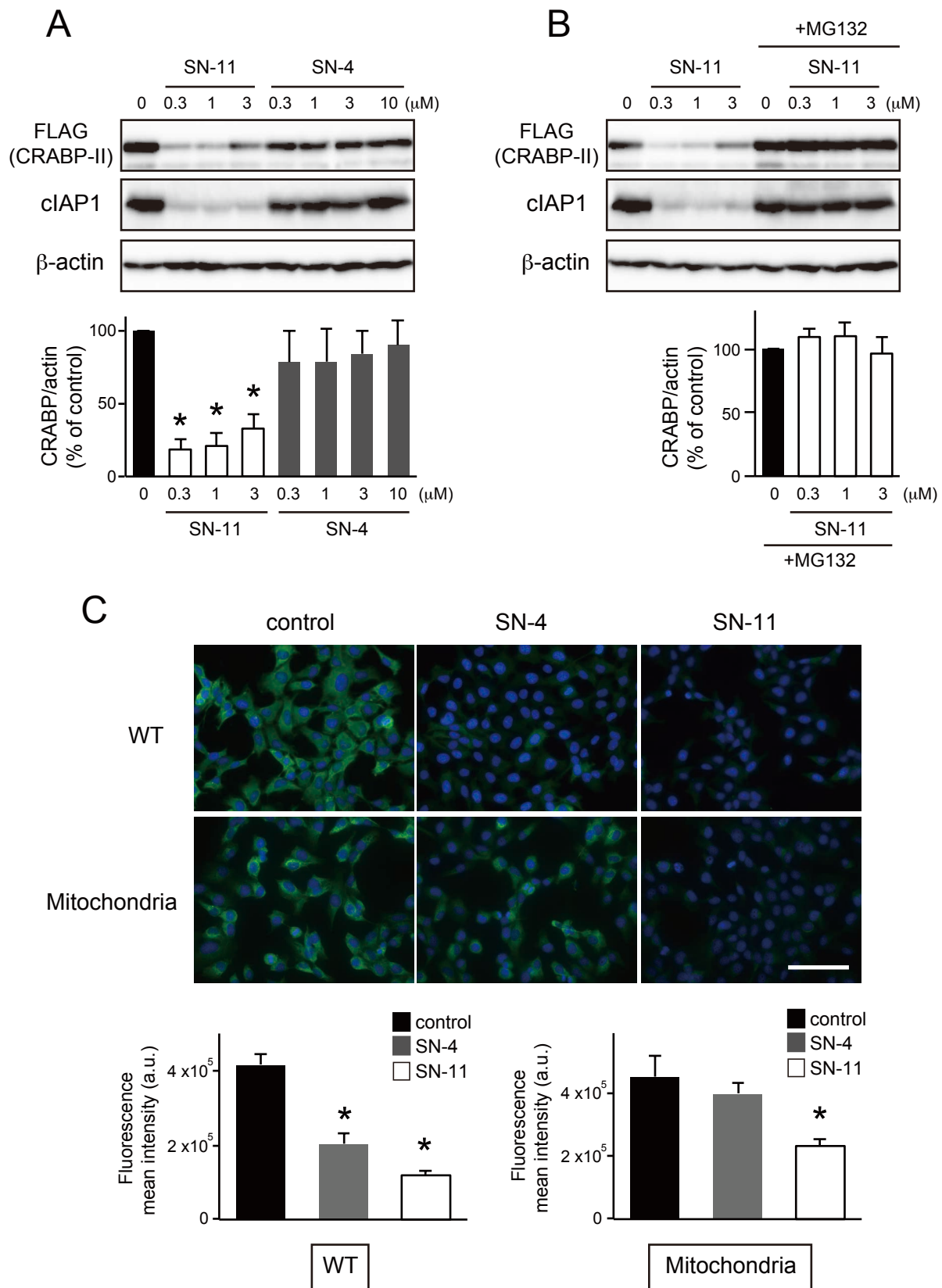


Fig.3

