lated without antibody binding for selection. Ideally, this combination should be used to confirm the results of the present study. Nonetheless, the use of Sia modification to avoid non-cognate T-B interactions in GCs may result in optimal selection of GC B cells, in addition to soluble antigen-mediated apoptosis (48), interzonal migration between the light and dark zones (49), and/or competition for antigen (50).

We also showed that T-cell activation increased the levels of Sn ligands, which may function as *trans*-ligands to regulate cell-cell interactions. Sn/CD169 is expressed principally on macrophages resident in regions exposed to body fluids (51) and interacts with T cells (41, 52, 53). *Sn* knock-out ($Sn^{-/-}$) mice exhibit increases in the proportions of $CD8^+$ T cells in the spleen and lymph nodes (54), indicating that Sn-mediated cellular interaction(s) may attenuate peripheral $CD8^+$ T-cell activities, although the underlying molecular mechanism remains to be elucidated. Indeed, $CD8^+$ T cells seem to express the Sn ligand preferentially in both the mouse (Fig. 7*A*) and human (51), suggesting that this may be of functional importance. Sn/$CD169^+$ macrophages have been reported to present antigen to $CD8^+$ T cells (55). More recently, it was suggested that Sn/$CD169^+$ macrophages target $CD8^+$ T cells in terms of presentation of dead cell-associated antigens during development of anti-tumor immunity (56), and target iNKT cells with lipid antigen (39). Sn/CD169 may be transferred, as a bleb, from macrophages to lymphocytes to participate in transfer of non-cognate antigen(s) (57). Collectively, many aspects of antigen presentation may involve Sn/$CD169^+$ cells, although the roles played by Sn/CD169 and the relevant ligand in terms of such presentation remain unknown.

To explore the functional aspects of ligand expression preference, we measured the level of CTL activity using OVA-conjugated latex bead-borne antigen, a model used to explore $CD8^+$ T-cell functions (Fig. 7, *C* and *D*). The latex-conjugated antigen is acquired by antigen-presenting cells expressing Sn/CD169 (42) and subsequently triggers CTL activation (32), although the Sn/$CD169^+$ cell type involved is not known. We found that the CTL activity of *Cmah* Tg T cells lacking the Sn ligand was enhanced (Fig. 7*D*). Consideration of the spatiotemporal aspects of events leading to development of CTL is helpful when seeking to understand such enhancement in the context of Sn/CD169 ligand induction upon activation of T cells (Fig. 1*B*). Sn ligand induction occurs after activation, and resting T cells of either wild-type or *Cmah* Tg mice do not express the Sn ligand. Thus, it is rather unlikely that Sn ligand interaction is involved in initial activation of naive T cells. Although both $Sn^{-/-}$ and *Cmah* Tg mice exhibited normal thymic T-cell development, the levels of peripheral $CD8^+$ T cells increased only when Sn was deficient. This indicates that optimal-ligand-independent Sn function is important in terms of $CD8^+$ T-cell homeostasis, although the mechanism remains elusive. In general, antigen presentation by DCs is considered to activate naive T cells, and macrophages present antigens to T cells primarily to trigger the effector functions of such cells. Activated DCs migrate from the marginal zone to the T-cell zone of the spleen. Therefore, T cells must migrate to the T-cell zone if antigen presentation by DCs is to be effective. Forced suppression of Sn ligand expression may allow T cells to migrate from the marginal zone to the T-cell zone in *Cmah* Tg spleens, thus hyperactivating CTL activity toward the OVA peptide (Fig. 7*D*).

We showed that both $CD4^+$ and $CD8^+$ T cells induced Sn ligand expression after activation (Fig. 7*B*). A previous study explored the interaction of Sn/$CD169^+$ macrophages with $CD4^+$ T cells in an experimental autoimmune encephalomyelitis model. Binding of Sn/$CD169^+$ macrophages to regulatory T cells suppressed proliferation of regulatory T cells, whereas $CD4^+$ effector T cells lacking the Sn ligand were not affected (58). Therefore, proliferation of cytotoxic $CD8^+$ T cells of *Cmah* Tg mice, which cannot induce Sn ligand synthesis, may render it possible to escape Sn-mediated suppression, enhancing CTL activity. It is also possible that constitutive Neu5Gc expression in *Cmah* Tg T cells alters cellular functions during the developmental "educational" step, although the $CD4^+$/$CD8^+$ ratio was normal in unimmunized Tg mice (Fig. 2*C*). Tg-derived CMAH expression in the Tg mouse is driven by leaky promoter activity, and we cannot rule out the possibility that not only B and T cells but also other cells express Tg-derived CMAH. Such expression could influence the *in vivo* phenotype. Further work is needed to clarify whether Sn/CD169 is functionally involved in antigen presentation event(s) or simply serves as a marker of cells involved in such event(s). However, the *Cmah* Tg mouse is the first functional Sn ligand knockdown animal model that allows investigation of the ligand functionality of Sn/CD169.

In the present study, we explored the physiological significance of activation-dependent Neu5Gc suppression via both T cell-autonomous and heterocellular interaction-mediated mechanisms. Activation-dependent Neu5Gc suppression is common to both B and T cells. Regulation of the sialoglycan-siglec interaction via alteration of Sia species seems to modulate selection of the target cells with which B and T cells interact. Binding to $T_{FH}$ cells in an antigen-dependent manner is important in the context of GC B cell activity. $T_{FH}$ cells select GC B cells that have already been activated by antigen as binding partners but do not select non-activated B cells. The results of the CTL assay (Fig. 7*D*) indicate that activated $CD8^+$ T cells may choose activated DCs rather than marginal zone macrophages as binding partners. Siglec-F ligand induction may also be involved in regulation of binding partner choice.

Lymphocytes of *Cmah* Tg mice did not exhibit activation-mediated Neu5Gc suppression *in vitro*. Induction of the GL7 epitope was suppressed in LPS-activated B cells (data not shown), and induction of the Sn ligand was suppressed in activated T cells (Fig. 2*E*). However, GC-like B cells, induced *in vitro* using feeder cells (59), expressed the GL7 epitope to some extent, regardless of the level of Tg-mediated CMAH expression.[3] Therefore, remodeling of Sia species to avoid Neu5Gc suppression in GC B cells completely is not yet possible. The molecular mechanism(s) that is responsible for additional Neu5Gc suppression and compromises attempts to express Tg-derived CMAH remains unknown. Both B and T cells may use the same mechanism of transcriptional regulation and/or control of protein degradation to this end. It is important to deter-

[3] Y. Naito-Matsui and H. Takematsu, unpublished observations.

mine how Neu5Gc synthesis is suppressed in GC cells; this is a major challenge in the field. The answer may shed light on the apparent silencing of Neu5Gc expression in neural tissues.

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# Aspirin augments the expression of Adenomatous Polyposis Coli protein by suppression of IKKβ

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ABSTRACT

Aspirin has been widely used as analgesic, antipyretic and anti-inflammatory medicine for long. In addition to these traditional effects, clinical studies suggest that aspirin can protect against cancer, but its mechanism has not been explored. To unveil it, we identified the proteins up- or down-regulated after incubation with aspirin by using proteomics analysis with Nano-flow LC/MALDI-TOF system. Interestingly, the analysis identified the protein of Adenomatous Polyposis Coli (APC) as one of the most up-regulated protein. APC regulates cell proliferation or angiogenesis, and is widely known as a tumor-suppressing gene which can cause colorectal cancer when it is mutated. Western blots confirmed this result, and real-time PCR indicated it is transcriptionally regulated. We further tried to elucidate the molecular mechanism with focusing on IKKβ. IKKβ is the essential kinase in activation of nuclear factor-kappa B (NF-κB), major transcriptional factors that regulate genes responsible for inflammation or immune response. Previous reports indicated that aspirin specifically inhibits IKKβ activity, and constitutively active form of IKKβ accelerates APC loss. We found that aspirin suppressed the expression of IKKβ, and the deletion of IKKβ by siRNA increases the expression of APC in HEK294 cells. Finally, we observed similar effects of aspirin in human umbilical vein endothelial cells. Taken together, these results reveal that aspirin up-regulates the expression of APC via the suppression of IKKβ. This can be a mechanism how aspirin prevents cancer at least in part, and a novel link between inflammatory NF-κB signaling and cancer.



## 1. Introduction

Aspirin has been widely used as analgesic, antipyretic and anti-inflammatory medicine for more than a century. In addition to these traditional effects, recent clinical studies revealed that aspirin has protecting effect against colorectal cancer [1] and several common cancers [2]. Although considerable heterogeneity exists within and between studies, the magnitude of the effect is remarkably consistent, despite differing dosing regimens, duration of use, study populations and geographical locations.

The pharmacological mechanism of aspirin has been intensively explored by researchers. Aspirin inhibits the cyclooxygenase (COX) enzymes COX-1 and COX-2, which synthesize inflammatory mediators like prostaglandins and thromboxanes [3]. Aspirin can also inhibit the transcriptional factors NF-κB pathway involved in the pathogenesis of the inflammatory response [4,5]. Yin et al. have reported that aspirin is the specific inhibitor of IKKβ, which is the essential kinase for NF-κB activation [4]. These reports can account for the classical analgesic effect of aspirin, but the mechanism for its pleiotropic effects of preventing cancer has not been fully explored.

In this study, we identified proteins that are up- or down-regulated by incubation with aspirin using proteomics analysis. Interestingly, treatment with aspirin remarkably increased the expression of APC protein. APC is tumor-suppressing gene and can cause colorectal cancer when it is mutated [6,7]. We further tried to explore the mechanism how aspirin regulates the expression of APC protein, and found IKKβ is the molecule bridging between aspirin and APC. These results could be a new mechanistic insight how aspirin prevents cancer, and reveal a new link between inflammatory NF-κB signaling and tumorigenesis.