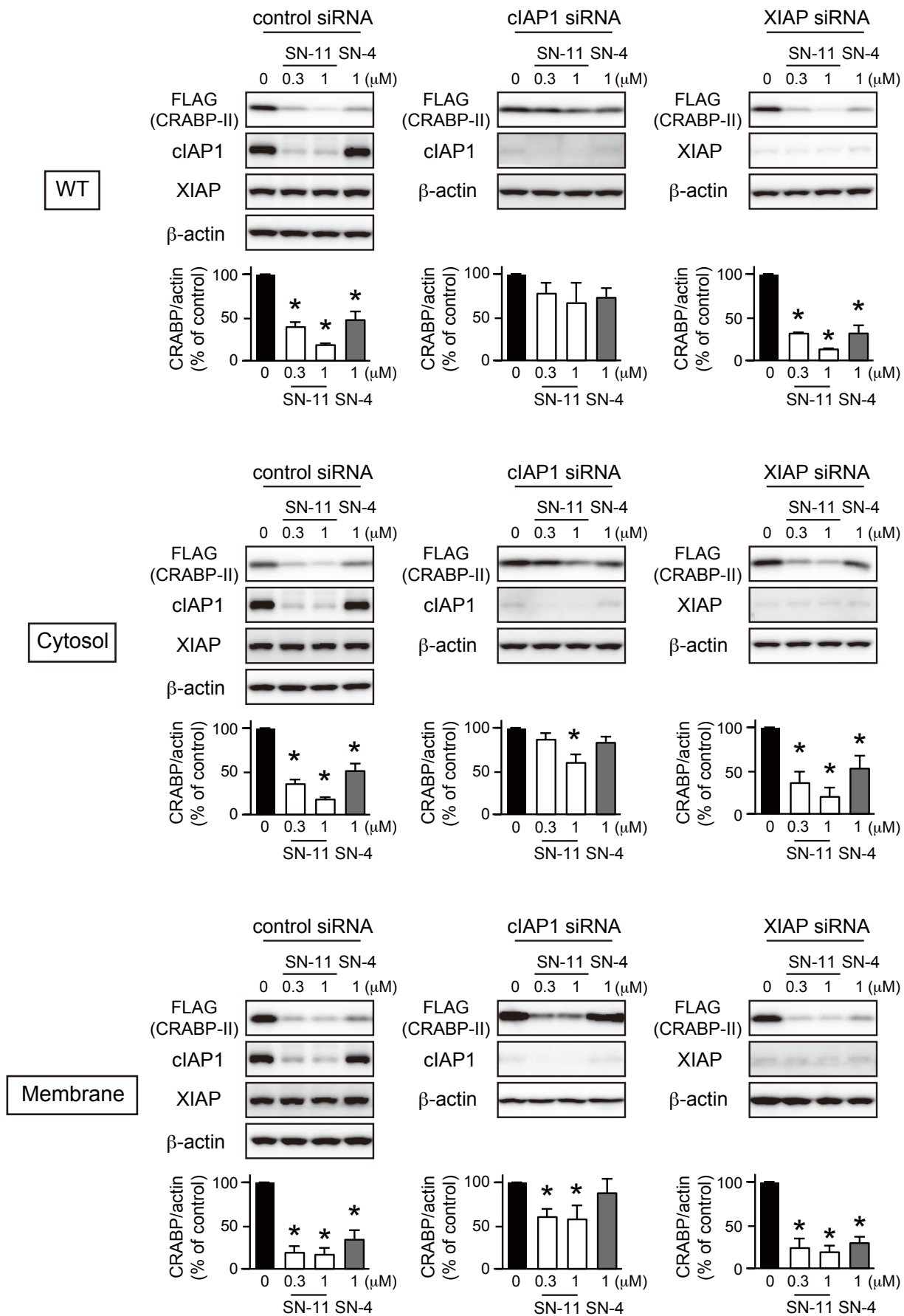


Fig.4

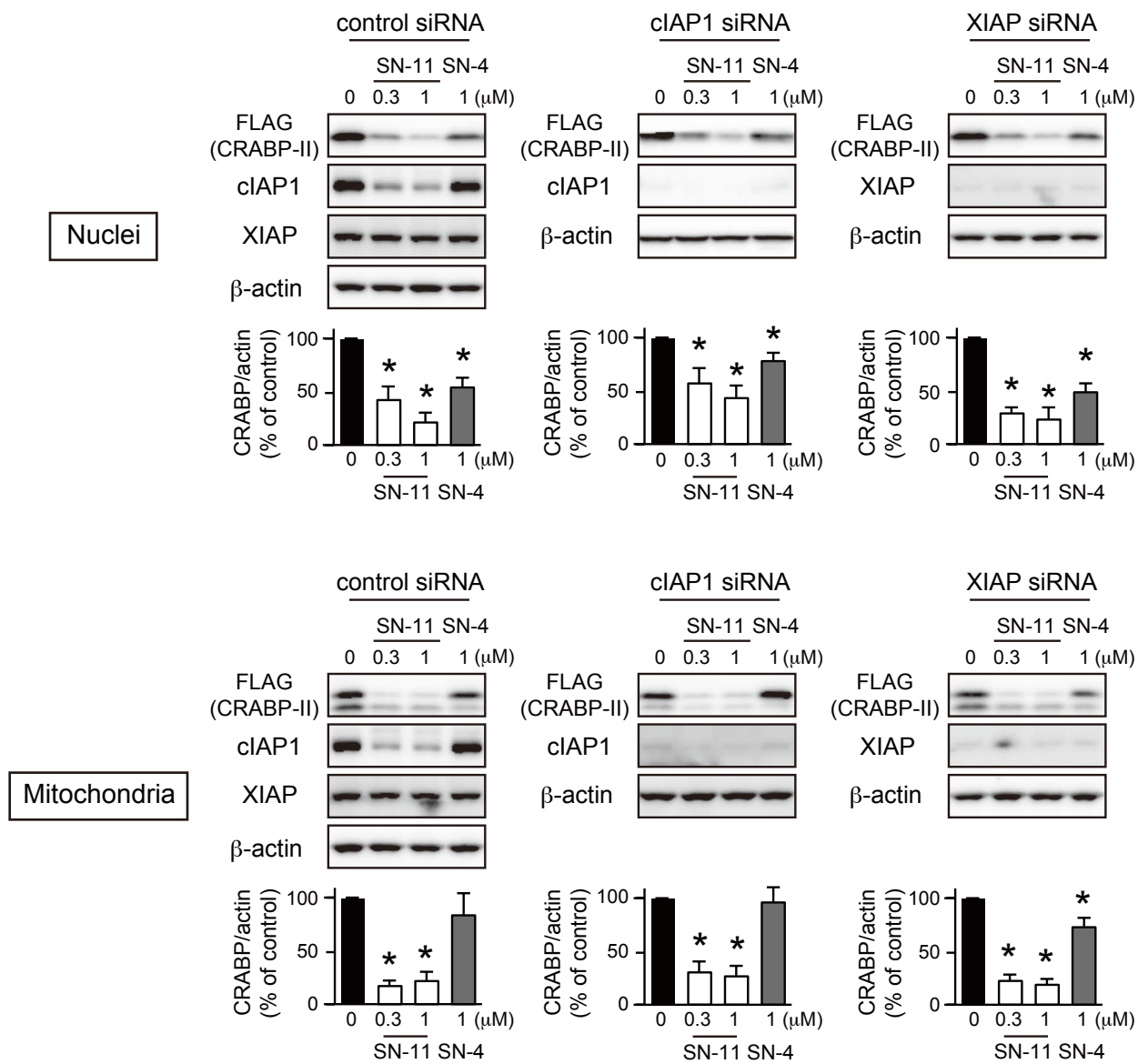


Fig.5