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## 2. Materials and methods

### 2.1. Antibodies and reagents

The antibodies that were used included anti-IKKβ (Millipore, Billerica, MA, USA), anti-APC (Millipore), anti-APC (Abnova, Taipei City, Taiwan), a series of antibodies to anti-GAPDH, anti-mouse IgG HRP-linked antibody and anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology, Boston, MA, USA (CST)); The reagents that were used acetylsalicylic acid (Aspirin), and cycloheximide (Sigma–Aldrich, St. Louis, MO, USA (Sigma)). Real-time reagents, TaqMan Gene Expression Master Mix and TaqMan Gene Expression Assay were purchased from Applied Biosystems (Foster City, CA, USA (ABI)). Small interfering RNA (siRNA) expression constructs for silencing IKKβ (pKD-IKKb-v3) and control siRNA (pKD-NegCon-v1) were purchased from Upstate (NY, USA). LIVE/DEAD viability/cytotoxicity kit (Thermo Scientific, Rockford, IL, USA).

### 2.2. Cell culture

The human embryonic kidney 293T cell line (HEK293T) was maintained in DMEM (Wako Pure Chemical Industries, Ltd., Osaka, Japan (Wako)), containing 10% FBS (Gibco, Foster City, CA, USA), 1% penicillin G/streptomycin (Sigma) at 37 °C under 5% $CO_2$. Human umbilical vein endothelial cells (HUVECs) were maintained in Endothelial cell Basal Medium-2 (EBM-2) (Lonza, Basel, Switzerland) at 37 °C under 5% $CO_2$.

### 2.3. Proteomics analysis

HEK293T cells were incubated with 10 mM aspirin for 48 h, and cell culture medium was subjected for analysis after freeze-dried. Sample was reduced with 45 mM of DTT (Wako), alkylated with 100 mM of iodoacetamide (Sigma), and digested with 2000 ng of trypsin (Promega, Madison, WI, USA). One-dimensional peptide fractionation was performed with a DiNa Direct Nano-flow LC/MALDI-TOF system (KYA Technologies, Japan) using a reverse-phase (RP) trap column (HiQ Sil C18-3, 0.8-mm i.d. × 3 mm) and an RP analytical column (HiQ Sil C18-3 Gradient, 0.15-mm i.d. × 50 mm). Peptides was subjected to the trap column and sequentially to the analytical column using a gradient of 0–50% solvent B in solvent A over 65 min [solvent A: 0.1% trifluoroacetic acid (TFA), 2% acetonitrile; solvent B: 0.1% TFA, 70% acetonitrile] and 50–100% solvent B for 15 min at a flow rate 200 nL/min. The RP column eluent was spotted onto a MALDI sample plate using a DiNa Direct Nano-flow LC/MALDI-TOF system (KYA tech.) and analyzed using a 4800 mass spectrometer (ABI). The peptides were fragmented under collision-induced dissociation conditions to give fragment ions that produce sequence information for the peptide. The software packages used for data acquisition and analysis were GPS explorer (ABI) and Mascot (Matrix science, Boston, MA, USA), respectively. Parameters of tolerance for the searches were set to 100 ppm for the MS and 0.2 Da for the MS/MS analyses, respectively.

### 2.4. Western blotting

Cells were cultured in the 6 cm-dishes and treated with aspirin or indomethacin for 24, 48, 72 h. Cells were scraped into PBS at 4 °C and pelleted (250 g for 5 min), and lysed by addition of the 1× Cell Lyses Buffer (CST) including 1 mM phenylmethylsulfonyl fluoride. After 20 min on ice, lysates were separated (13,000 g for 15 min), and protein content was determined using Dc Protein Assay Kit (Bio-Rad, Hercules, CA, USA). The protein in reducing sample buffer was heated at 95 °C for 5 min, and resolved in 4–20% mini-PROTEAN TGX Gel (Bio-Rad) for 35 min at 200 V. Then, the protein was transferred onto nitrocellulose membrane (Bio-Rad) at 100 V for 90 min. The membrane was pre-blotted in 5% milk-TBST buffer for 30 min, and blotted with first antibody for overnight and species-specific HRP-conjugated secondary antibody (CST) for 60 min. Detection of the bound antibody was performed using ECL reagent (GE Healthcare, Uppsala, Sweden).

To detect multiple signals from one membrane, the membrane was treated with a stripping buffer (Thermo Scientific) for 20 min at RT to remove the bound antibody. All the experiments were conducted for three or more times. Intensity of the immunoblot signal is analyzed quantitatively using ImageJ software.

### 2.5. Real-time PCR

Cells were scraped into PBS at 4 °C and pelleted (425 g for 3 min). Total RNA was extracted from cells using RNAqueous (Ambion, Foster City, CA, USA) according to the manufacturer's instructions. RNA (~2 μg) was converted to cDNA with the High Capacity RNA-to-cDNA kit (ABI). For quantitative real-time PCR analysis of APC, IKKβ and 18S rRNA reactions were performed using primer-probe sets (ABI). Using an ABI 7300 Real Time PCR System, PCR products were generated in cycle threshold (Ct) between the gene of interest and 18S rRNA using the equation $RQ = 2^{-\Delta\Delta Ct}$ and analyzed using SDS software version 1.4 (ABI).

## 3. Results

### 3.1. Proteomics analysis identified APC protein as one of the most up-regulated proteins by incubation with aspirin

To identify proteins affected by treatment with aspirin, HEK293T cells were incubated with aspirin and cell culture medium were subjected to proteomics analysis utilizing Nano-flow LC/MALDI-TOF system (Fig. 1A). Considering that all clinical reports indicated long-term use of high-dose aspirin is required for the anti-cancer effect, 48 h of incubation with high dose of 10 mM aspirin was applied. It was confirmed the treatment has no toxic effect on cells (Fig. 1B). Result indicated that 101 proteins were up-regulated after aspirin incubation, and APC protein was identified as one of the most up-regulated proteins.

### 3.2. Treatment with aspirin transcriptionally augments the expression of APC protein

To confirm the augmented expression of APC protein by aspirin, we checked the APC protein in HEK293T cells after incubation with aspirin. Cells were incubated with various concentrations of aspirin for 24 and 48 h, and cell lysate was subjected to Western blot. Interestingly, 24-h incubation with aspirin didn't increase the expression of APC protein, but 48-h incubation dramatically increased its expression in the dose of 10 mM (Fig. 2A), reproducing the result of proteomics analysis.

We also examined whether the change of its expression is transcriptionally or post-transcriptionally regulated. As shown in Fig. 2B, treatment with cycloheximide, inhibitor of protein synthesis, blocked the up-regulation of APC protein even after 48-h incubation of aspirin. Real-time PCR results indicated that treatment with aspirin increased mRNA for APC protein in a dose-dependent manner (Fig. 2C). These results indicate that aspirin transcriptionally up-regulates expression of APC.

### 3.3. Deletion of IKKβ augments the expression of APC protein

We tried to explore the molecular mechanism how aspirin augments the expression of APC protein. Yin et al. have reported

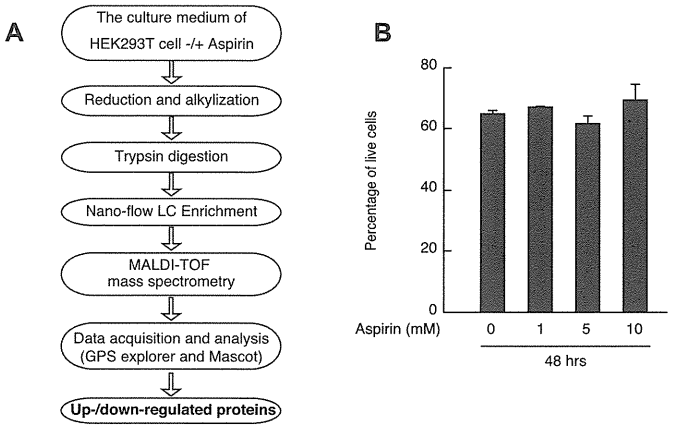


**Fig. 1.** Identification of up-/down-upregulated proteins by incubation with aspirin using proteomics analysis. (A) Proteomics analysis procedure. (B) The percentage of live HEK293T cells after incubated with various concentrations of aspirin for 48 was determined by LIVE/DEAD viability/cytotoxicity kit.

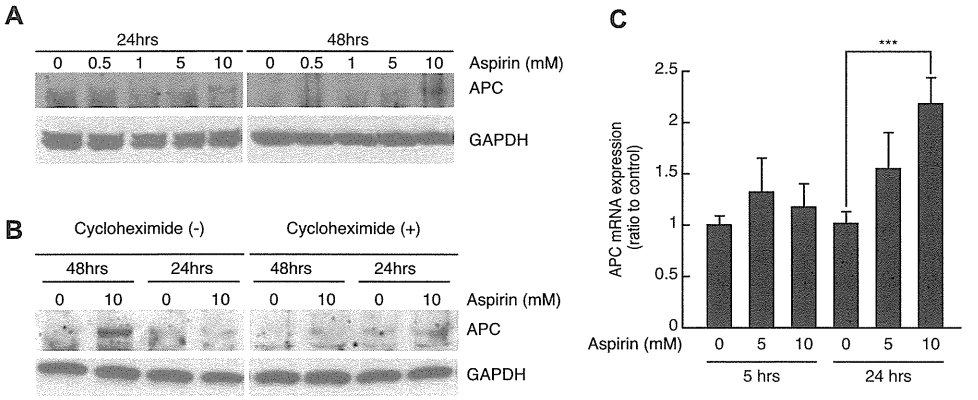


**Fig. 2.** Long-term incubation with aspirin transcriptionally increases the expression of APC protein. (A) APC immunoblots of HEK293T cells incubated with various concentrations of aspirin for 24 and 48 h. (B) APC immunoblots of HEK293T cells were incubated with 10 mM aspirin with or without cycloheximide (10 μg/mL) for 24 and 48 h. (C) Real-time PCR analysis of APC mRNA expression in HEK293T cells incubated with various concentrations of aspirin for 5 and 24 h. The data shown are from one representative experiment of the three that were performed. Error bar: ±SD. ***$P < 0.005$ in a two-sided, Student's *t*-test.

that aspirin inhibits the activity of IKKβ, which is the essential kinase in the NF-κB activation pathway [4]. Interestingly, Shaked et al. have reported that over-expression of constitutively active form of IKKβ accelerates APC loss [8]. Based on these reports, we checked the involvement of IKKβ in the up-regulation of APC protein by aspirin. We found that the deletion of IKKβ by siRNA dramatically increased the expression of APC protein (Fig. 3A) and aspirin suppresses the expression of IKKβ (Fig. 3B). These results implicated that the augmented expression of APC induced by aspirin is due to its inhibitory effect on IKKβ at least in part.

### *3.4. Aspirin increased the expression of APC and suppressed that of IKKβ in Human Umbilical Vein Endothelial Cells*

Next we examined whether those phenomena can be observed in Human Umbilical Vein Endothelial Cells (HUVECs). As shown Fig. 4, aspirin increased the expression of APC protein and suppressed the expression of IKKβ in HUVEC, similarly in HEK293T cells.

## 4. Discussion

Several lines of clinical studies have shown that aspirin has pleiotropic effect of preventing cancer, but its mechanism has

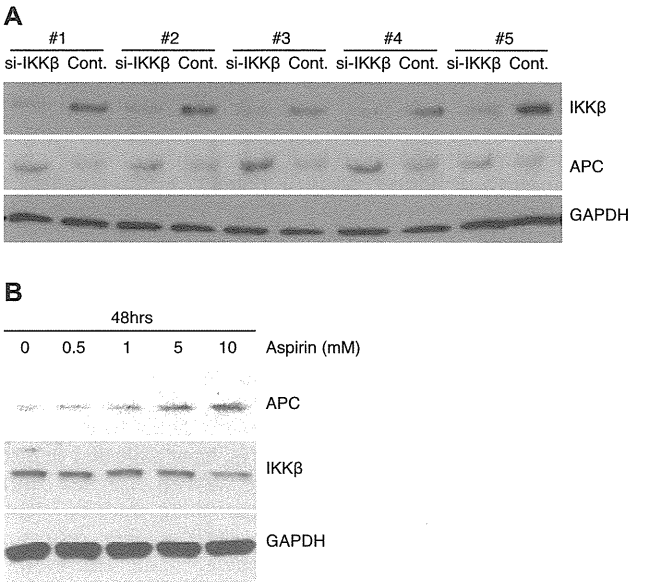


**Fig. 3.** Deletion of IKKβ increased the expression of APC protein. (A) Immunoblots of APC and IKKβ of HEK293T cells transfected with IKKβ siRNA (si-IKKβ or Control siRNA (Cont.). Five independent experiments are shown. (B) Immunoblots of APC and IKKβ of HEK293T cells incubated with various concentrations of aspirin for 48 h. The data shown are from one representative experiment of the three that were performed.

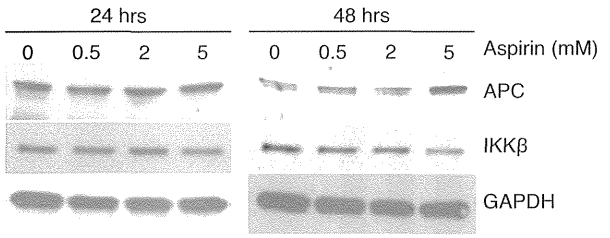


**Fig. 4.** Aspirin increased the expression of APC and suppressed that of IKKβ in HUVECs. Immunoblots of APC and IKKβ of HUVECs incubated with various concentrations of aspirin for 24 and 48 h. The data shown are from one representative experiment of the three that were performed.

not been explored yet. In this study, we found that the expression of tumor-suppressing APC protein is augmented by incubation with aspirin, possibly through suppression of IKKβ, an essential kinase in NF-κB signaling. Multiple clinical studies have reported that aspirin has preventing effect against cancer, but the effect is seen only when high dose of aspirin is used for long years [9]. It is good coincide with our results that the high dose and long incubation was required for the effect of aspirin.

The APC gene encodes a 310-kDa tumor suppressor protein which has multiple functional domains [10]. Clinical studies indicated that germ-line mutations in one allele of APC give rise to the intestinal polyp disorder, familial adenomatous polyposis (FAP), and mutation of both APC alleles occurs in tumors of FAP patients and the majority of sporadic colorectal cancers and is an early event in tumorigenesis [11]. The APC protein is expressed in most tissues and its expression in the colorectal epithelium contributes to normal growth and differentiation [12,13]. In particular, cells of the gut epithelium undergo cycles of proliferation, adhesion and migration and are sensitive to perturbations in these processes [14]. Functionally, the APC gene product modulates the oncogenic Wnt signal transduction cascade through its effects on cellular levels of β-catenin. In addition, dependent or independent of its ability to titrate β-catenin, APC affects diverse physiologic processes from cell growth to apoptosis in a number of cell types and organisms [15].

NF-κB is transcription factor playing pivotal role in inflammatory response. It generally exist in the cytosol bound to one of three inhibitory, IκB, subunits [16]. In response to a wide variety of inflammatory stimuli, NF-κB is classically activated through serine phosphorylation and degradation of IκB via the ubiquitin pathway, followed by translocation of NF-κB to the nucleus where it activates transcription. Serine phosphorylation of IκB is mediated by a large multi-unit complex containing two catalytic subunits (IKKα and IKKβ) [17,18], as well as the regulatory subunit IKKγ or NEMO, which has no kinase domain [19]. While IKKα plays an important role in skin development independent of its kinase activity [20] as well as a specialized role in the alternative pathway of NF-κB activation that induces specific genes in B cells via NF-κB2 and RelB [21], IKKβ appears to be the primary kinase mediating phosphorylation of IκB in most cell types [22].

Activation of IKKβ-NF-κB signaling in cancer has been reported by multiple groups. For example, Hu et al. have indicated that the expression of IKKβ correlates with poor survival of breast cancer [23]. In terms of its mechanism, the interesting work by Vlantis et al. demonstrates that constitutive IKKβ activity results in activation of the Wnt/β-catenin pathway [24]. However, it remained an open question whether IKKβ directly activates Wnt/β-catenin or indirectly activates Wnt/β-catenin via NF-κB or NF-κB target genes. The observation in this study that IKKβ has inhibitory effect on tumor-suppressing APC protein can be a missing piece to fit in.

However, it is still unclear how the loss of IKKβ up-regulated the expression of APC protein. Shaked et al. have shown that the over-expression of constitutively active of IKKβ accelerates APC loss through iNOS up-regulation [8]. Besides that, our proteomics analysis for identifying binding protein with IKKβ indicated that Cbl, and E3 ligase, binds with the constitutively active of IKKβ (data not shown) and Choi et al. have shown that APC protein is down-regulated by Ubiquitin-Proteinase Pathway by E3 ligase [25]. The further exploration of the mechanism would be of great interest.

## Acknowledgments

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ORIGINAL REPORT

# The increase in prescriptions of bisphosphonates and the incidence proportion of osteonecrosis of the jaw after risk communication activities in Japan: a hospital-based cohort study†

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## ABSTRACT

**Purpose** The purpose of this study was to investigate the impact of risk communication about bisphosphonate (BP)-related osteonecrosis of the jaw (ONJ) on the number of reported cases to the Drug Adverse Reactions Reporting System and on the incidence proportion of ONJ in a hospital-based cohort study in Japan.

**Method** We conducted a survey of the safety information on BP-related ONJ available from regulatory authorities, pharmaceutical manufacturers and academic associations. We also performed a trend analysis of a dataset from the Drug Adverse Reactions Reporting System and a sub-analysis, using previously constructed data from a retrospective cohort study.

**Results** Risk communication from pharmaceutical manufacturers and academic associations began within 1 year after revisions were made to the package inserts, in October 2006. Twenty times more cases of ONJ have been reported to regulatory authority since 2007, compared with the period before 2007. In our cohort, the incidence proportion of ONJ during and after 2009 was four times greater than before 2009. During this period, BPs were frequently prescribed, whereas there was no increase in the use of alternative agents, such as selective estrogen receptor modulators.

**Conclusion** ONJ was increasingly diagnosed after risk communication efforts, but the impact of the communications was not clear. Safety notifications were diligently disseminated after the package insert was revised. However, there was no surveillance for ONJ before the revision. © 2014 The Authors. *Pharmacoepidemiology and Drug Safety* published by John Wiley & Sons, Ltd.

KEY WORDS—risk communication; osteonecrosis of the jaw; oral bisphosphonates; pharmacoepidemiology

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## INTRODUCTION

Osteonecrosis of the jaw (ONJ), also called osteomyelitis of the jaw, is defined as the presence of exposed bone in the maxillofacial region that does not heal within 8 weeks.[1–3] ONJ has received increasing attention since case reports about patients exposed to bisphosphonates (BPs) were published in 2003.[4,5] In the United States of America (USA), regulatory authorities first indicated safety concerns about zoledronic acid and pamidronate with regard to osteonecrosis in 2003.[6] In 2004, the manufacturer of zoledronic acid revised the package insert in the USA and issued a "Dear Health Professional" letter.[7] Safety notifications regarding osteonecrosis were issued in other regions, such as Canada, Australia, New Zealand[7]

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and Japan, in 2004 and 2005. Early case reports were followed by the publication of epidemiological studies in 2005 and 2006.[8–10] Thereafter, position papers,[11] guidelines[12] and expert panel recommendations[3,13] were published in 2006 and 2007. Some of these papers cautioned patients receiving oral BPs.[3,11,13] The risk of ONJ for patients receiving oral BPs was considered much lower than the risk for patients receiving intravenous BPs.[11,13] However, the incidence proportion of an adverse reaction was not fully studied until later, when the risk associated with oral BPs was proved to be smaller than that for intravenous BPs.[14,15]

Although dissemination of safety information to health care professionals or patients is the most common method for minimizing risk when a novel safety concern is discovered, the impact of risk communication has remained unknown and cannot be guaranteed to result in the intended effect.[16,17] Few studies have addressed the long-term impact of risk communication on the incidence of adverse events and whether adverse events have been successfully reduced. Instead, the impact of risk communication is often assessed by measuring processes such as changes in drug use and by laboratory monitoring.[17] Because ONJ is uncommon in the general population and its background incidence rate is low, we attributed an increase in disease reports to greater recognition of the disease among BP-exposed patients after risk communication, if the characteristics of the patients and the use of BPs did not change substantially. We expected that the risk communication initiative would decrease the incidence proportion of ONJ among BP-exposed patients, after a temporary increase.

The purpose of this study was to investigate the impact of risk communication on oral BP-related ONJ in Japan; on the number of reported cases to the Japanese regulatory authority, the Drug Adverse Reactions Reporting System of the Pharmaceuticals and Medical Devices Agency (PMDA); and on the incidence proportion of ONJ in a hospital-based cohort study of 6923 osteoporosis patients at Kyoto University Hospital.

## METHODS

We surveyed safety information about oral BP-related ONJ that was produced by the PMDA, pharmaceutical manufacturers and academic associations. We also conducted a trend analysis of a dataset from the Drug Adverse Reactions Reporting System of the PMDA and a sub-analysis, using the previously constructed data from a retrospective cohort study that was conducted at Kyoto University Hospital from February 2011 to July 2012.[18] The protocol was approved by the Ethical Committee of the Graduate School of Medicine, Kyoto University (E1445).

### *Risk communication regarding oral BP-related ONJ*

First, we surveyed the safety information from the PMDA by searching the PMDA Web site for the words "jaw" or "BPs" (accessed June to July 2012). We extracted articles on periodic safety information and letters and guidance publications, and we listed the relevant information after removing duplicate information. Second, we surveyed the types of risk communication materials concerning oral BP-related ONJ that were released by manufacturers marketing oral BPs in Japan and how and when they were disseminated. Two pharmaceutical companies collected letters and guidelines from the 10 manufacturers marketing oral BPs in Japan between July 2012 and January 2013. Finally, we collected information on the risk communications materials (type, timing of dissemination and method of dissemination) that were released by two academic associations (the Japanese Society of Oral and Maxillofacial Surgeons and the Japanese Society for Bone and Mineral Research) between July and August 2012. One of the authors, a medical doctor, reviewed the collected communications materials and summarized the warnings and recommendations announced in the communications.

### *Reported cases of ONJ to the regulatory authority*

A dataset containing the adverse drug reactions reported to the Drug Adverse Reactions Reporting System of the PMDA between April 2004 and December 2011 was downloaded, and the cases of ONJ suspected to be adverse reactions to osteoporosis medications (including oral BPs) were counted. We used the preferred terms in the Standardized Medical Dictionary for Regulatory Activities (MedDRA) Queries for "osteonecrosis," with the exception of anatomically irrelevant terms, to retrieve the cases of ONJ. The list of drugs included in this study is shown in Appendix 1.

### *Cohort study*

We conducted a cohort study of outpatients and inpatients who were diagnosed with osteoporosis, using the International Classification of Diseases (ICD-10) code (Appendix 2), and who received at least one prescription for an osteoporosis medication at Kyoto University Hospital during a study period (November 2000 to October 2010).[18] The exclusion criteria were as follows: age younger than 20 years old; primary or

metastatic tumors in the maxillofacial region; history of trauma or radiation therapy in the maxillofacial region; and intravenous treatment with BPs.

We extracted the clinical data from the electronic medical records (EMRs) using an EMR retrieval system.[19] This system retrieves electronic data for outpatients and inpatients at Kyoto University Hospital, including demographic data, diagnoses and ICD-10 codes, medications and injections, laboratory tests and radiological and pathological studies. The median duration of oral BP administration, co-medications and comorbid conditions were also extracted using the EMR retrieval system.

The medications administered for osteoporosis between November 2000 and October 2010 in this cohort were collected by the retrieval system. The list of drugs included in the cohort study is shown in Appendix 3. The numbers of BP users, estrogen users and other osteoporosis drug(s) users in the cohort were calculated for each year, counting patients who were prescribed medications at least once during that year, regardless of the use of other osteoporosis medications.

To identify relevant ONJ cases, we reviewed the radiographic imaging and clinical records of the patients with a diagnosis of not only ONJ but also inflammatory conditions of the jaw that were possibly related to ONJ, as specified by the ICD-10 codes (Appendix 4). The diagnostic criteria were detailed in a previous report.[18] Briefly, ONJ was diagnosed independently by two oral and maxillofacial surgeons in accordance with the proposed criteria, using the findings from panoramic X-rays, technetium bone scans, computed tomography, histological images or surgery. We grouped the cases of osteomyelitis of the jaw with ONJ because we considered it difficult to distinguish between these two diseases. The radiographic findings for jawbone infections in patients treated with BPs are similar to those for ONJ related to BPs,[20–22] and the presence of osteonecrosis is a common histopathologic finding, both in ONJ and in osteomyelitis of the jaw related to BPs.[23]

The incidence proportion of confirmed ONJ was defined as the number of manually confirmed, newly developed ONJ cases in the cohort (e.g., BP group or non-BP group) in 2000–2002, 2003–2004, 2005–2006, 2007–2008 and 2009–2010, divided by the size of the cohort for each 2- or 3-year period. The BP group included the patients who were prescribed BPs at least once during the period and/or in the past, regardless of the use of other osteoporosis medications; the non-BP group included the patients who were prescribed osteoporosis medication(s) other than BPs and those who had never been prescribed BPs.

The distinction between BP users in the drug use survey and the BP group in the incidence proportion survey was as follows: we classified a patient who received both BPs and estrogen in the same year as one BP user and one estrogen user over the same time period in the drug use survey. However, we classified the patient into the BP group rather than the non-BP group in the incidence proportion survey. This distinction was made because the impact of osteoporosis medications other than BPs on the incidence proportion of ONJ was considered to be negligible.

We evaluated the proportions of the cases recorded as inflammatory conditions of the jaw and alveolitis of the jaw (specified by ICD-10 codes K10.2, K10.3 and K10.0 [Appendix 4] in the EMR); the proportions were defined as the number of newly recorded cases of the inflammatory condition of the jaw in the EMRs of the cohort (e.g., BP users or non-BP users) during each 2- or 3-year period, divided by the size of the cohort during the period.

## RESULTS

### *Risk communication regarding oral BP-related ONJ*

The risk communication materials regarding oral BP-related ONJ, released by the PMDA, pharmaceutical manufacturers and academic associations, are listed in Table 1. The pharmaceutical manufacturers revised the package inserts in October 2006. The case reports or epidemiological studies regarding ONJ were published after the package insert was revised. Six separate but overlapping guidance announcements, in addition to the package insert, were issued. An academic association held educational meetings for health professionals and patients during their annual meeting in April 2008.

### *Reported cases of ONJ to the regulatory authority*

An increasing number of cases of ONJ that were suspected adverse reactions to oral BPs were reported to the PMDA after 2007, immediately after the safety information was disseminated (Figure 1). These cases included those with a past history of ONJ (that is, cases of ONJ that occurred earlier were reported as cases of ONJ after 2004 in the system). There were nearly 20 times more reported cases of ONJ during and after 2007, compared with the number of cases during and before 2006. Reported cases of ONJ that are suspected to have been adverse reactions to osteoporosis medications other than BPs have been rare. For reference, the estimated numbers of patients taking oral BPs in Japan were 2 082 928 in 2007 and 2 470 979 in 2008.[24]

### *Cohort study*

The cohort consisted of 6923 osteoporosis patients; 4129 were prescribed oral BPs (59.6%; mean age,

Table 1. Risk communication about oral BP-related ONJ in Japan

| Date* | Organization | Content |
|---|---|---|
| Oct. 2006 | PMDA†, pharmaceutical manufacturers | Measure: revised package insert for alendronate and risedronate<br>"ONJ has been reported in patients receiving bisphosphonates. The majority of reported cases have been associated with dental procedures, such as tooth extraction, or with local infection. Physicians should fully disclose the adverse reactions to their patients and observe them closely." |
| Jan. 2007 | pharmaceutical manufacturers | Notices to hospitals and "Dear Health Professional" letters to inform them about the content of the revised package insert |
| June 2007 | academic association | Publication of a case report[33]<br>There was one case of osteoporosis diagnosed with oral BP-related ONJ; the other case, a case of multiple myeloma, was diagnosed with iv BP-related ONJ. |
| Sep. 2007 | PMDA, pharmaceutical manufacturers | Measure: revised package insert for etidronate |
| Oct. 2007 | academic association | Publication of an observational study[34]<br>Questionnaires were sent to 239 institutions, and 30 patients with osteonecrosis were reported. Of them, 20 patients received iv BPs, eight received oral BPs and one received both. |
| Jan. 2008 | academic association | News article entitled "osteonecrosis of the jaws induced by anti-osteoporosis treatment"<br>"Patients on BP therapy requiring dental procedures should tell their dentists that they are being treated with BPs, and physicians should fully explain the adverse reactions to their patients when prescribing BPs." |
| Jan. 2008 | academic association, pharmaceutical manufacturers | Announcement of a guidance publication, entitled "Bisphosphonates and osteonecrosis of the jaw"<br>A 20-page pamphlet, with the diagnostic criteria, clinical manifestations, risk factors and epidemiology of iv and oral BP-related osteonecrosis of the jaw and instructions for physicians, pharmacists, dentists and oral surgeons |
| Mar. 2008 | academic association | Announcement of guidance publication, entitled "management of patients on BP therapy"<br>A four-page pamphlet with the diagnostic criteria, management, risk factors, epidemiology of iv and oral BP-related osteonecrosis of the jaw and instructions for physicians, dentists and oral surgeons |
| Apr. 2008 | academic association | Public meeting for citizens: "The state of osteonecrosis of the jaw related to BPs" |
| Sep. 2008 | academic association | A pamphlet, entitled "Bisphosphonates and osteonecrosis of the jaw: clinical manifestations and guidelines for management, 2008" |
| Feb. 2009 | academic association | Training session for dentists, entitled "The state of osteonecrosis of the jaw related to BPs" |
| Feb. 2009 | academic association | News article, entitled "Bisphosphonates and osteonecrosis of the jaws" |
| May 2009 | PMDA, academic association | Announcement of a guidance publication, entitled "Bisphosphonate-Related Osteonecrosis of the Jaws"[35]<br>This official therapeutic manual for severe adverse reactions included the diagnostic criteria, clinical manifestations, risk factors and management methods for iv and oral BP-related osteonecrosis of the jaw for citizens and health care professionals |
| June 2009 | academic association | Public meeting for citizens, entitled "The state and the management of osteonecrosis of the jaws related to BPs" |
| July 2009 | academic association | Training meeting regarding BP-related osteonecrosis of the jaw for health care professionals |
| Nov. 2009 | academic association | Publication of an observational study[36]<br>The follow-up survey showed that surgical treatment might be useful for BRONJ when performed at the appropriate time, and BRONJ was shown to be refractory because only nine of 17 cases were cured in these 2 years. |
| May 2010 | academic association, pharmaceutical manufacturers | Publication of a position paper[37] |
| June 2010 | PMDA | Measure: revised package inserts for alendronate, risedronate and etidronate<br>"ONJ has been reported in patients receiving bisphosphonates, regardless of the route of administration. Treating physicians should advise their patients to undergo dental examinations and to finish any invasive dental procedures, such as tooth extraction, if necessary, prior to treatment with BPs. While on treatment with BPs, these patients should have regular dental consultations and avoid invasive dental procedures." |
| Sep. 2010 | academic association | Publication of a book, entitled "The utility and osteonecrosis of the jaw of BPs" |
| Sep. 2010 | PMDA | Release of safety measures ("The progress of assessments and measures regarding BP-related osteonecrosis of the jaw"), including a survey of the number of cases of BP-related osteonecrosis of the jaw and an outline of the individual case reports reported to PMDA |

*The date indicates the first dissemination of safety information.
†PMDA: Pharmaceuticals and Medical Devices Agency.

65.0), and 2794 patients received other osteoporosis drugs (40.3%; mean age, 65.5). The median durations of administration were 364.0 days for BPs and 439.5 days for other osteoporosis drugs. For the BP group and the other osteoporosis drugs group, the numbers of patients using concomitant steroids were 2934 (71.0%) and 1508 (53.9%), respectively; the numbers of patients treated with anti-cancer drugs were 551 (13.3%) and 256 (9.1%), respectively; and the numbers of patients with diabetes were 707 (17.1%) and 442 (15.8%), respectively.[18]

The number of BP users has been increasing steadily since 2000 (Figure 2). The number of estrogen users, including users of selective estrogen receptor modulators, has been low. The number of users of other osteoporosis medications, including active vitamin D3 or calcium,

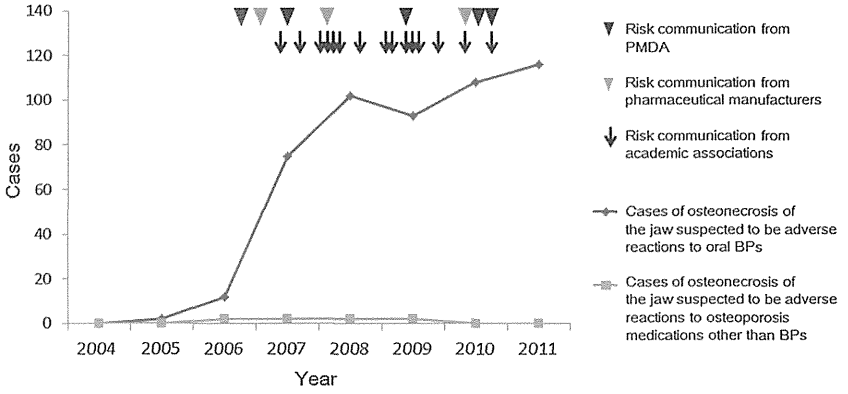



Figure 1. Trends in the number of ONJ cases per year reported to the Drug Adverse Reactions Reporting System of the PMDA and risk communication activities. Legend: The cases of ONJ that were suspected adverse reactions to oral bisphosphonates and those that were suspected adverse reactions to other agents for osteoporosis, reported to the Drug Adverse Reactions Reporting System of the PMDA, are shown as a dark gray line and a light gray line, respectively. Black arrowhead: risk communication from the PMDA; gray arrowhead: risk communication from pharmaceutical manufacturers; arrow: risk communication from academic associations

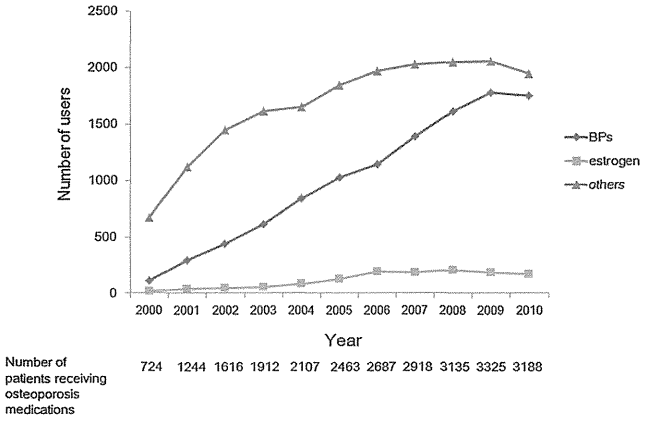



Figure 2. The number of patients prescribed each agent for osteoporosis in the cohort. Legend: The numbers of patients prescribed bisphosphonates, estrogen and a selective estrogen receptor modulator, as well as other agents for osteoporosis, each year in a cohort of 6293 osteoporosis patients are illustrated with a dark gray line of diamonds, a gray line of triangles and a light gray line of squares, respectively. The year 2000 contains 2 months, and the year 2010 contains 10 months. The numbers of patients receiving osteoporosis medications in each year are shown below the graph

increased before 2006 and since then has remained approximately constant.

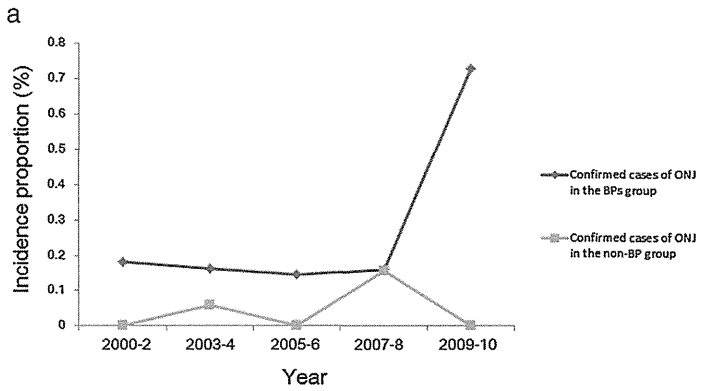
The EMRs of a total of 1987 patients with records of ONJ or inflammatory conditions of the jaw that were possibly related to ONJ were manually reviewed, and 46 patients were confirmed to have ONJ.[18]

The incidence proportion of confirmed ONJ in the BP group increased approximately four-fold in 2009 and 2010, compared with the pre-2009 level. The incidence proportion of confirmed ONJ in the non-BP group remained low (Figure 3a). Both of the incidence proportion of confirmed ONJ cases and that of inflammatory conditions of the jaw increased after 2009; however, the increase in inflammatory conditions of the jaw was not as high as that of confirmed cases (Figure 3b). This measure was therefore not a good surrogate for confirmed ONJ in this study.

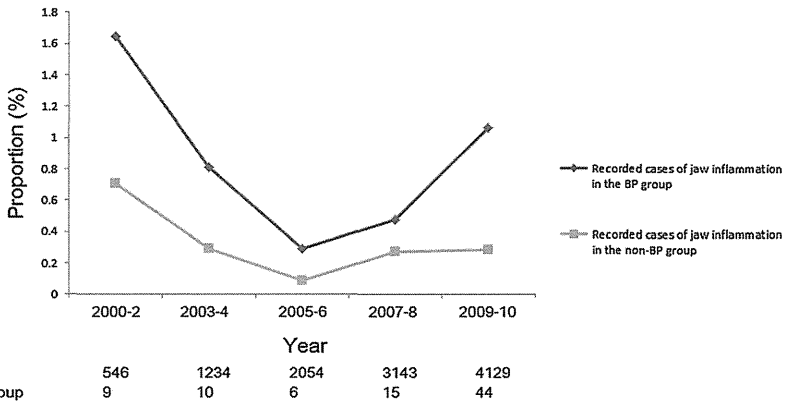
## DISCUSSION

Risk communication efforts by pharmaceutical manufacturers and academic associations began within 1 year after the package insert was revised in October 2006, and ONJ was increasingly reported to the PMDA within 1 year. In our cohort, the incidence proportion of ONJ, diagnosed according to standardized criteria, increased in 2009 and in later years. During this period, BPs were frequently prescribed, and there were no increases in the use of alternative agents, such as selective estrogen receptor modulators.

Physicians' case reports regarding ONJ in 2003[4,5] in the USA led to revisions of package inserts in 2004 to 2005.[7,25,26] In Japan, the pharmaceutical manufacturers revised the package inserts for intravenous BPs in 2005 and for oral BPs in 2006 and 2007, but the revision was delayed for 2 years after the revision in the USA. The physicians' case reports regarding ONJ were first published in 2007, 4 years after their publication in the USA; thus, the physicians' reports in Japan did not contribute to the increased suspicion of ONJ related to BPs or to the revision of the package insert. Academic associations were rather active in risk communication in the later dissemination phase. Physicians and academic associations have been able to detect new safety concerns for marketed drugs and to conduct epidemiological studies effectively, and we should reconsider academic associations, as well as the regulatory authority




| | | | | | |
|---|---|---|---|---|---|
| BP group | 546 | 1234 | 2054 | 3143 | 4129 |
| Confirmed cases of ONJ in the BP group | 1 | 2 | 3 | 5 | 30 |
| Non-BP group | 1272 | 1702 | 2236 | 2564 | 2794 |
| Confirmed cases of ONJ in the non-BP group | 0 | 1 | 0 | 4 | 0 |




| | | | | | |
|---|---|---|---|---|---|
| BP group | 546 | 1234 | 2054 | 3143 | 4129 |
| Recorded cases in the BP group | 9 | 10 | 6 | 15 | 44 |
| Non-BP group | 1272 | 1702 | 2236 | 2564 | 2794 |
| Recorded cases in the non-BP group | 9 | 5 | 2 | 7 | 8 |

Figure 3. (a). The incidence proportion of confirmed cases of ONJ in the cohort. Legend: The incidence proportions of the confirmed ONJ cases in 100 BP-group patients in 2000–2002, 2003–2004, 2005–2006, 2007–2008 and 2009–2010 are indicated by a dark gray line of diamonds. The incidence proportions of confirmed ONJ cases per 100 non-BP-group patients in each 2- to 3-year period are indicated by a light gray line of squares. The number of patients in the BP group, the number of confirmed ONJ cases in the BP group, the number of patients in the non-BP group and the number of confirmed ONJ cases in the non-BP group are shown below the graph. (b). The proportions of recorded ONJ cases in the cohort. Legend: The proportions of recorded cases of inflammatory conditions of the jaw in 100 BP-group patients in 2000–2002, 2003–2004, 2005–2006, 2007–2008 and 2009–2010 are indicated by a dark gray line of diamonds. The proportions of recorded cases of inflammatory conditions of the jaw in 100 non-BP-group patients in each 2- to 3-year period are indicated by a light gray line of squares. The number of patients in the BP group, the number of recorded cases of inflammatory conditions of the jaw in the BP group, the number of patients in the non-BP group and the number of recorded cases of inflammatory conditions of the jaw in the non-BP group are shown below the graph

and pharmaceutical manufacturers, as resources for monitoring and minimization of the risks of medicines and for ensuring the accuracy of information.

We evaluated the impact of risk communications by analyzing the prescriptions of medications for osteoporosis and the incidence proportion of ONJ. The use of BPs increased steadily, but the prescriptions for BPs were not influenced by the risk communications in this study. BPs are among the most established drug types for the treatment of osteoporosis in postmenopausal women,[27] and the gradual increase in the use of BPs over the periods, before and after the dissemination of the safety information, was reasonable considering the risk–benefit balance. We could not determine whether the physicians prescribed BPs after considering the risk–benefit balance or simply did not receive the safety information. Many confounding factors can influence the prescription of BPs, such as the active participation of academic associations or the perceptions of physicians and patients toward adverse events. Physicians might hesitate to change prescribing habits because of known obstacles, such as the lack of time during outpatient care and the desire to maintain trust in the physician–patient relationship.[28]

The rapid increase in the cases of ONJ that were suspected adverse reactions to oral BPs reported to the regulatory authority after the risk communications efforts might indicate that the primary cause of the increase was awareness of the disease because the increase was quite sharp. The incidence proportion of ONJ in the BP group increased in our cohort, although the increase occurred 3 years after the risk communications began. There would have been few missed or misdiagnosed cases of ONJ in our cohort because the cases were diagnosed based on an extensive manual review of the EMRs, using well-established criteria. There might have been other causes for the increase in the incidence proportion of ONJ in our cohort in addition to risk communication; one possibility is the longer exposure to BPs[8,29] in the cohort. Longer exposure and risk communication occurred simultaneously; therefore, we could not distinguish the impact of risk communication from that of longer exposure. There was a time difference between the increase in the number of cases of ONJ reported in the Drug Adverse Reactions Reporting System and the increase in the incidence proportion of ONJ in the cohort. The cases of ONJ reported to the Drug Adverse Reactions Reporting System include past cases of ONJ: cases that occurred before 2006 might be reported as cases of ONJ after 2006. Moreover, the diagnosis of ONJ is not standardized and might include other inflammatory conditions of the jaw. However, the number of ONJ patients in the cohort reflects the number of active ONJ patients diagnosed in the hospital. The difference between the recording and the diagnosis of ONJ most likely resulted in the time difference.

Previous reviews have found it difficult to estimate the average effect of risk communication on clinical practice[16,17,30] because of heterogeneity in the study designs, analyses, outcome measurements, therapeutic areas and types of communication. ONJ can be reduced with preventive measures, including clinical oral examinations and good oral hygiene.[31,32] Unfortunately, we did not observe a decrease in the incidence proportion of ONJ in our cohort during this study period, which would have been the clinical outcome. Additional appropriately designed research is warranted to understand the effects of past communications strategies and to estimate the impact of future communication.

The limitations of our study are described below. First, factors other than safety information collected in our study, such as pharmaceutical use, could have simultaneously influenced the incidence proportion of ONJ. Second, we did not consider the scale, the duration or the content of the risk communication; it is therefore not possible to evaluate the impact of each risk communication material quantitatively. Third, the data on drug use and on the incidence proportion of ONJ in Kyoto University Hospital were limited to a single institution in Japan; thus, the generalizability of the results cannot be assured. The much higher incidence of ONJ in our study compared to the published literature might be explained by the inclusion of numerous steroid users, older patients and inpatients. Moreover, the cohort study was subject to a referral bias toward the selection of more severe cases, given that our department is the lead institution for oral and maxillofacial surgery in Kyoto City, as discussed in our previous report.[18] We could not account for BP exposure that occurred before consultation at Kyoto University Hospital, which might have affected the incidence proportion of ONJ. Finally, this study was retrospective, using a database derived from the EMRs, and the data were not as accurate and consistent as they would have been in a prospective study.

## CONCLUSION

The use of oral BPs increased in osteoporosis patients, regardless of the safety notifications concerning ONJ related to BPs. ONJ was increasingly diagnosed after the dissemination of safety information about BP-related ONJ using repetitive and mixed communication methods; the impact of these communications materials was not clear. Our evaluation of the risk communication materials suggests that appropriate cooperation models involving the parties concerned with pharmacovigilance should be planned for the dissemination of safety information and for the delivery and evaluation of new safety concerns with marketed drugs.

## CONFLICT OF INTEREST

Eriko Sumi collected information on when, how and what type of risk communications regarding osteonecrosis of the jaw were released from pharmaceutical companies.

KEY POINT

- The use of oral bisphosphonates (BPs) in osteoporosis patients has increased regardless of safety concerns about osteonecrosis related to BPs. Osteonecrosis of the jaw was increasingly diagnosed after risk communication; however, the impact of the risk communication was not clear. Safety notifications were disseminated diligently after the package insert was revised. However, there was no surveillance for osteonecrosis of the jaw before the revision.

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site:

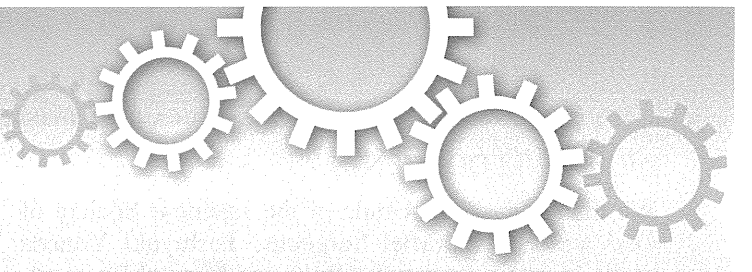
Appendix 1. List of drugs studied in cases of OMJ reported to the regulatory authority

Appendix 2. List of International Classification of Diseases (ICD-10) code for osteoporosis studied in the cohort study

Appendix 3. List of drugs studied in the cohort study

Appendix 4. List of International Classification of Diseases (ICD-10) code for inflammatory conditions of the jaw studied in the cohort study

SCIENTIFIC REPORTS



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# AMAP1 as a negative-feedback regulator of nuclear factor-κB under inflammatory conditions

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**NF-κB is a major transcriptional factor regulating many cellular functions including inflammation; therefore, its appropriate control is of high importance. The detailed mechanism of its activation has been well characterized, but that of negative regulation is poorly understood. In this study, we showed AMAP1, an Arf-GTPase activating protein, as a negative feedback regulator for NF-κB by binding with IKKβ, an essential kinase in NF-κB signaling. Proteomics analysis identified AMAP1 as a binding protein with IKKβ. Overexpression of AMAP1 suppressed NF-κB activity by interfering the binding of IKKβ and NEMO, and deletion of AMAP1 augmented NF-κB activity. The activation of NF-κB induced translocation of AMAP1 to cytoplasm from cell membrane and nucleus, which resulted in augmented interaction of AMAP1 and IKKβ. These results demonstrated a novel role of AMAP1 as a negative feedback regulator of NF-κB, and presented it as a possible target for anti-inflammatory treatments.**

Nuclear Factor-κB (NF-κB) consists of a family of transcription factors (p65 or RelA, p50, p52, c-Rel, and RelB) that play critical roles in inflammation, immunity, cell proliferation, differentiation and survival[1]. It generally exists as a homo- or heterodimer in the cytosol that is bound to the inhibitor of κB (IκB) (see review[2]). In response to a wide variety of stimuli, including inflammatory cytokines, IκB is phosphorylated and degraded via the ubiquitin pathway, which is followed by NF-κB translocation to the nucleus and activation of transcription. Serine phosphorylation of IκB is mediated by a large multi-unit complex containing two catalytic subunits (IKKα and IKKβ) and the regulatory subunit IKKγ or NEMO[3,4]. Among these subunits, IKKβ is the essential kinase mediating IκB phosphorylation in most cell types, and germline deletion of IKKβ results in embryonic lethality due to massive liver apoptosis, similar to the phenotype of p65-knockout mice[5].

NF-κB signaling proteins including these kinases are essential for maintaining living body as shown by IKKβ or p65 knockout mouse with phenotype of embryonic lethality, but they also have been implicated in the pathogenesis of many human diseases, including cancer[6–9]. Therefore, controlling their activities appropriately is of high importance. Although the mechanism for its activation was been well analyzed as described above, that for negative regulation is poorly explored.

We recently reported that IKKβ has a kinase-independent role in regulating widespread cellular responses and cell signaling[10], implicating that there are still undiscovered roles of IKKβ. To explore the roles more extensively, we identified A Multiple-domain Arf-GAP Protein 1 (AMAP1) as a binding partner of IKKβ by proteomic analyses. AMAP1, also called ASAP1 or DDEF1, is an Arf-GTPase-activating protein that functions on membrane surfaces to catalyze the hydrolysis of GTP bound to Arf, and plays major roles in the regulation of membrane remodeling and cytoskeletal organization, cellular migration and tumor invasion and metastasis[11–14]. More importantly, clinical studies have indicated that AMAP1 is dramatically up-regulated in advanced cancers[15–22]. The roles of AMAP1 in the inflammatory responses are of high interest considering the close relationship between caner and inflammation[2]; however, the roles have been poorly understood. Interestingly, Haque et al.

reported the inhibitory effect of AMAP1 (ASAP1) on the production of proinflammatory mediators[23], but the detailed mechanism remains unknown.

In this report, we carefully examined the involvement of AMAP1 in the regulation of NF-κB, and found that it has an unanticipated role to interfere the IKKβ−NEMO binding, which results in negative-feedback regulation of NF-κB activity. This report describes the detailed role of AMAP1 in the inflammatory responses, and AMAP1 can be a new target for developing anti-inflammatory treatments.

## Results

**AMAP1 interacts with IKKβ.** To discover novel roles of IKKβ, we started with identifying proteins that bind to IKKβ. Three kinds of FLAG-tagged IKKβ were overexpressed in HEK293T cells, and binding proteins were immunoprecipitated using anti-FLAG antibody. The samples were subjected to proteomic analyses using the nano-LC/MALDI-TOF system. Of the proteins identified repeatedly, we focused on AMAP1 since it is clinically important due to its augmented expression in cancer[15,16,19,22,24], which is one of the major inflammatory diseases[7,9]. To confirm the physical association of IKKβ and AMAP1, we performed co-immunoprecipitation (Co-IP) assays using the lysate of HEK293T cells co-overexpressed with HA-tagged AMAP1 and FLAG-tagged IKKβ. IKKβ and AMAP1 were detected in the precipitates with either the anti-HA antibody or the anti-FLAG antibody, respectively (Fig. 1a).

Next, we determined the binding sites of these proteins. Based on the functional modules of IKKβ, including an amino-terminal kinase domain (KD), a ubiquitin-like domain (ULD), an elongated α-helical scaffold/dimerization domain (SDD) and a carboxyl-terminal NEMO Binding Domain (NBD)[25], we divided IKKβ into 4 overlapping FLAG-tagged fragments. The constructs are IKKβ-Δ1 comprising the KD, ULD and SDD, IKKβ-Δ2 comprising the KD and ULD, IKKβ-Δ3 comprising only the KD, and IKKβ-Δ4 comprising the ULD, SDD and NBD (Fig. 1b), and each construct was expressed in HEK293T cells along with HA-tagged AMAP1. We found that AMAP1 was co-precipitated only with IKKβ-Δ4, but not with any other construct of IKKβ (Fig. 1c), indicating that AMAP1 binds to IKKβ at the C-terminal region containing the NBD. Further, we examined which region of AMAP1 is essential for its binding to IKKβ. Each of 6 GST-tagged fragments of AMAP1, which are Bar (Bin-Amphiphysin-Rvs), PH (Pleckstrin Homology), Arf-GAP, ANK (Ankyrin repeat), PRD (Proline Rich Domain) and SH3 (Src Homology3) domains (Fig. 1d), was expressed in HEK293T cells along with FLAG-tagged IKKβ. We found only SH3 domain construct was co-precipitated with IKKβ as shown in Fig. 1e. To confirm it further, we made construct of SH3-domain deleted AMAP1 (AMAP1ΔSH3). Cells were expressed with full-length AMAP1 or AMAP1ΔSH3 along with FLAG-tagged IKKβ, and we found only full-length AMAP1, not AMAP1ΔSH3, was co-precipitated with IKKβ (Supplementary Fig. S1 online). Taken together, these results indicated that the SH3 domain of AMAP1 is responsible for the binding with IKKβ, which implicates that the GTPase activity of AMAP1 is not involved in the interaction with IKKβ.

**AMAP1 negatively regulates NF-κB.** The C-terminal region of IKKβ identified as the binding site for AMAP1 contains the binding domain for NEMO/IKKγ, which is the regulatory subunit of the IKK complex[3]. It prompted us to verify the effect of AMAP1 on the association of IKKβ and NEMO because it is required for NF-κB activation[26]. As shown in Fig. 2a, overexpression of AMAP1 inhibited the binding of IKKβ and NEMO, and it was in a dose-dependent manner (Supplementary Fig. S2 online). In addition, the increased level of NEMO could mitigate the interaction between AMAP1 and IKKβ (Fig. 2b). Taken together, there is competition between AMAP1 and NEMO in IKKβ interaction. As expected from these results, overexpression of AMAP1 suppressed NF-κB activity (Fig. 2c), and it was confirmed by less translocation of p65 into nucleus after IL-1β stimulation in AMAP1-overexpressed cells (Fig. 2d). Additionally, suppression of AMAP1 by shRNA enhanced NF-κB activity (Fig. 2e) and augmented p65 translocation into the nucleus after IL-1β stimulation (Fig. 2f). Thus, AMAP1 interferes the binding of IKKβ and NEMO, and negatively regulates NF-κB.

**NF-κB activation induces AMAP1 translocation to cytoplasm and augments the AMAP1-IKKβ interaction.** In the experiments mentioned above, we sometimes observed that more amount of AMAP1 was detected after treatment with IL-1β (Supplementary Fig. S3a online). This observation coincides with previous report showing LPS significantly boosted AMAP1 protein levels within 15 min[23]. However, considering that 15 min is too short for synthesis or degradation of proteins, it was highly possible that AMAP1 might not be completely dissolved in cell lysis buffer because AMAP1 can be localized in lipid rafts[11–14] and nucleus[27], both of which might not be fully dissolved by 1% Triton X-100 of cell lysis buffer used in this study. Indeed, we also observed similar change of AMAP1 by LPS stimulation, but it disappeared when cells were lysed in 1% SDS cell lysis buffer (Supplementary Fig. S3b online). Therefore, we hypothesized that NF-κB activation induces AMAP1 translocation to the cytoplasm from cell membrane or nucleus, which could result in detection of more AMAP1 in cell lysate by 1% Triton X-100. As shown in Fig. 3a, fractionation by sucrose gradient indicated that IL-1β stimulation induced the translocation of AMAP1 from lipid rafts to the cytoplasm. We also performed confocal immunocytochemistry with using human umbilical vein endothelial cells (HUVECs) since HEK293T cells were not appropriate for observation of intracellular localization due to their large nuclei and thin cytoplasm. It was hard to see AMAP1 staining on cellular membrane, but we observed that IL-1β stimulation induced translocation of AMAP1 in nuclei to the cytoplasm (Fig. 3b). Finally, we repeated the sucrose gradient fractionation with HUVECs, and observed the translocation of AMAP1 from the membrane in response to IL-1β stimulation (Fig. 3c), similarly with HEK293T cells.

Considering that IKKβ is nearly entirely cytoplasmic protein[28,29], the translocation of AMAP1 to cytoplasm in response to IL-1β stimulation raised the question as to whether the activation of NF-κB would strengthen the interaction between AMAP1 and IKKβ. As shown in Fig. 3d and e, more IKKβ was co-precipitated with anti-AMAP1 antibody after IL-1β stimulation, and conversely, more AMAP1 was co-precipitated with anti-IKKβ antibody after IL-1β stimulation, revealing that NF-κB activation enhances the interaction between AMAP1 and IKKβ.

## Discussion

Most biological processes require positive and negative regulatory mechanisms to maintain equilibrium. The results from this study suggest a novel mechanism for NF-κB termination and contribute new knowledge to the mechanisms of NF-κB regulation. So far, the re-synthesis of IκB proteins induced by the activation of NF-κB is a widely accepted mechanism for terminating the NF-κB response. These proteins enter the nucleus, remove NF-κB from the DNA and re-localize it to the cytosol[30–33]. Here, we show that NF-κB activation induces AMAP1 translocation to cytoplasm from nucleus or lipid rafts and accelerates binding of IKKβ and AMAP1, which inhibits the association of IKKβ and NEMO, eventually leading to inactivation of NF-κB. Taken together, AMAP1 works as an inhibitory feedback mechanism for regulating the NF-κB pathway (Fig. 4).

Recent studies have established strong support for the critical role of NF-κB in cancer. Activation of NF-κB controls multiple cellular processes in cancer, including inflammation, transformation, proliferation, angiogenesis, invasion, metastasis, chemoresistance and

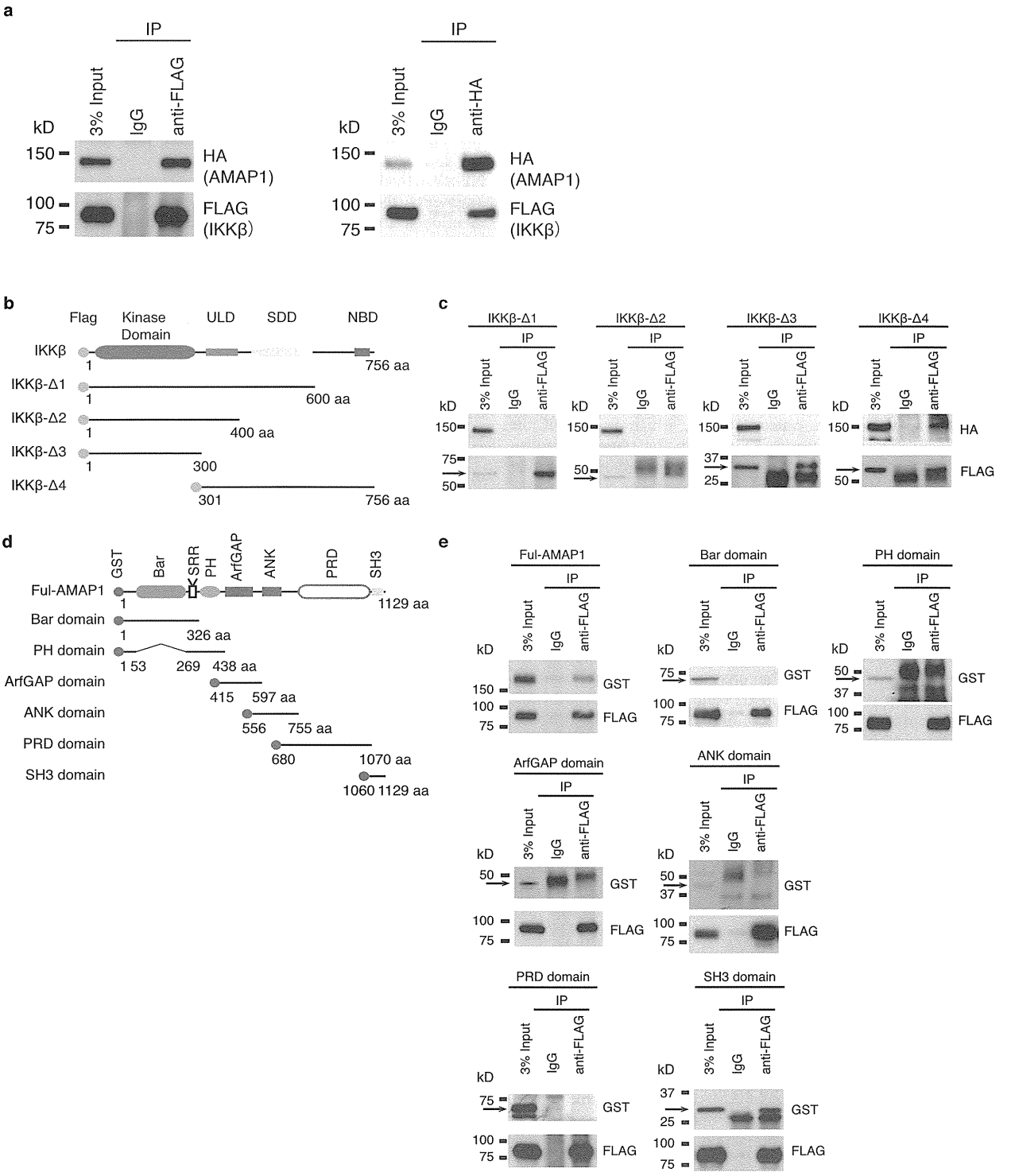


**Figure 1 | AMAP1 interacts with IKKβ *in vivo*.** (a) The binding of AMAP1 and IKKβ. FLAG-tagged IKKβ and HA-tagged AMAP1 were expressed in HEK293T cells and immunoprecipitated with anti-FLAG and anti-HA antibodies. AMAP1 and IKKβ were detected in the precipitate by immunoblotting with anti-FLAG and anti-HA antibodies. (b) Schematic representation of IKKβ and its mutants. (c) FLAG-tagged IKKβ/mutants and HA-tagged AMAP1 were expressed in HEK293T cells and immunoprecipitated with anti-FLAG antibody. AMAP1 was detected in the precipitate by immunoblotting with anti-HA antibody. (d) Schematic representation of AMAP1 and its mutants. (e) FLAG-tagged IKKβ and GST-tagged AMAP1/mutants were expressed in HEK293T cells and immunoprecipitated with anti-FLAG antibody. AMAP1 and its mutants were detected in the precipitate by immunoblotting with anti-GST antibody. The data shown are from one representative experiment of the three that were performed. Full-length blots are presented in Supplementary Figure 4.

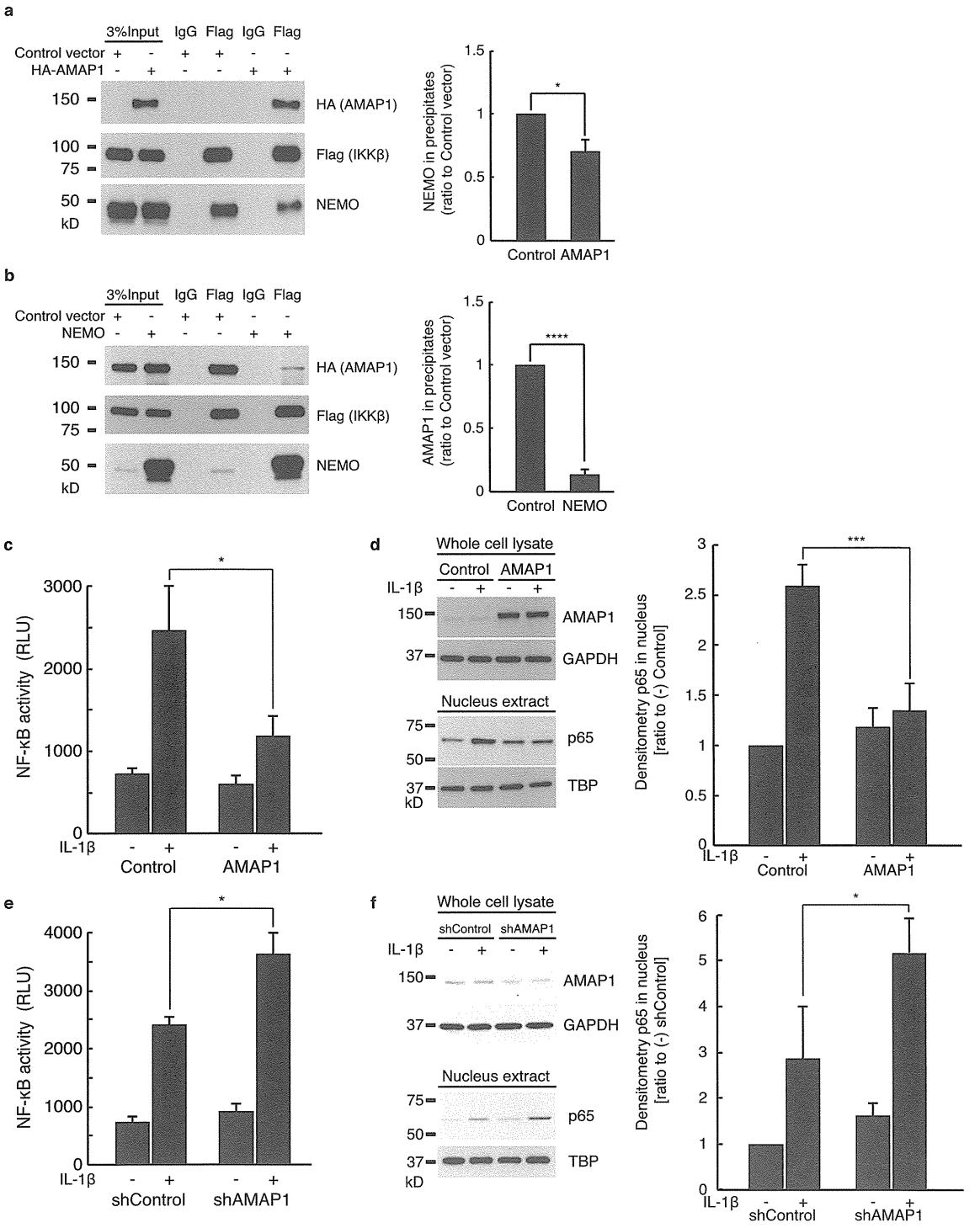


**Figure 2 | AMAP1 negatively regulates NF-κB.** (a) AMAP1 interfered with the association of IKKβ and NEMO. FLAG-tagged IKKβ and NEMO were expressed in HEK293T cells along with control vector or HA-tagged AMAP1 and immunoprecipitated with anti-FLAG antibody. AMAP1, IKKβ and NEMO were detected in the precipitates by immunoblotting with anti-FLAG, anti-HA and anti-NEMO antibodies. (b) The induction of NEMO reduced the interaction of IKKβ and AMAP1. FLAG-tagged IKKβ and HA-tagged AMAP1 were expressed in HEK293T cells along with control or NEMO vector and immunoprecipitated with anti-FLAG antibody. AMAP1, IKKβ and NEMO were detected in the precipitates by immunoblotting with anti-FLAG, anti-HA and anti-NEMO antibodies. (c) NF-κB activity from nuclear extracts of overexpressed AMAP1 and control cells with or without IL-1β (2.5 ng/mL) treatment for 30 min. (d) Immunoblots of AMAP1 (whole cell) and p65 (nuclear extract) of HEK293T cells transfected with pcDNA3.1-HA-AMAP1 (AMAP1 overexpression) or pcDNA3.1 (control) and the cumulative, quantitative densitometry data. (e) NF-κB activity from nuclear extracts of shAMAP1 and shControl cells with or without IL-1β (2.5 ng/mL) treatment for 30 min. (f) Immunoblots of AMAP1 (whole cell) and p65 (nuclear extract) of HEK293T cells transfected with AMAP1 shRNA (shAMAP1) or Control shRNA (shControl) with or without IL-1β (2.5 ng/mL) and the cumulative, quantitative densitometry data. The data from three independent experiments are shown. Error bar: ±SD. * $P < 0.05$, ** $P < 0.01$ in a two-sided, Student's *t*-test. Full-length blots are presented in Supplementary Figure 4.